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Protein minimization by random fragmentation and selection

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Protein–protein interactions are involved in most biological processes and are important targets for drug design. Over the past decade, there has been increased interest in the design of small molecules that mimic functional epitopes of protein inhibitors. BLIP is a 165 amino acid protein that is a potent inhibitor of TEM-1 β-lactamase (K_i = 0.1 nM). To aid in the development of new inhibitors of β-lactamase, the gene encoding BLIP was randomly fragmented and DNA segments encoding peptides that retain the ability to bind TEM-1 β-lactamase were isolated using phage display. The selected peptides revealed a common, overlapping region that includes BLIP residues C30–D49. Synthesis and binding analysis of the C30–D49 peptide indicate that this peptide inhibits TEM-1 β-lactamase. Therefore, a peptide derivative of BLIP that has been reduced in size by 88% compared with wild-type BLIP retains the ability to bind and inhibit β-lactamase.

Keywords: β-lactamase/BLIP/drug design/protein minimization/protein–protein interactions

Introduction

β-Lactam antibiotics such as the penicillins and cephalosporins are among the most often used antibacterial agents. The hydrolysis of β-lactam antibiotics, catalyzed by β-lactamases, is a common mechanism by which bacteria become resistant to β-lactam antibiotics. Based on primary sequence homology, β-lactamases have been categorized into four classes (A–D) of which all β-lactamases operate by hydrolyzing the amide bond of β-lactam antibiotics to generate ineffective antimicrobial agents (Matagne *et al.*, 1990; Petrosino *et al.*, 1998). The most prevalent plasmid-encoded β-lactamase in Gram-negative bacteria is the class A TEM-1 β-lactamase, which catalyzes the hydrolysis of both penicillins and cephalosporins (Bush, 1989; Wiedemann *et al.*, 1989; Matagne *et al.*, 1990; Frere *et al.*, 1999). Extended-spectrum cephalosporins have been introduced in an effort to circumvent the action of TEM-1 β-lactamase. The use of these agents, however, has resulted in the emergence of β-lactamases capable of hydrolyzing the extended spectrum antibiotics (Bush *et al.*, 1995; Medeiros, 1997; Sideraki *et al.*, 2001).

Another method to combat β-lactam resistance has been the use of small molecule β-lactamase inhibitors, such as clavulanic acid and sulbactam (English *et al.*, 1978; Page and Laws, 1998). These mechanism-based inhibitors protect the β-lactam drug from hydrolysis by β-lactamases and restore the therapeutic potential of the antibiotic (Charnas *et al.*, 1978; Fisher *et al.*, 1978; Therrien and Levesque, 2000). Variants of β-lactamase have now evolved, however, that resist these inhibitors while still maintaining the ability to hydrolyze β-lactam antibiotics (Imtiaz *et al.*, 1994; Henquell *et al.*, 1995; Palzkill, 1998).

Clavulanic acid was initially purified from the soil bacterium *Streptomyces clavuligerus*, which also produces a protein inhibitor of β-lactamases called β-lactamase inhibitor protein (BLIP) (Doran *et al.*, 1990). BLIP is a 165 amino acid protein composed of two-domains of ~78 residues each and is a potent inhibitor of TEM-1 β-lactamase (K_i = 0.1–0.6 nM) (Strynadka *et al.*, 1994; Petrosino *et al.*, 1999; Rudgers and Palzkill, 1999). In addition, BLIP inhibits β-lactamases from both Gram-positive and Gram-negative bacteria to varying degrees and also inhibits the cell-wall transpeptidase PBP5 from *Enterococcus faecalis* (Strynadka *et al.*, 1994).

The co-crystal structure of TEM-1 β-lactamase and BLIP reveals that BLIP binds just outside the active site pocket of β-lactamase and inserts two loops, one from each domain, into the active site of the enzyme (Figure 1) (Strynadka *et al.*, 1996). The majority of interactions with TEM-1 β-lactamase arise from domain-1 of BLIP, with Asp49 from the loop of domain-1 making four critical hydrogen bonds to four conserved residues in the active site pocket of the enzyme (Strynadka *et al.*, 1996). Together with Phe142 from the loop in domain 2, the residues from the two turns of BLIP mimic the binding of penicillin G in the active site pocket of the enzyme (Strynadka *et al.*, 1996).

