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Delivery of HIV-1 Nef Protein in Mammalian Cells Using Cell Penetrating Peptides as a Candidate Therapeutic Vaccine

Behnaz Sadat Jafarzade[1] · Azam Bolhassani[2] · Seyed Mehdi Sadat[2] · Ramin Yaghobi[3]

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Abstract The HIV-1 Nef protein expressed early in viral life cycle has been known as a potent candidate for therapeutic vaccine development. Due to different cell barriers, various cell penetrating peptides (CPPs) such as Pep-1 and CADY-2 have been known to deliver biologically active proteins to cytoplasmic compartments via the plasma membrane. In current study, we firstly evaluated the efficiency of lentiviral vector (pCDH-CMV-MCS-EF1-cGFP-T2A-puro) and eukaryotic expression vector (pEGFP-N1) for expression of HIV-1 Nef protein in HEK-293T cells using TurboFect transfection reagent. Our results showed that both vectors can effectively express the Nef proteins within the target cell. The pEGFP-N1 was more effective than pCDH-GFP for protein expression. Furthermore, Nef protein was expressed in *E. coli* as GST-Nef fusion and transfected by the amphipathic CPPs including Pep-1 and CADY-2 into HEK-293T cells. The size and morphology of the GST-Nef/CPP complexes were evaluated by scanning electron microscopy, and Zetasizer. Our data indicated that the recombinant GST-Nef protein generated in *BL21* strain migrated as a clear band of ~50 kDa in SDS-PAGE. The CPP/GST-Nef nanoparticles were formed with a diameter of below 200 nm and notably delivered into HEK-293T cells. Generally, the Nef protein was expressed in prokaryotic and eukaryotic expression systems using different vectors and efficiently transfected in mammalian cells using various delivery systems. The in vitro efficient delivery of HIV-1 Nef gene and also its protein supports the potential of Nef DNA constructs and CPPs as potent carriers of Nef protein for HIV vaccine design in Future.

Keywords HIV-1 · Nef · Cell penetrating peptide · Transfection · In vitro gene expression · Eukaryotic and prokaryotic expression systems

Abbreviations

AIDS	Acquired immunodeficiency syndrome
CPP	Cell penetrating peptide
HIV	Human immunodeficiency virus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide electrophoresis
SEM	Scanning electron microscopy

Introduction

The Nef protein of the human immunodeficiency virus (HIV) is a 25–30 kDa cytoplasmic phosphoprotein expressed at all stages of HIV-1 infections (Joseph et al. 2003). Its function in the progress of acquired immunodeficiency syndrome (AIDS) can be ascribed to three properties of Nef protein including: (a) enhancement of viral infectivity, (b) down-modulation of cell surface proteins such as CD4 and MHC I, and (c) induction of T cell activation pathways (Joseph et al. 2003). Several DNA constructs harboring HIV structural, regulatory or accessory genes as well as the recombinant proteins have been evaluated for development of preventive and therapeutic vaccines against HIV infections. Among HIV-1 accessory proteins, the Nef protein expressed early in viral life cycle

✉ Azam Bolhassani
azam.bolhassani@yahoo.com; a_bolhasani@pasteur.ac.ir

[1] Department of Microbiology, Fars Branch, Islamic Azad University, Shiraz, Fars, Iran

[2] Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

[3] Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

has been known as a potent candidate for development of therapeutic vaccine (Tahtinen et al. 2001). The studies indicated that the naked DNA enters cells rather poorly. Thus, a therapeutic gene is often delivered with a viral (e.g., *adenoviral*, *lentiviral*) or non-viral (e.g., electroporation, cationic polymers, cationic lipid) vectors (Eliyahu et al. 2005; Huang et al. 2015). Indeed, it is necessary for determination of suitable DNA delivery systems into normal cells or stem cells which are difficult to transfect (Huang et al. 2015). In order to find an efficient DNA construct based on the early proteins, we recently developed two DNA constructs containing HIV-1 Nef gene (i.e., pEGFP-Nef, pCDH-Nef-GFP) and compared their expression in mammalian cell line. Regarding to the reported data, the HEK cell line has been greatly used as an expression tool for recombinant proteins. Its biochemical machinery is capable of carrying out the post-translational folding and processing for generating functional protein from a wide range of mammalian and non-mammalian nucleic acids. The ability to generate transient HEK cell phenotypes by the introduction of exogenous nucleic acid depends on a range of transfection methods. Indeed, the transfection efficiency is particularly sensitive to the quality of the transfection reagents (Thomas and Smart 2005). An important variant of this cell line is the HEK-293T that contains the SV40 Large T-antigen, allows for episomal replication of transfected plasmids and expression of the desired gene products (Eliyahu et al. 2005; Thomas and Smart 2005). Herein, the expression of Nef protein in HEK-293T cells was analyzed by fluorescent microscopy, flow cytometry and western blot analysis. On the other hand, one approach to enhance the efficiency of the immune responses against HIV infection is the use of multiple highly conserved and immunogenic epitopes for vaccine design (Bolesta et al. 2005). For example, HIV-1 P24 and Nef proteins could induce immune responses due to their immunogenic and conserved epitopes (Zuniga et al. 2006; Addo et al. 2003). In this line, different transfection methods were used to deliver proteins and peptides. Among these approaches, cell-penetrating peptides (CPPs) comprise a family of peptides whose unique characteristic is their ability to insert into and cross biological membranes (Munoz-Morris et al. 2007). For instance, Pep-1 could deliver several proteins and peptides as non-covalent complexes into different cell lines in a fully biologically active form (Morris et al. 2001). In addition, a new cell-penetrating peptide known as CADY2 showed a high efficacy similar to Pep1 in delivering proteins (e.g., mRFP and GSTCdk2) into living cells. However, major differences were observed in their potency for different cargoes, dependent on physicochemical properties of cargoes (e.g., the nature of the cargo or high proportion of both positively and negatively charged residues within the cargo) and the carrier/cargo ratio (Kurzawa et al. 2010). The studies showed that these protein delivery systems represent several advantages such as rapid delivery of proteins into cells, stability in physiological buffers, and lack of sensitivity to serum and low cytotoxicity (Morris et al. 2001; Kurzawa et al. 2010; Gallo et al. 2002; Wu et al. 2002; Pratt and Kinch 2002; Aoshiba et al. 2003). In this study, HIV-1 Nef protein was generated in bacterial system. Then, the efficiency of the cell-penetrating peptides (Pep-1 and CADY-2) was evaluated to deliver Nef protein into the mammalian cells as compared to a commercial protein transfection reagent (TurboFect).

