Display of Functional β-Lactamase Inhibitory Protein on the Surface of M13 Bacteriophage

WANZHI HUANG,1 JOSEPH PETROSINO,1 AND TIMOTHY PALZKILL1-2*

Department of Microbiology and Immunology1 and Department of Biochemistry,2 Baylor College of Medicine, Houston, Texas 77030

Received 3 April 1998/Returned for modification 23 July 1998/Accepted 10 August 1998

The display of proteins on the surface of filamentous phage has been shown to be a powerful method to select variants of a protein with altered binding properties from large combinatorial libraries of mutants. The β-lactamase inhibitory protein (BLIP) is a 165-amino-acid protein that binds and inhibits TEM-1 β-lactamase-catalyzed hydrolysis of the penicillin and cephalosporin antibiotics. Here we describe the construction of a new phagemid vector and the use of this vector to display BLIP on the surface of filamentous phage. It is shown that BLIP-displaying phage bind to immobilized β-lactamase and that the binding can be competed off by the addition of soluble β-lactamase. In addition, a two-step phage enzyme-linked immunosorbent assay procedure was used to demonstrate that the BLIP-displaying phage bind β-lactamase with a 50% inhibitory concentration of 1 nM, which compares favorably with a previously published \( K_i \) of 0.6 nM. A system has therefore been established for protein engineering of BLIP to expand its range of binding to other β-lactamases and penicillin-binding proteins.

β-Lactam antibiotics, such as the penicillins and cephalosporins, are among the most often used antimicrobial agents. Because of their widespread use, bacterial resistance to these antibiotics has become an increasing problem (7). The most common mechanism of resistance is the production of β-lactamases. β-Lactamases are secreted to the periplasm of gram-negative bacteria, or extracellularly in gram-positive bacteria, where they hydrolyze β-lactam antibiotics to create ineffective antimicrobials. There are a large number of β-lactamases which are found to be encoded either on plasmids or on the bacterial chromosome (3). In gram-negative bacteria, the most common plasmid-mediated β-lactamase is the TEM-1 β-lactamase (30). This enzyme efficiently hydrolyzes penicillins and many cephalosporins and is therefore a widespread source of β-lactam resistance (18).

An effective means of combating TEM-1 β-lactamase-mediated resistance has been the clinical use of small-molecule β-lactamase inhibitors such as sulbactam and clavulanic acid (21). These molecules do not possess significant antimicrobial activity themselves but are used in conjunction with other β-lactam antibiotics, such as ampicillin. The inhibitor protects the antibiotic from the action of β-lactamase and thereby restores the therapeutic value of the antibiotic. However, in recent years, there have been many reports of resistance to β-lactam–β-lactamase inhibitor combinations (12). This resistance is due to mutations in β-lactamase that enable the enzyme to avoid inactivation by the inhibitor while retaining the ability to hydrolyze β-lactam antibiotics (15).

The small-molecule inhibitor clavulanic acid is a natural product from Streptomyces clavuligerus (19). In addition to clavulanic acid, S. clavuligerus also produces a protein inhibitor of β-lactamase, called β-lactamase inhibitory protein (BLIP) (8). BLIP is a 165-amino-acid protein encoded by the bli gene that binds and inhibits TEM-1 β-lactamase, with a reported \( K_i \) of 0.6 nM (28). BLIP also binds to other β-lactamases from both gram-negative and gram-positive bacteria, albeit with reduced affinity. In addition, BLIP has been reported to inhibit the Enterococcus faecalis PBP 5 with a \( K_i \) of 12 μM (28). The X-ray structure of BLIP has been solved both alone and in complex with TEM-1 β-lactamase and has revealed the residues making up the binding surface of BLIP (27, 28). Using this information, it may be possible to use site-directed mutagenesis techniques as a means of increasing BLIP binding and inhibition of β-lactamases and penicillin-binding proteins. If BLIP can be exploited as a molecular scaffold to engineer binding interactions, it may be possible to create new BLIP-based antibiotics and inhibitors.

Phage display has proven to be an effective methodology for the selection of binding partners of high affinity or altered specificity (26). Monovalent display of a protein of interest can often be achieved by fusing the gene encoding the protein to the N terminus of the M13 gene III coat protein (17). High-affinity variants can then be obtained by creating phage libraries containing mutants of the protein of interest and sequencing the corresponding DNA packaged in the phagemid particles after several rounds of binding selection (20). Here we describe the cloning of the BLIP gene into a new phagemid vector. The vector encodes chloramphenicol resistance and contains an amber codon at the 5′ end of gene III. BLIP is fused at its N terminus to the β-lactamase signal sequence and at its C terminus to the protein encoded by gene III of the phage (g3p). Transcription of the fusion is controlled by the constitutive β-lactamase promoter (4). Specific binding of phage containing the BLIP-g3p fusion to immobilized β-lactamase is demonstrated to occur with nanomolar affinity. This system can now be used to engineer new BLIP binding specificities.

