Biomaterials 35 (2014) 8416–8426

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

MLV based viral-like-particles for delivery of toxic proteins and nuclear transcription factors

Dai-Tze Wu, Monica J. Roth*

Department of Pharmacology, Robert Wood Johnson Medical School, Rutgers University, 675 Hoes Lane Rm 636, Piscataway, NJ, USA

ARTICLE INFO

Article history:
Received 30 May 2014
Accepted 2 June 2014
Available online 3 July 2014

keywords:
Protein transduction
Viral-like particles
Intracellular delivery
Transcriptional factors
Bacterial toxin

ABSTRACT

We have developed nanoparticles based on Murine Leukemia Virus virus-like-particles (VLPs) that efficiently deliver therapeutic bioactive proteins in their native state into target cells. Nuclear transcription factors and toxic proteins were incorporated into the VLPs from stable producer cells without transducing viral-encoded genetic material. Delivery of nuclear transcription factors required incorporation of nuclear export signals (NESs) into the vector backbone for the efficient formation of VLPs. In the presence of an appropriate targeting Env glycoprotein, transcription factors delivered and activated nuclear transcription in the target cells. Additionally, we show delivery of the bacterial toxin, MazF, which is an ACA-specific mRNA interferase resulted in the induction of cell death. The stable producer cells are protected from the toxin through co-expression of the anti-toxin MazE and continuously released MazF incorporating VLPs. This highly adaptable platform can be harnessed to alter and regulate cellular processes by bioactive protein delivery.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The transfer of therapeutic proteins to patients has great potential for treatment of diseases including genetic disorders and cancer. Protein delivery into target cells for protein therapeutics faces several significant limitations including the targeting and stability of the proteins *in vivo*. The limiting ability of proteins to cross the cellular membrane and escape the endosomal–lysosomal pathways are major barriers for intracellular delivery of macromolecules [1,2]. Improving the safety and efficacy of pharmaceutical protein production and intracellular delivery remains a big challenge.

Retroviral-like particles (VLPs) present an approach to avoid sequestration or degradation of target proteins within the endosomal–lysosomal pathway. The process of retroviral entry into a target cell is tightly regulated by the interactions between the retroviral envelope glycoprotein and a specific host cell receptor. This provides the potential to modify the envelope glycoprotein protein for targeted delivery of VLPs to specific tissues and cell types. Approximately 2000 copies of the Gag can be delivered into a transduced cell from each VLP [3]. Transient retroviral protein transduction (PT) systems have successfully delivered foreign functional proteins including Flp recombinase, cytosine deaminase, uracil phosphorybosyltransferase, and caspase to cells [4,5].

In this study, we develop a protein delivery system based on Murine Leukemia Virus (MLV) VLPs capable of continuous large-scale production and delivery of bioactive proteins. Unlike viral vectors, these particles do not package viral RNA. Cellular expression of the Gag protein generates replication-defective VLPs in the presence of targeting Env glycoproteins without the delivery of a viral genome [6]. Two classes of proteins were tested for incorporation into VLPs: nuclear transcription factors (TFs) and toxic proteins. In our system, the PT-VLPs are derived from a stable engineered mammalian producer cell line optimized for the release of high-titer VLPs, and tested for altered cellular behavior in the recipient cells.

2. Materials and methods

2.1. Plasmid construction

Construction of protein transduction vector (pPT) was base on pNCA-C (IN$^-$ D184N/K376A) proviral vector as backbone [7,8]. A new protease site (the nucleotide in bold, encoding RSSLY/PALTP indicates protease cleavage site) and three restriction sites: MluI, SwaI, and NotI (the nucleotides in italics) were incorporated into the C-terminus of matrix (MA) with overlapping PCR and the EcoRI/XhoI restriction sites (5′ CCGCCT**CGGTCTTCACTGTACCCCGCACTGACCCCC***ACGCGT*GGG*CATTTAAATT*GGG CG*CGGCCGC*TCCGATCC). The sequences encoding the foreign proteins were amplified by PCR and subcloned at the SwaI/NotI sites or MluI/NotI sites to generate pPT-Protein constructs. The MazF or MazF(E24A) sequences were amplified from pACyC-

* Corresponding author. Department of Pharmacology, Robert Wood Johnson Medical School, Rutgers University, 675 Hoes Lane Rm 636, Piscataway, NJ 08854, USA. Tel.: +1 732 235 5048.
E-mail address: roth@rwjms.rutgers.edu (M.J. Roth).

http://dx.doi.org/10.1016/j.biomaterials.2014.06.006
0142-9612/© 2014 Elsevier Ltd. All rights reserved.