Materials and Methods

Generation of Expression Vectors Harboring HIV-1 Nef

The pEGFP-N1 (Clontech, USA) and pCDH-GFP (pCDH-CMV-MCS-EF1-cGFP-T2A-puro, Invitrogen, USA) vectors were used for expression of HIV-1 Nef protein in mammalian cell line. In addition, the pEGFP-Nef expression vector was constructed by sub-cloning the complete *nef* open reading frame (pNL4-3, ~648 bp) from the synthetic pUC-Tat-Nef vector (Biomatik Co., Canada) as N-terminal fusion to green fluorescent protein (GFP) into the *Nhe*I/*Pst*I sites of pEGFP-N1 vector. For generation of pCDH-Nef-GFP, the Nef-GFP gene was sub-cloned from pEGFP-Nef (without stop codon) into the *Nhe*I/*Not*I cloning sites of pCDH expression vector. The DNA constructs containing Nef gene [pEGFP-Nef, and pCDH-Nef-GFP] were purified in large-scale using Midi-kit (Qiagen). DNA concentrations were determined by the absorbance measured at 260 nm. The presence of the inserted Nef fragment was confirmed by PCR and restriction enzyme digestion as detected on gel electrophoresis and also nucleotide sequencing. A schematic representation of the constructed Nef-GFP gene in two vectors was shown in Fig. 1.

Transfection of pEGFP-Nef and pCDH-Nef into the Mammalian Cells and Quantitation

Transient transfection in HEK-293T cells with two Nef expression vectors (pEGFP-Nef, and pCDH-Nef-GFP) and also pEGFP-N1 and pCDH-GFP as the positive controls was performed using TurboFect transfection reagent (Fermentas). Human HEK-293T cells were maintained in complete RPMI (Sigma) medium supplemented with 10 % fetal calf serum (FCS, Gibco) at 37 °C and 5 % CO_2 atmosphere. For transfection with TurboFect cationic polymer, 2 μg of each plasmid was pre-incubated with 4 μl of reagent in a final volume of 25 μl and incubated at room

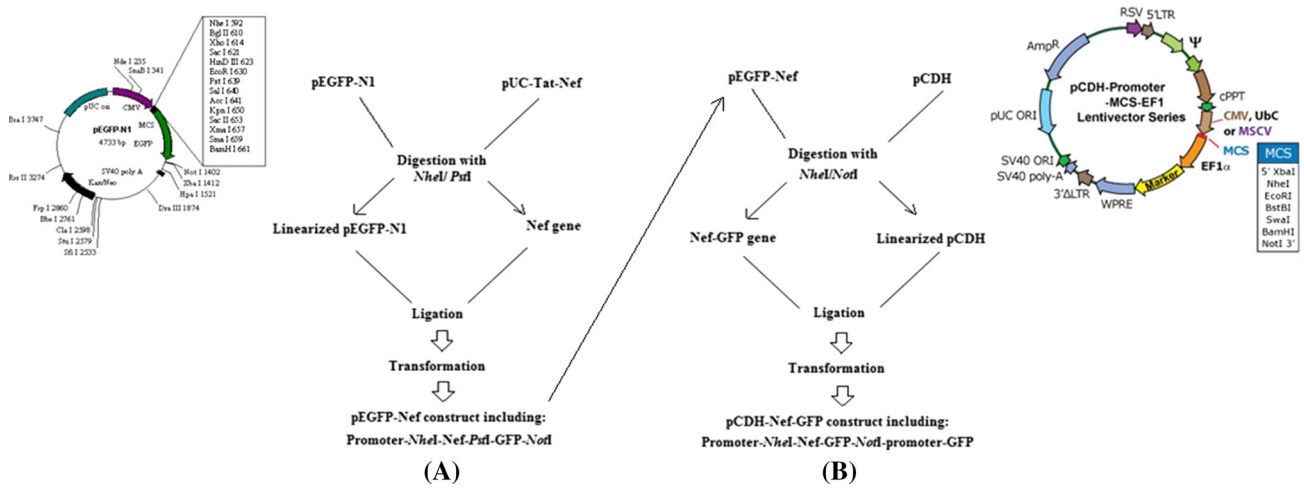

Fig. 1 A schematic representation of the constructed Nef-GFP gene in pEGFP-N1 (**a**) and pCDH (**b**) vectors

temperature for 20 min to allow the DNA/TurboFect complexes to form. The complexes were then added to 2×10^5 HEK-293T cells in each well for 12-well plate. The cells were harvested 48 h post-transfection, washed, and resuspended in PBS, to determine the proportion of fluorescent cells expressing Nef-GFP using flow cytometry analysis (Partec GmbH). The quality of protein expression was also detected by fluorescent microscopy and western blotting. HEK-293T cells were used as a negative control.

