Analytical Biochemistry 462 (2014) 35–43

Contents lists available at ScienceDirect

Analytical Biochemistry

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

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

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ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form 4 June 2014
Accepted 8 June 2014
Available online 19 June 2014

Keywords:
Phage display
Target-unrelated peptides
Propagation-related TUPs
M13
5′-Untranslated region

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.

http://dx.doi.org/10.1016/j.ab.2014.06.007
0003-2697/

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

Table 1
Summary of mutant phage clones identified in panning against Zn^{2+}.

Name of clone	Mutation	Experiment No.	Occurrences
Ph-HAIYPRH	G6813A	2	1/12
		11	1/12
		13	4/12
		14	1/12
Ph-HQLHHHL	C6810T	1	1/12
		6	1/12
Ph-HLHDTNH	A6809C	6	1/12
Ph-QLHRHHH	T6798C	1	1/12
		2	7/12
		3	5/12
		5	1/12
Ph-HSHHHSA	T6798C	15	1/12
Ph-HPPHHNT	T6798Δ	2	2/12
		5	1/12
Ph-HAFPHLH	G6793Δ	6	1/12
Ph-HDHRYPK	G6792T	3	1/12
		12	2/12
Ph-HTPQVHHPELTH	G6813A	18	2/20
Ph-HLHRLHTHEHSK	T6795Δ	18	2/20
Ph-TGITNWEVRTSR	C6794A	18	1/20
Ph-GWKSHLTHHHAE	G6793A	18	3/20
Ph-HLFHLTPYHGHE	G6748Δ	18	1/20
Ph-TLITFHHHEHVN	C6589T	18	1/20

The panning conditions for each experiment were as follows: Exp.1 (Tris–HCl, pH 7.5, negative selection before Round 2), Exp. 2 (Tris–HCl, pH 7.5, negative selection before Rounds 2 and 3), Exp. 3 (Tris–HCl, pH 7.5, negative selection before Rounds 2 and 3, zinc wash in every round), Exp. 5 (Tris–HCl, pH 7.5), Exp. 6 (Tris–HCl, pH 7.5, zinc wash in every round), Exp. 11 (sodium phosphate, pH 6.5, negative selection before Rounds 2 and 3), Exp. 12 (sodium phosphate, pH 6.0, negative selection before Rounds 2 and 3), Exp. 13 (sodium phosphate, pH 6.5, negative selection before Rounds 2 and 3), Exp. 14 (sodium phosphate, pH 6.0), Exp. 15 (sodium phosphate, pH 6.5), Exp. 18 (Tris–HCl, pH 7.5). Each fraction indicates the number of occurrences of the particular clone over the total number of clones sequenced.

described above using 10 μL of the amplified phage from the previous round. Plaques from the ultimate phage suspension were amplified and the viral DNA was purified and sequenced.

A 135-min screen of the amplified library

Twelve or more plaques from round 3 amplified phage were each used to infect 1 mL of early log ER2738 culture. After 135 min of shaking at 250 rpm and 37 °C, 10^5-fold and 10^6-fold dilutions of each growing culture were plated and the plaques were counted the next day. Clones of interest were identified by amplifying individual plaques, followed by purification and sequencing of the viral DNA.

Comparison of propagation rates

A stock solution of each phage clone was prepared by amplifying a plaque or approximately 1×10^8 pfu of the same phage (WT-M13, M13KE, or a mutant clone) in 20 mL of early log ER2738 cells. The phage solution was titered by standard methods [1,9]. All clone identities were carefully verified by purifying and sequencing the viral DNA from plaques. For each clone, 1×10^8 virions were combined with 20 mL of early log ER2738 culture and shaken at 250 rpm and 37 °C. At various time points, 10 μL of an appropriate dilution of the culture was plated. The next day the plaques were counted and the concentration of phage in the growing culture at each time point was determined in pfu/μL.

Results

Discovery of fast-propagating M13KE mutants

Panning experiments using immobilized Zn^{2+} as a target were aimed at identifying Zn^{2+}-binding peptides in the Ph.D.-7 and Ph.D.-12 libraries. As expected, most of the selected peptides contained at least three histidine residues. When their genomes were sequenced, several of the Zn^{2+}-binding phage clones were found to contain single mutations in the 5′-UTR of gene II (Table 1). Two phage clones from the Ph.D.-12 library instead had single mutations upstream of this region. Not surprisingly, most of these histidine-rich peptides demonstrated Zn^{2+}-binding in phage-ELISA experiments (data not shown). As previously reported, the gene II Shine-Dalgarno mutant Ph-HAIYPRH (G6813A) arose in several panning experiments, but did not show any affinity for Zn^{2+} [7]. It should be noted that several other Zn^{2+}-binding peptides discovered by panning (not shown) were displayed on phage with unmutated genome sequences.

