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Analytical Biochemistry 420 (2012) 68–72

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

A general insert label for peptide display on chimeric filamentous bacteriophages

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ARTICLE INFO

Article history:
Received 18 July 2011
Received in revised form 29 August 2011
Accepted 30 August 2011
Available online 8 September 2011

Keywords:
Combinatorial phage display
Recombinant protein VIII
Peptide display
Phage libraries

ABSTRACT

The foreign insert intended to be displayed via recombinant phage proteins can have a negative effect on protein expression and phage assembly. A typical example is the case of display of peptides longer than 6 amino acid residues on the major coat protein, protein VIII of the filamentous bacteriophages M13 and fd. A solution to this problem has been the use of "two-gene systems" generating chimeric phages that concomitantly express wild-type protein VIII along with recombinant protein VIII. Although the two-gene systems are much more permissive in regard to insert length and composition, some cases can still adversely affect phage assembly. Although these phages genotypically contain the desired DNA of the insert, they appear to be phenotypically wild type. To avoid false-negative results when using chimeric phages in binding studies, it is necessary to confirm that the observed lack of phage recognition is not due to faulty assembly and display of the intended insert. Here we describe a strategy for generating antibodies that specifically recognize recombinant protein VIII regardless of the nature of its foreign insert. These antibodies can be used as a general monitor of the display of recombinant protein VIII into phage particles.

Filamentous bacteriophages have proven to be an extremely useful tool for the study of protein–protein interactions [1,2] and have had a profound impact on the analysis of antibody–peptide binding [3–5]. Of the five structural proteins that make up filamentous phages, proteins III and VIII are most often used as N-terminal fusion proteins, displaying their foreign inserts on the phage surface [6]. Protein III most easily displays inserts and can express proteins hundreds of amino acids in length without a negative impact on assembly or titer of phages. Thus, protein III is routinely used in antibody phage display where either Fab or single-chain antibodies are expressed [7,8]. Although this system is quite efficient and widely used, the number of antibody or insert copies is limited to the five copies of protein III per phage. In situations where higher insert density is desirable, protein VIII should be considered. This is due to the fact that the filament structure is composed of some 2700 copies of protein VIII, a short 50-amino-acid protein that associates with the phage single-stranded DNA at its carboxy terminus and displays its free N terminus on the surface of the phage [6]. Peptide inserts can be introduced at the N-terminal aspect of protein VIII without disrupting the assembly so long as they are kept shorter than 6 to 8 residues [9,10]. Expression of longer peptides on all copies of protein VIII interferes with proper phage assembly. Thus, for instance, Cesareni and coworkers reported that only 20% of phage clones inserted with random octapeptides and 1% of clones inserted with random decapeptides produce viable phage particles [9]. Longer inserts can be displayed employing a "two-gene system" where two versions of protein VIII are expressed in the infected bacterium, one corresponding to unaltered wild-type protein VIII and the other being a recombinant that can display peptides even longer than 100 amino acids in its N-terminal aspect [11]. In this situation, assembly of the phage generates chimeric phages containing mostly wild-type protein VIII studded here and there with recombinant versions displaying their peptide insert at the phage surface [12]. Moreover, such chimeric phages may contain recombinant protein VIII displaying large inserts at extremely low levels [11,13]. To complicate matters, it has been shown that not only the length but also the sequence of the insert can affect incorporation levels into phage particles by affecting the critical steps of protein VIII membrane insertion and processing [13].

Although expression of peptides using this two-gene system is generally efficient and has only a marginal effect on the titer of chimeric phages, in some instances the particular nature of the peptide might be incompatible with functional phage assembly with two possible outcomes: (i) the recombinant protein VIII causes a block in assembly, leading to a dramatic drop in phage titer [9], or (ii) provided that a wild-type gene is expressed, the problematic recombinant protein VIII is simply not incorporated into the assembling phage, generating phages that phenotypically are uniformly wild type. Such phages are misleading in binding experiments because no binding is observed, not due to lack of peptide recognition but rather due to faulty display. To discriminate between these two options, it would be useful to have a specific

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doi:10.1016/j.ab.2011.08.050

general label for recombinant protein VIII (i.e., displaying inserts). Such a "general insert label" (GIL)[1] should be detectable without interfering with the expression of the foreign insert or phage assembly itself. Here we describe such a GIL and demonstrate its utility in measuring the presence of recombinant protein VIII in chimeric filamentous phages.

