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A general insert label for peptide display on chimeric filamentous bacteriophages
Gilad Kaplan, Jonathan M. Gershoni*

Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

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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.

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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 octa-peptides 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

* Corresponding author. Fax: +972 642 2046.
E-mail address: gershoni@tauex.tau.ac.il (J.M. Gershoni).

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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 (AEGQGRGC; see Fig. 1) were cloned into the pMalC-133-Aval 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-Aval vector contains the MBP gene modified to contain an N-terminal His tag and three inserted restriction sites: an asymmetric Aval restriction site (c/ctcgag) flanked by two SfiI 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 Aval-compatible overhangs were ligated into the Aval-digested pMalC-133-Aval vector. The addition of the Aval 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-Aval 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 OD₆₀₀nm = 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

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.

Expression of the wild-type and recombinant (Rec) copy of the protein VIII gene were 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 SfiI 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 SfiI 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 SfiI 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 SfiI restriction sites. The glycine marked in parentheses is optional because it is present only when the SfiI 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 SfiI-compatible ends were ligated into the SfiI-digested fth1 vector. For display on protein III, a modified fth1 vector was used where a BstXI 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 BstXI-compatible overhangs and ligated into the BstXI-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: BD016786.1) and binds gp120 specifically. Phage analysis conducted on the 1B6 mAb yielded a 14-amino-acid long peptide (CWGTCNLNKTTA(T)S) that has high affinity for the 1B6 mAb and is henceforth referred to as the 1B6 peptide. Sequences encoding for the 1B6 peptide were
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 (PQDKVFPPGQG) and core 2 (RTNTNRPOPDV) 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 (GRSWAQPGYPW-PLY) 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 monoclonal or rabbit polyclonal antibodies specific for M13 bacteriophage (RNTNRRPQDV) were coated with either murine or polyclonal antibodies specific for M13 bacteriophage (RNTNRRPQDV) were coated with either murine or polyclonal antibodies specific for M13 bacteriophage.

**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 SfiI 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 SfiI sites, no recombinant protein VIII is produced and the phages display only wild-type protein VIII. Due to the presence of the SfiI insertion cassette, recombinant protein VIIIIs 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 SfiI-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 AvaI site (CTCGGG) found in the modified MBP expression vector pMalC-133-Aval (see Materials and Methods). We previously demonstrated that cloning inserts into such Aval 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 S’ 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 immobilize 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 phases (Fig. 3): the wild-type phase and two types of phases displaying the 1B6 peptide (CWGTNCLNKTATNS) that is recognized by the 1B6 mAb (see Materials and Methods). The first phase expresses the 1B6 peptide on recombinant protein VIII and is a type 88 chimeric phase. The second phase 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 phases displaying the 1B6 peptide on recombinant protein VIII and shows no binding to phases displaying the same sequence on protein III or to the wild-type fth1 phases. The 1B6 mAb binds both phases 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 phases at similar levels, illustrating equal overall phase levels for all three phase types. These results indicate that all three phase types are present in equal amounts, that the 1B6 peptide is displayed on both the protein VIII and protein III phases, 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 phases. In this
of phages displaying the 1B6 peptide. As shown in Fig. 4, binding to recombinant total phage concentrations but with ever decreasing amounts of phages displaying the 1B6 peptide on protein VIII at a concentration of 10^{11} phages per ml. Homogeneous phages displaying the 1B6 peptide on all copies of protein VIII while containing only wild-type protein VIII (gray); recombinant 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.

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 which 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.

**Testing GIL–Ab as a general monitor of 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 VIIIIs 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.
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