MazF or pBAD33-MazF(E24A) (gifts from Dr. Masayori Inouye, Rutgers-RWJMS). The mouse TFs sequences were amplified from pMXs-TF purchased from Addgene. All primers are summarized in Table S1.

For the pL-GPTP (Gag-PT-Pol) vector construction, the 6-kb EcoRI-NdeI fragment from the pPT plasmid was subcloned into pSin-EF2-LIN28-Pur (Addgene 16580) to replace the Lin28 gene. The Gag N-terminal and IN C-terminal sequences were amplified from pPT-Oct4 plasmid using primers EcoRI 1010 fwd + XhoI 2118 rev and NdeI 5851 fwd + NsiI 6286 rev. The PCR products were subcloned in pL-GPTP by 0.5 kb EcoRI-MluI fragment exchange to replace the 5' LTR sequence and 0.5 kb NdeI-NsiI fragment exchanged to complete the IN sequences. Finally, the pPT-protein plasmid was subcloned into pL-GPTP by MluI/PshAI fragment exchange to generate the individual pL-GPTP plasmids. For pL-GPT2NP (Gag-PT-2NES-Pol) plasmids, the NES nucleotide sequence (encoding (NINELALKFAGLDL) was inserted into the p12 N-terminus and NC C-terminus in pL-GPTP vectors by overlapping PCR with EcoRI/PshAI and PshAI/XcmI restriction sites.

The *puromycin* gene within pSin-EF2-LIN28-Pur vector was replaced by KpnI fragment exchange with a *Zeocin* resistance gene using overlapping PCR. The *env* EA6-3X sequence was amplified from pHIT-EA6-3X plasmid [9], fragment exchanged using EcoRI/NdeI with Lin28 in pSin-EF2-LIN28-Zeo, generating pL-Env. EA6-3X chimeric Env, encodes the ecotropic M-MLV receptor binding domain bearing the N261I/E311V/G552R mutations and the amphotropic TM [9].

For pL-MazE-GFP construction, the IRES-Puro sequence was removed from the pSin-EF1-GFP-IRES-Puro backbone [10] by KpnI digestion followed by self-ligation. The MazE or truncated MazE sequences (MazE42-GFP and MazE61E-GFP) [11] were amplified from pCold-MazE (a gift from Dr. Masayori Inouye, Rutgers-RWJMS) by PCR and subcloned into pSin-EF1α-GFP-ΔKpnI at the SpeI restriction sites.

Inducible GFP reporter constructs with specific TRE elements were generated by modifying pSin-EF1α-GFP-IRES-Puro. TATA-specific TRE sequences were amplified from DNA within Qiagen Reporter Arrays (CCA-106L-2) with AgeI/SpeI restriction sites, replacing the EF-1α promoter sequences, generating pL-TF-TRE-GFP.

2.2. Cell culture

All of the cell lines were cultured as previously described [10]. The 293TCeB cells were maintained in DMEM containing 10 μg/mL Blasticidin S (Invivogen). The chimeric Gag VLPs producer cell lines in 293TCeB were maintained in DMEM containing 2.5 μg/mL puromycin, 400 μg/mL Zeocin (Invivogen), and 10 μg/mL Blasticidin S. HEK293T cell was purchased from American Type Culture Collection and the mouse embryonic fibroblast cell line (SNL) was ordered from Cell Biolabs, Inc. (CBA-316). HeLa MCAT and HEK293T MCAT cell line were created as previously described [12] and maintained in DMEM containing 10 μg/mL Blasticidin S.

2.3. Lentiviral production and generation of VLP producer cell lines

All lentiviral particles were produced as previously described [10]. Three days post-infection, puromycin and Zeocin selections were performed to obtain the stable VLP producer cell lines.

For generation of lentiviral particles that contained Gag-MazF-2NES-Pol sequences, HEK293T was first infected by pL-MazE-GFP lentiviral particles. HEK293T-MazE-GFP cells were used to transfect the pL-G-MazF-2NP, pCMV-ΔR8.2 Δvpr, and pHIT-G to generate Gag-MazF-2NES containing lentiviral particles.

2.4. Cell viability

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, M5655) assay was used to measure cell viability. Initially, 500 cells were plated into each well of a 96-well tissue culture plate and treated with 0–30 μg/mL of CA containing VLPs or 0–500 nM of methotrexate (Sigma, A6770) for one week. 100 μL medium contain 0.5 mg/mL MTT was added to each well and incubated at 37°C. After 4–6 h incubation, the medium was discarded and the 100 μL acidic isopropanol containing 0.04 N HCl and 0.1% NP40 was added to dissolve the crystals for 10 min at room temperature. The optical density was read at OD_{570} nm immediately.