Materials and methods

Construction of MBP fusion vectors

Sequences encoding for the GIL peptide (AEGGQRGC; see Fig. 1) were cloned into the pMalC-133-AvaI vector (see Results). This vector was adapted from the commercial pMALc system (New England Biolabs, Ipswich, MA, USA), which allows expression of maltose-binding protein (MBP) fusion proteins. The pMalC-133-AvaI vector contains the MBP gene modified to contain an N-terminal His tag and three inserted restriction sites: an asymmetric *Ava*I restriction site (c/tcggg) flanked by two *Sfi*I sites (the restriction sites were inserted, replacing the nucleotides corresponding to amino acids 134–143 of the native MBP gene). Oligonucleotides corresponding to the sequence encoding the GIL peptide flanked by *Ava*I-compatible overhangs were ligated into the *Ava*I-digested pMalC-133-AvaI vector (the addition of the *Ava*I complementary sequences adds a 3-amino-acid linker (Asp-Ser-Gly) that precedes and follows each GIL peptide).

Expression and purification of MBP fusion protein displaying GIL

Escherichia coli BL21 Rosetta (DE3) cells (Novagen Merck, Darmstadt, Germany) were transformed with the pMALc-131-AvaI plasmids containing multiple copies of the GIL peptide. The transformed cells were grown in lysogeny broth (LB) medium + 100 μg/ml ampicillin at 37 °C. When the culture reached OD_{600nm} = 0.7, isopropyl β-D-1-thiogalactopyranoside (IPTG, Ornat, Rehovot, Israel) was added (0.5 mM) and growth continued for an additional 4 h at 37 °C. Cells were harvested by centrifugation at 5000 rpm for 10 min. Cell pellets were resuspended in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Triton X-100 and lysed by sonication. The extracts were clarified by centrifugation at 20,000*g*. The MBP fusion protein was purified by ion metal affinity chromatography on Sepharose–nickel beads according to the supplier's instructions (Adar Biotech, Rehovot, Israel). Yields were approximately 4 mg of purified fusion protein per production/purification cycle.

Rabbits and immunization

Two New Zealand white female rabbits were immunized subcutaneously with 750 μg of the MBP fusion protein displaying three linear repeats of the GIL peptide suspended in complete Freund's adjuvant. Boosts were carried out at 2, 5, 10, and 13 weeks with 750 μg/boost of the above MBP fusion protein suspended in incomplete Freund's adjuvant. At 17 weeks, both rabbits were exsanguinated and the resulting sera were taken for analysis. Rabbits were purchased from and maintained by the Tel Aviv University animal care facility.