To rapidly identify additional fast-propagating phage clones independently of zinc-binding, serial amplifications of the Ph.D.-7 library were performed, omitting the intermediate panning steps. Three separate amplification regimens, each consisting of multiple rounds of amplification of the Ph.D.-7 library, were carried out, and the third and fourth rounds were mined for potential fast-propagating clones. The amplified phage was plated and a large number of plaques were picked for DNA purification and sequencing. The repeated appearance of a particular peptide sequence suggested a phage clone that was enriched by amplification. Several peptides came up more than once and were therefore sequenced upstream of gene II (Table 2). Ph-HAIYPRH (G6813A) had been identified in our earlier work [7] and Ph-AKIDART was now found to contain the same mutation. Ph-ANTLRSP had the A6809C mutation already observed in Ph-HLHDTNH, and Ph-KLPGWSG shared the G6792T mutation with Ph-HDHRYPK (Table 1). A few phage clones arose more than once across the three serial amplification experiments, but did not contain gene II 5′-UTR mutations (Ph-HPQLLRS, Ph-TAPYFPS, and Ph-YPGNLTG). Note that although Ph-HPQLLRS is predicted to be a streptavidin binder, there were no panning steps against streptavidin in this experiment.

In a third approach, a screen based on propagation rates was used to directly identify fast-propagating clones. The greatest difference in phage concentration between normal- and fast-propagating M13-based phage infecting *E. coli* is observed close to the 135-min mark (see Fig. 1 and figures in Ref. [7]). Randomly selected plaques from the amplified Ph.D.-7 and Ph.D-12 libraries were used to infect separate 1 mL cultures of *E. coli*. At 135 min, the cultures were diluted appropriately and plated, and the plaque counts were compared (Table 3). The clones giving the largest number of plaques were candidates for mutants and were characterized by sequencing the viral DNA. Clones having the smallest number of plaques were used as negative controls. Two novel mutations were found: A6802T (Ph-SNHAPRH) and C6799T (Ph-HEASQHAFSARL). The other fast-propagating clones were new in terms of the peptides displayed (Ph-ARPPASP,

Table 2
Summary of phage clones identified from the serial amplification of the Ph.D.-7 library.

Name of clone	Mutation	Serial amplification experiment	Occurrences
Ph-HAIYPRH	G6813A	Exp. 1, Round 3	9/54*
		Exp. 2, Round 3	1/36*
		Exp. 3, Round 3	4/44*
		Exp. 1, Round 4	1/24
Ph-AKIDART	G6813A	Exp. 1, Round 3	2/54
Ph-ANTLRSP	A6809C	Exp. 1, Round 3	1/54
		Exp. 1, Round 4	1/23
Ph-KLPGWSG	G6792T	Exp. 1, Round 3	2/54

Each fraction indicates the number of occurrences of the particular clone over the total number of clones sequenced.
* Results indicated with an asterisk were previously reported in our earlier work [7].

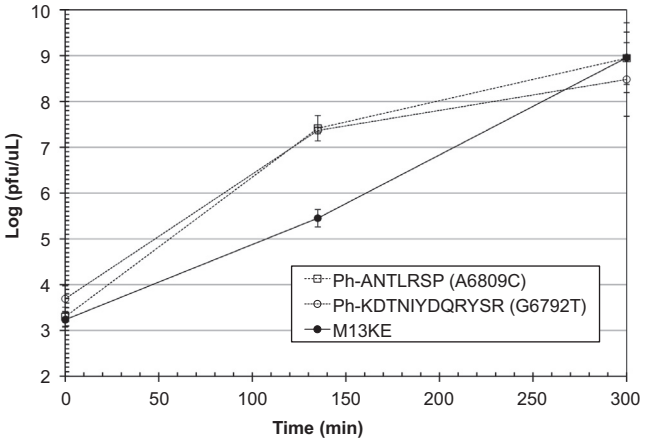

Fig.1. Time course for amplification of M13KE and two mutant phage clones. Each phage clone was amplified in three separate early log cultures of *E. coli* ER2738. From each culture, one aliquot was diluted and plated at the indicated incubation times, and the concentration of phage (pfu/μL) in each growing culture was determined based on plaque counts. Data points represent the average concentration of the three separate cultures for each type of phage, and the error bars show the 95% confidence interval. Statistical analysis indicated significant differences among the phage concentrations for the three sets of data at 135 min (ANOVA; $P < 0.0001$). Post hoc analysis showed that Ph-ANTLRSP and Ph-KDTNIYDQRYSR were not significantly different from each other (Tukey's HSD; $P = 0.7390$). Both mutant clones were significantly different from M13KE ($P < 0.0001$).

Ph-AMSPRMDGKVFA, Ph-GKPMPPM, Ph-KDTNIYDQRYSR, and one phage clone with no displayed peptide), but contained gene II 5′-UTR mutations that had already been identified in panning experiments (Table 1) or serial amplification (Table 2).

