Research in Pharmaceutical Sciences, August 2015; 10(4): 268-274
Received: Nov 2014
Accepted: Jan 2015

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Original Article

Expression of a biotin acceptor peptide-containing protein with potential incorporation on the lentiviral envelope as a viral surface engineering platform

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Abstract

Lentiviral vectors are among the promising viral based-vectors in gene therapy applications, but the efficiency of their targeting needs to be improved. (Strept)avidin-biotin adaptor system is a novel approach to modify the lentiviral envelope for better targeting properties. Herein, we describe utilization of this adaptor system by designing a candidate envelope protein-bearing biotin acceptor peptide (BAP) and evaluation of its expression in 293T cells. To this end, a DNA sequence containing flexible linkers, a 15-aminoacids BAP and specific membrane regions of a viral protein was designed and synthesized in tandem. The synthesized gene was amplified with polymerase chain reaction to include *Bgl*II and *Sal*I restriction sites and subcloned into the same sites of pDisplay vector in frame with HA-tag and myc epitope to construct the pDis-GS-BAP. 293T cells were transfected with pDis-GS-BAP and expression of resulting protein (dis-GS-BAP) was evaluated by Western blotting using anti-HA tag antibody. Efficiency of transfection procedure was evaluated by pEGFP-N1 vector and tracking for green fluorescent protein expression via fluorescence microscopy. Restriction analysis and DNA sequencing confirmed the precision of cloning steps. Fluorescence microscopy indicated above 70% transfection efficiency and Western blot analysis of pDis-GS-BAP-transfected 293T cells showed a protein band of approximately 17 kDa corresponding to the predicted size of dis-GS-BAP protein. These promising results indicate the possibility of cell surface expression and further biotinylation of dis-GS-BAP protein in ongoing studies.

Keywords: Lentiviral vectors; 293T Cells; (Strept)avidin-biotin; pDisplay; BAP; Gene therapy

INTRODUCTION

The success in gene therapy is highly dependent on the efficient transfer of the target gene to the target location. In this context, a number of studies have been conducted using different vectors to evaluate their efficiencies in transfer of the genetic materials (1,2). Among different modalities for gene transfer, viral-based vectors have been vastly exploited in clinical trials because of: *i)* Their ability to transfer their own genetic materials efficiently into the target cells *in vivo* and *ii*) Availability of versatile methods to manipulate and modify these vectors for gene delivery purposes (3,4). Among different viral vectors for gene therapy purposes, those derived from the lentivirus genus, which can transduce both dividing and non-dividing cells for stable expression of target genes, has gained so much attention. Lentiviral vectors have been extensively studied for both research and potential clinical applications (5). Accordingly, a number of prior studies have addressed different approaches to improve the production, safety and efficacy of lentiviral vectors (6-9).

One of the efficient approaches to improve lentiviral vectors for enhanced targeting to the aimed locations is through the modification of their envelope proteins (10). In fact, lentiviral vectors are able to incorporate cell membrane proteins into their envelope which confer new properties to the virions such as the ability to target specific subset of cells (11-13).

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Insertion of biotin acceptor peptides (BAP) into the extracellular domains of such membrane proteins is a relatively new strategy which provides a platform that allows the utilization of (strept)avidin-biotin system to modify the virions (11). To this end, BAP is covalently biotinylated by exogenous biotin ligase enzyme (metabolic biotinylation) and the resulting viral particles can then attach to different (strept)avidin-biotin based adaptor molecules (14). This system has been successfully used in purification and retargeting of lentiviral vectors (11,15). Of note, due to the high affinity between (strept) avidin and biotin (dissociation constants (K_d) of 10^{-15}), vectors using this adaptor system remain stable in the serum of immune competent of animals which is an important characteristics that implies the potentiality of this system for *in vivo* applications (16).

pDisplay is a mammalian expression vector which transports the protein or peptide of interest to the cell surface. By insertion of BAP in pDisplay, Niers and coworkers managed to metabolically biotinylate surface of different mammalian cells (17). In another studies, it was demonstrated that pDisplay can incorporate protein ligands into the envelope of lentiviral particles which provides a tool to modify these vectors (18,19). Rasbach and colleagues also showed that insertion of a linker between transmembrane domain and the displayed ligand can remarkably enhance the transduction efficiency of modified lentiviral vectors (18).