MATERIALS AND METHODS

Bacterial strains. Escherichia coli XL1-Blue (2) [F−; Tra+ proA+ B− lac+ Δ(lacZ)M15 recA1 endA1 gyr96 (NalR) thi hsdR17 (r− m−) supE44 relA1 lac], was used for transformations of ligations, and E. coli TG1 (10) [F− traD36− lac− Δ(lacZ)M15 proA+ B− supE4 (ΔhisM merB) (r− m− MmB−)], was used for production, amplification, and determination of the titer of bacteriophage.
FIG. 1. Maps of the plasmids used in this study. Restriction endonuclease sites used for the construction of the vectors (Materials and Methods) are also shown.
method of Lowman and Wells (17). Fifty milligrams of beads was suspended in 0.1 M sodium carbonate buffer (pH 9.6) and was incubated with purified TEM-1 β-lactamase at a concentration of 0.04 mg/ml for 24 h at 4°C. Unreacted oxirane groups were blocked by incubation with 10 mg of bovine serum albumin (BSA) per ml overnight at 4°C. The beads were then pelleted and washed several times with buffer A which is Tris-buffered-saline (25) containing 1 mg of BSA per ml and 0.5 g of Tween 20 per liter. The beads were stored in buffer A in a final volume of 0.5 ml. For panning, 10^11 phage were added to 5 mg of β-lactamase-conjugated oxirane beads in a final volume of 0.5 ml in buffer A. The binding mixture was incubated for 2 h at room temperature with rocking to reach equilibrium. The beads were then washed 10 times with 0.75 ml of buffer A. The bound phage were eluted from the beads by incubation with 0.2 ml of elution buffer (0.1 M glycine (pH 2.2), 1 mg of BSA per ml, 0.5 g of Tween 20 per liter, 0.1 M KCl) for 30 min. The elution mixture was neutralized with 25 pl of 1 M Tris-CI (pH 8.0). The phage titer of the elution mixture was determined as described above. The eluted phage were amplified by adding 0.15 ml of the neutralized elution mixture to 5 ml of E. coli TG1 cells. After 30 min of incubation at room temperature, 25 ml of 2YT medium (25) was added along with 0.15 ml of 2YT medium (25) was added along with 10^9 VCS M13 helper phage (Stratagene). The phage were precipitated as described above after overnight incubation with shaking at 37°C.

**Phage ELISA.** A two-step phage enzyme-linked immunosorbent assay (ELISA) (6) was performed to measure BLIP phage affinity for TEM-1 β-lactamase. Microtiter plates (Nunc; Maxisorp, 96 wells each) were coated with purified TEM-1 β-lactamase (at 10 μg/ml) in 50 mM sodium carbonate (pH 9.6) at 4°C overnight. The plates were then blocked with SuperBlock (Pierce) for 2 h at room temperature. Serial dilutions of the BLIP phage stock were added to the wells and incubated for 2 h at room temperature in buffer A at a final volume of 0.15 ml. The plates were then washed several times with buffer A, and the bound phage were probed with a sheep anti-M13 polyclonal antibody conjugated to horseradish peroxidase (HRP). To determine phage affinity, serial dilutions of β-lactamase and a subsaturating concentration of 1.3 × 10^11 BLIP phage were added to wells in 0.1 ml of buffer A. After 2 h at room temperature, the wells were washed multiple times with buffer A, and bound phage were probed as described above. Affinities (IC50) were calculated as the concentration of competing β-lactamase that resulted in half-maximal phage binding. The half-maximal concentration was calculated by converting the data in Fig. 3 from log to linear values and fitting the binding curve to the equation for a hyperbola.