Four additional mutant clones were discovered beyond the experiments described above. In a single amplification to prepare a concentrated stock solution of M13KE phage (no displayed peptide), two clones were found having the G6793A and G6793Δ mutations already corresponding to displayed peptides (Table 1). Two additional mutant clones were discovered as contaminants in concurrent but unrelated experiments involving recombination/mutagenesis of the M13KE genome. Ph-SLLSHNS (T6798C) and Ph-LSMTGRD (G6793A) had mutations we had already observed. All the phage clones containing mutations upstream of gene II are summarized in Table 4. To rule out additional mutations beyond the region indicated in Table 4, we sequenced the entire genome of a representative set of clones (M13KE, HAIYPRH, ANTLRSP, SNHAPRH, QLHRHHH, AMSPRMDGKVFA, LSMTGRD, GWKSHLTHHHAE, HAFPHLH, G6793Δ (no peptide), GKPMPPM, KLPGWSG, HLFHLTPYHGHE, and TLITFHHHEHVN). All sequences matched M13KE, with the exception of a T5091C mutation found in four clones: Ph-SNHAPRH, Ph-LSMTGRD, Ph-HAFPHLH, and Ph-KLPGWSG. This is a silent mutation in the third position of the Val290 codon in gene IV (GTT → GTC).

Characterization of propagation rates of M13KE mutants

Based on our earlier findings for Ph-HAIYPRH (G6813A) [7], we suspected that all of the mutants would have higher propagation rates than M13KE, the library vector having no displayed peptide and an M13mp19-like genome. To compare the propagation rates, a time course for phage amplification was performed using one putative propagation-enhanced clone each from the Ph.D.-7 and Ph.D.-12 libraries (Fig. 1). We grew separate cultures of ER2738 cells infected with each phage clone at identical multiplicity of infection (MOI) and monitored the amount of phage in the cultures throughout a 5-h incubation. All cultures began with the same concentration of phage (5×10^3 pfu/μL), but during the middle of the incubation (~135 min), the mutants Ph-ANTLRSP (A6809C) and Ph-KDTNIYDQRYSR (G6792T) had concentrations that were two orders of magnitude higher than that of M13KE. At the end of 5 h, all phage cultures leveled off at 10^8–10^9 pfu/μL, which is typical for M13 phage growing in *E. coli* cells [1]. These results indicate a dramatically higher rate of propagation during the earlier stages of incubation for phage containing gene II 5′-UTR mutations.

The propagation rates of all 27 mutant phage clones, as well as Ph-HAIYPRH [7], were compared using just the 135-min time point; Fig. 2 thus serves as a slice of the middle of such a time course as described above. Fig. 2 clearly demonstrates that each mutant phage has a concentration 1–2 orders of magnitude higher than M13KE at 135 min. According to statistical analysis, several groups of clones have no significant concentration differences within the group, but a few groups of clones are significantly different from other groups; e.g., HDHRYPK, KLPGWSG, and GKPMPMM are all significantly higher than SLLSHNS, QLHRHHH, HEASQHAFSARL, GWKSHLTHHHAE, HPPHHNT, SNHAPRH, HAFPHLH, and ARPPASP. When analyzed by mutation, the fastest mutations G6813A, A6809C, and G6792T, along with WT-M13, all have 135-min concentrations that are significantly higher than the slowest mutations C6799T, T6798C, and T6798Δ (refer to Table 4). Moreover, G6813A and G6792T had significantly higher concentrations than A6802T. All other mutations fall in between the highest and lowest concentrations, with statistical overlap among them.

The concentration of phage at 135 min of infection, as shown in Fig. 2, can identify a potential propagation-related TUP among clones selected by phage display. The assay only requires an accurate titer of a concentrated phage stock solution for each clone of interest as well as the M13KE control. The concentration range 5×10^6–2×10^7 pfu/μL, which can be prepared from a simple dilution of the amplified phage, will conveniently deliver 1×10^8 virions to an *E. coli* culture using a starting volume of 5–20 μL diluted

Table 3
135-min screens of the serially amplified (round 3) Ph.D.-7 and Ph.D.-12 libraries.

Amplified library	No. plaques (10^5 dilution)	No. plaques (10^6 dilution)	Mutation	Peptide
Ph.D.-7	9	1	Normal (negative control)	LRTDPIF
Ph.D.-7	29	2	Normal (negative control)	No Peptide
Ph.D.-7	641	85	A6802T	SNHAPRH
Ph.D.-7	2008	174	G6792T	GKPMPPM
Ph.D.-7	2208	253	T6798C	ARPPASP
Ph.D.-12	24	3	Normal (negative control)	VFCTTPQRPITS
Ph.D.-12	1536	103	A6809C	No Peptide
Ph.D.-12	1616	146	C6799T	HEASQHAFSARL
Ph.D.-12	2336	220	G6792T	KDTNIYDQRYSR
Ph.D.-12	3216	250	T6795Δ	AMSPRMDGKVFA

Each value given in the table is the number of plaques obtained when 10 μL of diluted phage-infected *E. coli* culture was plated at 135 min of incubation.

Table 4
Summary of mutations upstream of gene II in M13KE.

