Bifidobacterium Longum Modified Recombinant HU Protein as a Vector for Nonviral Delivery of DNA to HEK293 Human Cell Culture

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Creation of effective nontoxic highly specific systems for nonviral transportation of DNA is one of the priority problems in the development of genotherapeutic methods. Chimerical recombinant proteins consisting of Antp and Tat protein cell-penetrating domains and *Bifidobacterium longum* DNA-binding histone-like protein HU were obtained. The resultant recombinant proteins bind to plasmid DNA *in vitro* and provide intracellular delivery and expression of the reporter genetic construction with GFP gene in cultured HEK293 human cells.

Key Words: *nonviral delivery of DNA; PTD domains; cell-penetrating peptides; histone-like proteins; bifidobacteria*

Cell-penetrating protein domains, also called cell-penetrating peptides (CPP) or protein transduction domains (PTD), are short amino acid sequences (10-20 a.a) of some proteins in higher eukaryotes and viruses responsible for energy- and receptor-independent penetration of the corresponding proteins into the cytosol through the cytoplasmic membrane and even into the cell nucleus from the environment [2]. By the present time the best studied PTD domains are those of the *Drosophila melanogaster* homeoprotein *Antennapedia* (Antp), HIV-1 Tat transactivator protein, and Herpes simplex virus VP22 protein [4]. Recent studies have shown that artificial conjugation of PTD peptides with high-molecular-weight compounds provided their effective intracellular delivery *in vitro* and *in vivo* [1,7,9,10]. Due to their low cytotoxicity and biogenic nature PTD peptides are attractive candidate vectors for intracellular transportation of protein and gene therapeutic constructions in molecular medicine [2,3].

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The aim of our study was to obtain chimerical recombinant proteins consisting of Antp protein PTD domain or Tat protein mutant PTD domain [5], on the one hand, and DNA-binding histone-like HU protein from *Bifidobacterium longum*, on the other hand, providing transportation of plasmid DNA to human cell culture.

MATERIALS AND METHODS

E. coli strains XL-1Blue and BL21 (DE3) were cultured in LB nutrient medium under standard conditions. HEK293 cells were cultured at 37°C in DMEM with 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 5% CO_2.

Oligonucleotide primers were designed using PerlPrimer software. Oligonucleotides were synthesized by the amidophosphite method and purified by electrophoresis in PAAG.

PCR amplification of *B. longum* VMKB44 *hup* gene was carried out by nested PCR [6]. At stage 1, *hup* gene was amplified from genomic DNA using HiFidelity PCR kit (Qiagen) with Phup-F1 and Thup-R1 primers (1 μM each) by the following pro-

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tocol: 1) 20 sec at 94°C; 2) 20 sec at 60°C; 3) 45 sec at 72°C; 30 cycles. The second round of PCR was carried out with hup-R1 primer in combination with hupORF-F1, YM3-hup-F1, or Antp-hup-R1 primers (1 μM each; Table 1). PCR was carried out according to the above protocol. The resultant PCR products were purified by QIAquick PCR kit (Qiagen) and cloned in pET-28b(+) vector (Novagen) using *Ncol* and *Bam*HI restriction endonucleases (Fermentas) and bacteriophage T4 DNA ligases (Fermentas) by the standard method [8].

Nucleotide sequences of DNA inserts were verified by sequencing. The dideoxysequencing reaction was carried out with DTCS Starter kit (Beckman Coulter). The sequencing products were analyzed in a CEQ 8000 automated sequencer (Beckman Coulter).

E. coli BL21 (DE3) strain transformed with the resultant plasmids was incubated at constant shaking (180 rpm) to optical density (OD) of 0.7 at λ=600 nm. The cloned gene expression was then induced by adding isopropyl-β-D-thiogalactoside to the final concentration of 1 mM and culturing was continued for 3 h at 37°C and permanent shaking. Aliquots of the culture (1 ml) were centrifuged 3 min at 13,000*g* in a microcentrifuge and the precipitates were resuspended in 100 μl buffer (50 mM Tris hydrochloride, pH 6.8, 100 mM β-mercaptoethanol, 1% SDS, 0.0025% bromophenol blue, and 10% glycerol). After heating at 100°C (3 min) the samples were again centrifuged and 15 μl supernatant was applied onto 12.5% PAAG with SDS. The rest culture was used for isolation of the target protein.