Phage display

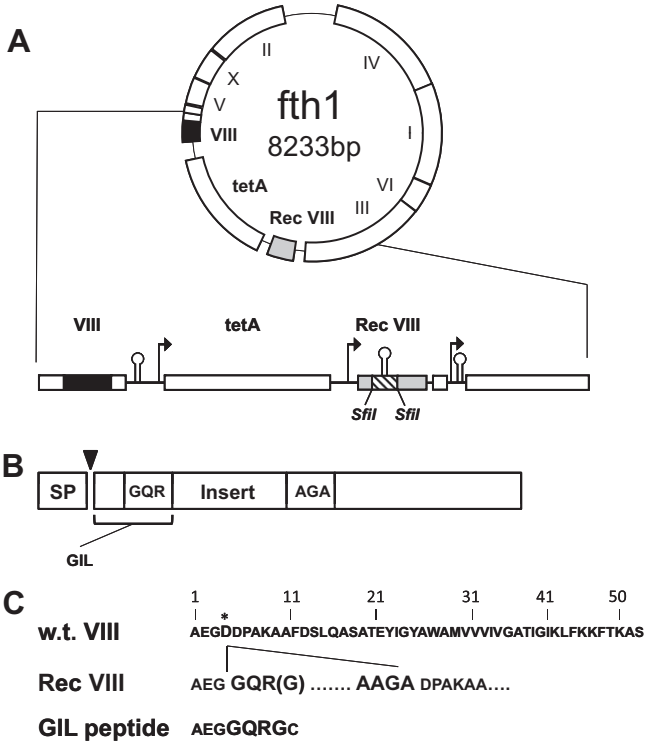

Fig.1. The fth1 vector and origin of the GIL. (A) Schematic of the fth1 vector [12]. Both the wild-type and recombinant (Rec) copy of the *protein VIII* gene are filled in (black and gray, respectively). The recombinant *protein VIII* gene contains a DNA stuffer that encodes for two stop codons and an RNA transcription terminator (hairpin structure). The stuffer is removed when an insert is cloned between the two indicated *Sfi*I sites. (B) Schematic of recombinant protein VIII showing the signal peptide (SP) and the cleavage site for signal peptidase (black arrowhead). The insert is marked and flanked by the sequences encoded by the *Sfi*I restriction sites. The N-terminal GIL sequence, which is recognized by GIL–Ab, is also marked. (C) Sequences of mature wild-type (w.t.) phage protein VIII (first line), mature recombinant (Rec) protein VIII (second line), and general insert label (GIL) (third line). First line: The fourth residue (Asp) marked with an asterisk is the residue that is replaced by the *Sfi*I insertion cassette in the recombinant fth1 protein VIII [12]. Second line: sequence of the N terminus of the recombinant fth1 protein VIII. Residues depicted in larger font are encoded by the *Sfi*I restriction sites. The glycine marked in parentheses is optional because it is present only when the *Sfi*I site is maintained during the cloning process. Third line: sequence of the GIL used to immunize rabbits.

All phage display constructs were produced using the fth1 vector as described previously [12,14]. Briefly, for display on protein VIII, oligonucleotides corresponding to the desired sequence flanked by *Sfi*I-compatible ends were ligated into the *Sfi*I-digested fth1 vector. For display on protein III, a modified fth1 vector was used where a *Bst*XI cloning cassette at the 5′ region of the *protein III* gene allows introduction of inserts between the codons of amino acid residues 1 and 2 of the mature protein. Hence, display on protein III was performed using oligonucleotides corresponding to the desired insert flanked by *Bst*XI-compatible overhangs and ligated into the *Bst*XI-digested modified fth1 vector.

Phage display of 1B6 antibody-binding peptide

The 1B6 antibody is a murine monoclonal antibody (mAb) isolated in-house from a BALB/c mouse immunized with recombinant HIV-1 gp120 (SF2 strain; GenBank ID: BD016766.1) and binds gp120 specifically. Phage analysis conducted on the 1B6 mAb yielded a 14-amino-acid long peptide (CWGTNCLNKTATNS) that has high affinity for the 1B6 mAb and is henceforth referred to as the 1B6 peptide. Sequences encoding for the 1B6 peptide were

[1] *Abbreviations used:* GIL, general insert label; MBP, maltose-binding protein; LB, lysogeny broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate-buffered saline; mAb, monoclonal antibody; HCV, hepatitis C virus; ELISA, enzyme-linked immunosorbent assay; GIL–Ab, general insert label antibodies/GIL-specific serum; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylbenzidine.

cloned to produce both protein VIII and protein III N-terminal fusions using the fth1 vectors described above.

Phage display of sequences from HCV core protein

Sequences encoding for three linear epitopes found in the hepatitis C virus (HCV) core protein were cloned into the fth1 vector as described above. Sequences core 1 (PQDVKFPGGG) and core 2 (RNTNRRPQDV) correspond to amino acid positions 19 to 28 and 13 to 22, respectively, on the core protein from the HCV subtype 1b (GenBank ID: ADG27648.1), whereas core 3 (GRSWAQPGYPWPLY) corresponds to amino acid positions 73 to 86 on the same protein from the subtype 3a virus (GenBank ID: ADG27631.1).

