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Identification and characterization of mutant clones with enhanced propagation rates from phage-displayed peptide libraries

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ABSTRACT

A target-unrelated peptide (TUP) can arise in phage display selection experiments as a result of a propagation advantage exhibited by the phage clone displaying the peptide. We previously characterized HAIYPRH, from the M13-based Ph.D.-7 phage display library, as a propagation-related TUP resulting from a G → A mutation in the Shine-Dalgarno sequence of gene II. This mutant was shown to propagate in *Escherichia coli* at a dramatically faster rate than phage bearing the wild-type Shine-Dalgarno sequence. We now report 27 additional fast-propagating clones displaying 24 different peptides and carrying 14 unique mutations. Most of these mutations are found either in or upstream of the gene II Shine-Dalgarno sequence, but still within the mRNA transcript of gene II. All 27 clones propagate at significantly higher rates than normal library phage, most within experimental error of wild-type M13 propagation, suggesting that mutations arise to compensate for the reduced virulence caused by the insertion of a lacZα cassette proximal to the replication origin of the phage used to construct the library. We also describe an efficient and convenient assay to diagnose propagation-related TUPS among peptide sequences selected by phage display.

Phage display is a powerful and popular technique used to identify peptide ligands for a variety of protein and nonprotein molecules, including antibodies, cell surface receptors, enzymes, small molecules, and inorganic materials [1–5]. A library of peptide or protein sequences is displayed on the outer surface of the bacteriophage virions as a result of a randomized DNA insert fused to the gene of a coat protein. Peptide ligands specific for a given target can be identified from the phage display library in an iterative selection process called panning. Phage that bind to the target are amplified, and subsequent rounds of panning and amplification enrich the pool in favor of phage bearing peptides that have the desired binding properties. Because each displayed peptide is physically linked to the gene that encodes it, identification of each selected peptide follows directly from sequencing the viral DNA. The widely used Ph.D.-7 and Ph.D.-12 libraries display random sequences of heptapeptides or dodecapeptides, respectively, at the N-terminus of the M13 bacteriophage minor coat protein pIII, which is present in 5 copies at one end of the phage virion [1]. The pentavalent display of peptides on the M13 virion does not measurably affect the infectivity of the phage [6,7]. These libraries are constructed using the simple M13mp19 derivative M13KE [8], which carries the lacZα cassette cloned proximal to the phage replication origin, for blue/white screening of phage clones on X-gal indicator plates. Each library has a complexity in excess of 2 billion independent clones, displaying a wide diversity of sequences with no significant positional biases apart from the expected censoring of proline and positively charged residues at the N-terminus of the secreted peptide-pIII fusion, and single cysteines throughout the displayed peptide [9].

Target-unrelated peptides (TUPs)[1] are phage-displayed peptides that are enriched in the pool of phage during the rounds of panning, but are not actually selected as a result of their affinities for the target. Because they can dominate the pool of selected peptides at the conclusion of a phage display experiment, they can be falsely

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[1] *Abbreviations used:* WT-M13, wild-type M13 bacteriophage; TUP, target-unrelated peptide; Ph-*PeptideSequence*, M13 phage displaying the peptide with the given sequence; 5′-UTR, 5′-untranslated region; LB, Luria-Bertani; pfu, plaque-forming units; MOI, multiplicity of infection; ori, origin of replication; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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identified as target-binding peptides. TUPs can be classified as "selection-related" or "propagation-related" [7,10]. Selection-related TUPs are displayed peptides that bind to a component of the screening system other than the target itself [11,12], such as polystyrene (e.g., microtiter wells or Petri dishes, even when blocked), streptavidin (the capturing agent for biotinylated targets), or bivalent metal ions (used to immobilize His-tagged protein targets) [11,12]. For example, selection-related TUPs may contain the WXXW motif, which can bind to polystyrene [13–16], or the HPQ motif, which binds to streptavidin [17–20]. It can be difficult to recognize selection-related TUPs since the assays used to confirm target-binding often employ the same components as the initial panning experiment.

In contrast, a propagation-related TUP is a peptide that is coincidentally displayed on a library phage clone that contains an advantageous mutation or genetic rearrangement, allowing it to propagate faster than the rest of the library [7,10]. During the amplification steps carried out in *Escherichia coli* between rounds of panning, the concentration of phage having a propagation advantage increases more rapidly than that of the normal library phage [21,22]. At the end of the phage display experiment, the peptides displayed by these faster phage clones may dominate the pool of selected sequences even though they are not target-binding peptides. We characterized the first known propagation-related TUP in our work with the Ph.D.-7 library [7]. This phage clone, displaying the peptide HAIYPRH, contains a single G → A mutation in the 5′-untranslated region (5′-UTR) of gene II in M13. The mutation allows the HAIYPRH phage clone to propagate significantly faster than the rest of the phage. HAIYPRH has arisen as a TUP in several phage display experiments employing various targets [12]. Smith and coworkers have also identified three propagation-related TUPs in a phage display library constructed in the vector fd-tet [10]. These clones resulted from the spontaneous restoration of the minus-strand ori, which had been disrupted by the tetracycline resistance cassette in the construction of fd-tet. With the minus-strand ori no longer compromised, the rearranged clones propagate faster than the rest of the library phage.