With the aim of providing an efficient platform for biotinylation of lentiviral vector surfaces, we designed a plasmid, encoding BAP sequence based on pDisplay containing the stem region of the vesicular stomatitis virus (VSV) glycoprotein as a candidate linker to improve the surface accessibility of BAP. As the first step of the project, we initially investigated the expression of the candidate protein by western blot in 293T cells, which is the mostly used cell line for the production of lentiviral vectors (20).

MATERIALS AND METHODS

Construction of the recombinant plasmid

The pDisplay vector was purchased from Life Technologies (USA). The DNA Sequence, comprising of flexible linkers (21), 15-aminoacid BAP (22,23), and membrane-proximal stem region of the VSV glycoprotein ectodomain (24) (NCBI Nucleotide database accession number: NC_001560.1) was synthesized in tandem by Biomatik (Canada) (Fig. 1A).

The synthesized DNA sequence was employed as the template in polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Fermentas, Lithuania) for insertion of *Bgl*II and *Sal*I restriction sites at the head and tail of the insert respectively (Fig. 1B).

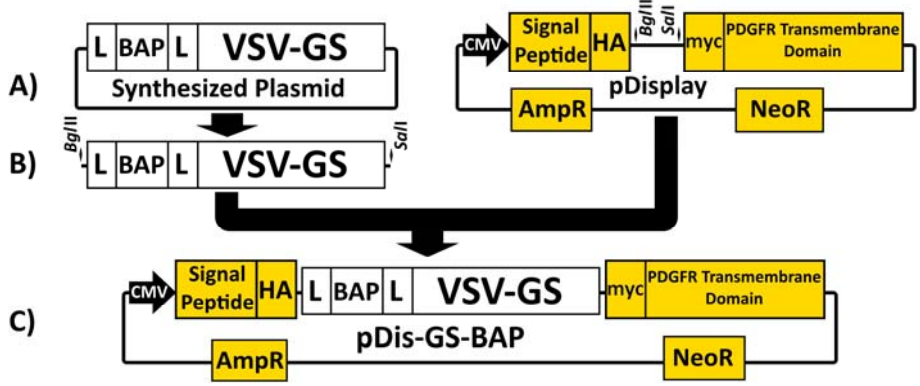

Fig. 1. Schematic presentation of cloning steps for construction of pDis-GS-BAP plasmid. A; plasmid purchased from Biomatik, harboring the synthetic fused gene comprising the flexible linkers, 15-aminoacid BAP and membrane-proximal stem region of the vesicular stomatitis virus (VSV) glycoprotein protein ectodomain. B; PCR amplicon with *Bgl*II and *Sal*I restriction sites at the head and tail of the insert respectively. C; The constructed pDis-GS-BAP. L: flexible linker, BAP: biotin acceptor peptide, VSV-GS: membrane-proximal stem region of the vesicular stomatitis virus G protein ectodomain, HA: HA-tag, myc: *myc* epitope, CMV; Cytomegalovirus promoter, AmpR; Ampicillin resistance gene, NeoR; Neomycin resistance gene.

PCR was performed using forward primer: 5′-ACT**AGATCT**GGCGGCGGCGGATCCGGC CT (containing *Bgl*II restriction site; bold sequence) and reverse primer: 5′-CTT**GTCGAC**TTTCCAACTACTGAACCA ACCTTCTAC (harboring *Sal*I restriction site; bold sequence). The initial denaturation step was 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C (denaturing), 30 s at 60 °C (annealing), and 1 min at 72 °C (extension), and a final extension at 72 °C for 5 min. Then the PCR product was evaluated by agarose gel electrophoresis.

For cloning of the amplicon, both pDisplay and GS-BAP DNA fragments were digested by *Bgl*II and *Sal*I (Takara, Japan) and the corresponding bands were gel purified by GeneJET™ Gel Extraction Kit (Fermentas, Lithuania). Subsequently, the purified insert fragment (GS-BAP) was cloned into the *Bgl*II-*Sal*I restriction sites of pDisplay, downstream of HA-tag and in frame with *myc* epitope by T4 DNA ligase to make the final pDis-GS-BAP plasmid (Fig. 1C). All the cloning steps were performed according to the standard protocols in *Escherichia coli* DH5α (25). Primary confirmation of the recombinant plasmid was performed by restriction digestion analysis using FastDigest *Sal*I and FastDigest *Hind*III (Thermo scientific, Lithuania) and the selected clone was sequenced at the sequencing facility of Pasteur Institute of Iran using T7-promoter and BGH-Reverse universal primers.