Mimicking the binding and inhibition activity of BLIP via a small peptide would be very beneficial for the development of novel β-lactamase inhibitors and antibiotics. However, the rational design of an effective inhibitor of β-lactamase based on the structure of BLIP would be a daunting process because of the large interface between TEM-1 β-lactamase and BLIP that consists of 2636 Å^2 (Strynadka *et al.*, 1996). In addition, although the co-crystal structure of TEM-1 β-lactamase with BLIP reveals residues in direct contact, a definitive role in binding has not been assigned for a majority of these positions. Finally, residues that have been shown to be involved in binding and inhibition of β-lactamase, such as Asp49 and Phe142, lie in different domains that are distant in primary sequence from one another (Figure 1) (Petrosino *et al.*, 1999).

Phage display is an effective method for sorting large, randomized libraries of mutants for specific proteins or peptides that bind a target molecule with high affinity (Lowman *et al.*, 1991; Dennis *et al.*, 1995; Rudgers and Palzkill, 1999; Huang *et al.*, 2000). To aid in the development of BLIP-based peptide inhibitors of β-lactamases, we have expanded on this property of phage display to select small, contiguous regions of BLIP that retain the ability to bind and inhibit TEM-1 β-lactamase from a large pool of randomly fragmented BLIP peptides. The results obtained suggest that random fragmentation of a gene followed by phage display selection of fragments that encode peptides that retain binding function is an efficient method to identify peptide mimics of a protein–protein interaction.

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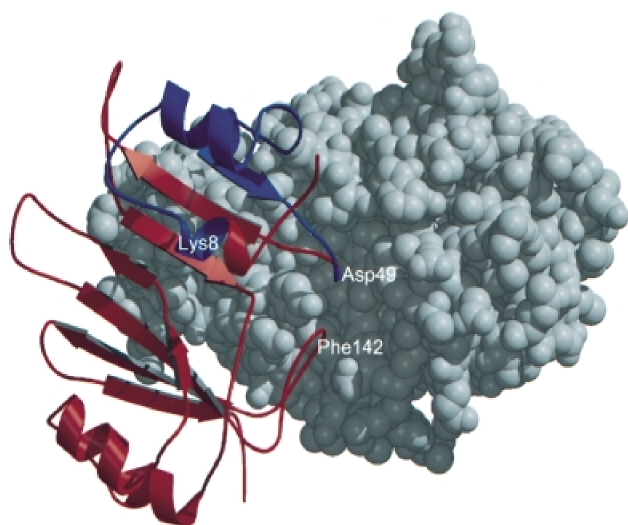

Fig. 1. Structure of complex between BLIP and TEM-1 β-lactamase. TEM-1 β-lactamase is shown in space-fill and is colored gray. The BLIP structure is presented in ribbon format and colored red. The K8–D49 peptide region of BLIP is indicated in blue. The position of the BLIP Asp49 and Phe142 turn regions that insert into the active site of TEM-1 β-lactamase are labeled. The figure was produced using the Molscript (Kraulis, 1991) and Raster3d (Merritt and Murphy, 1994) programs.

Materials and methods

Bacterial strains and plasmids

Escherichia coli XL1-Blue (Bullock *et al.*, 1987) (Stratagene) was used for transformation of the ligation reaction of the pGR48 library and to generate the initial unpanned bacteriophage stocks of the pGR48 library. *E.coli* TG1 (Gibson, 1984) was used for all other transformations of ligation reactions and also for production, amplification and determination of the titer of bacteriophage stocks. The *bli* gene was PCR amplified from the pTP154 plasmid that has been described previously (Huang *et al.*, 2000). To display randomized peptide libraries on the surface of M13 bacteriophage, the pTP167 phagemid was used (T.Palzkill, unpublished data). This vector expresses proteins under the control of the constitutive β-lactamase promoter as fusions to the His6-tag at their N-terminus and the M13 gVIIIp at their C-terminus. The vector encodes chloramphenicol resistance allowing for the selection of bacteria harboring the phagemid on medium containing chloramphenicol.

