Cell Wall-targeting Domain of Glycylglycine Endopeptidase Distinguishes among Peptidoglycan Cross-bridges*5

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ALE-1, a homologue of lysostaphin, is a peptidoglycan hydrolase that specifically lyases Staphylococcus aureus cell walls by cleaving the pentaglycine linkage between the peptidoglycan chains. Binding of ALE-1 to S. aureus cells through its C-terminal 92 residues, known as the targeting domain, is functionally important for staphylocytic activity. The ALE-1-targeting domain belongs to the SH3b domain family, the prokaryotic counterpart of the eukaryotic SH3 domains. The 1.75 Å crystal structure of the targeting domain shows an all-β fold similar to typical SH3s but with unique features. The structure reveals patches of conserved residues among orthologous targeting domains, forming surface regions that can potentially interact with some common features of the Gram-positive cell wall. ALE-1-targeting domain binding studies employing various bacterial peptidoglycans demonstrate that the length of the interpeptide bridge, as well as the amino acid composition of the peptide, confers the maximum binding of the targeting domain to the staphylococcip peptideoglycan. Truncation of the highly conserved first 9 N-terminal residues results in loss of specificity to S. aureus cell wall-targeting, suggesting that these residues confer specificity to S. aureus cell wall.

Lysostaphin, a peptidoglycan hydrolase, is secreted by Staphylococcus simulans biovar staphylolyticus to preferentially lyse the interpeptide bridge of the S. aureus cell wall (1–3). The enzyme can potentially be used as an antibiotic against drug-resistant S. aureus as it has been shown by both in vitro and in vivo studies to act against staphylococcal infections (4–7). The lysostaphin proenzyme displays a three-domain modular design: an N-terminal domain of tandem repeats, a central zinc-containing metalloprotease catalytic domain, and a C-terminal targeting domain. Upon maturation, the tandem repeats are removed, leaving only the catalytic domain and the targeting domain in the mature lysostaphin (8). The C-terminal portion of lysostaphin, consisting of 92 amino acids, is thought to be the targeting domain that directs the interaction of lysostaphin with S. aureus cell walls (9). A mutant lysostaphin lacking the targeting domain loses both its abilities to bind to staphylococci and to distinguish between host cells and target cells (9). This kind of targeting domain can be found among other types of peptidoglycan hydrolases, for example, the C terminus of N-acetylmuramyl-l-alanine amidases and the N terminus of glucosaminidase, both of which are products of the S. aureus autolysin proenzyme Atl (10). These domains have been shown to direct autolysis to the equatorial surface ring of S. aureus (11). Fragments of this consensus sequence can also be mapped to some proteins secreted by other bacterial species, such as zoocin A produced by Streptococcus equi subsp. zooepidemicus, which is thought to be involved in cell wall recognition and binding (12).

The targeting domain belongs to the recently identified prokaryotic SH3b domain family, the prokaryotic counterpart of the well-characterized SH3’s (Src homology 3) domains found in eukaryotes and viruses (13). Eukaryotic SH3s are important, modular protein domains that mediate protein-protein interactions upon binding to a proline-rich peptide sequence (14) and are commonly involved in signal transduction and membrane trafficking pathways. A subset of bacterial invasion proteins containing the SH3b domains are thought to bind the receptors of their target cells or utilize the SH3-like modulating pathways to promote their survival in the invaded cells (13). However, SH3b domains display low sequence similarity to eukaryotic SH3 domains, indicating that their function may differ from eukaryotic SH3s (15). Indeed, another subset of SH3b domains found in the bacterial peptidoglycanolytic enzymes is thought to mediate the binding to bacterial cells (15).

ALE-1 is a close lysostaphin homologue produced by Staphylococcus capitis EPK1 (16) and possesses a modular structure similar to lysostaphin. It is composed of an N-terminal 13 amino acid repeat domain followed by a central catalytic domain and a C-terminal targeting domain of 92 amino acids (92AA) that is extremely similar to that of lysostaphin. Unlike lysostaphin, ALE-1 does not undergo post-translational processing of its N-terminal repeats. In this report, we describe the crystal structure of the targeting domain of ALE-1, which represents an SH3b domain, as well as insights into its recognition of the S. aureus peptidoglycan.

EXPERIMENTAL PROCEDURES

Materials—Bacterial strains used in this study are listed in supplemental Table S1. Staphylococcus was grown in trypticase soy broth. Streptococcus was grown in Berman broth. Bacillus was grown in Nutrient broth. Escherichia coli was grown in Luria-Bertani broth. When necessary, ampicillin was added to a final concentration of 50 µg/ml.

