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Original article

HVJ-E/importin-β hybrid vector for overcoming cytoplasmic and nuclear membranes as double barrier for non-viral gene delivery

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ABSTRACT

In order to enhance the nuclear import of the transgene, we prepared plasmid DNA/importin-β conjugates consisting of biotinylated poly(ethylenimine)s and recombinant streptavidin-fused importin-β. Hemagglutinating virus of Japan-envelope vector containing the PEI polyplex/importin-β conjugate showed high transfection efficiency not only in vitro but also in vivo. We showed that novel HVJ-E/importin-β-conjugated PEI polyplex hybrid vector could overcome plasma and nuclear membrane barriers to achieve effective transfection.

1. Introduction

In order to express the protein encoded in an exogenous gene, one has to deliver the gene into the nucleus of desired cells. Since non-viral vectors lack the ability to import into the nucleus by themselves, their efficiency of transgene expression is generally lower than viral vector's one. Indeed, in non-dividing cells, transgene expression by non-viral vectors is very difficult [1–3]. Miyake et al. showed that the non-viral gene expression with polyplex and lipoplex exactly depended on cell division by examining the morphology of cells and the dynamics of GFP gene expression [4,5]. When the herpes simplex virus thymidine-kinase gene was microinjected into the nucleus of a mouse cell line deficient in thymidine-kinase activity, it was expressed by 50–100% of the injected cells. On the contrary, when the gene was microinjected into cytosol, enzymatic activity was not determined [6]. Furthermore, the expression efficiency by non-viral vector is strongly dependent on the cell-cycle phase at transfection. When cells were transfected during or just before mitosis with non-viral vectors, transfection efficiency was increased, suggesting that transfection close to M-phase is facilitated by nuclear envelope breakdown [7–9]. These studies obviously demonstrate that the nuclear membrane is a serious barrier for non-viral gene delivery system.

Over the past decade a great deal of effort has been directed towards the enhancement of the nuclear import of exogenous genes [10–12]. Trafficking of nuclear proteins through the nuclear pore complex (NPC) of the nuclear envelope (NE) is mediated by the presence of nuclear localization signal (NLS) on proteins [13–15]. Then, major advance have been made in the field of macromolecular nucleocytoplasmic trafficking among with recent development of molecular and cellular biology. Several groups reported that NLS non-covalently or covalently bound to DNA enhanced the nuclear import [16–19], while opposite results were obtained [20–23]. Attempts to improve the nuclear import of exogenous gene through the utilization of NLS have seemed to achieve limited success.

The NLS-mediated strategy for nuclear import of a transgene often requires the contribution of the importin-α adapter, which reduces the nuclear import efficiency of transgenes. Therefore, the direct conjugation of importins to plasmid DNA was expected to promote nuclear import of plasmid DNA. First, the importin-β-binding domain of importin-α (IBB) was coupled to plasmid DNA covalently. However, this peptide did not enhance either the nuclear import of plasmid DNA or the efficiency of cationic-lipid-mediated transfection [23]. More recently, chimeric fusion protein between human high-mobility group (HMG) protein and IBB was prepared and estimated as gene carrier. Although IBB of the chimeric protein increased transfection efficiency, the effect on nuclear import of plasmid DNA was uncertain [24]. In nucleocytoplasmic trafficking of nuclear proteins, importin-β superfamily proteins has frequently been shown to play an essential role by interacting with the NPC and transporting directly the bioactive

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compounds into nucleus by itself [25–32]. Then, we previously prepared a plasmid DNA/importin-β conjugate consisting of biotinylated plasmid DNA and a recombinant streptavidin-importin-β chimeric protein. The nuclear import of a biotinylated plasmid was enhanced with stereptavidin-fused importin-β, while expressed protein from biotinylated plasmid was increased moderately due to the transcriptional inhibition [33]. On the other hand, hemagglutinating virus of Japan (HVJ; Sendai virus) envelope vector (HVJ-E) is useful for both in vitro and in vivo gene expression because of directly and efficiently envelope-cell fusion [34–36]. Therefore, we prepared plasmid DNA/importin-β conjugates (pDNA/b-PEI/βS) consisting of biotinylated polyethylenimine (PEI) and recombinant streptavidin-fused importin-β (βS). Moreover, a HVJ-E containing pDNA/b-PEI/βS was evaluated as an artificial virus gene carrier in vitro and in vivo. In this study, the polyplex from an intact plasmid and a biotinylated polycation was used to avoid the decrease of transcriptional efficiency.

2. Materials and methods

2.1. Cell culture

NIH3T3 cells (mouse embryo fibroblast) were grown in Dulbecco's modified Eagle's medium (Nissui) containing 10% fetal bovine serum (Invitrogen) supplemented with 100 unit/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO_2 atmosphere. Cultured cells were plated on glass bottom dish (Matsunami Glass Ind., Ltd.) 24 h before use for nuclear import assays and intracellular trafficking.

2.2. Reagents

For in vitro transfection studies, an expression plasmid, pGL3-control (pGL3) in which the *Photinus pyralis* luciferase gene was under the control of the SV40 promoter was purchased from Promega. The supercoiled plasmid DNA was amplified in *Escherichia coli* strain JM109 and purified by EndoFree Plasmid Kit (Qiagen). The plasmid DNA encoding streptavidin (pGSH) was kindly gifted from Prof. Housaka (Japan Advanced Institute of Science and Technology) [37]. The synthetic oligonucleotides for the primer of PCR were obtained from Nisshinbo. All dephosphorylations were carried out as described in the protocol of Shrimp Alkaline Phosphatase Kit (Promega). Biotinylating reagent in which biotin linked N-hydroxysuccimidyl ester residue with PEG spacer containing average molecular weight of 3400 Da (Biotin-PEG-NHS) was purchased from Nektar.