Construction of minimized BLIP phage-display library

To construct phage display libraries of randomly fragmented BLIP peptides, *bli* was fragmented using a nebulizer as described (http://dna1.chem.ou.edu/protocol_book/protocol_partII.html). The BLIP gene was PCR amplified from pTP154 using the top strand primers PD-bla1 (5′-CGGGGGAGCTCGTTTCTTAGACGTCAGGTGGC-3′) and the bottom strand primer BLIP-Xba (5′-CGGGCCGTCTAGAATACAAGGTCCCACTGCCGCTTG-3′). Since the PCR primers used to amplify *bli* are complementary to regions outside the gene of interest, an additional 251 nucleotides upstream and eight nucleotides downstream of *bli* were also amplified, generating a 760 bp PCR product. Approximately 20 μg of the PCR product in 2 ml of ice-cold sterile 40% glycerol, 10 mM Tris, pH 7.5 was added to a nebulizer cup (IPI Medical Products, Part No. 4207) and placed on ice. The nebulizer was modified as described (http://dna1.chem.ou.edu/protocol_book/protocol_partII.html). The DNA was fragmented by applying 45 p.s.i. of nitrogen gas to the DNA sample at 5 min intervals for a total of 30 min. After each interval, the nebulizer was centrifuged at 150 *g* for 1 min at 4°C to collect the DNA sample in the bottom of the nebulizer. A 40% glycerol, 10 mM Tris, pH 7.5 solution was added to the nebulized sample after each centrifugation to bring the final volume of the sample back to 2 ml. The nebulized DNA was concentrated and cleaned using a QIAquick PCR purification kit (Qiagen) and resuspended in 50 μl of sterile, distilled, deionized water, pH 7.0.

The sheared BLIP DNA fragments were treated with T4-DNA polymerase according to the manufacturer (New England Biolabs) and incubated at 11°C for 1 h. The resulting DNA fragments were gel purified from a 0.8% agarose gel using a QIAquick (Qiagen) column to remove DNA fragments outside the desired range of 100–500 bp.

The BLIP fragment library gVIII phage display vector, pGR48, was constructed by replacing the 780 bp *Sal*I–*Xba*I fragment of pTP167, which contains the $bla_{\text{TEM-1}}$ gene, with fragmented *bli* DNA. A 3 μg amount of restriction enzyme-treated pTP167 vector was gel purified to remove the $bla_{\text{TEM-1}}$ DNA fragments and treated with calf-intestinal phosphatase followed by Klenow as described by the manufacturer (New England Biolabs). A 25 μl volume of the treated fragmented *bli* DNA was ligated into the digested pTP167 vector. Plasmid DNA was electroporated into *E.coli* XL1-Blue according to the manufacturer (Stratagene) and transformed bacteria were selected on LB agar containing 12.5 μg/ml chloramphenicol. A library size of ~40 000 inserts was obtained as determined by colony polymerase chain reaction (PCR) using the primer Blasig (5′-CAACATTTCCGTGTCGCCC-3′) and G8bamHI (5′GGGCGGATCCTATCAGCTTTCGAGG-TGAATTTCTT-3′) (Hanke and Wink, 1994). The resulting colonies were pooled using 1 ml of LB medium. A 1/50 volume of the pooled colonies was used to prepare bacteriophage library stocks.

Preparation, enrichment and titering of phage particles

The naive phagmid library was packaged and titered as described previously (Huang *et al.*, 2000). Panning was performed in 96-well microtiter plates coated with 0.2 ml of a 10 μg/ml solution of purified TEM-1 β-lactamase or BSA as described (Huang *et al.*, 2000). Phage amplification and phage titer determinations were performed as described (Huang *et al.*, 2000).

Expression and purification of β*-lactamase*

β-Lactamase was purified to >90% homogeneity using a zinc chelating Sepharose (fast flow) column (Pharmacia) and Sephadex G-75 gel filtration chromatography as described previously (Cantu *et al.*, 1997). His-BLIP was expressed using vector pGR32 and purified to >90% homogeneity as described previously (Petrosino *et al.*, 1999).

