Preparation of peptide-targeted phagemid particles using a protein III-modified helper phage

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Ligand or peptide-targeted phagemid particles are being pursued as vehicles for receptor-mediated gene delivery. Here we describe a helper phage in which the protein III (pIII) protein is modified by the addition of a ligand peptide sequence at the amino terminus. Phagemid particles can be prepared with the help of this modified helper phage and should display the ligand peptide in most of the pIII proteins on the phagemid surface. Using such a method, it is not necessary for the phagemid to encode the pIII protein, which leaves a larger space for cloning genes of interest. In addition, the technique should allow for the rapid testing of peptide ligands selected from phage display libraries using phagemids encoding various reporter genes (e.g., green fluorescent protein, luciferase, β -galactosidase) and therapeutic genes.

INTRODUCTION

In recent years, modified filamentous bacteriophage vectors were found to be capable of delivering genes to mammalian cells using targeting ligands such as growth factors (1-4), antibodies (5), or viral capsid proteins (6). Therefore, it seems feasible to develop specifically targeted phage vectors expressing peptide ligands for gene therapy (7). Some studies have shown that the level of ligand display plays an important role in determining the efficiency of transduction and suggests that phagemid particles might be more appropriate than phages for gene delivery. Most filamentous phage libraries have been constructed using protein III (pIII) as a genetic and structural base for the display of peptides and proteins (8-13). To prepare phagemid particles containing the selected peptide ligand, two methods are commonly used. One is to clone the modified pIII expression cassette into a phagemid, followed by rescue with widely used helper phages such as R408 (14) and VCSM13 (Stratagene, La Jolla, CA, USA). Consequently, the phagemid particles contain mixtures of

wild-type and modified pIII proteins. The other strategy was more complicated (15): phage shock protein (psp) promoter was fused to the sequence encoding pIII (gIII). Cells containing the psp-gIII fusion were used to prepare helper phages with a gIII deletion. The resulting helper phage was then used to package phagemid encoding peptide-modified pIII. Both of the above strategies used the phagemid to encode the modified pIII, which occupied a large space on the phagemid and left limited space for cloning additional genes. In addition, reconstruction of vectors was necessary to test the gene delivery ability of selected ligands using phagemids encoding different reporter genes. To circumvent these problems, we present a new method to prepare ligand peptide-displaying phagemid particles.

MATERIALS AND METHODS

Cell Culture

COS-1 cells (ATCC, Manassas, VA, USA) were cultured at 37°C in medium consisting of 90% Dulbecco's modified Eagle's medium (DMEM)

and 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO₂.

Construction of Modified Helper Phage M13KO7P1

M13KO7P1 was obtained by subcloning the *Sna*I and *Pac*I fragments of phage clone M13P1 (displaying a 12-residue peptide, PKLLPWQSLRMH) selected from Ph.D.-12™ Phage Display Peptide Library (New England Biolabs, Beverly, MA, USA) into the M13KO7 (Amersham Biosciences, Uppsala, Sweden) *Sna*I/*Pac*I-digested backbone (for a detailed plasmid map, see Supplementary Figure S1 available online at www.BioTechniques.com).

Phage Preparation

M13KO7 and M13KO7P1 helper phages were prepared as previously described (16) except that the ER2738 cell line (New England Biolabs) was used as the host instead of Nm522 (for a detailed plasmid map, see Supplementary Figure S1). Phage titers were determined by infecting ER2738 cells with phage dilutions and counting the number of kanamycin-resistant clones obtained.

Preparation of Phagemid Particles

Phagemid particles were prepared following a previous protocol (17), with some modification. Briefly, a clone of pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA)-transformed ER2738 was picked and transferred into 1 mL of Luria Bertani (LB) media containing 70 μ g/mL kanamycin. The culture was grown at 37°C for 5 h, after which 100 μ L of the culture were transferred into 1 mL of LB media containing 70 μ g/mL kanamycin. Helper phage [1×10^9 plaque-forming units (pfus)] was added and vortex mixed immediately. After shaking at 37°C for another 2 h, all cultures were transferred into 1 L LB containing 70 μ g/mL kanamycin and shaken at 37°C for 14-16 h. Phagemid particles were purified with polyethylene glycol (PEG)/NaCl and washed several times.

Quantification of Phagemid Particles

Phagemid particles were quantified by enzyme-linked immunosorbent assay (ELISA) as previously described (18). Briefly, serial dilutions of phagemid particles in coating buffer (0.1 M NaHCO₃, pH 9.1) were coated in microtiter plates (Corning®; Corning, NY, USA) at 4°C overnight. After blocking with 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS), the bound phages were stained with mouse anti-M13 antibody conjugated with horseradish peroxidase (HRP; Amersham Biosciences) diluted 1:200 in PBST (PBS containing 0.1% Tween® 20). The signal was developed using 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate and quantitated in an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). M13KO7 helper phage of known number of pfus was used for standardization.