HCV antigens were detected using polyclonal serum from an HCV-positive human patient obtained commercially from BBI Diagnostics (West Bridgewater, MA, USA).

Phage ELISA

Enzyme-linked immunosorbent assay (ELISA) plates (cat. no. 3590, Corning, Corning, NY, USA) were coated with either murine monoclonal or rabbit polyclonal antibodies specific for M13 bacteriophages. Blocking was carried out using 5% nonfat dry milk and 20% bovine serum in PBS. Phage concentrations ranged from 10^{10} to 10^{12} phages per milliliter. All washes were with 0.05% Tween 20 in PBS. Captured phages were detected with one of the following reagents diluted in blocking solution: GIL–Ab serum (1:1000), polyclonal rabbit anti-M13 antibodies (1:1000), HCV-positive human serum (1:64), or the 1B6 mAb (2.5 μg/ml). All secondary antibodies used were standard horseradish peroxidase (HRP)-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA, USA), which were detected by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB/E solution, Millipore, Billerica, MA, USA). The reaction was stopped by the addition of 1 M H_2SO_4, and the plates were read by a BioTek ELx808 microplate reader at 450 nm. All figures depict single experiments where error bars represent standard deviations of duplicate or triplicate measurements. Each ELISA experiment was repeated at least three times.

Results and discussion

Production of GIL-specific polyclonal serum

The fth1 "88" phage display vector contains two copies of *protein VIII* genes, one wild type and the other recombinant (Fig. 1; see Ref. [12]). Peptide display is based on insertion of foreign sequences into the recombinant copy of the *protein VIII* gene between two *Sfi*I sites separated by a DNA sequence coding for two stop codons followed by a *trpA* transcription terminator, all of which displace the reading frame by 1 nucleotide [12] (Fig. 1). Hence, prior to insertion of a foreign sequence between the two *Sfi*I sites, no recombinant protein VIII is produced and the phages display only wild-type protein VIII. Due to the presence of the *Sfi*I insertion cassette, recombinant protein VIIIs contain a discriminating sequence not found in wild-type proteins (Fig. 1). In view of this, it was proposed that the N-terminal 7 residues of recombinant protein VIII (AEGGQRG), which contain the first *Sfi*I-encoded unique sequence (GQRG), could serve as a universal endogenous antibody tag for all recombinant versions of the protein regardless of length or composition of the peptide being displayed. Because most of our random peptide libraries often contain flanking cysteine residues to generate constrained looped random peptides, we decided to include the first cysteine residue as amino acid 8 in the GIL to be tested (AEGGQRGC). Once designed, it was then necessary to produce fusion proteins that display the GIL to be used as immunogens. For this, the GIL-encoding sequences (GIL inserts) were cloned into the asymmetric *Ava*I site (CTCGGG) found in the modified MBP expression vector pMalC-133-AvaI (see Materials and Methods). We previously demonstrated that cloning inserts into such *Ava*I sites drives the formation of tandem repeats of the inserts in the proper reading frame and orientation [15]. This is due to the fact that the internal asymmetry of the restriction site generates two different 5′ overhangs, thereby forcing compatible inserts to clone in only one orientation. Clones containing two, three, and four linear GIL insert repeats were isolated and confirmed by sequencing (Fig. 2). The clone containing three tandem repeats was selected and used for MBP–GIL fusion protein production, which in turn was used to immunize rabbits. The resulting polyclonal serum was tested for specific binding to the GIL.