The proteins were purified by metal chelate chromatography using His-Spin Protein Miniprep (ZymoResearch) according to manufacturer's instruction. In order to obtain DNA-protein complexes, the protein preparations were mixed with plasmid DNA in PBS and incubated for 15 min at 37°C. Semiquantitative evaluation of DNA binding to proteins was carried out by inhibition in agarose gel. Agarose gels with 1.5% concentration were prepared on TAE buffer with ethidium bromide (0.5 μg/ml); electrophoresis was carried out at field intensity of 10 V/cm over 30 min, the results were visualized by photography of the gel plates on a transilluminator.

Protein electrophoresis was carried out in denaturing PAAG with SDS in a step buffer system [8] with 5% concentrating and 15% separating gels (29:1 acrylamide:bis-acrylamide ratio) at the electric field intensity of 15 V/cm. The gels were stained with R250 Coumassie brilliant blue and photographed.

DNA-protein complexes for cell culture transfection were prepared as follows: 0.5-20 μg affinity-purified proteins in a buffer (50 mM phosphate buffer, pH 7.7, 300 mM NaCl, 250 mM imidasole) were mixed with 1 μg pEGFP-N1 plasmid and brought to a final volume of 100 μl with PBS. Plasmid DNA in 100 μl PBS served as the negative control. The DNA-protein complexes were incubated for 30 min at 37°C and applied to washed HEK293 culture in wells of a 24-

TABLE 1. Oligonucleotide Primers Used in the Study

Primer	Primer 5'-3' sequence (recognition sites for *Nco*I, *Nde*I, and *Bam*HI restriction endonucleases are underlined)
Phup-F1	CGGTACCTACTGGCTGCGTATTCCG
Thup-R1	CGAATTCGCTGAACTAGTCCGGA
hupORF-F1	GGCGCGCCCATGGCATACAACAAGTCTGA
YM3-hup-F1	ATATCCATGGGCACCCATCGCCTGCCGCGCCGTCGTCGCCGCCGTGGCCATATGGCATACAACAAGTC
Antp-hup-F1	ATATCCATGGGCCGCCAGATTAAAATTTGGTTTCAGAACCGCCGCATGAAATGGAAAAAAGGCCATATGGCATACAACAAGTC
hup-R1	GCGCGGATCCCTCGGTGACGGCCTTCT

TABLE 2. Fragments of Amino Acid Sequences of Chimerical Proteins

Protein	Amino acid sequence (N-C)
YM3-HU	MG-**THRLPRRRRRR**-GH-(HU-9 kDa)-DPNSSSVDKLAAALE*HHHHHH*
Antp-HU	MG-**RQIKIWFQNRRMKWKK**-GH-(HU-9 kDa)-DPNSSSVDKLAAALE*HHHHHH*
HU	MG-(HU-9 kDa)-DPNSSSVDKLAAALE*HHHHHH*

Note. Polypeptide sites corresponding to PTD domains are shown with bold type, hexahistidine epitope labels with italic type.

well plate. After 30-min incubation at 37°C, complete growth medium (400 µl) was added into the wells and incubation was carried out over 18 h. The expression of GFP was evaluated by fluorescent microscopy.

RESULTS

The delivery of plasmid DNA molecules into living cells with the cell-penetrating PTD domains was evaluated using recombinant chimerical proteins consisting of YM3 and Antp N-terminal PTD domains fused with the amino acid sequence of low-molecular (9 kDa) histone-like DNA-binding protein HU from *B. longum* and with C-terminal hexahistidine epitope label providing rapid purification of the target protein by metal chelate affinity chromatography (Table 2).