Purification of Single-Stranded DNA from Phage and Phagemid Particles

Single-stranded DNA (ssDNA) was extracted from the helper phages or phagemid particles following the supplied protocol (Ph.D.-12 Phage Display Peptide Library Kit). Briefly, phages or phagemid particles were precipitated by PEG/NaCl. The pellet was then suspended with 100 µL of iodide buffer. Two hundred and fifty microliters of ethanol were added and incubated for 10 min to allow for ssDNA precipitation. The pellet was collected after centrifugation at 12,000× *g* for 10 min, washed in 70% ethanol, and air-dried for 10 min. It was finally dissolved in 30 µL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and analyzed by agarose gel electrophoresis.

Western Blot Analysis

Purified phages or phagemid particles were boiled in Tris-glycine-sodium dodecyl sulfate (SDS) sample buffer containing 5% 2-mercaptoethanol for 5 min prior to loading on

a 12% SDS-PAGE gel. Following separation, proteins were transferred to nitrocellulose filters (0.2-mm pore; Bio-Rad Laboratories) in 96 mM glycine, 12 mM Tris base, 0.01% SDS, and 20% methanol. The filter was blocked in 5% skimmed milk in PBST for 2 h and incubated with mouse anti-M13 pIII monoclonal antibody (1 µg/mL; New England Biolabs) for 1 h at room temperature, followed by the addition of goat anti-mouse immunoglobulin G (IgG) HRP-streptavidin conjugate (ImmuClub Labs, Sunnyvale, CA, USA) at a 1:10,000 dilution in 1% BSA in PBST. After a 1-h incubation, the filters were washed with PBST, developed using a SuperSignal® West Pico kit (Pierce Biotechnologies, Rockford, IL, USA) for 5 min, and exposed to X-ray film.

Internalization of Phagemid Particles

We assessed the internalization of various phage particles by COS-1 cells using a modified procedure of Poul and Marks (5). Briefly, phages were added to cells grown in 24-well tissue culture

plates in RPMI medium with 10% FBS and incubated for 2 h at 37°C. Cells were washed for 3 × 10 min in PBST at room temperature, and fixed with 4% formaldehyde for 30 min at room temperature. Cells were then washed for 3 × 10 min in PBS, permeabilized with cold methanol for 10 min and again washed for 3 × 10 min in PBS. They were blocked with 5% normal goat serum, 1% BSA in PBS for 1 h at room temperature, and incubated with anti-M13 antibody diluted at 1:200 in PBS containing 1% BSA. After another wash of 6 × 10 min in PBS,

Figure 1. Analysis of phage and phagemid particles. (A) DNA released from phage and phagemid particles was loaded on a 1% agarose gel. (B) Western blot analysis of peptide-pIII fusion proteins in phage and phagemid particles. Lanes 1 and 7: M13KO7P1 helper phage; lanes 2 and 8: phagemid particles rescued by M13KO7P1 helper phage; lanes 3 and 9: M13KO7 helper phage; lanes 4 and 10: phagemid particles rescued by M13KO7 helper phage. Lane 6: M13P1. Lane 5: λ DNA/HindIII marker (Takara, Dalian, China).

Figure 2. Internalization of peptide-targeted phagemid particles in COS-1 cells. Cells were incubated with phages or pEGFP-N1 phagemid particles [1×10^{10} plaque-forming unit (pfu)/mL] for 2 h and stained for internalized phage. (A) Phagemid particles (rescued by M13KO7P1); (B) M13P1; (C) phagemid particles (rescued by M13KO7).

fluorescein-labeled affinity purified antibody to mouse IgG (H+L; KPL, Guildford, UK) diluted at 1:50 in PBS containing 1% BSA was added and incubated for 30 min. Following a final rinse for 6×10 min in PBST, the cells were visualized under a fluorescence microscope (Axioskop 2; Carl Zeiss, Göttingen Germany). In some experiments, 0.5 mM synthesized P1 peptide (PKLLPWQSLRMH) was added simultaneously as a competitor.

In Vitro Phagemid Particle Transduction

COS-1 cells (2000 cells/well) were plated in a 24-well plate for 24 h before phagemid particle addition. Phages were added at 10^{11} pfu/mL in medium containing 10% FBS. After 48 h, the cells were visualized under a fluorescence microscope.

