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Corruption of phage-display libraries by target-unrelated clones: Diagnosis and countermeasures

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Abstract

Phage display is used to discover peptides or proteins with a desired target property—most often, affinity for a target selector molecule. Libraries of phage clones displaying diverse surface peptides are subject to a selection process designed to enrich for the target behavior, and subsequently propagated to restore phage numbers. A recurrent problem is enrichment of clones, called target-unrelated phage (TUPs), that lack the target behavior. Many TUPs are propagation-related; they have mutations conferring a growth advantage, and are enriched during the propagations accompanying selection. Unlike other filamentous phage libraries, fd-tet-based libraries are relatively resistant to propagation-related TUP corruption. Their minus strand origin is disrupted by a large cassette that simultaneously confers resistance to tetracycline and imposes a rate-limiting growth defect that cannot be bypassed with simple mutations. Nonetheless, a new type of propagation-related TUP emerged in the output of *in vivo* selections from an fd-tet library. The founding clone had a complex rearrangement that restored the minus strand origin while retaining tetracycline resistance. The rearrangement involved two recombination events, one with a contaminant having a wild-type minus strand origin. The founder's infectivity advantage spread by simple recombination to clones displaying different peptides. We propose measures for minimizing TUP corruption.

Keywords

Phage display; target-unrelated peptides; phage propagation; filamentous phage libraries; affinity selection; recombination

Introductory statement

Phage display is a suite of techniques for surveying large populations of peptides, proteins or protein domains (here referred to as peptides regardless of molecular size) for some desired target behavior [1]. Surveys employ selective strategies in which peptides are made accessible on the outer surface of phage particles (virions) by genetic fusion to phage coat proteins. The inputs to selection are libraries of peptide-bearing virions. A library can have 10 billion or more distinct peptides, each corresponding to a distinct phage clone. Each clone, thus distinct peptide, is represented by thousands to millions of identical copies. Selection culls the input phage population to yield an output subpopulation enriched for

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clones whose displayed peptides exhibit the desired target behavior. After selection, output phage must ordinarily be amplified by infecting bacteria, batch-propagating the infected cells, and partially purifying the secreted virions from the medium. Amplified outputs from selection serve as inputs for further rounds of selection. The amplified or unamplified outputs from final rounds of selection are infected into a bacterial host for propagating and characterizing individual clones. The clones that are most abundant in the output population are judged the most promising.

Unfortunately, clones may come to predominate in selection outputs for reasons unrelated to the desired target behavior. Such clones, called target-unrelated phage or peptides (TUPs) [2;3], can be categorized as selection-related or propagation-related. A selection-related TUP is favored during the selection process itself. In affinity selections, for example, the desired clones bind a target selector, while TUPs may bind the selection apparatus instead [3]. A propagation-related TUP, in contrast, is favored because of increased infectivity or productivity during the propagations that are a necessary adjunct to selection. The propagation advantage may be intrinsic to the peptide itself for example, if the peptide somehow increases efficiency of infection or assembly; or extrinsic to the peptide—for example, if a phage clone's propagation advantage stems from a mutation elsewhere in the genome [2].

Phage-display libraries based on popular fd-tet-derived vectors such as fUSE5 (type 3 as defined [4]) and f88-4 (type 88; Table 1) are inherently resistant to corruption by propagation-related TUPs. Their resistance stems from disruption of the minus-strand replication origin by a tetracycline resistance cassette [5;6]. Initiation of minus-strand synthesis on the viral plus strand (the circular single strand delivered by the virion) to make the double-stranded replicative form (RF) is consequently very inefficient [7]. Minus-strand synthesis becomes severely rate-limiting not only for RF replication, but also for initiation of gene expression in a newly-infected cell. The replication-defective phage are propagated in the presence of tetracycline, and infectious units are ordinarily quantified as colony-forming units (cfu; filamentous virions are secreted without killing the host) rather than as plaque-forming units. Given their severe replication rate limitation, these phage do not generally gain an overall growth advantage from small incremental propagation improvements due to simple mutations. The obvious way for fd-tet-derived phage to revert to a functional minus strand origin is by deletion of the tetracycline resistance cassette, but such revertants don't survive in the tetracycline-containing cultures used for propagation. Contaminating wild-type phage with intact minus-strand origins also don't survive, as they are tetracycline sensitive. The replication defect has key advantages apart from TUP resistance [8], offsetting the inconvenience of low RF copy number in initial library construction.