Due to the continuing popularity of phage display, there has been much interest in target-unrelated peptides in recent years. Compilations of known or suspected TUP sequences [11,12] and the online database SAROTUP [23,24] have made it possible to identify many of the TUPs that arise in panning experiments. Numerous TUPs have been assigned as selection-related or propagation-related, and are accompanied by the underlying causes for their respective appearances in phage display experiments. However, several TUPs such as LPLTPLP and SVSVGMKPSPRP (from the Ph.D.-7 and Ph.D.-12 libraries, respectively) became apparent simply because they have arisen in multiple panning experiments employing various and unrelated targets [12]. They have not been demonstrated to be selection-related, and yet no propagation advantage (or genetic cause thereof) has been identified either. Conversely, numerous propagation-related TUPs are suspected to be present in phage display libraries based on their abundances in next-generation sequencing analysis of naïve and amplified libraries [22], but they generally have not been identified by any ubiquity across phage display experiments in various laboratories, nor has the genetic basis for their relative abundances been characterized. Peptides not reported in databases and not found in the phage display experiments of other labs can still arise as TUPs in new experiments, especially when newly constructed libraries are used.

Here we report 24 new peptides from the Ph.D.-7 and Ph.D.-12 libraries that are displayed on fast-propagating phage bearing 14 different mutations either in the 5′-UTR of gene II or just upstream of it, as well as three fast-propagating clones harboring such mutations but not displaying peptides. Some of these clones were found by deliberately mining the libraries, while others were discovered serendipitously. Some of these peptides have been previously reported in the literature as true ligands selected by phage display, albeit with no knowledge that their selection may be due, at least in part, to propagation advantages. We also describe a simple assay that can efficiently characterize the relative propagation rate of a potential target-binding phage to determine whether it may carry a propagation-related TUP.

Materials and methods

Materials

The Ph.D.-7 Phage Display Peptide Library (lot 3), Ph.D.-12 Phage Display Peptide Library (lot 8), *E. coli* ER2738 (*F′ proA⁺B⁺ lacI^q Δ(lacZ)M15 zzf::Tn10(Tet^R)/fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5*), M13KE phage, and the -96 gIII sequencing primer S1259S 5′d(CCCTCATAGTTAGCGTAACG) were supplied by New England Biolabs, Inc. (Ipswich, MA). Custom primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Chelating Sepharose Fast Flow resin was from Amersham Biosciences (Piscataway, NJ). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were from American Bioanalytical, Inc. (Natick, MA). Polyethylene glycol 8000 (PEG) was from Sigma–Aldrich, Inc. (St. Louis, MO). All other materials and reagents were from Fisher Scientific.

General methods

All methodology for the use of the Ph.D. libraries, including preparation of media and solutions, ER2738 strain maintenance, phage amplification and titering, and purification of single-stranded M13 viral DNA is described in the Ph.D.™ Phage Display Libraries Instruction Manual [9] and in the literature [1]. DNA sequencing was performed by the New England Biolabs Sequencing Core Facility with an Applied Biosystems 3730xl DNA Analyzer, using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). For sequencing of the randomized peptide region of the Ph.D. libraries, either the -96 gIII sequencing primer or the custom primer 5′-d(CCGTAACACTGAGTTTCGTCACC) was used. The custom primer 5′-d(GGCCGGAGACAGTCAAATCACC) was used to sequence the 5′-untranslated region of gene II. A panel of 20 custom primers was applied to the full Sanger dideoxy sequencing of the M13KE genome. All statistical computations were carried out using JMP v. 11.0.

Panning of the Ph.D.-7 and Ph.D.-12 libraries against Zn^{2+}

Panning of the Ph.D.-7 and Ph.D.-12 libraries was performed as described previously [7]. Two modifications were used in some of the experiments to diversify the identity and number of Zn^{2+}-chelating side chains in the selected peptides. First, various pH values were obtained by buffering the solutions with 0.1 M sodium phosphate (pH 6.0, 6.5 or 7.5). Second, after washing the resin with incubation buffer, it was also washed with 1 mL of 1 mM zinc chloride ("zinc wash") before the usual treatment with washing buffer. The particular conditions for each experiment are outlined in the footnote to Table 1.

Serial amplification of the Ph.D.-7 and Ph.D.-12 libraries

For the first round of serial amplification, 10 μL of Ph.D.-7 or Ph.D.-12 library was combined with 20 mL of early log ER2738 culture and shaken at 250 rpm for 4.5 h at 37 °C. The amplified phage solution was obtained from sequential PEG precipitations, following standard protocols [1,9]. Rounds 2 and 3 (and in some cases Round 4) of serial amplification were carried out exactly as