For obtaining this protein construct, the *hup* gene encoding HU protein was amplified from genome DNA of *B. longum*; during the second round of PCR the fragments encoding the minimum PTD domains

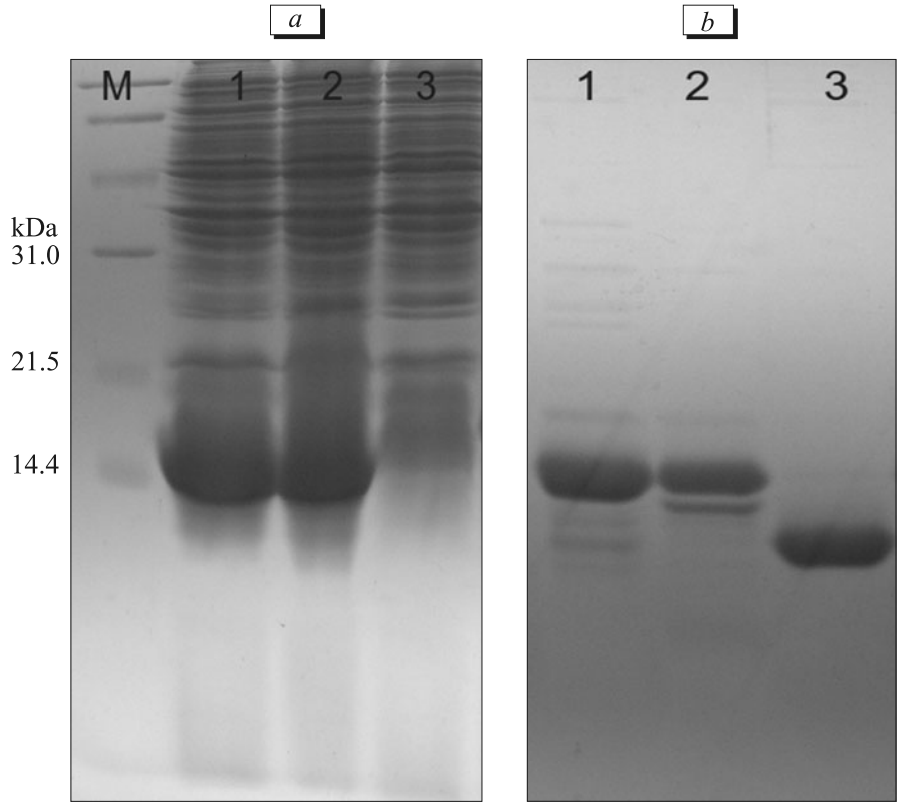

Fig. 1. Denaturating SDS-PAAG electrophoresis of lysates of *E. coli* BL21 (DE3) strain transformed with pET28/Antp-hup and pET28/YM3-hup plasmids after induction (*a*), and electrophoresis of affinity-purified YM3-HU, Antp-HU, HU proteins (*b*). For *a*: M: molecular weight marker; *1*) pET28/Antp-hup plasmid; *2*) pET28/YM3-hup plasmid; *3*) nontransformed strain (negative control). For *b*: *1*) YM3-HU; *2*) Antp-HU; *3*) HU.