Cell line, the transfection procedure and fluorescence microscopy

293T cells (National Cell Bank of Iran) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS (PAA, Austria), 2 mM stable glutamine (PAA, Austria), and penicillin/streptomycin (PAA, Austria) in humidified incubator at 37 °C and 5% CO_2. One night prior to transfection, 293T cells were seeded in 6-well plates (400000 cells/well). The next day, transfection was performed by TurboFect transfection reagent (Thermoscientific, Lithuania) according to the manufacturer's protocol. In brief, 6 µl of TurboFect were added to 400 µl DMEM containing 4 µg of pDis-GS-BAP. After 15 min of incubation at room temperature, the mixture was added drop-wise to the wells. Forty eight hours later cells were harvested for analysis of expression. As control for tracking the transfection efficiencies, the 293T cells were also transfected with 4 µg of a green fluorescent protein (GFP)-expressing vector, pEGFP-N1 (Clontech Laboratories, Inc. USA) by the same procedure in separate experiments and wells. 24 h post-transfection, expression of GFP was evaluated by inverted fluorescence microscope (INVERSO TC100 Epi Fluor, Medline Scientific, UK).

Western blotting

Western blotting was performed according to standard procedures and protocols (25,26). In brief, for each sample, approximately 1 × 10^6 cells were harvested and washed twice by phosphate buffer saline (PBS). Cell pellets were resuspended in PBS followed by addition of equal amount of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and incubated in boiling water for 10 min and were loaded into a 12% SDS polyacrylamide gel. At the end of electrophoresis, protein bands were transferred to a polyvinylidene difluoride membrane (Roche, Germany) in wet condition and blocked by 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). Blots were incubated with anti-HA tag mouse monoclonal antibody (Cell Signaling, USA) as primary antibody, and after several washing steps with anti-mouse IgG HRP-linked antibody (Cell Signaling, USA) in TBST-skim milk 2% (1:1000 and 1:2000 respectively). Expression of the recombinant protein (disGS-BAP) was detected via addition of Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences, UK) followed by visualization with the Kodak Image Station (Kodak, Rochester, NY).

RESULTS

Construction of pDis-GS-BAP

Agarose gel electrophoresis of PCR-amplified GS-BAP showed the proper DNA band size of 222 bp (Fig. 2A). The result of double digestion of pDisplay vector with *Bgl*II and *Sal*I enzymes indicated a DNA band with

the size of 5303 kb corresponding to the linearized vector body (Fig. 2B). As illustrated in Fig. 1, the purified 222 bp DNA band of GS-BAP and the 5303 bp vector corresponding to the linearized form of pDisplay were used for constructing pDis-GS-BAP. Restriction digestion analysis of pDis-GS-BAP by FastDigest *Sal*I and FastDigest *Hind*III was performed and agarose gel electrophoresis showed the insertion of GS-BAP fragment into the pDisplay vector (Fig. 2C). Finally, sequencing results of the selected clone confirmed the DNA sequence and proper reading frame of pDis-GS-BAP plasmid.

Transfection and Western blot analysis of disGS-BAP expression

Microscopic Evaluation of transfection control group indicated that approximately 70%-80% of the 293T cells expressed GFP (Fig. 3). Western blot analysis of 293T cells transiently expressing disGS-BAP protein indicated a single band around 16-17 kDa corresponding to the expected size of the BAP-encoding protein, as detected by the antibody against the N-terminal HA tag. No protein band was observed in the untransfected (control) 293T cells (Fig. 4).