Characterization of GIL-specific serum

GIL-specific serum (GIL–Ab) was tested for binding against three phages (Fig. 3): the wild-type phage and two types of phages displaying the 1B6 peptide (CWGTNCLNKTATNS) that is recognized by the 1B6 mAb (see Materials and Methods). The first phage expresses the 1B6 peptide on recombinant protein VIII and is a type 88 chimeric phage. The second phage type displays the 1B6 peptide on all copies of protein III. It should be noted that only insert expression on recombinant protein VIII generates the GIL sequence. Fig. 3 depicts a representative ELISA experiment illustrating that GIL–Ab efficiently binds only the phages displaying the 1B6 peptide on recombinant protein VIII and shows no binding to phages displaying the same sequence on protein III or to the wild-type fth1 phages. The 1B6 mAb binds both phages displaying the 1B6 peptide, on both protein VIII and protein III, but to a lesser degree corresponding to the fewer copies of protein III as compared with recombinant protein VIII. Polyclonal rabbit anti-M13 antibodies bound all three phages at similar levels, illustrating equal overall phage levels for all three phage types. These results indicate that all three phage types are present in equal amounts, that the 1B6 peptide is displayed on both the protein VIII and protein III phages, yet only recombinant protein VIII is recognized by GIL–Ab.

To further demonstrate the correlation between GIL–Ab binding to recombinant protein VIII and 1B6 mAb binding to the inserted 1B6 peptide, the following experiment was performed. Phages displaying the 1B6 peptide on protein VIII were serially diluted twofold into a solution containing wild-type fth1 phages. In this

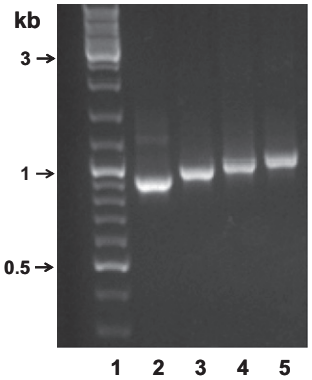

Fig.2. Polymerase chain reaction (PCR) analysis of GIL insert containing pMalC-133-AvaI clones. PCR was performed using primers upstream and downstream of the *Ava*I site. Lane 2: amplification of the unmodified vector results in a 938-bp fragment; lanes 3, 4, and 5: amplifications of vectors containing 2, 3, and 4 GIL inserts, respectively. Each extra GIL insert adds 33 bp.

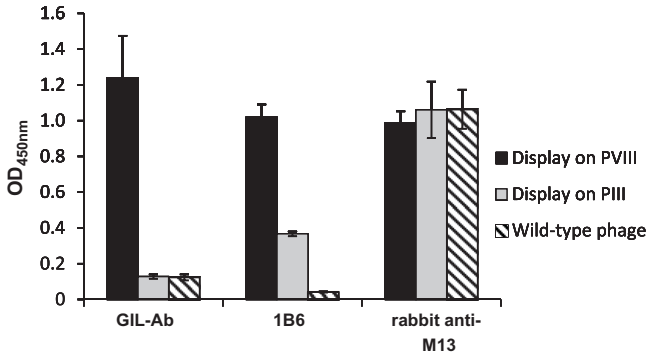

Fig.3. Determining GIL–Ab specificity for recombinant protein VIII by ELISA. ELISA wells coated with anti-M13 antibodies were used to capture three different phages: chimeric phages displaying the 1B6 peptide on protein VIII while containing both recombinant and wild-type protein VIII (black); homogeneous phages displaying the 1B6 peptide on all copies of protein III while containing only wild-type protein VIII (gray); and wild-type fth1 phages (hatched). The captured phages were reacted against GIL–Ab, 1B6 mAb, and rabbit anti-M13 polyclonal antibodies. Bars represent standard deviations of duplicate measurements from a single experiment.

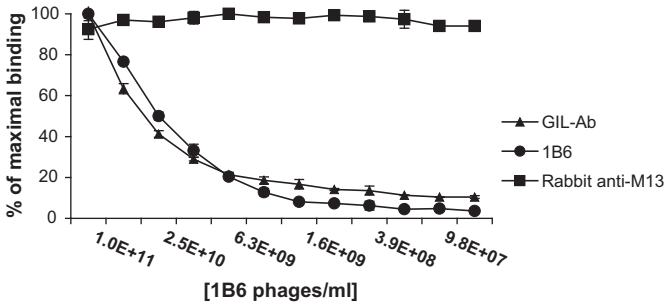

Fig.4. Testing the detection threshold of GIL–Ab by ELISA. Chimeric phages displaying the 1B6 peptide on protein VIII at a concentration of 10^{11} phages per milliliter were serially diluted 1:2 into wild-type fth1 phages at the same concentration. The phage dilutions were then added to ELISA well plates coated with anti-M13 antibodies. Captured phages were detected using GIL–Ab (triangles), 1B6 mAb (circles), or polyclonal rabbit anti-M13 (squares) antibodies. Bars represent standard deviations of triplicate measurements from a single experiment.