2.3. Purification of recombinant protein

The expression vector of recombinant mouse importin-β, pGEX-2T-importin-β, was generated as described previously [33]. To construct the expression vectors (pGEX-4T-3-imp-β-S.avidin) of recombinant mouse importin-β (1∼641 aa)-streptavidin (1∼170 aa) chimera, the BamHI–BglII fragment of streptavidin was amplified from pGSH [37] by PCR using the synthetic oligonucleotides (5′-AAAATCGGATCCCATATGGACCCGTCCAAG-3′ and 5′-CT- TGGAAGATCTTTAGTGGTGGTGGTGGTG-3′). After digesting with BamHI and BglII, this PCR product was inserted into BamHI site of dephosohrylated pGEX-4T-3 (Amersham pharmacia biotech) to prepare the expression vector (pGEX4T-3-S.avidin) of GST-streptavidin. The importin-β BamHI-BamHI fragment was amplified from pGEX2T-importin-β [33] by PCR using the synthetic oligonucleotides (5′-ATCTCCGGATCCATG-GAGCTCATAACCATC-3′ and 5′-CTATATGGATCCA- CCACCCAA-CACTTCCAC-3′). After digesting with BamHI, this PCR product was inserted into BamHI site of dephosphorylated pGEX-4T-3-S.avidin. To confirm the insertion of the fragment, obtained pGEX-4T-3-imp-β-S.avidin was digested with BamHI. Furthermore, the direction of the insert was checked with XhoI followed by electrophoresis on 1.0% agarose gels. Expression and purification of recombinant mouse importin-β-streptavidin (βS) were performed as described previously [33]. The expression vector of βS was transformed into *E. coli* strain BL21. The *E. coli* were grown in LB medium 100 μg/mL ampicillin at 37 °C to a density of 1.0∼1.5 (OD_{550}). The expression of βS was induced by 0.1 mM IPTG for 14 h at 20 °C and was purified with Glutathione Sepharose™ 4B and PD-10 column (GE Healthcare).

2.4. Preparation of biotinylated polyethylenimine (b-PEI)

Branched polyethylenimine (PEI) with molecular weight of 2.5×10^4 was purchased from Aldrich. To a dry DMSO solution (1 mL) containing PEI (25 mg) was added 5 mg of biotin-labeling reagent (Biotin-PEG-NHS). The mixture was stirred at room temperature for 20 h and poured into H_2O (5 mL). Purification was carried out with ultrafiltration with Centricon Plus-20 (NMWL 5000, Amicon). The white powder (14.3 mg) was obtained after lyophilization. The number of introduced biotin-appended PEG chain was determined as ca. 2 by the elemental analysis and ^{1}H-NMR spectroscopy. Anal. Calcd. for (biotin-PEG)$_2$-PEI·230H_2O: C, 49.20; H, 11.03; N, 22.10. Found: C, 49.23; H, 11.02; N, 22.05%. ^{1}H-NMR(400 MHz, 25 °C) δ(D_2O) 2.30-2.75 (m, 2324H, -NCH_2-) and 3.52 (s, 568H, -OCH_2-)ppm.

2.5. Microinjection

NIH3T3 cells were grown on marked coverslips in the incubator at 37 °C with 5% CO_2 atmosphere. The recombinant protein (βS) was injected into cytoplasm with glass micropipettes. Injections were performed under visual control on a fixed stage of inverted phase contrast microscope IX-70 (Olympus, Japan) by using a micromanipulator MMO-202 N (Narishige, Japan) and a microinjector IM-16 (Narishige). The recombinant protein (βS) was immuno-stained by using anti-GST antibody (1:250 dilution; #2624, Cell Signaling Technology). Samples were observed by fluorescence microscope IX-71 (Olympus) attaching a confocal scan unit CSU-10 (Yokogawa) with ORCA-ER CCD camera (Hamamatsu Photonics).

2.6. In vitro transfection

To prepare plasmid DNA/importin-β conjugate (pDNA/b-PEI/βS), 1 μg of pGL3 was mixed into 6.9 mM βS (1 μL), 1.33 mM b-PEI (2.5 μL), and 13.3 mM PEI (3.75 μL). The N/P ratios of the complexes were adjusted for 10. As a result, 12 b-PEI and 24 βS molecules were bounded per a plasmid calculatedly.

NIH3T3 cells were seeded at a density of 5×10^4 cells/well in 1 mL of the growth medium in 24-well plates and incubated for 24 h prior to transfection. At the time of transfection, the culture medium was replaced with 200 μL/well of the fresh medium. The solution (50 μL) containing one μg of plasmid DNA was added into a well. After 24 h of incubation time, quantification of total protein and measurement of luciferase activity were estimated. Total protein content in cells was quantified by Protein Quantification Kit (Dojindo, Japan) according to the manufacturer's instructions using a Multiskan Ascent BIF microplate reader (Thermo Labsystems). Separately, the luciferase activity in cells was measured by Steady-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions using a Fluoroskan Ascent FL microplate luminometer (Thermo Labsystems). The measurements of the luciferase activity and protein