Phage ELISA

Phage ELISA was performed in 96-well microtiter wells as described (Huang *et al.*, 2000). A total of 5×10^{11} phage displaying one of the eight selected BLIP peptides in 0.2 ml of blocking buffer [2% milk powder in 1× Tris-buffered saline, pH 7.5 (Sambrook *et al.*, 1989)] were added in duplicate to the blocked BSA, β-lactamase or anti-His6-tagged antibody (New England Biolabs) wells. Bound phage were detected

using anti-M13 phage antibody conjugated to horseradish peroxidase (Pharmacia).

Peptide synthesis and inhibition assays

Peptides K8–D49 and C30–D49 were synthesized by Research Genetics (Huntsville, AL). The K8–D49 peptide was N-terminal biotinylated, cyclized and purified by HPLC to >90% purity. The C30–D49 peptide was cyclized and purified by HPLC to >90% purity. No modification of the C-terminus was performed for either peptide. Disulfide bonds were removed in the K8–D49 and C30–D49 peptides by the addition of 10 mM DTT to each peptide stock prior to performing inhibition assays. Inhibition assays were performed as described previously by incubating various concentrations of the cyclized or reduced K8–D49 peptide, C30–D49 peptide or a control peptide (protein kinase C substrate [PLSRTLSVAAKK]) (Sigma) with TEM-1 β-lactamase for 2 h at 25°C to establish equilibrium. Enzyme assays were performed in 0.05 M phosphate buffer (pH 7.0) containing 1 mg/ml BSA. Following incubation, 70 μM cephaloridine was added and hydrolysis of the substrate was monitored at 260 nm. The final volume of the reaction was 0.05 ml and the extinction coefficient used for cephaloridine was $\Delta\varepsilon = 10\,200\ \mathrm{m}^{-1}\ \mathrm{cm}^{-1}$ (Petrosino *et al.*, 1999). DTT concentrations were adjusted such that all samples contained equal concentrations of the reducing agent. No significant loss of wild-type TEM-1 β-lactamase activity was detected in assays containing DTT alone.

Results

Fragmentation of BLIP for phage display

The minimum sequence and structural requirements of BLIP to inhibit TEM-1 β-lactamase are unknown and difficult to predict. To identify the minimum peptide sequence of BLIP that is sufficient to inhibit TEM-1 β-lactamase, random peptide fragments of BLIP ranging in size from ~33 to 166 amino acid residues were fused to the gene VIII protein (gVIIIp) of M13 bacteriophage. To generate the fragmented BLIP peptides for phage display, the PCR-amplified BLIP gene, *bli*, was randomly fragmented into 100–500 bp segments (see Methods). The sheared DNA fragments were ligated into the phage display vector, pTP167, which is designed to express the peptides as fusion proteins to a His6-tag at their N-terminus and to the M13 gVIIIp at their C-terminus. A library size of ~40 000 independent inserts was generated. Analysis of fragments within the library revealed that a range of DNA inserts from 100 to 500 bp were present with no evidence of bias towards a particular insert size (data not shown). Sequence analysis of 13 randomly chosen inserts revealed that all the inserts between the His6-tag and M13 gVIII consisted of *bli* DNA, none of which contained in-frame BLIP sequences with both the His6-tag and gVIIIp.

Although there was no preference for the size of DNA inserts in the phage display library, there was a bias for the insertion of fragments containing the extreme 3′ terminus of the *bli* gene. Eleven of the 13 inserts sequenced contained the same 3′ terminus but different 5′ starting termini. The bias may be due to insufficient treatment of the sheared PCR fragments by T4-DNA polymerase. This would favor DNA inserts that required only one terminus to be filled by the DNA polymerase, such as fragments harboring the original, blunt-end 5′ or 3′ terminus of the PCR product. However, it is not known why the 3′ end of the PCR fragment was so strongly favored over the 5′ terminus.