**RESULTS**

**Construction of a BLIP phage display vector.** A new phagemid vector, pG3-C3, was constructed for the display of BLIP (Fig. 1). The pG3-C3 phagemid encodes chloramphenicol resistance and is designed to express heterologous proteins as fusions at their N terminus to the signal sequence of TEM-1 β-lactamase. In addition, the heterologous proteins are fused at their C terminus to the g3p of M13 for display on the surface of the bacteriophage. The fusion protein is expressed under the control of the constitutive β-lactamase promoter. Because there is an amber codon at the fusion junction between the heterologous protein and g3p, it is necessary to express the fusion in an amber suppressor-containing strain of E. coli. The pG3-C3 g3p plasmid was constructed by insertion of a SacI-I-XbaI DNA fragment containing the promoter and gene encoding TEM-1 β-lactamase into the pG3-C3 plasmid, as shown in Fig. 1. The **bla**_TEM-1_**gene** that was inserted was previously engineered to contain a SalI linker insertion at the codon for the third amino acid position beyond the cleavage site of the β-lactamase signal sequence (14). The SalI linker provides a site to fuse a heterologous gene encoding a protein that will be secreted under the control of the β-lactamase signal sequence. pG3-BLIP was constructed by PCR amplification of the **bla** gene by using primers containing XhoI and XbaI sites. The **bla** fragment was then inserted into the **SalI-,XbaI-digested pG3-C3 vector** (Fig. 1). The resulting phagemid is designed to express the BLIP-g3p fusion, which, after cleavage of the β-lactamase signal sequence, contains the entire amino acid sequence of the wild-type BLIP. Two two-round panning with serial dilutions of purified β-lactamase, and serial dilutions of phage displaying wild-type BLIP were allowed to bind to the immobilized protein in the presence of a large excess of BSA. After washing, bound phage were stained with HRP-conjugated anti-M13 antibody. The binding curve in Fig. 3A demonstrates that the pG3-BLIP phage bind β-lactamase. To quantify binding, ELISA plates were coated with wild-type and a constant saturating concentration of pG3-BLIP phage was added with serial dilutions of purified β-lactamase. As seen in Fig. 3B, the pG3-BLIP phage were competed off of the immobilized β-lactamase with soluble β-lactamase at an IC50 of 1 nM. This affinity compares favorably to the published **Ki** value of 0.6 nM for the BLIP-β-lactamase interaction (28). These results show that BLIP is expressed on the surface of the bacteriophage in a form that binds tightly and specifically to β-lactamase.

**Specific enrichment of BLIP phage by panning on β-lactamase.** In order to use the pG3-BLIP phagemid to engineer BLIP for altered binding properties, it is necessary to be able to select binding phage by panning on an immobilized substrate. This was tested by attaching purified β-lactamase to oxirane-acryl beads and incubating the beads with 10^11 phage from the pG3-BLIP phage stock in the presence of a large excess of BSA and a constant saturating concentration of pG3-BLIP phage was added with serial dilutions of purified β-lactamase. Two two-round panning with serial dilutions of purified β-lactamase. As seen in Fig. 3B, the pG3-BLIP phage were competed off of the immobilized β-lactamase with soluble β-lactamase at an IC50 of 1 nM. This affinity compares favorably to the published **Ki** value of 0.6 nM for the BLIP-β-lactamase interaction (28). These results show that BLIP is expressed on the surface of the bacteriophage in a form that binds tightly and specifically to β-lactamase.

**Specific enrichment of BLIP phage by panning on β-lactamase.** In order to use the pG3-BLIP phagemid to engineer BLIP for altered binding properties, it is necessary to be able to select binding phage by panning on an immobilized substrate. This was tested by attaching purified β-lactamase to oxirane-acryl beads and incubating the beads with 10^11 phage from the pG3-BLIP phage stock in the presence of a large excess of BSA and a constant saturating concentration of pG3-BLIP phage was added with serial dilutions of purified β-lactamase. As seen in Fig. 3B, the pG3-BLIP phage were competed off of the immobilized β-lactamase with soluble β-lactamase at an IC50 of 1 nM. This affinity compares favorably to the published **Ki** value of 0.6 nM for the BLIP-β-lactamase interaction (28). These results show that BLIP is expressed on the surface of the bacteriophage in a form that binds tightly and specifically to β-lactamase.
which produced the nondisplaying phage, was constructed by inserting a gene cassette encoding spectinomycin resistance (23) into the chloramphenicol resistance gene of pG3-C2 (Fig. 1). The advantage of this system is that the extent of enrichment of nondisplaying phage versus BLIP-displaying phage can be determined simply by determining the titer of the phage recovered from the oxirane beads on spectinomycin-containing agar plates as well as chloramphenicol-containing agar plates. The results in Table 1 illustrate that 27-fold more pG3-BLIP phage were recovered from the oxirane beads on spectinomycin-containing agar plates as well as chloramphenicol-containing agar plates.