Detection of Nef Protein Expression in HEK-293T Using Western Blot Analysis

HEK-293T cells were washed with PBS and lysed in whole-cell lysis buffer (10 % glycerol, 0.5 mM EDTA, 1 mM DTT, 2 mM natrium fluoride, 0.2 % Triton X-100 in PBS; pH 7.4) supplemented with protease inhibitor (Sigma). Proteins were separated on 12.5 % (w/v) polyacrylamide gel and transferred to nitrocellulose membrane (Millipore). The peroxidase conjugated-anti-GFP antibody (Acris, USA, 1:5000 v/v) was used to confirm Nef-GFP protein expression under standard procedures. The immunoreactive protein bands were visualized using peroxidase substrate named 3,3′-diaminobenzidine (DAB, μSigma).

Statistical Analysis

Statistical analysis (Student's *t*-test) was performed by Prism 5.0 software (GraphPad, San Diego, California, USA) to analyze the percentage of Nef-GFP, and GFP expression using flow cytometry analysis. The value of $p < 0.05$ was considered statistically significant. Similar results were obtained in two independent experiments.

Peptides

The cell penetrating peptides including Pep-1 (Ac-KETWWETWWTEWSQPKKKRKV-Cya) and Cady-2 (Ac-GLWWRLWWRLRSWFRLWFRA-Cya) were purchased from Biomatik Corporation (Cambridge, Canada). Pep-1 peptide was composed from three components including a hydrophobic N-terminal domain (a tryptophan rich motif) for interaction with proteins and efficient targeting to the cell membrane, a hydrophilic C-terminal domain (KKKRKV) for solubility and intracellular delivery, and a short linker (SQP) for improvement of the integrity of both domains (Kurzawa et al. 2010). Several modifications of Pep-1 sequences at the N-/C-terminal regions such as addition of acetyl (Ac) and cysteamide (Cya) groups have been also proposed to stabilize the cargo–carrier complexes and its transduction mechanism. CADY2 (Ac-GLWWRLWWRLRSWFRLWFRA-Cya) is also a highly hydrophobic and positively charged peptide with the modified terminals (Kurzawa et al. 2010).

Production of the Recombinant HIV-1 Nef Protein in Prokaryotic Expression System

For the generation of Nef-expressing plasmid, Nef gene was amplified by polymerase chain reaction (PCR) using the designed primers as following:

Forward Primer: AT CGA ATT CGA CAT ATG GGT GGC AAG TGG TC (*Eco*RI)

Reverse Primer: TGG ACT AGC GGC CGC TTA TCA GAA TTC CTG C (*Not*I)

The following program was used for amplification of HIV-1 Nef gene: hot start at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The

program followed by a final extension at 72 °C for 10 min. The PCR product was digested with *Eco*RI/*Not*I and ligated to the linearized pGEX6p2. After transformation of *E. coli* DH5α with the ligated solution, the positive colonies harboring pGEX-Nef were grown in LB broth medium, supplemented with ampicillin. Then, the extraction of plasmids was performed using mini-kit DNA extraction (Qiagen). The presence of the inserted Nef fragment was confirmed by restriction enzyme digestion and PCR as detected on gel electrophoresis and also the fidelity of the construct was confirmed by DNA sequencing. After confirming the recombinant pGEX-Nef, the *E. coli BL21* was transformed with the plasmid DNA and a single colony was grown in 5 ml LB medium containing ampicillin at 37 °C with constant agitation in shaking incubator. The next day, 500 µl of bacterial culture was inoculated in 50 ml Ty2x medium. The culture was grown in an OD_{600} of 0.7–0.8 with vigorous shaking (~200 rpm) at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for Nef expression. The incubation period continued for another 4 h at 37 °C with shaking at 200 rpm. Protein samples were analyzed by SDS-PAGE in 12.5 % (W/V) polyacrylamide gel followed by staining with coomassie brilliant blue. An imidazole-SDS-Zn reverse staining method was used for the protein purification as described by Richard J. Simpson (Simpson 2007). The purified protein was dialyzed with PBS (pH 7.2) at 4 °C overnight. The quality and quantity of the purified fusion protein with a GST tag at N-terminus (GST-Nef) was determined by a 12 % SDS-PAGE gel electrophoresis, and NanoDrop spectrophotometry, respectively. The primary structure of HIV-1 Nef protein (Accession No.: AF324493_9) is as following:

MGGKWSKSSVIGWPAVRERMRRAEPAADGVGA VSRDLEKHGAITSSNTAANNAACAWLEAQEEEEVG FPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHS QRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTF GWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMD DPEREVLEWRFDSRLAFHHVARELHPEYFKNC.

Preparation of CPP-Protein Complexes and Their Physicochemical Characterization

The stock solution of the synthetic PEP-1 and Cady-2 peptides were prepared in sterile water at a final concentration of 2 mg/ml. Pep-1/Nef-GST and CADY-2/Nef-GST complexes with a molar ratio of 20:1 (1 Âµg of Nef protein) were formed in 50 µl phosphate-buffer saline (PBS) and incubated for 30 min at room temperature to allow complete electrostatic interaction between CPPs and protein, ensuring the formation of complex. TurboFect was used as a protein transfection reagent. TurboFect/Nef complexes were prepared according to manufacturer's instruction (Pro-Ject™ Reagent, Fermentas). In addition, 0.5 µg of FITC-antibody control protein diluted in 25 µl of PBS was delivered by TurboFect, Pep-1 and CADY-2. The size and morphology of the nanoparticles (Pep-1/GST-Nef, CADY-2/GST-Nef) were analyzed at a molar ratio of 20:1 using a scanning electron microscope (SEM; KYKY-EM3200 model, China) and also Zetasizer Nano ZS instrument (Malvern Instruments, UK) at room temperature.

