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A polystyrene binding target-unrelated peptide isolated in the screening of phage display library

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ABSTRACT

Phage display is a powerful methodology for the identification of peptide ligands binding to any desired target. However, the selection of target-unrelated peptides (TUPs) appears as a huge problem in the screening of phage display libraries through biopanning. The phage-displayed peptide TLHPAAD has been isolated both in our laboratory and by another reserach group on completely different screening targets prompting us to hypothesize that it may be a potential TUP. In the current study, we analyzed the binding characteristics and propagation rate of phage clone displaying TLHPAAD peptide (SW-TUP clone). The results of ELISA experiment and phage recovery assay provided strong support for the notion that SW-TUP phage binds to polystyrene with a significantly higher affinity than control phage clones. Furthermore, this polystyrene binding was demonstrated to occur in a concentration- and pH-dependent mode. Characterization of the propagation profile of phage clones within a specified time course revealed no statistically significant difference between the amplification rate of SW-TUP and control phages. Our findings lead us to the conclusion that SW-TUP phage clone with the displayed peptide TLHPAAD is not a true target binder and its selection in biopanning experiments results from its bidning affinity to the polystyrene surface of the solid phase.

1. Introduction

Phage display is a versatile and powerful technology for the discovery of ligands which bind to any desired target. These targets consist of a wide variety of structures including inorganic materials, proteins, carbohydrates, cells, organs, and even whole organisms. In this technique, randomized foreign DNA sequences are genetically fused to one of the bacteriophage coat proteins. This allows the guest peptides encoded by exogenous inserts to be displayed on the outer surface of the phage virion. In this manner, a library of variant peptides is generated in which each phage clone represents a distinct amino acid sequence [1–3]. A phage display library can be screened against any target through an iterative cyclic selection procedure called biopanning. Phages displaying peptide ligands that bind specifically to the target are selected and then amplified. Several rounds of affinity selection and amplification progressively enrich the phage population in favor of peptides that have strong target binding characteristics [4,5].

The successful identification of high-affinity binding ligands for a variety of targets has been reported in the literature. However, phage display screenings suffer from a great limitation and that is the nonspecific selection of false positive peptides. These ambiguous results are referred to as target-unrelated peptides (TUPs) and can potentially sneak into and even become predominant in the output pool of biopanning [6,7]. In general, TUPs are placed in two categories of selection-related and propagation-related. Selection-related TUPs may prevail in biopanning owing to their binding to one component of the screening system other than the target such as solid phase, capturing reagents, and blocking substances. Propagation-related TUPs are selected because they are displayed on phage clones with faster propagation rates [6,8–11]. A review of the literature reveals that some sequences and motifs have previously been confirmed as binders to the different constituents of the screening system. For obvious reasons, TUPs are not proper candidates for potential application in the establishment of targeted diagnostics and therapeutics. Moreover, their misidentification as specific target-binding peptides could mislead further analysis and misdirect future research. Thus, these false positive peptides should be recognized and distinguished from true positive binders [9]. This discrimination between false binders and true ones can play a

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determining role in greater integrity of phage display selections. Recently, the development of bioinformatic tools and databases specialized for phage display has contributed enormously to the identification of possible TUPs in a set of peptides that arise in biopanning experiments. SAROTUP (Scanner and Reporter Of Target Unrelated Peptides) is the most important and comprehensive bioinformatics source for phage display community with multiple analysis tools and an extensive compilation of known or suspected TUP sequences that have been extracted from published phage display studies [12].

We isolated a phage clone called SW-TUP displaying TLHPAAD peptide through *in vitro* screening of a phage display library of 7-mer randomized peptides on SW480 (colorectal adenocarcinoma cell line) as the target. A database search through SAROTUP indicated that this peptide has also been reported previously by another research group. They selected TLHPAAD-displaying phage via screening of Ph.D.™-7 (the same library we used in our work) against epoxy [13]. The survey of accumulating evidence from phage display studies highlights the fact that an individual peptide is far less likely to be capable of binding specifically to unrelated targets with thoroughly different identities and structures. This raised suspicion that TLHPAAD sequence may not be a true binder for both targets and led us to advance the hypothesis that it may be a nonspecific target-unrelated peptide. Based on this, we addressed in detail the binding and propagation characteristics of SW-TUP clone. Our findings suggested that TLHPAAD is a TUP which binds to the polystyrene surface of the microtiter plates.

2. Materials and methods

2.1. Phage display library screening

SW480 adenocarcinoma cells were seeded (6×10^5 cells/well) onto polystyrene six-well plates (Corning Incorporated, Corning, NY, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM). After cells reached 80–90% confluency, they were kept in serum-free medium for 1 h. Cells were blocked with 2% bovine serum albumin (BSA) solution and then incubated with an aliquot of the phage display library containing 10^{11} pfu with gentle shaking. After 1 h, the unbound phages were removed by multiple washes with PBS and PBST. The cell-bound phages were harvested by adding acidic elution buffer and then neutralized with 1 M Tris-HCl. A small amount of the recovered phage was used for titering and the rest was amplified for the next round of *in vitro* selection. The same polystyrene microwell plates used in the procedure of library affinity selection were also exploited for all binding assays.