Name of Clone	Disc.	Mutation	5'-UTR of gene II mRNA	log(pfu/μL) at 135-min	Lit.	Mimo-DB	SAROTUP (PhD7Faster)	Naïve Abund.
Normal M13KE Phage Wild-type M13			***GUUUUUGGGGCUUUUC***UGAUUAUC**AACCGGGGUA**CAUAUG...	5.38 7.51				
Ph-HAIYPRH Ph-AKIDART Ph-HTPQVHHPELTH	a, b b a	G6813A	----------A----------...	7.45 7.45 7.52	x	x	x	0.0005 0.00023 0.00016
Ph-HQLHHHL	a	C6810T	----------U----------...	7.18			x	
Ph-ANTLRSP Ph-NoPeptide Ph-HLHDTNH	b c a	A6809C	----------C----------...	7.50 7.13 7.26	x	x	(x)	0.00004
Ph-SNHAPRH*	c	A6802T	----------U----------...	6.91				
Ph-HEASQHAFSARL	c	C6799T	----------U----------...	6.71				
Ph-ARPPASP Ph-QLHRHHH Ph-HSHHHSA Ph-SLLSHNS	c a a e	T6798C	----------C----------...	7.01 6.68 7.36 6.59	x	x	x x (x)	$8x10^{-7}$
Ph-HPPHHNT	a	T6798Δ	----------Δ----------...	6.86			(x)	
Ph-AMSPRMDGKVFA Ph-HLHRLHTHEHSK	c a	T6795Δ	----------Δ----------...	7.11 7.04			(x)	$3x10^{-6}$
Ph-TGITNWEVRTSR	a	C6794A	----------A----------...	7.05				
Ph-LSMTGRD* Ph-GWKSHLTHHHAE Ph-NoPeptide	e a d	G6793A	----------A----------...	7.19 6.84 7.32			x	
Ph-HAFPHLH* Ph-NoPeptide	a d	G6793Δ	----------Δ----------...	6.95 7.33	x			
Ph-GKPMPPM Ph-HDHRYPK Ph-KLPGWSG* Ph-KDTNIYDQRYSR	c a b c	G6792T	--------U----------...	7.69 7.68 7.69 7.49	x x x	x x x	(x) (x)	0.0014 0.00007 0.00008
Ph-HLFHLTPYHGHE	a	G6748Δ	Upstream of 5'-UTR (gene II promoter)	7.15			(x)	
Ph-TLITFHHHEHVN	a	C6589T	Upstream of 5'-UTR (lacZα insertion)	7.17				

In the normal M13KE sequence, the Shine-Dalgarno (SD) region is in bold, the gene II RNA operator sequence is italicized + bold, and the start codon is underlined. Phage clones are named according to the displayed peptide; some phage clones in the table have no peptide insert as indicated. Mutant clones were discovered as follows: [a] in Ph.D.-7 or Ph.D.-12 phage display experiments using Zn^{2+} as the target, [b] in serial amplification of the Ph.D.-7 library, [c] in a 135-min screen of the serially amplified Ph.D.-7 or Ph.D.-12 library, [d] in the amplification of M13KE, [e] as a contaminating clone in a concurrent experiment in our lab. An asterisk (*) indicates the concomitant mutation T5091C. The log(pfu/μL) at 135 min of incubation with *E. coli* cells is the same value plotted in Fig. 2. Each "x" indicates that the peptide has been identified in panning experiments reported in the literature and/or is present in the databases MimoDB, SAROTUP, and/or PhD7Faster, which is part of the SAROTUP suite (see details under 'Discussion'). The last column contains the abundances of certain clones in the naïve library as determined through deep sequencing (number of occurrences divided by 4×10^6 clones analyzed) as reported by Derda and coworkers [22] or by personal communication with Ratmir Derda. Clones without abundances indicated were not detected in Derda's study.

phage suspension. The concentration of phage 135 min into the infection of the cells can be determined by plating a 10^4–10^6-fold diluted aliquot of the cell culture. If the phage concentration is significantly higher than that of M13KE (10- to 100-fold, or 1–2 on the log scale), it is very likely that the phage clone possesses a propagation advantage due to a mutation or other genetic rearrangement.

Discussion

A new repertoire of propagation-related TUPs

Several different approaches have led us to discover 27 new M13KE clones carrying single-base mutations. Including the first discovered mutation, G6813A in Ph-HAIYPRH [7], this collection consists of 14 unique mutations and 25 different peptides (three clones have no displayed peptide). The majority of the mutations were identified in panning experiments using Zn^{2+} as the target. Table 1 contains 14 different clones, but because of duplication for G6813A and T6798C, there are only 12 unique mutations in this group. Serial amplification revealed the new clones Ph-AKIDART, Ph-ANTLRSP, and Ph-KLPGWSG (Table 2), but all three phage had mutations previously seen. The 135-min screen, which identified fast-propagating mutants by increased plaque count during the exponential phase of propagation, yielded seven new clones including the two new mutations A6802T and C6799T (Table 3). Table 4 summarizes all the mutant phage clones arranged according to their mutations. Seven out of 14 mutations are represented by at least two different phage clones, and 5 of these mutations have 3–4 different clones. Our extensive mining of the Ph.D.-7 and Ph.D.-12 libraries has focused this repertoire to a fairly small number of unique mutations, with several redundancies, in a very specific region of the M13KE genome. While the phage display libraries are likely to contain additional mutant clones having peptides we have not yet seen, we suspect that additional mutations conferring a fast-propagation phenotype will be confined to this region of the M13KE genome.