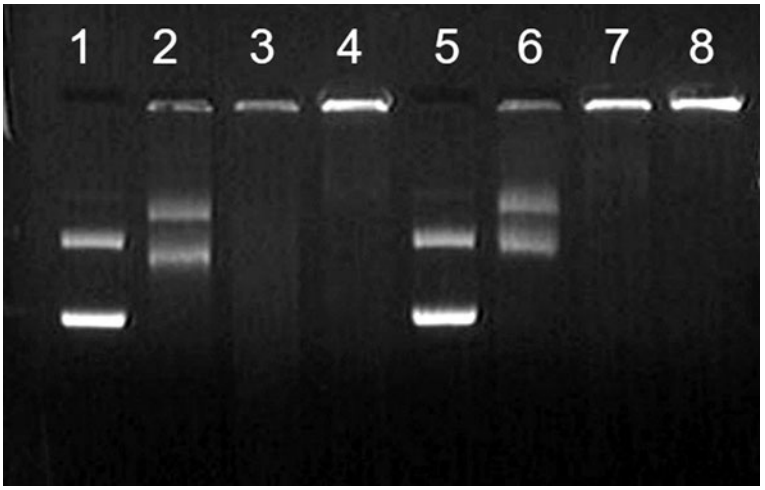

Fig. 2. Reduction of electrophoretic mobility of DNA-protein complexes in gel. *1-4*: 0.5 µg plasmid pEGFP-N1 DNA treated with 0, 2, 5, and 10 µg recombinant chimerical protein Antp-HU, respectively. *5-8*: 0.5 µg plasmid DNA treated with 0, 2, 5, and 10 µg recombinant protein HU, respectively.

