Protein Expression and Purification 110 (2015) 52–56

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression and characterization of myristoylated preS1-conjugated nanocages for targeted cell delivery

Masaharu Murata [a,b,*], Jing Shu Piao [a], Sayoko Narahara [a,b], Takahito Kawano [a,b], Nobuhito Hamano [a], Jeong-Hun Kang [c], Daisuke Asai [d], Ryo Ugawa [e], Makoto Hashizume [a,b]

[a] Department of Advanced Medical Initiatives, Faculty of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Innovation Center for Medical Redox Navigation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[c] Department of Biomedical Engineering, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan
[d] Department of Microbiology, St. Marianna University School of Medicine, Sugao 2-16-1 Miyamae, Kawasaki 216-8511, Japan
[e] Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 22 October 2014
and in revised form 2 December 2014
Available online 10 December 2014

Keywords:
Protein cage
Ferritin
Myristoylation
Hepatitis B virus
Targeted delivery

ABSTRACT

Lipid modification of proteins plays key roles in cellular signaling pathways. We describe the development of myristoylated preS1–nanocages (myr–preS1–nanocages) that specifically target human hepatocyte-like HepaRG cells in which a specific receptor-binding peptide (preS1) is joined to the surface of naturally occurring ferritin cages. Using a genetic engineering approach, the preS1 peptide was joined to the N-terminal regions of the ferritin cage *via* flexible linker moieties. Myristoylation of the preS1 peptide was achieved by co-expression with yeast N-myristoyltransferase-1 in the presence of myristic acid in *Escherichia coli* cells. The myristoylated preS1–nanocages exhibited significantly greater specificity for human hepatocyte-like HepaRG cells than the unmyristoylated preS1–nanocages. These results suggest that the lipid-modified nanocages have great potential for effective targeted delivery to specific cells.

Introduction

Post-translational modifications allow the incorporation of chemicals and new molecular functions that cannot be directly encoded by genetics [1]. In particular, post-translational lipid modifications of proteins involved in signaling pathways are crucial for biological activities. Many signaling proteins are modified by covalent links to fatty acids and/or prenyl groups. These hydrophobic moieties, which include myristate, palmitate, farnesyl, and geranylgeranyl, are more than just fats because they modulate the specificity and efficiency of signal transduction pathways [2,3].

We recently reported the design, synthesis, and biological characterization of several types of protein nanocages that were composed of small heat-shock proteins [4,5]. Genetic incorporation and chemical modification of the exterior surface of the nanocages with the addition of a peptide ligand, for example, allowed the protein–nanocages to target specific cells, depending on the type of ligand [6,7]. Furthermore, our nanocages can encapsulate a variety of molecules, including anticancer drugs and imaging agents [8,9]. Indeed, the specificity of engineered nanocages carrying the preS1 peptide for liver cells *in vitro* was similar to that of HBV[1] [6,10]. We chose the preS1 peptide, which is located on the outermost part of the envelope protein of hepatitis B virus (HBV), because it possesses a hepatocyte receptor-binding site involved in the initial step in HBV infection. This strategy was effective for producing nanocages that were directed to and entered specific cell types or cancer cells through ligand-mediated active binding. Nevertheless, it is necessary to improve their specificities for target cells for use *in vivo* as drug delivery systems [11–13], vaccine, or in diagnostic imaging [14,15].

Here, we describe the simple preparation of myristoylated preS1–nanocages using a genetic engineering approach. Our strategy for the functional expression of the nanocages in *Escherichia coli* is based on the co-expression of recombinant human ferritin light chain (rh-ferritin-L) fused to preS1 (preS1–nanocages) and yeast N-myristoyltransferase-1 (rNMT-1), myristoyl-CoA:protein. Ferritin generally contains 24 subunits of spherical capsids, with different ratios of heavy chain (H) to light chain (L), an outer

* Corresponding author at: Department of Advanced Medical Initiatives, Faculty of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6251; fax: +81 92 642 6252.
E-mail address: m-murata@dem.med.kyushu-u.ac.jp (M. Murata).

[1] *Abbreviations used:* HBV, hepatitis B virus; rh-ferritin-L, ferritin light chain; rNMT-1, yeast N-myristoyltransferase-1; H, heavy chain; L, light chain; IPTG, isopropyl thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DLS, dynamic light scattering; TEM, transmission electron microscopy.

http://dx.doi.org/10.1016/j.pep.2014.12.001
1046-5928/

diameter of 125 Å and an inner hollow core diameter of 80 Å [16,17]. Our results indicated that engineered ferritin-based nanocages formed similar structures under physiological conditions regardless of their surface modifications. Furthermore, myristoylation of the preS1 moieties on the exterior surface of the nanocages promoted ligand-receptor interaction on the hepatocyte-like HepaRG cells (Fig. 1).

Materials and methods

DNA cloning, expression, and purification of myristoylated preS1–nanocages

The amino acid sequences of the recombinant plasmids encoding preS1–nanocages linked to the preS1 peptide *via* a linker moiety and of the control nanocage are shown in Fig. S1. Plasmid pET–preS1–ferritin-L, a bacterial expression vector containing the preS1 gene fused in frame with rh-ferritin-L at the amino-terminus, was cloned by PCR, as previously described [6]. The expression cassette for yeast NMT-1 was amplified by PCR using genomic DNA from yeast strain S288C as a template. For recombinant yeast NMT-1, we used a 5′ primer (5′-GGGACGCCCATGGGCTCAGAAGAGGATAAAGCGAAAAAATTAGAGAATTTATTG-3′) tagged with an *Nco*I site (underlined) and a 3′ primer (5′-GGGGGATCCCTACAACATAACAACACCGACATTGCTACGC-3′) tagged with a *BamH*I site (underlined). The PCR-generated 1.37-kb fragment was digested with *Nco*I and *BamH*I and the resulting DNA fragment was ligated into the same sites of pACYCDuet-1 vector (Novagen/Merck, Darmstadt, Germany). The resulting vector (pACYC–rNMT1) was sequenced to verify correct insertion of the fragment.