Transfection of the Recombinant GST-Nef Protein in the Mammalian Cells

HEK-293T cells (1×10^5 cells/well) were seeded onto 24-well microtiter plates in complete RPMI-1640 medium (Sigma) supplemented with 10 % heat-inactivated fetal calf serum (FCS, Gibco) at 37 °C in an atmosphere containing 5 % CO_2. After growth of the HEK-293T cells to 80 % confluency, the medium was replaced by serum-free medium and then 100 µl of each complex (i.e., Pep-1/GST-Nef, and CADY-2/GST-Nef at a molar ratio of 20:1; TurboFect/GST-Nef; TurboFect/FITC-Ab; Pep-1/FITC-Ab; CADY-2/FITC-Ab) was applied to each well. After 1 h incubation at 37 °C, the cells were supplemented with fresh RPMI, 5 % FCS in a total volume of 200 µl medium without removal of the Pep-1/GST-Nef, and CADY-2/GST-Nef, Pep-1/FITC-Ab, and CADY-2/FITC-Ab overlay, and were further incubated for 4 and 24 h. It should be noted that the cells were overlaid with 200 µl complete medium after 4 h incubation at 37 °C with TurboFect/GST-Nef and TurboFect/FITC-Ab complexes in medium without FCS. Then, the cells were treated with trypsin-EDTA, harvested by centrifugation and resuspended in PBS 1×. The delivery of GST-Nef protein was confirmed by SDS-PAGE and western blotting. In addition, the transfected cells with FITC-Ab were monitored by fluorescence microscopy (Envert Fluorescent Ceti, Korea). For western blot analysis, the cell pellets were scraped from their dishes and washed in PBS 1× at 4 and 24 h after transfection. The extracted protein samples were separated by SDS-PAGE in a 12.5 % (w/v) polyacrylamide gel. The proteins were resolved on gel and transferred onto protran nitrocellulose transfer membrane (Schleicher and Schuell Bioscience, Dassel, Germany). The membrane was pre-equilibrated with Tris-buffered saline Tween-20 (TBST) solution containing 2.5 % bovine serum albumin (BSA) for overnight and reacted with anti-Nef monoclonal antibody (1:10,000 v/v, USBiological) under standard procedures for 2 h at 25 °C. After three washes with TBS, the membrane was incubated with anti-mouse IgG-HRP (1:5000, Sigma, USA) for 1.5 h at room temperature. The immunoreactive protein bands were visualized using peroxidase substrate 3,3′-diaminobenzidine (DAB, Sigma, St. Louis, MO).

Results and Discussion

Expression of HIV-1 Nef Protein in Transfected Target Cells

In order to construct the expression vectors, the Nef gene was digested and inserted into the pEGFP-N1 and pCDH-GFP vectors. PCR analysis and digestion indicated the correct cloning of the Nef gene into each expression vector. The Nef gene migrated as ~648 bp in agarose gel. Generally, both pEGFP-Nef and pCDH-Nef vectors were correctly constructed as shown in Fig. 2. For in vitro delivery of the Nef DNA construct (pEGFP-N1, pEGFP-Nef, and pCDH-Nef-GFP), TurboFect cationic polymer was used as the transfection reagent. Nef-GFP expression was evaluated by fluorescence microscopy and flow cytometry at 48 h after transfecting HEK-293T cells. Our data showed that the levels of protein expression were 82, 55, 52, and 78 % for pEGFP-N1, pEGFP-Nef, pCDH-GFP, and pCDH-Nef-GFP, respectively (data not shown). The transfection efficiency using TurboFect delivery system showed that the pEGFP-N1 was more potent than pCDH-GFP vector for Nef protein expression. Because the pCDH-GFP vector has two promoters which one of them controls GFP expression and another controls the Nef-GFP fusion expression. Thus, the expression of Nef-GFP protein without GFP expressed from vector will be 26 %, respectively. In order to confirm the expression of Nef protein in HEK-293T cells, western blot analysis was performed after harvest the cells transfected with two expression vectors. The extracts of HEK-293T cells transfected with pEGFP-Nef and pCDH-Nef-GFP indicated the presence of Nef fluorescent protein, when incubated with anti-GFP antibody, whereas the extracts of untransfected HEK-293T cells and transfected with expression vectors alone (pEGFP-N1 or pCDH-GFP) did not show any band in these regions upon incubation with the same antibody. The specific bands with expected size of ~50 and 27 kDa were detected for Nef-GFP and GFP expressed from pEGFP-Nef, and pEGFP-N1 vectors in the transfected cells as shown in Fig. 3. Our data indicated that two expression vectors effectively express the Nef protein within the target cells.