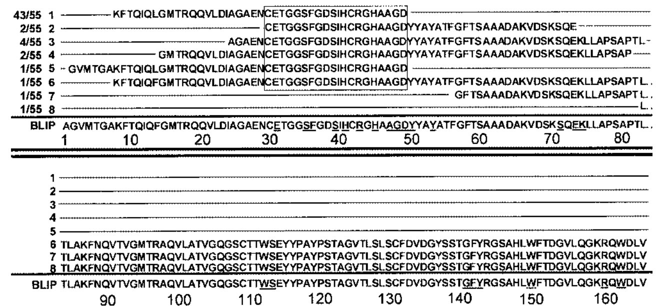

Fig. 2. BLIP amino acid sequence of peptides selected by phage display for binding TEM-1 β-lactamase. The total number of times each peptide was found among 55 inserts sequenced is indicated to the left of the eight selected peptides. The C30–D49 region common to six peptides is boxed. The wild-type BLIP sequence is numbered and shown below the selected peptide sequences. Underlined residues in the BLIP sequence indicate residues that contact TEM-1 β-lactamase according to the co-crystal structure of β-lactamase and BLIP (Strynadka *et al.*, 1996).

Enrichment of BLIP peptides that bind β-lactamase

To identify BLIP peptides that bind to β-lactamase, the phage display library was enriched for phage particles that display peptides that bind to immobilized TEM-1 β-lactamase. After extensive washing to remove unbound phage, the bound phage were eluted from the immobilized β-lactamase and used to infect *E.coli*. The phage were amplified in the infected bacteria and used for another round of binding enrichment. After each round of binding enrichment, representative clones were randomly selected to determine if the library was converging on a particular BLIP insert. Colony PCR allowed the rapid determination of the DNA insert size by using primers that flanked the BLIP inserts. After one round of panning, the enriched library remained highly diverse with inserts ranging from 100 to 500 bp, similar to the starting library. However, by round two the library began to converge on a 120 bp insert and by round three 15 of the 16 selected clones examined contained a single 120 bp insert (data not shown). The panning experiment was repeated with the naive phage display library and the library again began to converge on a 120 bp insert during the third round of binding enrichment. By round four the library had completely converged on a 120 bp DNA insert.

DNA inserts from clones isolated from the final two rounds of each binding enrichment experiment were sequenced to determine the identity of the enriched fragments. All inserts were BLIP sequences in-frame with both the His6-tag and the M13 gVIIIp. Out of 55 clones sequenced, inserts encoding a total of eight different BLIP peptides were identified, with 43 of the inserts encoding a BLIP peptide consisting of residues K8–D49 and also containing the mutation F14L (Figure 2). The convergence of the library on the K8–D49 sequence in two independent panning experiments suggests that the K8–D49 peptide binds to β-lactamase. Six peptides were derived from domain 1 of BLIP and had in common the BLIP C30–D49 residues (Figure 2). The fact that all of the clones isolated contained in-frame BLIP fragments with the His6-tag and gVIII in the proper orientation whereas the starting library did not suggest that functional peptides were selected from the naive library. The finding of peptides derived from domain 1 after phage display is also striking considering the bias in the naive library towards clones containing domain 2.

Phage ELISA was used to verify that the eight different peptides identified by the phage display experiments bind to immobilized TEM-1 β-lactamase. Two-fold more phage

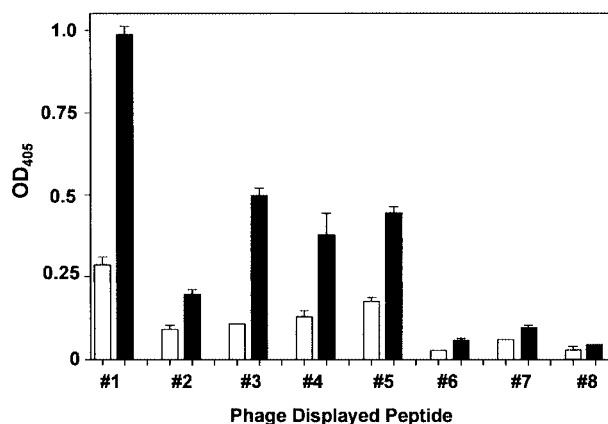

Fig. 3. ELISA measurements of the TEM-1 β-lactamase binding capacity versus the BSA binding capacity of the eight selected BLIP peptides displayed on the surface of M13 bacteriophage. TEM-1 β-lactamase measurements are shown in black and BSA measurements in white for each phage-displayed peptide. Peptides are numbered as in Figure 2.