5 The abbreviations used are: SH3, Src homology 3; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
Crystal Structure of ALE-1-targeting Domain

Protein Expression and Purification—The full ALE-1, ALE-1 truncated for N-terminal repeats (residue 115–362, ΔN-term), ALE-1 truncated for C-terminal targeting domain (residue 36–270, ΔC-term), ALE-1 truncated for both N-terminal repeats and C-terminal targeting domain (ΔNΔC-term), and C-terminal targeting domain (residue 271–362) were expressed as His$_6$ tag fusion proteins (Fig. 1). The corresponding sequences were amplified with PCR using S. capitis EPK1 chromosomal DNA as a template with the primer pairs (supplemental Table S1) and cloned in-frame downstream of pQE30 vector (Qiagen) to generate respective expression plasmids. E. coli M15 cells (pREP4) were transformed, grown to mid-log phase in 500 ml Luria-Bertani broth at 37 °C, induced with 1 mM isopropyl 1-thio-D-galactopyranoside and further incubated for 13 h. The cells were harvested by centrifugation, suspended in 30 ml of buffer B (8M urea, 0.1M NaH$_2$PO$_4$, 0.01M Tris-HCl, pH 8.0), and incubated for 30 min at room temperature, and then disrupted by ultrasonication. After removing undisrupted cells by centrifugation at 25,000 × g for 40 min at 4 °C, the supernatant was applied onto a nickel-nitrotriacetic acid affinity column (Qiagen) pre-equilibrated with buffer B. The column was washed with 5× bed volumes of buffer C (8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl, pH 6.3), and the protein was eluted with buffer E (8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl, pH 4.5). The eluted fraction was stepwise dialyzed against 4 M urea (0.1 M phosphate buffer, pH 6.8) then 0.1 M phosphate buffer at pH 6.8.

The selenomethionine enriched C-terminal targeting domain was expressed as a FLAG-tagged fusion protein. The corresponding sequence was amplified with the primers ALEU6 and ALEL2 (supplemental Table S1) and cloned into pGEM-T Easy vector. The insert was cut with HindIII and EcoRI and cloned in-frame downstream of pFLAG MAC expression vector (Sigma) to generate pF92AA. pF92AA was transformed into E. coli BL21 cells. The recombinant E. coli was grown in 3 liters of M9 medium to mid-log phase, then supplemented with 150 mg of seleno-L-methionine, 300 mg of lysine hydrochloride, 300 mg of threonine, 300 mg of phenylalanine, 150 mg of leucine, 150 mg of isoleucine, and 150 mg of valine and incubated further for 15 min at 37 °C. The culture was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and further incubated for 16 h. The cells were harvested by centrifugation, suspended in 30 ml of buffer A (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 μg/ml sodium azide), and mixed with 3 ml of buffer C (0.1 M CaCl$_2$, 0.1 M MgCl$_2$, 1 mM phenylmethylsulfonyl fluoride, 20 mM dithiothreitol). The cells were then disrupted by sonication. After centrifugation at 25,000 × g for 40 min at 4 °C, the supernatant was treated with SDS-heating killed S. aureus FDA209P for 1 h at 4 °C. The S. aureus cells were washed with 0.1 M phosphate buffer, pH 6.8, six times, and the bound FLAG-tagged 92AA was eluted with 8 M urea (0.1 M phosphate buffer, pH 6.8). The eluted fraction was extensively dialyzed against 0.1 M phosphate buffer (pH 6.8) and used for crystallization.

Protein Crystalization and Structure Determination—Crystals of the ALE-1-targeting domain (92AA, residues 271–362) were grown by the hanging drop, in which 4 μl of protein at 12 mg/ml in 10 mM phosphate buffer at pH 6.2 was mixed with 4 μl of the reservoir solution. The reservoir solution contained 100 mM sodium acetate, 32–35% polyethylene glycol (PEG) 3350, and 100 mM MES, pH 6.5, or 100 mM HEPES, pH 7.5, as buffer. The crystals belong to the space group P2$_1$2$_1$2$_1$ (a = 45.2 Å, b = 58.5 Å, c = 85.1 Å) with two molecules per asymmetric unit.

The successful incorporation of two selenium atoms into a truncated form of the targeting domain was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (data not shown). Crystals of the Se-protein were grown by hanging drop, in which 4 μl of 18 mg/ml protein in water was mixed with 4 μl from the reservoir identical to that used for 92AA. The Se-protein crystals belong to the space group 1432 (a = b = c = 105 Å) with one molecule per asymmetric unit.

Diffraction data from 92AA and multiwavelength anomalous diffraction data from three crystals of the Se-protein were collected at 17-ID IMCA port at the Advanced Photon Source (Table 1, showing one of the three selenomethionine data sets) and were processed using HKL2000 (17). Two selenium sites were identified and refined in all three MAD data sets with CNS (18). MAPMAN (19) was used to average these three MAD-derived electron maps, yielding a clean map. Iterative cycles of model building and refinement were performed in XtaView (20) and CNS, respectively. Resolution was calculated using 5% of the data.

The structure of the Se-protein (residues 279–362) was refined to 1.80 Å resolution against the data collected at peak wavelength (0.97917 Å) with unmerged Friedel pairs. Subsequently, the structure of 92AA was determined by molecular replacement and refined to 1.75 Å resolution. Two copies of 92AA were referred to as molecules A and B. Based on the electron density and protein sequence, a long stretch of residues were built at the N termini, which belong to the FLAG-tag (D$_{260}$YKDDDDKVKL$_{270}$) arbitrarily assigned sequence number for model continuity. Molecule A (264–362) consists of 99 residues and molecule B (260–362) of 103 residues, and both models show good geometry.