The amplification time course shown in Fig. 1 compares the propagation of M13KE to two different mutant clones, Ph-ANTLRSP (A6809C) from the Ph.D-7 library and Ph-KDTNIYDQRYSR (G6792T) from the Ph.D.-12 library. These two clones were chosen for full characterization because together they represent both Ph.D. libraries as well as two mutations that have significant redundancy in the collection (see Table 4). Additionally, the A6809C mutation is in the gene II Shine-Dalgarno sequence, while the G6792T is in the gene II mRNA operator sequence. Both mutant phage clones

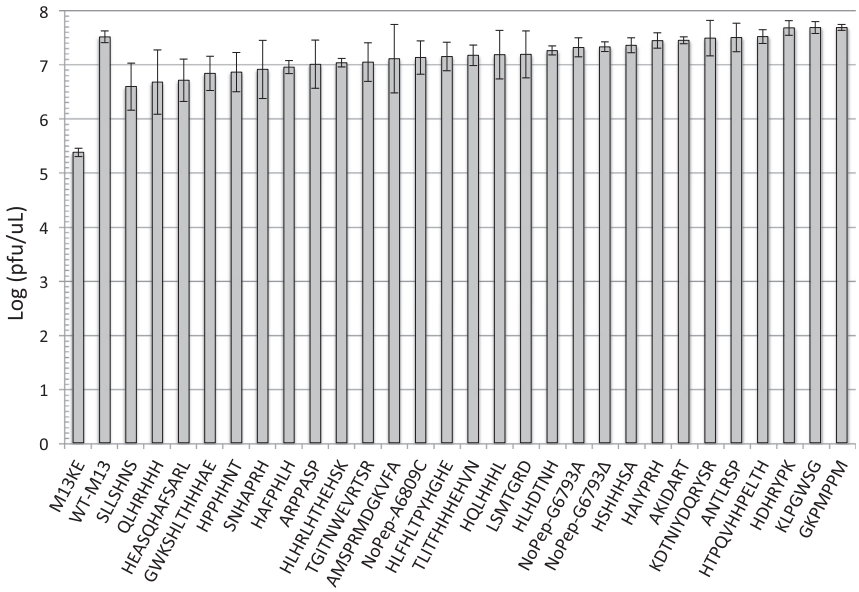

Fig.2. Phage concentrations of all mutant phage clones at 135 min of incubation. Each phage clone was amplified separately in an ER2738 culture. At 135 min, three aliquots from each flask of growing culture were diluted and plated, and the concentration of phage (pfu/μL) was determined based on plaque counts. The M13KE control was run 20 times for a total of $n = 60$ platings, and each other phage clone was run twice for a total of $n = 6$ platings. Each bar represents the average concentration of all platings for a given clone, and the error bars show the 95% confidence interval. Mutant clones are arranged in order of increasing phage concentration. Every clone (mutants and WT-M13) was found to be significantly different from M13KE (ANOVA; $P < 0.0001$). Post hoc analysis (Tukey's HSD) showed that several groups of clones have no significant concentration differences within the group, but a few groups of clones are significantly different from other sets; e.g., HDHRYPK, KLPGWSG, and GKPMPMM are all significantly higher than SLLSHNS, QLHRHHH, HEASQHAFSARL, GWKSHLTHHHAE, HPPHHNT, SNHAPRH, HAFPHLH, and ARPPASP. When analyzed by mutation, the fastest mutations G6813A, A6809C, and G6792T, along with WT-M13, all have 135-min concentrations that are significantly higher than the slowest mutations C6799T, T6798C, and T6798Δ. Moreover, G6813A and G6792T had significantly higher concentrations than A6802T. All other mutations fall in between the highest and the lowest concentrations, with statistical overlap among them.

propagated significantly faster than M13KE in the early stages of incubation, resulting in phage concentrations that were 100-fold higher at 135 mins. The relative propagation rates of all the mutant clones are compared in Fig. 2, which plots the phage concentration at 135 min of incubation in *E. coli*. Every mutant clone has a concentration at least 10 times higher than that of M13KE, and many clones approach the concentration of the wild-type M13.

In a culture containing mixed clones, higher propagation rates give mutant phage clones sufficient evolutionary advantage over phage with normal genome sequences, as was demonstrated for Ph-HAIYPRH [7]. In phage display experiments, such fast-propagating clones are enriched during the amplification steps that are performed in between the rounds of target panning, making it more likely for their displayed peptides to appear multiple times at the end of the experiment [21,22].

Origin of propagation-related TUPs in the Ph.D. libraries

The Ph.D. libraries are constructed using the vector M13KE, in which KpnI and EagI cloning sites have been inserted at the start of gene III in M13mp19 such that the randomized peptides can be appended to the N-terminus of the coat protein pIII [8]. M13mp19 contains the lacZα gene and polylinker embedded in the intergenic region near the origin of replication to allow blue/white screening for recombinant clones. The proximity of this large insert near the origin of replication causes the propagation of M13KE-based phage to be significantly reduced compared to that of wild-type M13 phage (see first two bars in Fig. 2).