The advantage of this system is that the extent of enrichment of nondisplaying phage versus BLIP-displaying phage can be determined simply by determining the titer of the phage recovered from the oxirane beads on spectinomycin-containing agar plates as well as chloramphenicol-containing agar plates. The results in Table 1 illustrate that 27-fold more pG3-BLIP phage were recovered from the oxirane beads on spectinomycin-containing agar plates as well as chloramphenicol-containing agar plates.

The results in Table 1 illustrate that 27-fold more pG3-BLIP phage were recovered from the oxirane beads on spectinomycin-containing agar plates as well as chloramphenicol-containing agar plates. The data in Table 1 indicate that 13-fold more BLIP phage were bound to β-lactamase than to the BSA control (compare pG3-BLIP in the column with 0 μM β-lactamase added versus that with BSA beads). In addition, there was only a twofold difference in the number of BLIP phage versus nondisplaying phage recovered from the BSA beads (compare pG3-BLIP and pG3-SPT in the BSA bead column). This is in contrast to the 27-fold difference between pG3-BLIP phage and nondisplaying phage recovered from β-lactamase beads described above. These data provide further evidence that BLIP phage bind specifically to immobilized β-lactamase.

**DISCUSSION**

Phage display is a powerful method for engineering the binding affinity and specificity of many different molecules, including antibody fragments, enzymes, hormones, protease inhibitors, and DNA binding proteins (reviewed in references 5 and 20). The ability to use phage display to alter the binding properties of a protein depends on whether a polypeptide can be expressed and displayed on the surface of the bacteriophage in a folded, functional form. Here we have displayed BLIP as a fusion to g3p and have shown that bacteriophage containing the fusion bind specifically and with high affinity to β-lactamase.

Display of functional BLIP was accomplished by constructing a new phage display vector that allowed BLIP to be expressed under the control of the constitutive TEM-1 β-lactamase promoter and secreted under the direction of the β-lactamase signal sequence. Many phage display vectors use the lac promoter for expression of the heterologous protein-g3p fusion (1, 13). Exposure of E. coli to IPTG can induce the lac promoter to high levels of transcription. However, in the absence of IPTG, there is still significant transcription because the bacterial cell usually does not contain enough lac repressor to bind all of the lac promoters from a multicopy plasmid (9). For phage production, IPTG is not used because of toxicity of the g3p fusion proteins and the resultant low phage titer (13). The β-lactamase promoter is weak and is not inducible (4). The low levels of transcription of the BLIP-g3p fusion are not toxic to E. coli, and titers of 10^12 to 10^13 phage/ml were routinely obtained in the experiments reported here.

Because of the high frequency of frameshift mutations in the BLIP gene found among transformants after insertion of the BLIP gene into the pG3-C3 vector, it was necessary to obtain

<table>
<thead>
<tr>
<th>Phage</th>
<th>No. of phage particles recovered from beads:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-Lactamase-coated beads + β-lactamase</td>
</tr>
<tr>
<td></td>
<td>0 μM</td>
</tr>
<tr>
<td>pG3-BLIP</td>
<td>2.3 × 10^6</td>
</tr>
<tr>
<td>pG3-SPT</td>
<td>8.6 × 10^5</td>
</tr>
</tbody>
</table>

*Results represent the number of phage particles recovered from beads after the application of 10^11 phage in the binding reaction mixture. Note that each column represents a binding experiment, and in each experiment, 10^11 pG3-BLIP phage and pG3-SPT phage were added simultaneously to the beads.
a nonmutant sequence by functional selection for BLIP phage that bound to immobilized β-lactamase. It is unclear why such a high frequency of clones contained frameshift mutations in BLIP in the nonselected population. Presumably it is due to a high frequency of polymerase errors during PCR. A possible reason for this is the very high G-C content of the BLIP gene. The fragment of BLIP cloned into the pG3-C3 vector is 69% G-C. G-C DNA templates are often difficult to amplify by PCR (11). The difficulty may be due to the higher melting temperature of G-C-rich template DNA or to extensive secondary structure in the template (24). The finding of a high frequency of frameshift mutations in the amplified BLIP DNA may indicate that PCR of G-C-rich DNA has reduced fidelity.

The development of the BLIP phage system will now be exploited to determine which residues on BLIP are critical for TEM-1 β-lactamase binding and to select for variants that bind tightly to other β-lactamases or penicillin-binding proteins. To achieve this, libraries of random mutants of BLIP will be created in the pG3-BLIP vector. The phage libraries will then be panned on purified TEM-1 β-lactamases and other β-lactamases as described here.

ACKNOWLEDGMENTS

We thank Gary Rudders for comments on the manuscript. This work was supported in part by NIH grant AI32956.

REFERENCES