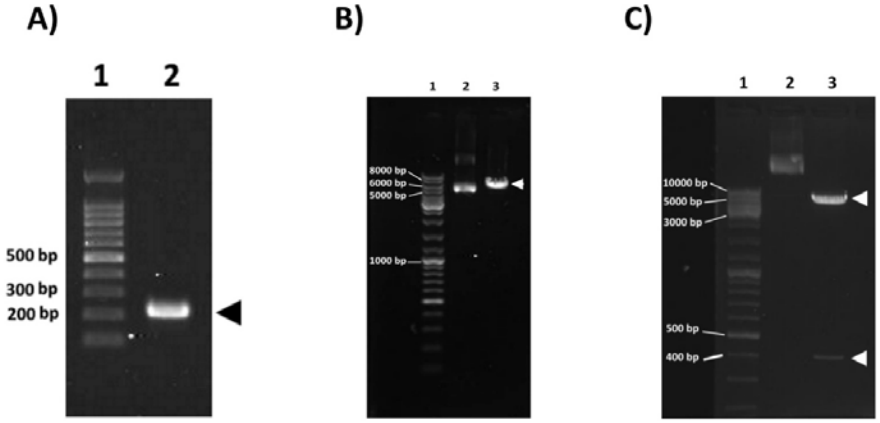

Fig. 2. Agarose gel electrophoresis of A; PCR product of GS-BAP fragment amplification; Lane 1: DNA ladder (YTA 100 bp DNA ladder, Yekta Tajhiz Azma, Iran), Lane 2: GS-BAP fragment (220 bp). B; Double digestion of pDisplay by *Bgl*II and *Sal*I; Lane 1: (GeneRuler DNA Ladder Mix, Fermentas, Lithuania), Lane2: undigested pDisplay, Lane 3: digested and linearized pDisplay (5303 bp). C; Primary confirmation of pDis-GS-BAP by restriction enzyme digestion using *Sal*I and *Hind*III; Lane 1: (GeneRuler DNA Ladder Mix, Fermentas, Lithuania), Lane 2: undigested pDis-GS-BAP, Lane 3: digested pDis-GS-BAP which resulted in two DNA fragments (5125 bp and 398 bp)

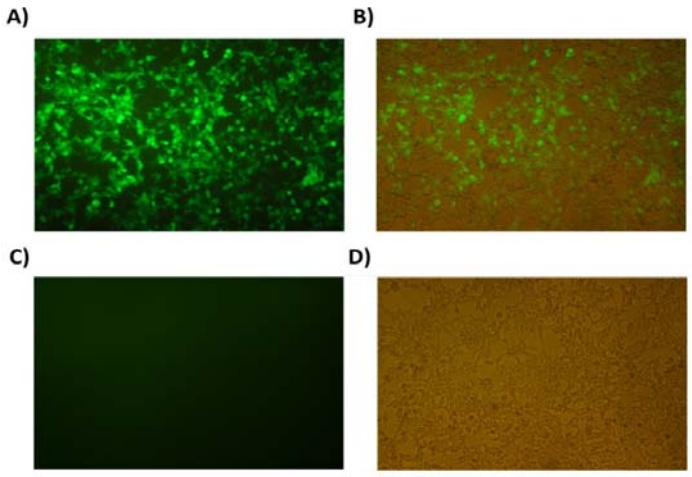

Fig. 3. Evaluation of transfection efficiency by visualization of GFP expression in 293T cells using inverted fluorescence microscope. A; Observation of GFP expressing cells under the dark field. B; The same cells under the bright field along with UV exposure. C; Un-transfected cells exposed to UV light and under the dark field. D; The same un-transfected cells exposed to UV light and under the bright field.

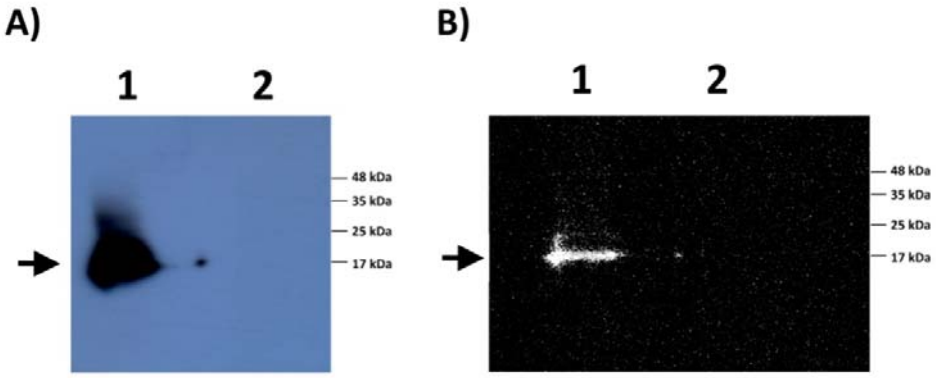

Fig. 4. Western blot analysis of dis-GS-BAP protein expression in 293T cells visualized by A; X-ray film photography and B; Kodak image station. Lane 1: transfected cells, Lane 2: non-transfected cells. The location of 16-17 kD band is shown by arrows.