The mutations we have found may compensate for the replication defect of M13KE. This type of effect was observed by Zinder and coworkers in f1 bacteriophage, the sibling of M13 in the Ff class of bacteriophages (M13, f1, and fd are 98% homologous [25]). When small insertions were made in the f1 origin of replication, mutations cropped up to compensate for the compromised ori [26–28]. These mutations, some of which correspond to the G6792T, G6793A, C6794A, T6798C, and C6799T mutations in M13KE, lie in the gene II mRNA operator sequence, to which pV binds to repress the translation of gene II [29,30]. Zinder showed that when a mutation is present, binding of the mRNA by pV is decreased [31] and the consequently reduced pV repression of gene II results in increased production of pII [26,28]. pII plays a key role in the life cycle of M13 following *E. coli* infection when it nicks the replicative form of the viral genome to initiate rolling circle replication. Zinder's findings suggest that overproduction of pII enhances the replication stage of the infection cycle and thereby compensates for severely reduced origin function.

Additionally, the propagation-related TUPs found by Smith and coworkers in the fd-based phage display library arose to compensate for an ori disrupted by the tetracycline resistance gene, albeit in this case by spontaneous restoration of the minus-strand ori rather than by point mutations [10]. Interestingly, the phage display vector fUSE5 (also based on fd-tet) [32,33] already carries the mutation corresponding to our C6810T (verified using http://www.ncbi.nlm.nih.gov/pubmed and Refs. [33–35]). This mutation presumably arose during the construction of fd-tet to compensate for the attenuation caused by the insertion of the tetracycline resistance cassette in the ori. In contrast to M13KE, fd-tet picked up the compensatory mutation in the gene II 5′-UTR prior to library construction and therefore conferred the mutation to every clone in the library. With the ori defect already compensated for by the C → T mutation in the gene II 5′-UTR, the fd-tet-based library is resistant to the other mutations to which the M13KE-based libraries are susceptible.

The mutations in M13KE are clustered primarily in the 5′-untranslated region of the gene II mRNA transcript. Eight mutations fall in the gene II operator sequence, suggesting that reduced

repression by pV and consequently increased production of pII compensate for a less effective ori, as per the findings of Zinder and coworkers. Three mutations fall in the Shine-Dalgarno (SD) sequence, which is the primary binding feature recognized by the ribosome [36,37]. We previously suggested that the G6813A mutation (Ph-HAIYPRH) increases the complementarity of the SD sequence for the ribosomal RNA by one base, and thereby enhances pII synthesis by the ribosome [7]. However, the other two SD mutations, C6810T and A6809C, do not create a more complementary ribosome-binding site. Moreover, the A6802T mutation (Ph-SNHAPRH) falls in neither the gene II operator nor the SD sequence, but rather in a section found by Zinder and coworkers to be deletable without affecting translational repression by pV [28]. While primary sequence is important in the recognition of the SD sequence by the ribosome as well as the gene II operator by pV, the influence of secondary structure in these binding events cannot be underestimated [38–43]. It is likely that any single mutation in the 5′-UTR alters the three dimensional structure of the mRNA, indirectly affecting ribosome and/or pV binding.

Four gene II 5′-UTR mutant clones also differ from M13KE at position 5091. This change is a silent mutation in the third position of the Val290 codon in gene IV (GTT → GTC). Interestingly, this mutation is a reversion back to the original wild-type M13 codon for Val290. Apparently this reversion is not required for fast propagation; although Ph-KLPGWSG carries both the G6792T and T5091C mutations, Ph-GKPMPMM has only the G6792T mutation and propagates with a comparably high rate. Two other clones carrying the T5091C mutation (Ph-LSMTGRD and Ph-HAFPHLH) also propagate at statistically identical rates to their corresponding clones without T5091C. The particular importance of the T5091C mutation, if any, will be the subject of further work.

Finally, two propagation-enhancing mutations upstream of gene II are not in the 5′-UTR; i.e., they do not fall within the mRNA transcript for pII. The G6748Δ mutation (corresponding to position 5933 in wild-type M13) is in the gene II promoter site for *E. coli* RNA polymerase [34,44] and thus may affect the transcription of gene II. The C6589T mutation falls in the lacZ insertion of M13KE, resulting in a Pro116Leu mutation in the α-fragment of β-galactosidase. Given that this clone still produces dark blue plaques on X-gal plates, and that the lacZ insertion is obviously not required for phage function, it is not clear how this mutation improves phage propagation. Sequencing of the entire phage genome for these two clones failed to identify any additional deviations from M13KE, not even T5091C.