Here we show that even fd-tet-based libraries can be corrupted by propagation-related TUPs. The TUPs reported in this article emerged in the output of a typical *in vivo* selection for tumor-homing peptides. They have a complex chimeric rearrangement that restores a functional minus strand origin without eliminating tetracycline resistance. While such TUPs are presumably rare compared to less complex TUPs in other libraries, it is evident that their threat cannot be ignored. In light of these findings, general strategies for diagnosing and avoiding such TUPs will be discussed.

¹Abbreviations used: TUP, target-unrelated phage or peptide; RF, double-stranded intracellular replicative form of phage DNA; cfu, tetracycline-resistant colony-forming units; Tet, tetracycline.

Materials and methods

In vivo selections

In vivo selections [9] from the f3-15mer library (Table 1) in SCID mice harboring MDA-MB-435eb.1 tumor xenografts were performed as described [10] to discover peptides that home to breast tumors. A portion of the input library was subjected to a round of positive selection on adherent tumor cells in tissue culture. The amplified output of that selection and the unselected input library were subjected separately to three rounds of in vivo selection in tumor-bearing mice. Randomly-chosen clones from each output were sequenced in order to profile the selected phage-borne peptides [1].

Virological properties of output clones

Phage clones were propagated, and virions were purified and quantified spectrophotometrically, as described [8;11]. Infectivities were calculated as tetracycline-resistant cfu [8] per virion. Plaques [8] were photographed with a microscope-mounted camera, and the areas measured using a Wacom pen tablet (© 2010).

Results and discussion

Following in vivo selection from the f3-15mer phage-display library (Table 1) in mice harboring tumor xenografts (Materials and methods), two phage clones, IV1 and IV2, predominated in final outputs. These clones had no discernible affinity for the tumor cell line in vivo or in vitro (results not shown) but had unusually high infectivities, prompting further investigation.

The chimeric parentage of TUPs

The genome of wild-type filamentous phage (strains fd, f1 and M13) has two origins of DNA replication, one for the minus strand and one for the plus strand ([12]; “Wild-type” in Fig. 1). In normal f3-15mer clones, a tetracycline resistance cassette disrupts the minus-strand origin (Fig. 1, “Disrupted”). Clones IV1 and IV2 have an inversion of the tetracycline resistance cassette in combination with a fully restored minus strand origin of replication identical in sequence to that of wild-type fd (Fig. 1, “Restored”). Apart from their displayed peptides, IV1 and IV2 are genetically identical, indicating common descent. Sequence alignment reveals two hybrid junctions, corresponding to two recombination events in the lineage of their common ancestor (Fig. 2). One of those recombinations evidently involved a genome with wild-type fd replication origins. Phage or phagemids with such origins are common contaminants of phage-display libraries. Although tetracycline strongly counterselects such contaminants in fd-tet-derived libraries, co-infection can maintain a low level of contamination transiently, providing an opportunity for chimeric phage to arise. Once a founder chimeric clone had arisen in a rare chain of events of this kind, its replication proficiency could readily spread to other clones with other displayed peptides via a single recombinational exchange [13] mediated by extensive homology (Fig. 2 legend). Thus a single founder is presumed to have given rise to both IV1 and IV2, as well as to a third chimeric clone, IV6, which is identical in structure to IV1 and IV2 except for its displayed peptide, and which was independently selected from the same input library (Table 1; J.R. Newton, unpublished).