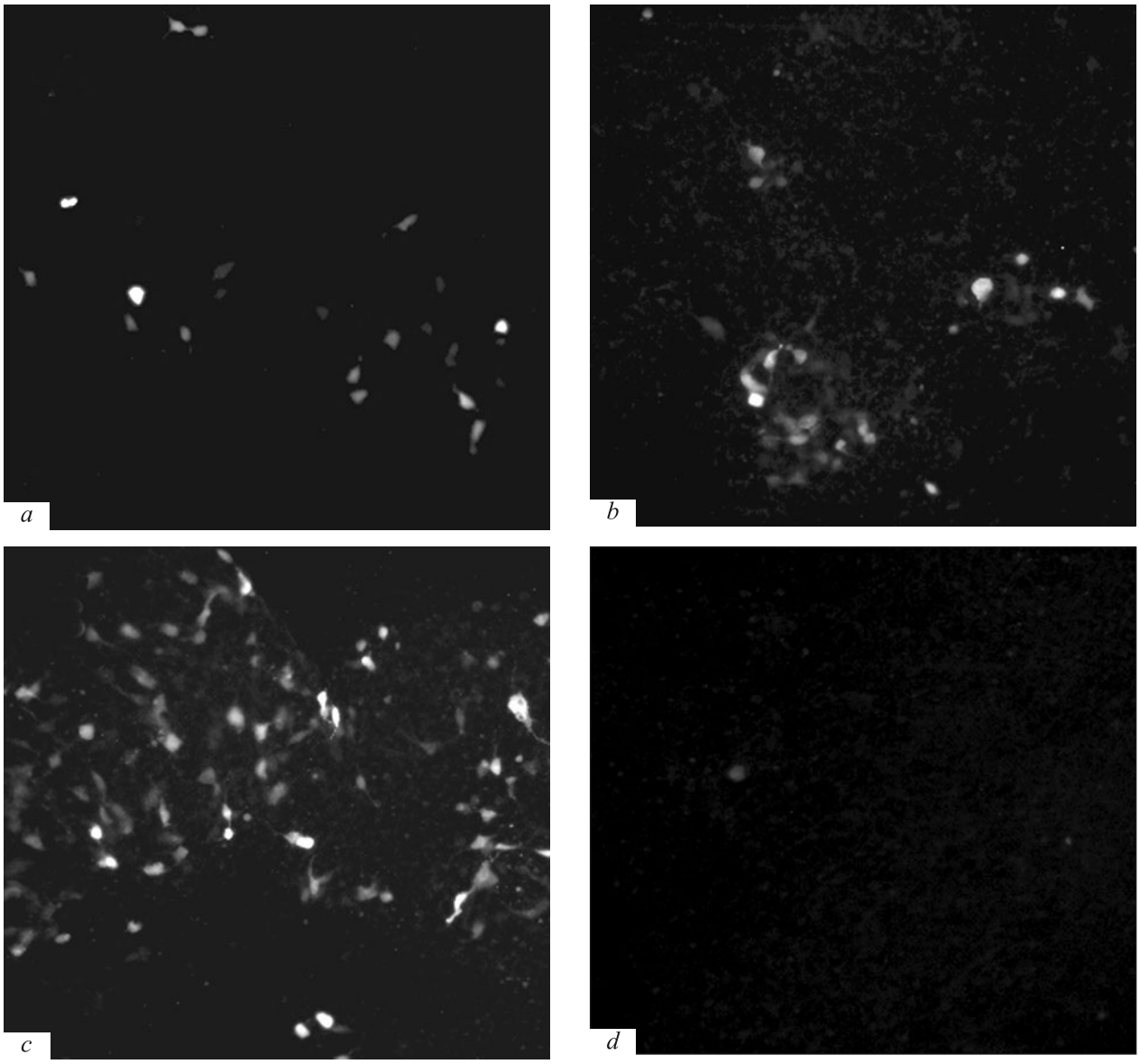

Fig. 3. Transfection of HEK293 cells with DNA-protein complexes consisting of pEGFP-N1 reporter plasmid (2 μg/ml) and recombinant chimerical proteins HU (*a*), YM3-HU (*b*), or Antp-HU (*c*) in a concentration of 20 μg/ml. *d*) control transfection with DNA without proteins.

of YM3 (product of spontaneous mutagenesis of Tat protein PTD domain) and Antp were added. The resultant PCR products were cloned in pET-28b(+) plasmid vector with the formation of translation fused chimerical genes, YM3-hup-6xHis, Antp-hup-6xHis, and hup-6xHis. The resultant vector plasmids were named pET28/YM3-hup, pET28/Antp-hup, and pET28/hup, respectively.

Expression of recombinant plasmids in *E. coli* strains transformed with plasmids pET28/YM3-hup, pET28/Antp-hup, and pET28/hup led to production of proteins with expected molecular weights (Fig. 1, *a*). Purification of hexahistidine-labeled proteins from transformed strains showed that the greater part of proteins were soluble and effectively bound to the affinity carrier (Fig. 1, *b*). Hence, due to purification by affinity chromatography 95% pure protein preparations were obtained. We therefore concluded that the resultant producer strains can be used for the production of chimerical proteins containing the PTD domain and the DNA binding domain of HU protein.

Specific reaction of the resultant proteins with plasmid DNA molecules was evaluated by retention of DNA-protein complexes during agarose gel electrophoresis. The experiment demonstrated binding of the resultant proteins to DNA in mixtures of different proportions and the formation of complexes with the corresponding electrophoretic mobilities (Fig. 2).

The next stage of the study was evaluation of penetration of DNA-protein complexes containing pEG-

FP-N1 plasmid with GFP gene and chimerical Antp-HU, YM3-HU, and HU DNA-binding proteins, into cultured HEK293 cells. Experiments with variants of the DNA/protein weight proportions in the complexes have shown that the percentage of transfected cells was maximum after addition into the plates of the complex containing 1 μg plasmid DNA pEGFP-N1 and 10 μg chimerical recombinant DNA-binding protein. Interestingly, a certain percent of GFP-positive cells was seen after treatment with recombinant HU protein without PTD domain. On the other hand, the percentage of GFP-positive cells was significantly higher after treatment with HU protein fused with YM3 and even more so to the Antp PTD domain (Fig. 3).

For semiquantitative analysis of GFP expression in cell cultures transfected with DNA-protein complexes, the mean values of GFP fluorescence were measured in a series of microphotographs. The level of GFP fluorescence was maximum after addition of chimerical Antp-HU protein to the cultures (8.83±3.56). The level of fluorescence after using YM3-HU was approximately 2-fold lower (4.33±2.17). Fluorescence recorded after using HU protein was only 0.96±0.48, which was however significantly higher ($p<0.01$) than the fluorescence of control specimens (0.05±0.04).

No nonspecific toxicity caused by membrane damage was detected at the chimerical protein concentrations (4-20 μg/ml) used in our experiments.

Hence, our findings indicate that the chimerical proteins containing the *B. longum* minor histone-like protein HU domain and the YM3 and Antp PTD domains provide rather effective and nontoxic transfection of mammalian cell cultures with plasmid DNA. However, further *in vitro* and *in vivo* studies are needed before using the resultant DNA-binding proteins as means for vector delivery of DNA in gene therapy.

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