Mutations in M13KE were either randomly acquired during Ph.D. library construction or already existed as a very small fraction of the M13KE vector originally used to make the libraries. Indeed, the two peptideless clones G6793A and G6793Δ were discovered in a sample of M13KE rather than in one of the libraries. The propagation advantage possessed by a given mutant clone presumably allowed it to be enriched during the primary amplification following library construction. Using deep sequencing of 4×10^6 clones from the Ph.D.-7 library, Derda and coworkers have recently reported that about 28% of the peptide sequences in the naïve library have higher than theoretical abundances [22]. They hypothesized that these peptides are displayed on fast-propagating phage clones, and indeed demonstrated that the abundances increased upon amplification of the library. Among our set of fast-propagating mutants, seven Ph.D.-7 clones and two Ph.D-12 clones were found by Derda and coworkers to have relatively high abundances (Table 4), although the rest did not appear in their screen. The highest abundances were found for GKPMPPM (0.0014) and HAIYPRH (0.0005). (Note that >99% of the population was expected to have a single copy number, thus 2.5×10^{-7} abundance [22].) Grouped by mutation, the highest abundances are represented by G6813A, A6809C, and G6792T. It is remarkable that these are the exact same three mutations that demonstrate the fastest propagation in our assay (and not significantly different from wild-type M13; see Fig. 2).

Propagation-related TUPs in databases and in the literature

We cross-referenced the peptides associated with our fast-propagating mutants against the MimoDB and SAROTUP databases. MimoDB (http://immunet.cn/mimodb/) compiles the peptide sequences from published phage display experiments [24,45]. HAIYPRH, ANTLRSP, QLHRHHH, GKPMPPM, HDHRYPK, and KLPGWSG were found in MimoDB (Table 4). Also developed by Huang and coworkers is SAROTUP (*Scanner and Reporter Of Target-Unrelated Peptides*, http://immunet.cn/sarotup/), a web server that compiles known and strongly suspected target-unrelated peptides or sequence motifs (both selection-related and propagation-related) [23]. It can be used for data cleaning as it can identify and exclude target-unrelated peptides from a set of peptides identified in phage display. HAIYPRH, HQLHHHL, QLHRHHH, HSHHHSA, and GWKSHLTHHHAE were found in SAROTUP. HAIYPRH is categorized as a propagation-related TUP [7], but all the other peptides are simply flagged as divalent metal ion binders. As part of the SAROTUP suite, PhD7Faster (http://immunet.cn/sarotup/cgi-bin/PhD7Faster.pl) predicts propagation-related TUPs based on the sequence of the displayed peptide [46]. The design of this predictor employed training sets generated by the sequencing of 7 million phage clones from the amplified Ph.D-7 library [47], but it analyzes clones based on displayed peptide sequence motifs. Seven of our clones are classified as propagation-related TUPs in PhD7Faster (see Table 4), but we have presented very strong evidence that the enrichment of these clones in the library is due to mutations upstream of gene II rather than their displayed peptide sequences.

When we reported HAIYPRH in 2008 as the first TUP known to be propagation-related, it had already arisen in the panning experiments of at least six laboratories employing various unrelated targets (including our own experiments with Zn^{2+}; see references within [7]). In a recent review of target-unrelated peptides, Vodnik and coworkers compiled 19 different targets for which HAIYPRH has been identified [12], and the list continues to grow [48,49]. In many of these cases, authentic target binding by HAIYPRH has been confirmed. In our own phage-ELISA experiments, HAIYPRH was not found to bind to our target Zn^{2+} [7], but several of the other phages in Table 1 do so (phage-ELISA data not shown). In these cases, the displayed peptides on the phage contain 3 or 4 histidine residues (unlike HAIYPRH), so it is not surprising that they bind to Zn^{2+}. It is certainly possible for a propagation-enhanced clone to be also a true target binder, and it is probably not pure coincidence. The enrichment of propagation-enhanced clones during amplification ensures high relative concentrations of certain peptides, making them more likely than other clones in the library to be selected for their genuine affinity for the target [21]. In cases involving notorious propagation-related TUPs like HAIYPRH, reliable target-binding assays become particularly important.

A few of our new fast-propagating clones have arisen in the phage display experiments of other laboratories. ANTLRSP (A6809C) was identified in the panning of the Ph.D.-7 library against a poly(L-lactide) (PLLA) crystalline film [50]. While phage-ELISA confirmed that the ANTLRSP-displaying phage does indeed bind to the PLLA crystal film (α form), it was the only one of eight studied peptides that was not specific for the crystal film over the reference amorphous film. QLHRHHH (T6798C) and HAIYPRH (G6813A) each appeared as one clone out of 48 in phage display using the cell surface of highly metastatic human hepatocellular carcinoma as the target [49]. Both of these phage showed enhanced binding efficiency to the cells compared to phage lacking a displayed peptide, but neither clone was nearly