Generally, we utilized a transient transfection assay for the HIV-1 *nef* gene in the HEK-293T cell line using TurboFect cationic polymer. Our results indicated that both expression vectors (pCDH-GFP and pEGFP-N1) capable of expressing the Nef protein in the target cell. In addition, we observed lower level of Nef protein expression relative to expression of the GFP reporter protein, when similar plasmid DNA concentrations were used. This result is similar to the previous reported data about the co-transfected *nef* and *gfp* plasmid constructs into the monkey kidney cell line (COS-1) for analysis of Nef protein expression (Hartz et al. 2003). Moreover, the Nef expression could be increased by incubation with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (Kienzle

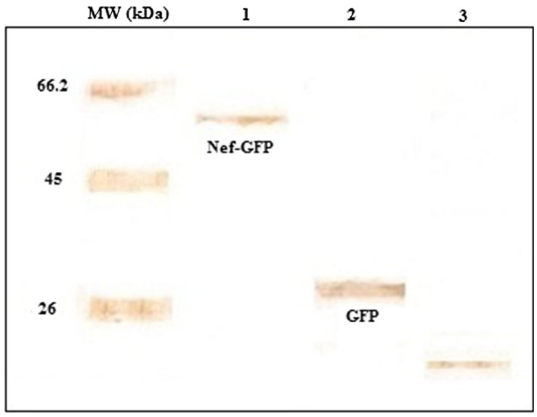

Fig. 3 Western blot analysis using an anti-GFP antibody: The results showed the expression of the full-length Nef-GFP (~50 kDa) and GFP (~27 kDa) proteins for the cells transfected with pEGFP-Nef (*lane 1*) and pEGFP-N1 (*lane 2*), respectively. Any detectable band was not observed in untransfected cells (*lane 3*) as a negative control

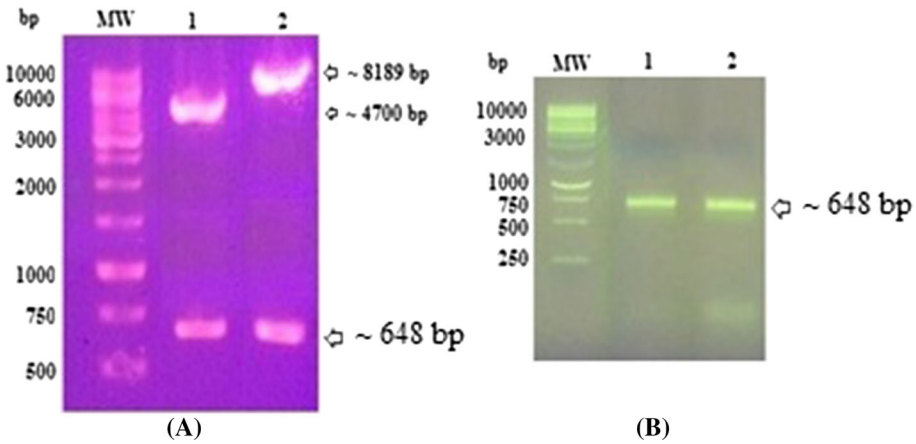

Fig. 2 Cloning of HIV-1 Nef into the multiple cloning sites (MCS) of pCDH-GFP and pEGFP vectors **a** Confirmation of Nef gene (~648 bp) by digestion from pEGFP-N1 vector (*lane 1*), and pCDH vector (*lane 2*) **b** PCR analysis of Nef from pCDH-GFP (*lane 1*) or pEGFP-N1 (*lane 2*) vector. *MW* is molecular weight marker (Fermentas)

et al. 1992). According to our results, the pEGFP-N1 and pCDH-GFP plasmids are potent expression vectors as only 2 μg of each plasmid transfected into cells produced the efficient levels of Nef protein. On the other hand, pEGFP-N1 showed high expression of Nef protein compared to pCDH-GFP expression vector with two individual promoters in HEK-293T cells. Some studies indicated that the lentiviral vectors are potentially useful tools for gene delivery (Kim et al. 2012). Our study indicated that their transfection efficiency without packaging was lower than a typical expression vector such as pEGFP-N1 likely due to the large size of the lentiviral vectors. Thus, these vectors need to package for expression of genes with high efficiency. According to the reports, for producton of viral particles, expression and packaging vectors are transiently co-transfected into producer mammalian cells (e.g., HEK 293 cells) (Machida 2003; Federico 2003; Heiser 2004). In addition, TurboFect as a cationic polymer could efficiently deliver the plasmid DNAs in the cells. TurboFect reagent demonstrates superior transfection efficiency and minimal toxicity when compared to lipid based or other polymer-based transfection reagents (Burkhart et al. 2015; AbdEllah et al. 2014; Abdul Halim et al. 2014).

Expression and Purification of the Recombinant Nef Protein

The Nef gene was cloned into the *Eco*RI/*Not*I sites of the expression pGEX6p2 vector, which enables the expression of a fusion protein with a GST tag at N-terminus. The results of PCR and digestion showed a clear band of ~648 bp for Nef in agarose gel (Fig. 4). The recombinant Nef protein expressed in *BL21* strain migrated as a clear band of ~50 kDa in SDS-PAGE that was identified using anti-Nef antibody in western blotting. The purification of Nef protein was done using reverse staining method and visualized by SDS-PAGE (Fig. 5). Finally, the concentration of the purified GST-Nef protein was determined by NanoDrop spectrophotometry (~0.6–0.8 mg/ml).

Identification of Nef-Based Nanoparticles Delivery by Western Blotting

The formation of Pep-1/GST-Nef or CADY-2/GST-Nef complexes in a molar ratio of 20:1 was confirmed by SEM as the spherical shape with an average size of 120–200 nm (Fig. 6). These data were compatible with the particle