manner, we generated a series of phage mixtures containing identical total phage concentrations but with ever decreasing amounts of phages displaying the 1B6 peptide. As shown in Fig. 4, binding signals from polyclonal rabbit anti-M13 antibodies remained constant, confirming the equal total phage concentrations. In contrast, however, binding signals from both GIL–Ab and 1B6 mAb dropped in a very similar manner as the concentration of 1B6 peptide presenting phages decreased, illustrating the similar detection sensitivity of both reagents and the correlation between the presence of the 1B6 peptide and the generation of the GIL in recombinant protein VIII. In experiments where both antibodies are reacted simultaneously to 1B6 displaying phages, binding of 1B6 mAb competes and interferes with GIL–Ab binding and vice versa, as to be expected (data not shown).

Testing GIL–Ab as a general monitor of recombinant protein VIII

For GIL–Ab to be useful, one must confirm the generality of its binding activity. Table 1 is a list of 12 different peptide inserts validated for display on protein VIII using GIL–Ab and the relevant anti-insert antibodies. The peptides range in length from 7 to 43 residues, some of them contain flanking cysteine residues and others do not, and together they represent reasonable cover of amino acid compositions. Validation of peptide expression was carried out by phage ELISA (see Materials and Methods). Each of the phages displaying the listed peptides was tested for binding to both GIL–Ab and the anti-insert antibody listed as compared with wild-type fth1 phages. All 12 peptide displaying phages gave high binding signals to both GIL–Ab and the corresponding anti-insert antibodies, whereas only background binding was observed using the wild-type fth1 phages (data not shown). The fact that peptides not flanked by cysteine residues are recognized by GIL–Ab shows that the cysteine residue is not essential for binding. In addition, peptide 12 is also missing Gly7 of the GIL sequence. Nevertheless, GIL–Ab binds this construct efficiently. Hence, the first 6 GIL residues (AEGGQR) are the core GIL–Ab epitope, with some flexibility as to the sequence at the C terminus of the GIL. Over the past year, we have successfully used GIL–Ab as a general insert label detector for a wide variety of inserted sequences, showing GIL–Ab to be a general detector of recombinant protein VIII regardless of the nature of the displayed sequence.

Use of GIL–Ab to monitor recombinant protein VIII levels

The use of GIL–Ab as a unique monitor of functional expression and assembly of recombinant protein VIII is shown in the following experiment. In studying the polyclonal serum response against HCV core protein, we used three recombinant phages, each expressing a different peptide on protein VIII and designated cores 1, 2, and 3 (see Materials and Methods). Sequencing confirmed that all three phages contained the expected DNA inserts in the proper reading frame. However, when tested against polyclonal serum from a patient positive for HCV (Fig. 5), core 1 consistently gave a strong signal, core 3 gave a weaker but clearly detectable signal, and core 2 gave no response at all. The initial conclusion could be that the core 2 peptide is apparently not an efficient antigen and little activity against this epitope is detectable in the patient's serum. However, lack of activity could be explained by inappropriate peptide conformation despite its being displayed efficiently by phage protein VIII [16–19]. Testing the binding of GIL–Ab to these phages is extremely revealing. Phages cores 1 and 3 gave strong GIL–Ab responses, illustrating that although the peptides are equally well expressed and assembled in the phages, the HCV serum response to each is quite different. This is not surprising because the HCV serum was derived from a patient infected with HCV subtype 1a (core 3 represents a subtype 3 peptide). Surprisingly, no GIL–Ab response could be demonstrated for the core 2 phage, whose signal was no better than that of wild-type phage. Hence, the absence of signal with the HCV serum is first and foremost due to lack of expression, assembly, and display of the core 2 peptide, and conclusions regarding immunogenicity or proper peptide conformation cannot be drawn based on this phage system. Here GIL–Ab proved to be a critical reagent—a sensor of sorts for the confirmation that recombinant protein VIII is expressed and assembled.