RESULTS AND DISCUSSION

While performing a screen for epidermal growth factor receptor (EGFR) binding peptide ligands, we discovered that the phage clone M13P1 from the Ph.D.-12 Phage Display Peptide Library could bind to and be internalized by COS-1 cells. We attempted to use the ligand sequence from this clone to modify the pIII gene of the filamentous helper phage M13KO7. In a previous study, it was demonstrated that the pVIII of helper phages could be modified by the addition of a peptide (19). Phagemids without the coding sequence of pVIII can be encapsulated by the helper phage. It was therefore hypothesized that the peptide could be adapted to be displayed on the pIII protein of

M13KO7. The modified phages were transformed into ER2738 host cells. The transformed cells formed smaller plaques compared with those transformed with M13KO7. The propagation rate of the M13KO7P1-transformed cells in liquid LB medium was also slower. The yield of M13KO7P1 was approximately 4.3×10^9 pfu/mL culture, while for M13KO7, it was approximately 3.8×10^{11} . These data indicated that the insertion of the extra peptide sequence into the amino terminal of pIII in M13KO7 may have affected helper phage propagation to some extent, but the effect was still tolerable.

The peptide-modified helper phages were then used for packaging phagemid pEGFP-N1, which encodes the green fluorescent protein (GFP) reporter gene. Unexpectedly, the titers of the resulting phagemid particles with M13KO7P1 helper or M13KO7 helper were similar (1.2×10^{12} and 1.8×10^{12} pfu/mL, respectively). It appears that the peptide-modified helper phage could package phagemid efficiently, even though its growth was somewhat compromised.

To assess the efficiency of phagemid packaging, we extracted ssDNA from the phagemid particles and analyzed it by gel electrophoresis. As shown in Figure 1A, both M13KO7 and M13KO7P1 can rescue phagemid DNA to form phagemid particles. There was only a small fraction of phage DNA detected in the packaged viral particles (Figure 1A). Furthermore, we have also prepared phagemid particles of the pGL3 control vector (Promega, Madison, WI, USA), which contains luciferase genes, using these two helper phages (data not shown). Therefore, this method constitutes a new and easy way of packaging various phagemids encoding reporter genes or therapeutic genes for gene therapy applications.

Immunoblot analysis was done using anti-pIII antibodies to evaluate the valency of peptide displays on the phage coat.

As shown in Figure 1B, the bands of M13P1, M13KO7P1, and phagemid particles rescued by M13KO7P1 had a slightly higher molecular weight than that of M13KO7 and phagemid particles rescued by M13KO7. This is as expected because the addition of the peptide ligand sequence followed by a linker (GGGS) should result in a molecular weight increase of about 1.76 kDa. No wild-type pIII was detected in the particles packaged by M13KO7P1, even after longer exposures (data not shown), indicating that most of the pIII proteins on the phagemid particles contained ligand peptide displays.

We also examined whether the assembled phagemid particles could be internalized by COS-1 cells. As shown in Figure 2, M13P1 and the phagemid particles packaged by M13KO7P1 were actively endocytosed by the cells after a 2-h incubation (Figure 2, A and B), while the addition of the excessive free peptide P1 completely inhibited the internalization, resulting in no fluorescent signal (data not shown). Additionally, the internalization of phagemid particles rescued by M13KO7 was barely detectable (Figure 2C). These data further demonstrate that the peptide was displayed on the coat of the phagemid particles and that it was the peptide that mediated the internalization of the phagemid particles. However, substantial GFP expression was only observed in about 3% of the cells (Figure 3) transfected with phagemid particles packaged by M13KO7P1. This suggested that there are other issues that need to be resolved, including endosome release, translocation into nucleus, DNA release, and transformation from ssDNA into double-stranded DNA (dsDNA).

In this study, we have described a quick and efficient method for preparing pIII-modified phagemid particles. By constructing and using helper phages, the process was completed in approximately 1 week, without the need to synthesize primers or sequence inserts. Moreover, it was shown that a phagemid could be constructed without the expression cassette of pIII, freeing more space for genes of therapeutic or other interest. Because the phagemid particle size is directly proportional to the size of the phagemid genome as

Figure 3. In vitro transfection of phagemid particles. COS-1 cells were transfected with pEGFP-N1 phagemid particles rescued by M13KO7P1. (A) Cells visualized under a fluorescence microscope. (B) Phase contrast image of the cells in the same field as panel A.

previous described (19), the resulting phagemid particles were expected to be smaller than those prepared using other methods. The small size of the gene delivery particle might enhance its penetration capacity when used in *in vivo* gene delivery. In addition, the expression cassette of pVIII may be further modified with an endosomal lytic or other peptide that can facilitate phagemid transduction. Taken together, the strategy used here should be useful for the development of new, targeted gene delivery vectors using phagemid particles containing ligand peptide.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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