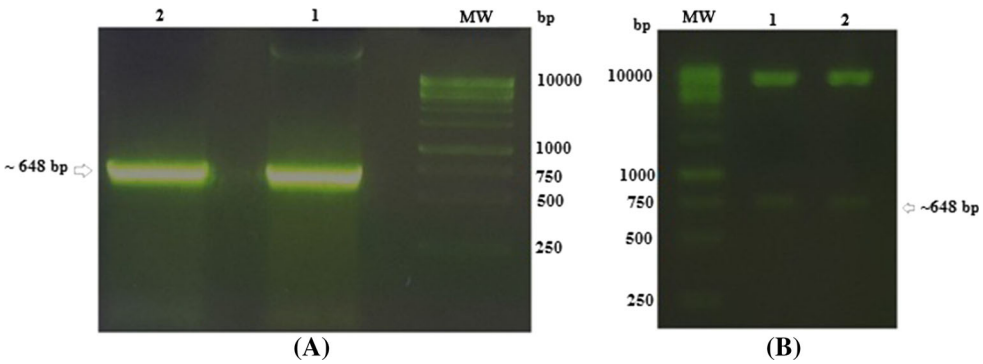

Fig. 4 Confirmation of Nef gene cloned in pGEX6p2 vector using PCR analysis (**a**) and digestion with *Eco*RI/*Not*I (**b**). *Lanes 1 and 2* show two confirmed clones. *MW* is molecular weight marker (Fermentas)

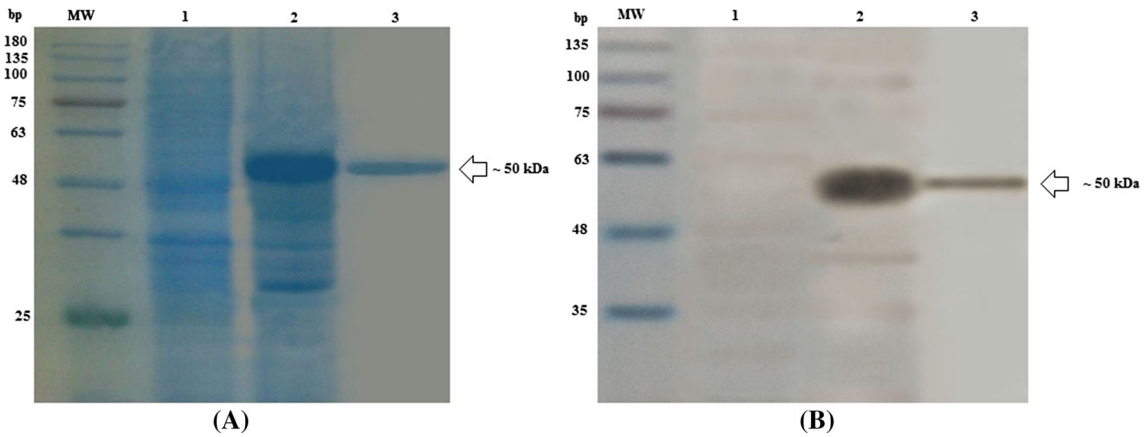

Fig. 5 SDS-PAGE (**a**) and Western blot analysis (**b**) of the purified HIV-1 GST-Nef protein using reverse staining method *lane 1* before induction, *lane 2* after induction, *lane 3* the purified GST-Nef protein. *MW* is molecular weight marker (Prestained protein ladder, 10–170 kDa, Fermentas)

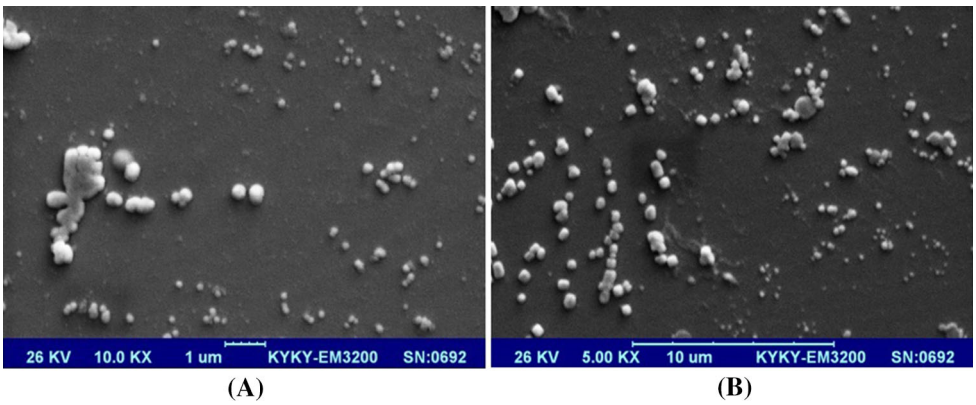

Fig. 6 The SEM micrograph of the spherical nanoparticles formed at molar ratio of 20:1. **a** CADY-2/GST-Nef complex. **b** Pep-1/GST-Nef complex

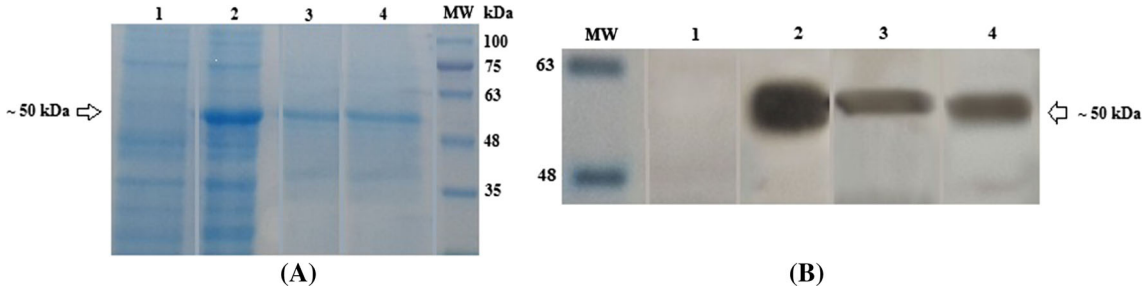

Fig. 7 Transfection efficiency of GST-Nef using Pep-1, and CADY-2 at a molar ratio of 20:1 in the HEK-293T cells after 4 h by SDS-PAGE (**a**) and western blot analysis (**b**) *lane 1* before induction, *lane 2* after induction, *lane 3* delivery of GST-Nef by Pep-1, *lane 4* delivery of GST-Nef by CADY-2. *MW* is molecular weight marker (Prestained protein ladder, 10–170 kDa, Fermentas)