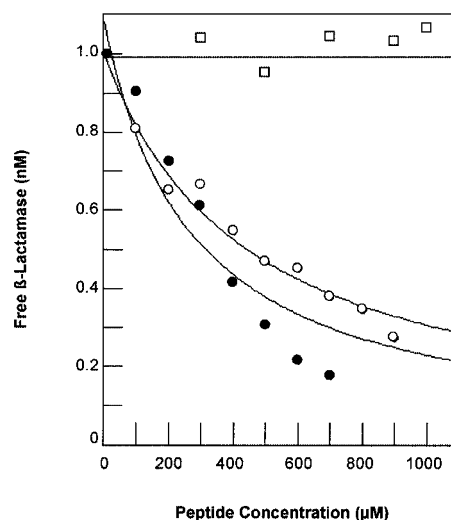

Fig. 4. Inhibition assays of TEM-1 β-lactamase using the K8–D49, C30–D49 and control peptides. Peptide inhibitory activity is expressed as the remaining concentration of free β-lactamase at various peptide concentrations. For each set of data points a non-linear regression fit was calculated. Filled circles, reduced K8–D49 peptide; open circles, reduced C30–D49 peptide; squares, control peptide (protein kinase C substrate). Each data point represents the average of two independent experiments.

displaying the K8–D49 peptide were found to bind β-lactamase compared with the other phage-displayed peptides tested. In addition, only phage-displaying peptides that included the C30–D49 BLIP sequence were found to bind β-lactamase (Figure 3).

To verify further that the full-length BLIP peptides were displayed on the surface of the M13 phage, the phage displaying the eight different peptides were tested for binding to anti-His6-tag antibody by phage ELISA. Since a His6-tag is fused to the N-terminus of each phage-displayed peptide, only phage displaying full-length peptides would be expected to bind the coated ELISA wells. The results indicated that phage displaying all eight peptides tested bound the antibody-coated wells, confirming that the full-length peptides are displayed on the surface of the M13 phage particles (data not shown).

Inhibition of β-lactamase by minimized BLIP peptides

Although the K8–D49 peptide was selected based on the ability to bind TEM-1 β-lactamase, it is possible that the peptide could bind without inhibiting function. To establish if the K8–D49 peptide inhibits TEM-1 β-lactamase, the full-length peptide was synthesized and used for *in vitro* inhibition studies. Since the crystal structure of BLIP reveals a disulfide bond between residues C30 and C42, it was hypothesized that this bond formation may be important in the inhibition of β-lactamase by the K8–D49 peptide and therefore the peptide was oxidized to form the disulfide bond. The inhibition assay involved incubating various concentrations of the K8–D49 peptide in the presence of TEM-1 β-lactamase until equilibrium was achieved. After the incubation phase, the β-lactamase substrate, cephaloridine, was added and the hydrolysis of the substrate was monitored to determine the concentration of peptide required to inhibit TEM-1 β-lactamase. The K_i for the BLIP peptide was determined as 352 μM, indicating that the selected peptide retained the ability both to bind and to inhibit TEM-1 β-lactamase.

To determine the importance of the disulfide bond in the inhibition of β-lactamase by the K8–D49 peptide, the disulfide bridge between the C30 and C42 residues was reduced. The inhibition assay using the reduced peptide and TEM-1 β-lactamase revealed a small increase in binding with K_i = 294 μM, indicating that the disulfide bond is not critical for the inhibition of β-lactamase by the K8–D49 peptide (Figure 3). Inhibition of β-lactamase also appears specific to the K8–D49 peptide in that no inhibition of TEM-1 β-lactamase was observed when using the 12 amino acid protein kinase C substrate peptide (Figure 4).