Chimeric TUPs have a propagation advantage

Restoration of the minus strand origin of replication is expected to boost infectivity, a key determinant of propagation efficiency. This prediction was confirmed by the triplicate infectivity assays in Fig. 3A, which show that chimeric clones IV1 and IV2 have infectivities 3 to 5 times higher than those of normal library clones IV3 and IV4, and of the

library's parent fd-tet. Plaque size and turbidity are sensitive indicators of infectivity, since plaque formation requires repeated rounds of infection. Chimeric phage IV1 and IV2 make plaques that are distinctly larger and less turbid, hence more visible, than those of normal library phage and fd-tet, though less visible than those of wild-type fd (Fig. 3 panels B–H).

Why did TUPs emerge in the selections described here?

Three factors may have contributed. 1) Serial replenishment. Our input library had been replenished several times by serial re-propagation since its original construction in order to restore adequate virion numbers. In each successive re-propagation, a small number of input virions—just enough cfu to represent the primary 2.5×10^8 -clone library 30 to 1000 times over—were infected into large bacterial cultures, yielding vastly larger numbers of output virions. These are exactly the conditions that favor propagation-related TUPs. 2) Amplification after negative selection. Before positive selections, the library was subjected to negative selection by passage through non-tumor-bearing mice. This “mouse depletion” aimed to reduce the background of non-target-specific phage that bind normal tissues. To restore adequate virion numbers, output virions had to be amplified by propagation before positive selection. Again, propagation-related TUPs would be favored by this post-selection amplification. 3) Weak or ineffective positive selection. In a successful positive selection, a few clones with the desired target behavior (affinity for tumor tissue in our case) predominate in the output. Propagation-related TUPs are likely to be culled by the resulting population bottleneck unless they have already risen to prominence in the input as a result of prior propagations. In contrast, an ineffective positive selection does not impose a severe population bottleneck, allowing even rare TUPs to survive in the output. The absence of strongly tumor-avid virions in the outputs of our positive selections suggests that those selections may have been ineffective, allowing propagation-related TUPs to gain a further advantage during the post-selection amplifications.

Recommendations

We suggest that the risk of TUP corruption can be minimized in a few simple ways. 1) Libraries can be replenished by parallel re-propagation through transfection with primary library RF DNA, rather than by serial re-propagation through infection with virions from the previous propagations. 2) Negative selections that require post-selection amplification (as was the case with our mouse depletions) should be undertaken with caution, in the knowledge that they could favor propagation-related TUPs. 3) IV1 and IV2 give plaques that are visually very distinct from those of normal library members like IV3 and IV4 (Fig. 2, B–H). This suggests that plaque assays might be an effective way to monitor library populations for the emergence of TUPs with a propagation advantage in the course of selection. 4) Improved vector design might reduce the likelihood of corruption by new propagation-related TUPs. For instance, the IV4nm construct (Table 1) is identical to IV4 except for a deletion that removes all but a small remnant of the disrupted minus-strand origin and eliminates the imperfect inverted repeats that mediated inversion of the tetracycline resistance cassette (Fig. 2, “Deleted”). The deletion has no apparent effects on infectivity or plaque size (Fig. 3A and B). It narrows the scope for emergence of new complex recombinational rearrangements, not only by eliminating unnecessary nucleotides, but also by blocking pathways that depend on inversion of the tetracycline resistance cassette—including the pathway that gave rise to the TUPs reported here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic diagram of alternative structures at the minus- and plus-strand origins. Functional minus- and plus-strand origins are labeled *ori-* and *ori+*, respectively; plus-strand synthesis proceeds from left to right as shown, minus-strand synthesis in the opposite direction. The symbols are not drawn to scale: the minus- and plus-strand origins span only a few dozen nucleotides each, while the Tn10-derived tetracycline resistance cassette (Tet) that disrupts the minus-strand origin in the second line spans 2774 nucleotides. The names to be used in this article for the four minus-strand origin structures are listed on the right.