hydrodynamic diameter and zeta potential analysis of both complexes. SDS-PAGE also showed a dominant band of ~50 kDa related to GST-Nef protein along with Pep-1 or CADY-2 peptide band. The efficiency of GST-Nef delivery in HEK-293T cell line was investigated by CADY-2, Pep-1 and TurboFect as compared to GST-Nef delivery alone for 4 and 24 h after transfection. The extracts of HEK-293T cells transfected with the recombinant GST-Nef protein complexed with CPPs indicated the presence of GST-Nef (~50 kDa) in western blotting after incubation with anti-Nef antibody, whereas the extracts of untransfected HEK 293T cells or the transfected cells with the recombinant GST-Nef protein alone did not show any band in these regions upon incubation with the same antibody as shown in Fig. 7. The results indicated a similar band of GST-Nef at both times of post-transfection by Cady-2, Pep-1 and TurboFect delivery systems. It has been established that cell-penetrating peptides (CPPs) are short cationic/amphipathic peptides which facilitate cellular uptake of various molecular cargoes and therefore have great potentials in vaccine design (Ma et al. 2014; Hassane et al. 2010). Pep-1 and also CADY-2 peptides have been documented to interact with and deliver a variety of peptides and proteins into cells (Gros et al. 2006; Crombez et al. 2009). Our study showed the formation of the non-covalent complexes of Pep-1 and CADY-2 with HIV-1 GST-Nef and also the same ability of Pep-1 and CADY2 to deliver the recombinant protein into mammalian cells. The capability of protein condensation into small compact particles for the efficient delivery of protein into cells was analyzed by SEM. Our results showed spherical particles with a size range of 120–200 nm in diameter at molar ratio of 1:20 (Protein/Peptide). Efficient delivery of CADY-2, and Pep-1 peptides was shown to transfer some proteins into the mammalian cells independently of their nature and size, including 120–150 kDa proteins such as beta-galactosidase and antibodies, protein kinase A, caspase 3, mRFP, and GST-Cdk2, p27Kip (Munoz-Morris et al. 2007; Kurzawa et al. 2010; Morris et al. 2008; Heitz et al. 2009). Current study indicated that Pep-1/GST-Nef, CADY-2/GST-Nef, and TurboFect/GST-Nef nanoparticles were stable for transfection in HEK-293T cells and overcame the intracellular barriers based on their physicochemical properties. The studies also showed that cell-penetrating peptides that form non-covalent complexes with their cargo are a strong alternative to conjugate or covalent forms for delivery of biological macromolecules into cells (Kurzawa et al. 2010). The transfection efficiency of GST-Nef protein using Pep-1, CADY-2 and also TurboFect reagent showed that Pep-1, CADY-2, and TurboFect-based nanoparticles

facilitate uptake of GST-Nef fusion protein into the cells. Some studies have shown that transport efficiency of CPPs depends on the properties of both CPP and cargo as well as on the transfection conditions and the cell lines (Munoz-Morris et al. 2007; Kurzawa et al. 2010). A study also indicated that Pep-1 fused to Methionine sulfoxide reductase A (Pep-1-MsrA) effectively enter cells and plays a protective role against oxidative stress and inflammation. Indeed, Pep-1-MsrA may be improved as a potent therapeutic agent for decreasing atherosclerosis related cardiovascular diseases (Wu et al. 2015).

Conclusion

In this paper, we have represented that both vectors (pCDH-CMV-MCS-EF1-cGFP-T2A-puro and pEGFP-N1) can effectively express the Nef protein within the target cell; although the pEGFP-N1 vector was more effective than pCDH-GFP for protein expression. Furthermore, the Nef protein expressed in *E. coli* as GST-Nef fusion was transfected by Pep-1 and CADY-2 CPPs into HEK-293T cells. Our data indicated that the CPPs/GST-Nef nanoparticles were formed with a diameter of below 200 nm and notably delivered into the cells. However, there are few studies for using CPPs as protein delivery system for vaccine design. Briefly, we showed the efficiency of two CPPs for generation of small nanoparticles with GST-Nef protein as a vaccine candidate against HIV infections. The potent transfections into cells open the way to design a more efficient protein-based therapeutic vaccine against HIV infections.

Compliance with Ethical Standards

Conflict of interest The authors confirm that this article content has no conflicts of interest.

References

AbdEllah NH, Taylor L, Ayres N, Elmahdy MM, Fetih GN, Ibrahim ESA, Pauletti GM (2014) Increased transfection efficiency of lipid coated methacrylate-based gene delivery systems. Int J Pharm Sci Res (IJPSR) 5:812–816

Abdul Halim NS, Fakiruddin KS, Ali SA, Yahaya BH (2014) A comparative study of non-viral gene delivery techniques to human adipose-derived mesenchymal stem cell. Int J Mol Sci 15:15044–15060

Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, Johnston MN, Corcoran C, Wurcel AG, Fitzpatrick CA, Feeney ME, Rodriguez WR, Basgoz N, Draenert R, Stone DR, Brander C, Goulder PJ, Rosenberg ES, Altfeld M, Walker BD (2003) Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 77:2081–2092

Aoshiba K, Yokohori N, Nagai A (2003) Alveolar wall apoptosis causes lung destruction and amphysematous changes. Am J Respir Cell Mol Biol 28:555–562