The prevalence of the common overlapping region C30–D49 in six of the eight selected peptides suggests that this region is important for binding and inhibition of TEM-1 β-lactamase. To investigate the importance of this region, the C30–D49 peptide was synthesized and oxidized to form a disulfide bond between the C30 and C42 residues. Inhibition assays using both the oxidized and reduced forms of the C30–D49 peptide revealed the peptides inhibit TEM-1 β-lactamase with K_i values of 683 and 446 μM, respectively (Figure 4). The fact that the C30–D49 peptide inhibits TEM-1 β-lactamase to approximately the same degree as the K8–D49 peptide suggests that the C30–D49 region is critical for binding and inhibition of TEM-1 β-lactamase. The removal of the disulfide bond resulted in only a slight change in inhibition of TEM-1 β-lactamase, similar to that in the K8–D49 peptide, indicating that the disulfide bond is not important for the inhibition of β-lactamase by the peptide.

Discussion

Phage display has been shown to be an effective means of sorting large collections of random sequence peptides and mutant libraries of proteins for variants that bind a target molecule with high affinity (Lowman *et al.*, 1991; Dennis *et al.*, 1995; Rudgers and Palzkill, 1999; Huang *et al.*, 2000). Here we fused random fragments of BLIP to the M13 gVIIIp and enriched for peptides that bind to TEM-1 β-lactamase. From the panning results, a total of eight in-frame BLIP-derived sequences were identified. Of these selected BLIP peptides, 96% were found to contain a common overlapping region of BLIP consisting of residues C30–D49. The strong bias for peptides containing this region of BLIP suggests that this is an important region for binding TEM-1 β-lactamase. Because the phage library was constructed such that the

smallest BLIP peptide displayed on the phage surface would be expected to be ~30 amino acids, the 20-residue C30–D49 peptide is probably not represented in the original library. This may explain why a smaller peptide containing the C30–D49 region was not identified.

Of the BLIP peptides that were identified, the K8–D49 peptide represented 78% of the insert population. It is of interest that both the K8–D49 and the G2–D49 peptides identified by phage display contained the F14L substitution (Figure 2). This mutation was most likely introduced during PCR amplification of the BLIP gene during the construction of the phage-display library. The occurrence of the substitution in two independent peptide sequences suggests that it may act to stabilize the peptides from proteolysis of bacterial enzymes or contribute to binding to TEM-1 β-lactamase.

It has been demonstrated that peptides fused to M13 gVIIIp vary widely in efficiency of display on the surface of the bacteriophage (Malik *et al.*, 1996). For example, the addition of just nine residues to a well-displayed hexapeptide greatly decreased the efficiency of display (Malik *et al.*, 1996). The K8–D49 peptide described here may be displayed more efficiently on the surface of the M13 phage compared with other peptides or wild-type BLIP. This may explain why the G2–D49 and K8–V165 peptides (Figure 2), despite containing all of the amino acids found in the K8–D49 peptide, are not substantially enriched in the phage display experiments. The additional residues on these peptides may decrease the amount of peptide displayed on the phage, giving a slight advantage to the K8–D49 peptide that could quickly result in the K8–D49 peptide overtaking the phage population and providing for the observed enrichment.

In virtually all the peptides selected by phage display, the 20-residue sequence, C30–D49, was identified, suggesting that this region contains several contact residues with TEM-1 β-lactamase. If contact residues in the wild-type BLIP-β-lactamase complex are retained in a peptide β-lactamase complex, it is possible that peptides containing the highest density of contact residues would be selected in the phage display experiment. To test this possibility, a window encompassing 20 contiguous amino acids was scanned through the BLIP sequence and scored for the number of residues that contact TEM-1 β-lactamase based on the contact residues reported for the X-ray structure of the BLIP-β-lactamase complex (Strynadka *et al.*, 1996) (Figure 5). It is evident that domain 1 of BLIP contains the windows with the highest density of contact residues. Note that the C30–D49 window resides within a cluster of windows that contain 10–11 contact residues (Figure 5). This suggests that peptides containing domain 1 sequences including positions 30–49 were the most prevalent in the phage-display enrichment because they contain the highest density of contact residues. This result also implies that the peptides selected by phage display use the same set of contact residues as those found in the wild-type BLIP-β-lactamase crystal structure. In contrast, the residues located in domain 2 of BLIP that make direct contact with TEM-1 β-lactamase are widely dispersed in small clusters of two to four residues (Figure 5). Therefore, phage-displaying peptides derived from domain 2 may not have been enriched because domain 2 does not contain a high density of contact residues.