2.2. Phage amplification and titering

The Ph.D.™-7 phage display peptide library kit was purchased from New England BioLabs (Beverly, Massachusetts, USA). This library is a derivative of M13mp19 vector and its phages display on their surface a diverse pool of randomized 7-mer peptides fused to the N-terminus of the minor coat protein (pIII) of M13KE phage [14].

Escherichia coli strain ER2738 (New England BioLabs, USA) was used to amplify phages. The strain was grown overnight in liquid LB medium to obtain log-phase bacterial culture (OD = 0.5). The phage suspension was added to 20–25 mL of the culture and the mixture was incubated for 4.5 h at 37 °C with vigorous shaking. Bacterial cells were removed from the mixture by centrifugation. To precipitate phage particles, 1/6 volume of 20% polyethylene glycol (PEG)-8000/2.5 M NaCl was added to the supernatant and incubated overnight at 4 °C or for 2 h on ice. The next day, phages were harvested by centrifugation at 13,000 rpm for 15 min. Two successive precipitation steps were applied to obtain a pure phage suspension. The resulting phage pellet was dissolved in Tris Buffered Saline (TBS) and stored at 4 °C [14].

For phage titering and plaque assay, the phage suspensions were serially diluted in LB medium. The phage dilutions were added to log-phase cultures of ER2738 bacterial strain. Following incubation for 5–10 min at room temperature to allow the infection of bacterial cells by phage virions, 2–3 mL of melted top agar (45 °C) was added to the mixture. The suspension was then poured onto the surface of LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 40 mg/mL) (AppliChem, Germany) and isopropyl β-D-thiogalactopyranoside (IPTG; 50 mg/mL) (AppliChem, Germany). The plates were incubated overnight at 37 °C. The titer of each phage suspension was determined as pfu/mL by counting the number of blue plaques appeared after 12–16 h [15].

2.3. ELISA assay for target binding

SW480 colon adenocarcinoma cells were grown in DMEM at 25 cm^2 cell culture flasks. The cultured cells were counted and 1×10^4 cells were seeded onto each well of a multi-well plate (Corning Incorporated, Corning, NY, USA) followed by an overnight incubation. The next day, when cells reached 80% confluency they were exposed to serum-free medium for 1 h and washed three times with PBS. To fix the cells, they were incubated with ice-cold 4% paraformaldehyde (PFA) for 10–15 min. The fixed cells were washed with PBS containing 0.05% Tween 20 (PBST) and then blocked by adding 200 μL of 2% BSA at 37 °C for 1.5–2 h 1×10^9 pfu of each phage clone (SW-TUP and control phage clones) was added to the wells containing blocked cells and the plate was incubated at 37 °C for 1 h followed by washing five times with washing buffer. Washing steps were performed in order to remove phages not bound to cells. 100 μL of anti-M13 mouse monoclonal antibody (Abcam Inc, MA, USA) was added to each well and incubated for 1 h. The wells were washed five times with 200 μL of PBST, incubated with 100 μL of rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam Inc, MA, USA) for 1 h and washed 5 times with PBS. To detect the remaining phages, 100 μL of freshly prepared TMB (3,3′,5,5'-tetramethylbenzidine) substrate was added to each well and the plate was incubated for 5 min in the dark place. The reaction was terminated by adding 50–100 μL of 0.5 M H_2SO_4 and the absorbance values were recorded by an automated ELISA plate reader (BioTek, USA) at 450 nm [16].

2.4. Polystyrene binding

Polystyrene binding was investigated as described previously [17]. To evaluate the binding affinity of SW-TUP phage clone to the polystyrene surface of the microtiter plate, the effect of different washing reagents and blocking buffers was examined on phage binding. The wells of the microtiter plate were blocked with 200 μL of 1% BSA, 1% nonfat milk (NFM), PBS, PBST, TBS, and TBST for 2 h. 1×10^9 pfu of SW-TUP and control phages was applied to the wells and incubated for 1 h. The wells were then washed five times with 200 μL of corresponding buffer to remove the unbound phages. Each wash lasted for 5 min. Phages were then incubated with primary anti-M13 antibody and secondary antibody. To detect phages that remain attached to polystyrene, color development was carried out by using TMB as described previously.

2.5. Phage recovery assay

1×10^9 pfu of each phage clone (diluted in PBS) was poured into separate wells of a 96-well microtiter plate and incubated for 1 h at room temperature with gentle agitation. The wells were washed