2.5. Immunofluorescence & confocal microscopy

In brief, cells seeded on poly-L-lysine coated glass coverslips were fixed and permeabilized with −20°C methanol, blocked by 5% BSA, and stained as previous described [13]. For consecutive antibody studies, the donkey anti-goat antibody was used prior to either the goat anti-mouse or rabbit antibodies. HEK293T & 293TCeB cells expressing the chimeric Gag protein were imaged on a Zeiss LSM510 META confocal microscope with a 63 × water immersion objective at the Robert Wood Johnson Medical School Confocal and Electronic Imaging Center.

2.6. Western blot and antibodies

VLPs of Gag-TF chimeras were harvested from producer cell supernatants and concentrated by centrifugation at 15,000 × g for 30 min. All antibodies used for the Western blots and immunofluorescence staining are listed in Table S2.

2.7. TF Activity assays

For TF activity assays, the specific pL-TFTRE-GFP lentiviral vector was produced as described [10] and introduced into SNL cell to establish the stable sensor cell line in the absence of drug selection. The bioactivity of PT was determined by measuring the percentages of GFP-positive cells by flow cytometry [10] at day 8 post Gag-TF-2NES VLPs treatment.

2.8. Quantitative PCR assays

cDNA templates from viral RNA or cellular mRNA were synthesized as previous described [8,10]. For genomic DNA purification, infected cells were collected at 10 days post-infection, genomic DNA was purified using DNeasy Blood & Tissue Kit (Qiagen) and used as template for PCR.

qPCR reactions were carried out using the Power SYBR Green PCR Master Mix (4367659, Applied Biosystems) on a Mastercycler ep realplex real-time PCR system (Eppendorf). qPCR reactions were performed in a total volume of 20 μl with 2 μl of template cDNA, plasmid DNA (for standard curve), or genomic DNA (~300 ng) with 250 nM of each primer. qPCR reactions were performed under the following conditions: 1 cycle at 95°C for 10 min, followed by 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles. The primers and probe used for quantification are shown on Table S1.

3. Results

3.1. Generation of non-infectious VLPs from stable producer cells

Gammaretroviruses are nanoparticles capable of specifically delivering sequestered proteins into target cells [4,5]. Fig. 1 outlines the approach to modify VLPs to assemble target proteins of interest, specifically nuclear transcription factors and toxic proteins. Viral particles (VPs) are assembled on the plasma membrane through interaction of four viral components, the precursor Gag and Gag-Pol, Env plus RNA (Fig. 1a). In this scheme, the proteins of interest are incorporated into the Gag precursor protein. VLP release is independent of an Env protein or specific viral RNAs [14–17] and particles are thus non-infectious but require an optimal ratio of Gag to Gag-Pol precursor proteins. Upon particle formation, the viral protease cleaves the precursor proteins, releasing the individual viral proteins and the proteins of interest, which are then delivered into the target cells and can traffic independently.

Fig. 1b outlines the vectors developed to establish high efficiency producer cells for continuous production of non-infectious protein transduction particles. The protein transduction system differs from VPs in that the VLPs are used for delivering protein rather than a gene. Therefore, incorporation of the viral RNA into the particles or inclusion of functions required for the establishment of an integrated provirus is not required, and are in fact, disadvantageous [18,19]. Proteins of interest were inserted within the *gag* gene between the MA and p12 proteins, bracketed by MLV PR cleavages sites (MLV Gag-PT-Pol) [4]. The MLV *gag-pol* bearing mutations in the integrase (IN) (D184N/K376A) was expressed from an internal elongation factor 1α (EF1α) promoter within the lentiviral vector (pL-GPTP). The MLV genome contained deletion in the sequences of 5' and 3' LTR, as well as the packaging site and MLV splice donor site. Thus, even if RNA from this construct is packaged it cannot be reverse-transcribed, and sites required for integration of the virus are absent. An *env* is expressed from an independent lentiviral vector (pL-Env) (Fig. 1c). Transduction of this PT sequence and *env* using a lentiviral packaging system into HEK293T cells and selection for puromycin and Zeocin resistance allows for the identification of producer cells [18–20]. For PT studies, a modified ecotropic MLV Env (EA6-3X) was utilized because of its properties of high titer with decreased syncytia formation in mouse cells or human cells expressing the MCAT receptor [9].

3.2. Incorporation of nuclear TFs within MLV based VLPs

Initial Gag constructs (Fig. 1b) aimed at studying the delivery of nuclear transcription factors (Gag-TF), including mouse Oct4 (Gag-

Download English Version:

https://daneshyari.com/en/article/10227304

Download Persian Version:

https://daneshyari.com/article/10227304

Daneshyari.com