as interesting as the prevailing sequence AWYPLPP, which was the focus of follow-up studies. One of our Zn^{2+}-binding peptides, HAFPHLH (G6793Δ), was identified as a ligand for a recombinant antibody previously selected in panning against the hepatitis C virus (HCV) core protein [51]. Heptapeptides selected by phage display against the recombinant antibody were used to inform the characterization of the epitope on the HCV protein. While HAFPHLH was able to inhibit the binding of the antigen by the antibody, it was 100 times less effective than the other peptide (SAPSSKN) selected in the same experiment. Puddu and Perry selected KLPGWSG (G6792T) in panning against silica nanoparticles [52]. Extensive binding studies showed KLPGWSG to have the highest affinity for silica among all their selected peptides, possibly as a result of its net positive charge. KLPGWSG was also identified by Caprini and coworkers as a highly prevalent sequence in the panning against murine neural stem cells (NSC), and follow-up studies strongly suggested that this peptide enhances neuronal differentiation [48]. Interestingly, negative selection on Petri dishes was followed by amplification prior to panning against the NSC. Thus the input library was likely enriched in the fast-propagating mutant KLPGWSG as well as HAIYPRH and GKPMPPM (G6792T), which were the second and third most commonly selected sequences in the same experiment. HAIYPRH, GKPMPPM, and HDHRYPK (G6792T) were identified in the third round of panning against a syndiotactic poly(methyl methacrylate) film that was conditioned to expose surface ester groups (conditioned st-PMMA). These clones disappeared in the fourth round of panning while several other clones survived eight rounds of panning [53]. While an ELISA assay did confirm some target binding for HAIYPRH, GKPMPPM, and HDHRYPK, the ratio of the association constant for the target to the reference film (conditioned st-PMMA/unconditioned st-PMMA) was less than half of that for several other selected clones. GKPMPPM was also found in the panning of the library against the PPIase domain of Parvulin17, but only when the target was at 10 nM concentration [54]. At much higher Par17 concentration (6 μM), a strong and completely different consensus sequence surfaced. The authors recognized that GKPMPPM was most likely a TUP that either is overrepresented in the library or is a nonspecific binder. It should be noted that when all the peptides above were selected by their respective targets, none was known as a propagation-related TUP.

It is noteworthy that we have not found some of the most common TUPs in the Ph.D. libraries. GETRAPL, LPLTPLP, SILPYPY, and SVSVGMKPSPRP have been tagged as TUPs based on their ubiquity in reported results of phage display experiments [12,24]. These elusive Ph.D.-7 clones do have relatively high abundances in the naïve library according to Derda and coworkers: 0.00029 for GETRAPL [22], 0.00015 for SILPYPY, and 0.00006 for LPLTPLP (Ratmir Derda, personal communication). We expect that these TUPs are propagation related and each carries a mutation similar or identical to one of our mutations; we simply have not happened upon them in our own studies.

Avoiding, identifying, and managing propagation-related TUPs

It is important to note that in phage display experiments, propagation-related TUPs often arise alongside true target binders, and indeed can be target binders themselves. The majority of the fast-propagating mutants in Table 4 were identified while panning against Zn^{2+}, but many of them are also Zn^{2+} binders. The interplay of two factors ultimately determines the diversity of successive pools of phage during panning and amplification: (i) the selection of peptides with an affinity for the target during the panning steps, and (ii) the enrichment of clones having higher propagation rates during the amplification steps [21]. When binding to a target that is relatively weak, the preferential enrichment of particular clones in the pool of phage is more likely to be caused by propagation advantages than by strong selection by the target. Experiments that employ a strong target with a generally high likelihood of selecting peptides are not apt to be dominated by propagation-related TUPs. The loss of diversity in the pool of phage can be minimized by avoiding or decreasing the number of amplification steps in a phage display experiment [21] or by performing amplification in isolated compartments such as monodisperse droplets [55].

Selected sequences can be examined using data-cleaning tools such as peptide databases [56]. The peptides compiled in SAROTUP are known to be target unrelated. MimoDB can also be used to determine if a peptide has been identified in phage display using other targets—if such is the case, it is possible that the peptide is target unrelated. The true test in distinguishing a propagation-related TUP from a target-binding peptide is not only to characterize the binding of the selected peptide to the target (e.g., by phage ELISA), but also to measure the propagation rate of the phage clone itself. We have demonstrated a very efficient procedure that clearly differentiates between a normal-propagating library phage (like M13KE) and a fast-propagating mutant with the potential to be a TUP (Fig. 2). This assay involves infecting an *E. coli* culture with an appropriate amount of the amplified phage of interest and comparing its concentration at 135 min of infection with that of M13KE. At this point in the infection, there is a very marked difference between the concentration of normal-propagating phage and phage carrying a advantageous mutation (1–2 orders of magnitude).

It is important to bear in mind that while the selection of propagation-enhanced clones may be unavoidable in the screening of certain targets, it does not exclude the selection of authentic target binders. Careful awareness of the possibility of TUPs and thorough assays for binding verification allow the effective identification of useful ligands by phage display.

Acknowledgments

At New England Biolabs, we thank Laurie Mazzola, Joanna Bybee, and Nancy Badger for DNA sequencing and Beth Paschal for technical assistance. We recognize Stonehill College students Heidi Weinreich, Evan Tallmadge, and Michelle Houle for preliminary work on this project. At Stonehill, we also thank Prof. Maura Tyrrell for assistance with statistical analysis. We are grateful to Ratmir Derda (University of Alberta) for sharing sequencing data prior to publication. We thank Beth Paschal and Daniel Hall for helpful discussion and critical reading of the manuscript. This work was funded by the National Science Foundation under Grant 0622540 (K.T.H.N., C.B.M-R.), the Stonehill Undergraduate Research Experience (H.R.B., C.M.M.), and Stonehill College Undergraduate Research Grants (M.A.A., L.E.H., E.M.Z.). We dedicate this paper to the memory of our friend and colleague, Carlos F. Barbas III.

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