Conclusions

The fth1 recombinant protein VIII has a unique N terminus that is exploited as a GIL. Polyclonal antibodies that specifically bind the N terminus of recombinant protein VIII (GIL–Ab) were produced and shown to be capable of binding a variety of recombinant protein VIIIs displaying different peptide inserts. Thus, the use of GIL–Ab provides a single reagent that enables the monitoring of chimeric phages produced using the fth1 vector. Because phage display libraries are used extensively for diagnostic purposes and as tools for vaccine research [20–22] (reviewed in Refs. [23,24]), validating the presence of the displayed peptide is of utmost importance for minimizing false-negative results caused by difficult-to-display sequences.

Table 1
List of epitopes displayed on protein VIII and validated for expression using GIL–Ab.

	Inserted sequence	Length[a]	Binding antibody	Antibody target
1	SSGKLIS	7	HIVIg	HIV envelope protein
2	CSGKLIC	7	HIVIg	HIV envelope protein
3	QWWAPTA	7	11A	SARS coronavirus spike protein
4	PQDVKFPGGG	10	HCV-positive human serum	HCV core protein
5	APAGFAILSAP	11	4H3	HIV envelope protein
6	CVPDHWWTPQRC	12	11A	SARS coronavirus spike protein
7	CSGLRNETFLRC	12	17b	HIV envelope protein
8	CMTHVGWVTPKC	12	CG10	HIV envelope protein
9	GRSWAQPGYPWPLY	14	HCV-positive human serum	HCV core protein
10	CWGTNCLNKTATNS	14	1B6	HIV envelope protein
11	CVKLTP TPV TSVITQAC	17	17b	HIV envelope protein
12	STG . . . YTT	43	80R	SARS coronavirus spike protein

Note: HIVIg, pooled human immunoglobulin G from individuals positive for human immunodeficiency virus; SARS, severe acute respiratory syndrome; HCV, hepatitis C virus.

[a] Length in amino acids.

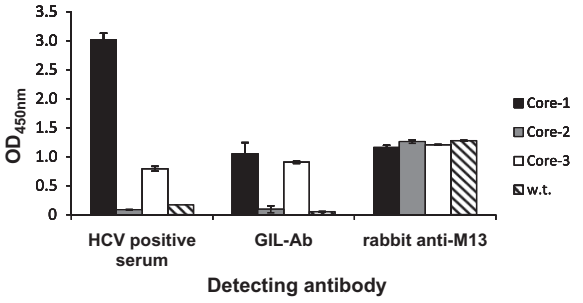

Fig.5. Binding ELISA of chimeric phages displaying different linear epitopes from HCV on protein VIII. Phages displaying three different sequences from the HCV core protein were captured in duplicate wells coated with anti-M13 antibodies. The captured phages were then reacted against HCV-positive human serum, GIL–Ab, and polyclonal rabbit anti-M13 antibodies. Black bars: phages displaying core 1; gray bars: phages displaying core 2; open bars: phages displaying core 3; hatched bars: wild-type (w.t.) fth1 phages. Bars represent standard deviations of duplicate measurements from a single experiment.

An additional potential use of GIL–Ab could be as an affinity reagent to enrich phage display libraries for insert displaying phages, thereby increasing their specific activity.

Although the GIL–Ab produced in this study is specific for the fth1 vector, the outlined strategy can be adapted for any phage display system in which several unique residues can be found at the N terminus of either recombinant protein VIII or recombinant protein III.

Acknowledgments

We thank Dr. Dror Siman Tov for his advice and assistance with the HCV experiments, Ms Larisa Smelyanski for her expert technical support, and Prof. Itai Benhar for providing the modified MBP expression vector. This study was supported by the Israel Science Foundation and the U.S.–Israel Binational Science Foundation. The authors wish to thank Mrs. Edna Grinberg for her generous support of this research. G.K. is the recipient of the Jakov, Mirianna, and Jorge Saia Doctoral Prize.

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