DISCUSSION

Heterologous incorporation of membrane proteins on the surface of lentiviral vectors happens via active or passive mechanisms. In active mechanism, the presence of interaction between cytoplasmic tails of cellular proteins and viral core determine the incorporation. The availability on the cell surface especially at the virus budding sites, and absence of incompatibility between cytoplasmic tail and viral assembly are enough for passive incorporation of a protein on lentiviral vectors (10).

Among the available tools for passive incorporation of membrane proteins into lentiviral envelope, pDisplay plasmid seems to be a preferred tool. This is due to its capability of cell surface expression of the desired protein (via a murine Ig κ-chain signal peptide and the transmembrane domain of platelet derived growth factor receptor (PDGFR)) (27) and the presence of a short (8-residues) truncated segment of PDGFR cytoplasmic tail which reduces the likelihood of incompatibly and hence increases their chance of packaging on the lentiviral virions. By using pDisplay, Goyvaerts and coworkers, managed to develop a nanobody display technology system for lentiviral vectors which enabled them to specifically transduce dendritic cells and macrophages (19).

Lentiviral vector metabolic biotinylation starts with tagging the desired proteins with a BAP. Two types of biotin acceptor domains are currently available (14) including; *i*)a natural biotinylation substrates (like 1.3S subunit of *Propionibacterium shermanii* transcarboxylase domain (PSTCD)) (28,29) and *ii*) a 15-residue BAP also known as AviTag which has been isolated by library screening (22,30). In agreement with previous studies, due to its higher rate and efficiency of biotinylation, the AviTag was employed in the current study (31,32).

In a previous study, to biotinylate the cell surface, Niers and colleagues directly cloned AviTag into pDisplay (17). On the other hand, Rasbach and coworkers, demonstrated that the distance between cell membrane and the displayed ligand could play an important role in accessibility of the displayed ligand. Accordingly, they employed and inserted a spacer peptide (linker) between cell membrane and the displayed ligand (18). In this context and to address the same strategy, we hypothesized that a naturally occurring extracellular domain of a membrane glycoprotein would be a good candidate linker. Therefore, in the present study, we inserted the 42 amino acid of the juxtamembrane region of VSV glycoprotein ectodomain between AviTag and transmembrane domain. In addition, by flanking the AviTag with two sets of flexible $(Gly)_4Ser$ linker (33), we reduced possible hindrances, and further ensured the accessibility of BAP to the biotin ligase (34). In summary, in the current study a sequence

bearing AviTag and truncated form of VSV glycoprotein membrane-proximal ectodomain was cloned into pDisplay and their presence were confirmed by restriction enzyme digestion and DNA sequencing. 293T cells (a common cell line for the production of lentiviral vector) were transfected with the resulting plasmid (pDis-GS-BAP) and GFP expression in the control group confirmed the transfection efficiency. Western blot analysis of the cells transfected with the pDis-GS-BAP showed a single band of approximately 16-17 kDa which was in accordance with the predicted size of the disGS-BAP protein based on the total size of its components (16.3 kDa).

CONCLUSION

Results of this study showed the successful expression of pDis-GS-BAP in 293T cells as the first step in production of a surface-engineered lentiviral vector. However, since for the final aim of this study, disGS-BAP protein must be delivered to the cell membrane for incorporation on viral envelope, therefore, further studies are required to demonstrate the membrane transfer of the disGS-BAP. Finally the ability of BirA enzyme to recognize BAP and biotinylated dis-GS-BAP on the cell surface and the effect of included spacer domain on the accessibility of BAP must also be investigated in further studies.

ACKNOWLEDGEMENTS

The present study was supported by Pasteur Institute of Iran in fulfillment of the Ph.D. thesis of Dr. H. Etemadzadeh.

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