Bolesta E, Gzyl J, Wierzbicki A, Kmieciak D, Kowalczyk A, Kaneko Y, Srinivasan A, Kozbor D (2005) Clustered epitopes within the Gag-Pol fusion protein DNA vaccine enhance immune responses and protection against challenge with recombinant *vaccinia* viruses express ing HIV-1 Gag and Pol antigens. Virology 332:467–479

Burkhart A, Thomsen LB, Thomsen MS, Lichota J, Fazakas C, Krizbai I, Moos T (2015) Transfection of brain capillary endothelial cells in primary culture with defined blood brain barrier properties. Fluid Barrier CNS 12:1–14

Crombez L, Morris MC, Dufort S, Aldrian-Herrada G, Nguyen Q, Mc Master G, Coll JL, Heitz F, Divita G (2009) Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. Nucl Acid Res 37:4559–4569

Eliyahu H, Barenholz Y, Domb AJ (2005) Polymers for DNA delivery. Molecules 10:34–64

Federico M (2003) Methods in molecular biology, lentivirus gene engineering protocols. Humana, Totawa, p 229

Gallo G, Yee HF, Letourneau PC (2002) Actin turnover is required to prevent axon retraction driven by endogenous actomyosin contractility. J Cell Biol 158:1219–1228

Gros E, Deshayes S, Morris MC, Aldrian-Herrada G, Depollier J, Heitz F, Divita G (2006) A non-covalent peptide-based strategy for protein and peptide nucleic acid transduction. Biochim Biophys Acta 1758:384–393

Hartz PA, McMiller T, Scott-Wright D, Samuel KP (2003) Low level expression of the HIV1 Nef protein in transiently transfected COS-1 cells in culture. Cell Mol Biol 49:1101–1107

Hassane FS, Saleh AF, Abes R, Gait MJ, Lebleu B (2010) Cell penetrating peptides: overview and applications to the delivery of oligonucleotides. Cell Mol Life Sci 67:715–726

Heiser WC (2004) Methods in molecular biology, gene delivery to mammalian cells. Humana, Totawa, p 246

Heitz F, Morris MC, Divita G (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharm 157:195–206

Huang NC, Ji Q, Ariga K, Hsu S (2015) Nanosheet transfection: effective transfer of naked DNA on silica glass. NPG Asia Mater 7:1–9

Joseph AM, Ladha JS, Mojamdar M, Mitra D (2003) Human immunodefficiency virus-1 Nef protein interacts with Tat andenhances HIV-1 gene expression. FEBS Lett 548:37–42

Kienzle N, Bachmann M, Müller WEG, Müller-Lantzsch N (1992) Expression and cellular localization of the Nef protein from human immunodeficiency virus-1 in stably transfected B-cells. Arch Virol 124:123–132

Kim JH, Lee SH, Sohn HJ, Lee J, Chwae YJ, Park S, Kim K, Shin HJ (2012) The immune response induced by DNA vaccine expressing *nfa1* gene against *Naegleria fowleri*. Parasitol Res 111:2377–2384

Kurzawa L, Pellerano M, Morris MC (2010) PEP and CADY-mediated delivery of fluorescent peptides and proteins into living cells. Biochim Biophys Acta 1798:2274–2285

Ma J, Xu J, Guan L, Hu T, Liu Q, Xiao J, Zhang Y (2014) Cell-penetrating peptides mediated protein cross-membrane delivery and its use in bacterial vector vaccine. Fish Shellfish Immunol 39:8–16

Machida CA (2003) Viral vectors for gene therapy: methods and protocols. Humana, Totowa

Morris MC, Depollier J, Mery J, Heitz F, Divita G (2001) A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 19:1173–1178

Morris MC, Deshayes S, Heitz F, Divita G (2008) Cell-penetrating peptides: from molecular mechanisms to therapeutics. Biol Cell 100:201–217

Munoz-Morris MA, Heitz F, Divita G, Morris MC (2007) The peptide carrier Pep-1 forms biologically efficient nanoparticle complexes. Biochem Biophys Res Commun 355:877–882

Pratt RL, Kinch MS (2002) Activation of the EphA2 tyrosine kinase stimulates the MAP/ERK kinase signaling cascade. Oncogene 21:7690–7699

Simpson RJ (2007) Zinc/Imidazole procedure for visualization of proteins in gels by negative staining. CSH protocols 4:pdb-prot4701

Tahtinen M, Strengell M, Collings A, Pitkanen J, Kjerrstrom A, Hakkarainen K, Peterson P, Kohleisen B, Wahren B, Ranki A, Ustav M, Krohn K (2001) DNA vaccination in mice using HIV-1 *nef, rev* and *tat* genes inself-replicating pBN-vector. Vaccine 19:2039–2047

Thomas P, Smart TG (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. J Pharm Toxicol Method 51:187–200

Wu Y, Wood MD, Yi T, Katagiri F (2002) Direct delivery of bacterial a virulence proteins into resistant Arabidopsis protoplasts leads to hypersensitive cell death. Plant J 33:130–137

Wu Y, Xie G, Xu Y, Ma L, Tong C, Fan D, Du F, Yu H (2015) PEP-1-MsrA ameliorates inflammation and reduces atherosclerosis in apolipoprotein E deficient mice. J Transl Med 13:1–13

Zuniga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, Hernandez A, Sanchez H, Frahm N, Linde CH, Hewitt HS, Hildebrand W, Altfeld M, Allen TM, Walker BD, Korber BT, Leitner T, Sanchez J, Brander C (2006) Relative dominance of Gag p24-specific cyto toxic T lymphocytes is associated with human immunodeficiency virus control. J Virol 80:3122–3125