Transformation competent BL21(DE3) cells (Agilent Technologies, Phoenix, AZ, USA) were separately transformed with pET–preS1–ferritin-L and pACYC–NMT1 plasmid pairs. Transformed *E. coli* cells were selected using LB-agar plates coated with chloramphenicol/ampicillin (pACYC/pET) double antibiotics. The transfected cells containing the expression vector were grown in 2× YT medium (Sigma–Aldrich, St. Louis, MO, USA) containing 100 μg/mL ampicillin and 30 μg/mL Chloramphenicol. At an OD_{600} of 0.5, 1.5 mL stock solution containing 5 mM myristic acid (Sigma–Aldrich, St. Louis, MO, USA) and 0.6 mM bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA) was added and cells were grown for another hour. Recombinant protein production was initiated by the addition of isopropyl thiogalactopyranoside (IPTG; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to a final concentration of 1 mM and incubation at 37 °C for 4 h. The cells were pelleted by centrifugation at 4 °C, the pellet was resuspended in 8 mL of sample buffer (25 mM KH_2PO_4–KOH, pH 7.0, 2 mM dithiothreitol, and 1 mM ethylenediaminetetraacetic acid), and the resulting suspension was ultrasonicated (Bioruptor™ UCD-300; Tosho Denki, Yokohama, Japan) on ice for 4 min (H-amplitude, 20 s sonication at 20-s intervals). DNase I and RNase A were then added to final concentrations of 5 and 1 mg/mL, respectively, and the cell lysate mixture was incubated at 4 °C for 30 min. Insoluble material was removed by centrifugation at 20,000×*g* for 20 min at 4 °C. The recombinant protein was purified by ion-exchange chromatography, in which the supernatant was loaded on a HiLoad 16/10 Q Sepharose HP™ anion-exchange column (GE Healthcare, Little Chalfont, United Kingdom). The target protein was eluted in sample buffer containing 1 M NaCl.

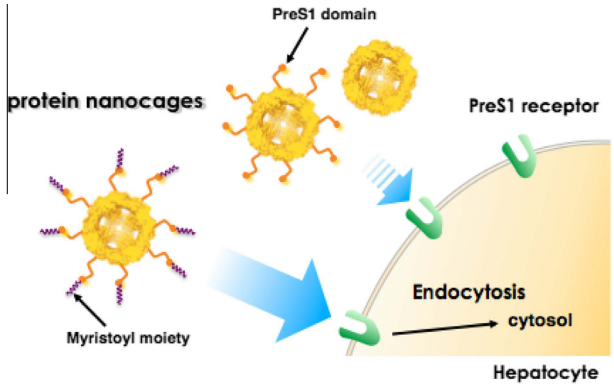

Fig. 1. Schematic illustration of genetically engineered nanocages for hepatocyte selective targeting.

Characterization of the nanocages

Size-exclusion chromatography confirmed that monodisperse nanocages were successfully obtained with sufficient purity. The fractions containing the purified nanocages were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using 15% gels according to a standard protocol. The sizes of the nanocages in physiological conditions were measured by dynamic light scattering (DLS) (Malvern Nanosizer ZS; Malvern Instruments Ltd., Malvern, UK). The molecular weight of the purified nanocages was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Autoflex speed; Bruker Corp., Billerica, MA, USA) with a sinapinic acid matrix. The structure of the nanocages was assessed by transmission electron microscopy (TEM). First, the protein nanocages were applied to carbon-coated copper grids. After allowing the nanocages to absorb, the grids were rinsed with droplets of deionized water and stained with 2% uranyl acetate. The samples were observed using a TECNAI 20 and Eagle 2k CCD camera (FEI, Hillsboro, OR, USA) at an accelerating voltage of 200 kV.

Chip-based analysis

Chip-based analysis was performed with the Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Briefly, for the Protein 80 kit, the samples (1–4 μl) were diluted in sample buffer with 1 M DTT. The samples were denaturated by placing the vials in heating block at 95 °C for 5 min, cooled, and centrifuged for 15 s. Then 84 μl of deionized water was added to the protein ladder and each sample. After filling the chip with a gel/dye mix and destaining solution, 6 μl aliquots of the samples and protein ladder were loaded onto the chip. The separated proteins were detected with laser-induced fluorescence. The sample buffer included an upper and a lower marker of known molecular weights.

Immunoblotting

The SDS polyacrylamide gel of the control nanocage (i.e. ferritin-L), preS1–nanocages, and myristoylated PreS1–nanocages were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) for western blotting. After protein transfer, the filter was blocked with 2.5% skimmed milk at room temperature for 1 h and incubated with an anti-ferritin light chain antibody (dilution 1:1000; ab69090; Abcam, Cambridge, MA, USA) or an anti-myristic acid (conjugated) antibody (dilution 1:50; ab37027; Abcam) overnight at 4 °C. The bound primary antibody was detected with peroxidase-conjugated anti-rabbit immunoglobulin G (WB-1000; Vector Laboratories, Burlingame, CA, USA) using an ECL luminescence kit (GE Healthcare).

Flow cytometry

Before flow cytometric analysis, the nanocages were labeled with fluorescent dye. These nanocages were incubated with an