Fig. 2.

Recombination events giving rise to the chimeric f3-15mer clone, as inferred from its hybrid junction sequences. The chimeric clone is hypothesized to have arisen from two homologous recombination events involving double-stranded RF DNAs from the f3-15mer library itself and from a contaminant with wild-type phage fd replication origins. **A.** Nucleotide sequence alignments of parental genomes with the hybrid junctions of the chimeric clone. Parental nucleotides identical to those of the chimeric clone are in bold type. **Junction 1** is formed by recombination between the imperfect inverted repeats (shading) that define the stem of hairpin **[B]**, an essential element of the minus-strand origin [12]. Sequence differences bracketing the junction limit the exchange to within the 4 nucleotides marked with the bar. Because the inverted repeats flank the Tet cassette in the disrupted f3-15mer origin, the exchange inverts the Tet cassette. At **Junction 2**, an f3-15mer minus strand is joined near one end of its Tet cassette to a wild-type fd plus strand just upstream of its intact minus-strand origin. The hybrid junction occurs within a short string of chance sequence identities (shading); bracketing differences limit the exchange to within the 5 nucleotides marked with the bar. **B.** A recombinant molecule with the exchanges depicted in **A** would resolve during replication into the chimeric f3-15 clone. Replication origins and the Tet cassette are represented as in Fig. 1; the cross-hatched symbol represents the coding sequence for the displayed peptide. Initially, a recombinant molecule would have two same-sense oriented plus-strand origins. The next round of plus-strand synthesis starting at the left-hand origin would terminate at the right-hand origin, circularizing the unit genome in between, which corresponds to the observed chimeric clone. Subsequently, a single exchange between a chimeric clone and a non-chimeric f3-15mer clone anywhere in the 4013 nucleotides of sequence identity between the peptide coding sequence and Junction 1 would resolve at the next cycle of plus-strand synthesis into a new circular chimeric genome bearing the peptide sequence from the non-chimeric clone.

Fig. 3. Infectivity parameters of phage clones. **A.** Percent infectivity measured as tetracycline-resistant colony-forming units (cfu) per virion; open triangles, filled circles and open diamonds mark data-points from three independent titerings. **B.** Scattergram of plaque areas. **C–H.** Photographs of IV1–IV4, fd-tet and fd plaques, respectively.

Table 1

Provenance and salient characteristics of phage clones and library

Clone or library	Displayed peptide ^a	Minus-strand origin structure ^b	GenBank accession	Provenance
fd	None	Wild-type	J02451.1	Wild type
fd-tet	None	Disrupted	AF217317.1	Constructed from fd
f88-4	NVP stuffer	Disrupted	AF218363.1	Constructed from fd-tet
fUSE5 vector	Frameshifting stuffer	Disrupted	AF218364.1	Constructed from fd-tet
f3-15mer library	X ₁₅ (2.5 × 10 ⁸ peptides)	Disrupted	AF246445.1	Constructed in fUSE5 vector
IV1 ^c	RGGRCLLCCLCLWWA	Restored	HM355482	Chimeric member of f3-15mer library
IV2	AVAGGRSVVDARVAR	Restored		Chimeric member of f3-15mer library
IV6	RTEVPVLSFTSPLTG	Restored		Chimeric member of f3-15mer library
IV3	PFARAPVEHHDVVGL	Disrupted		Normal member of f3-15mer library
IV4 ^c	RVPPRYHAKISPMVK	Disrupted	HM355481	Normal member of f3-15mer library
IV4nm	RVPPRYHAKISPMVK	Deleted	HM453030	Constructed from IV4

^a f88-4 is a type 88 vector and displays peptides on pVIII; all other clones or libraries listed except fd and fd-tet are type 3 and display peptides on pIII.

^b See Fig. 1.

^c Additional differences found in the complete sequences of IV1 and IV4 outside their replication origins are catalogued in the supplementary material.