By identifying a 20-residue region of BLIP that binds and inhibits TEM-1 β-lactamase, we have reduced the region of BLIP to assay for the development of a novel β-lactamase inhibitor by 88%. Further mutagenic studies can now be used

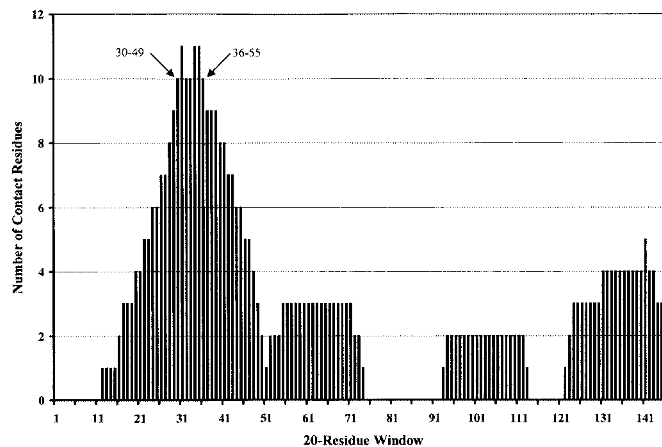

Fig. 5. Position of BLIP amino acid residues that contact TEM-1 β-lactamase in the X-ray structure of the BLIP–TEM β-lactamase complex (Strynadka *et al.*, 1996). A window of 20 contiguous BLIP residues was scanned across the BLIP sequence starting at position 1. Each set of 20 contiguous residues was scored for the number of residues that make contact with TEM-1 β-lactamase based on the contact residues reported for the co-crystal structure of TEM-1 β-lactamase and BLIP (Strynadka *et al.*, 1996). Two 20-residue windows, 30–49 and 36–55, are indicated. The starting residue for every tenth window is indicated below the graph.

to identify residues in this region that are critical for binding and inhibiting the enzyme. By using phage display, it should be possible to improve further the binding of the minimized peptide for TEM-1 β-lactamase. In addition to designing inhibitors for TEM-1 β-lactamase, this method can also be used to generate novel inhibitors of other class A and potentially class C and D β-lactamases that currently are not inhibited by the existing small-molecule β-lactamase inhibitors.

Protein–protein interactions are important events in most biological processes and are important targets for drug design. Over the past decade, there has been increased interest in the design of small molecules that mimic functional epitopes of proteins (Leatherbarrow and Salacinski, 1991; Dennis *et al.*, 1995; Li *et al.*, 1995; Huang *et al.*, 2000). However, interfaces between two binding partners may consist of as many as 40 contact residues, making it difficult to construct small peptide inhibitors of such large and often discontinuous regions. Recent studies indicate that only a small subset of the contact residues in the interface contribute substantially to binding, making the design of small molecular inhibitors potentially less daunting (Jin *et al.*, 1992; Nuss *et al.*, 1993; Clackson and Wells, 1995). In addition, several potent inhibitors of biologically important proteins have been identified in which the critical residues involved in binding the target molecule cluster together in a single domain of the inhibitor (Leatherbarrow and Salacinski, 1991; Buckle *et al.*, 1994; Dennis and Lazarus, 1994; Li *et al.*, 1995). However, identification of these domains has relied on crystal structure analysis and extensive mutagenic studies. Here we used phage display to identify a small domain of BLIP that contains a cluster of residues that are sufficient for binding and inhibition of TEM-1 β-lactamase. This method can easily be applied to other protein–protein complexes to identify critical binding residues in either protein partner. Although a co-crystal structure of the inhibitor–enzyme complex has been previously solved and used in the method described here (Strynadka *et al.*, 1996), this information is not required for these experiments.

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