The full coordinates (1R77) of the ALE-1 cell wall-targeting domain have been released in the Protein Data Bank.

Preparation of Bacterial Cells for Binding Assay—Several bacterial preparations were used for the binding assay, including intact cells, heat-killed cells, SDS-heated cells, and SDS-trypsinized cells. S. aureus FDA209P was grown in trypticase soy broth to mid-log phase,
centrifuged, and then resuspended in 0.1 M phosphate buffer and used as intact cells. Alternatively the cells were suspended in 0.1 M phosphate buffer without or with SDS (final 4%) and incubated in boiling water for 30 min, washed at least 10 times with 0.1 M phosphate buffer. The obtained cells were used as heat-killed cells or SDS-heat-killed cells. The SDS-heat-killed cells were further incubated with trypsin (100 μg/ml) in 10 mM Tris-HCl, 10 mM CaCl₂ at 37 °C for 15 h, washed several times with distilled water containing 0.5 mM phenylmethylsulfonyl fluoride, suspended in 0.1 M phosphate buffer, pH 6.8, and used as SDS-trypsin cells.

Preparation of Peptidoglycan (PG)—PG was prepared essentially as described by Stranden et al. (21). The cultured cells were washed with PBS, suspended in 10 ml of 1 M NaCl, and mixed with glass beads (6 g/10 ml in 0.1 M phosphate buffer, pH 6.8) and homogenized with a cell homogenizer (output level 4/min7 repeats; B. Braun Biotech). Unbroken cells and glass beads were removed by centrifugation at 625 × g for 15 min at 4 °C, and the supernatant was centrifuged again at 5625 × g for 10 min at 4 °C. The obtained cell pellets were resuspended in 0.5% SDS and incubated at 60 °C for 30 min to remove any non-covalently bound components. The cell walls were isolated by centrifugation and washed six times with water and further washed with 1 M Tris-HCl, pH 7.0. To remove Protein A, samples were incubation with 0.2 mg/ml trypsin in 1 M Tris-HCl, pH 7.0, with 10 mM CaCl₂ for 24 h at 37 °C. Samples were centrifuged and washed several times with buffer and water. To remove teichoic acids, samples were incubated with 1 ml of 40% (w/v) aqueous hydrofluoric acid for 18 h at 4 °C. Purified peptidoglycan was isolated by centrifugation, washed more than four times with water, and lyophilized.

Assay of Staphyloytic Activity—Lytic activity was assayed by following the rate of decrease in the turbidity of the cell suspension as described previously (22). In brief, heat-killed S. aureus FDA209P cells were incubated with (+92AA) or without (−92AA) excess amount of the targeting domain for 1 h and extensively washed with PBS. The cells were then suspended in 0.1 M phosphate buffer (1 mg of [dry weight]/ml, pH 6.8). Truncated forms of ALE-1 at appropriate concentrations were mixed with 2 ml of the cell suspension and the mixture was incubated at 37 °C. Specifically, full ALE-1 and ΔN-ALE were added to the cells at 0.05 μM, and the C-terminal truncated forms were at 0.4 μM. The rate of decrease in turbidity was measured at 595 nm (ΔA₅₉₅) on a spectrophotometer.

Binding Assay—Bacterial cells or PGs were suspended in 0.1 M phosphate buffer, pH 6.8, containing 100 mM iodoacetic acid (A₅₉₅ = 1.0). Proteins of interest were incubated with 100 μl of the suspension for 1 h at 4 °C. Samples were then washed three times with 0.1 M phosphate buffer, pH 6.8, containing 10 mM iodoacetic acid, and the bound proteins were eluted by incubating with 30 μl of 4% SDS. The eluted proteins were separated by SDS-PAGE (15% acrylamide) and stained with Coomassie Brilliant Blue. In some experiments, the amount of protein was estimated by NIH image version 1.52 using scanned protein bands.

Detection of Bound Protein to Bacterial PG by ELISA—Polystyrene enzyme immunoassay 96-well plates (Nalge Nunc) were coated with 100 μl per well of sonicated bacterial PG diluted in PBS, pH 7.2, to the concentration of 12.5 μg/ml and left overnight. Sonication was performed to ensure a homogeneous coating suspension of the insoluble PG. After coating, the wells were washed three times with distilled water, then 1% bovine serum albumin in PBS containing 20 μg/ml human IgG was added to the wells and left overnight. After blocking, the wells were washed three times with distilled water, then 7.0 μg/ml protein (100 μl) diluted in PBS was added to the wells and incubated at 4 °C for 1 h. After incubation with protein, the wells were washed three times with PBS containing 0.05% Tween 20. Anti-ALE-1 serum (100 μl) diluted in PBS containing 0.1% bovine serum albumin was added to the wells and incubated at 37 °C for 2 h. After three washes with PBS-Tween 20, 100 μl of diluted horseradish peroxidase-conjugated rabbit immunoglobulin anti-goat IgG antiserum was added and incubated for 2 h at 37 °C. Unbound conjugate was removed by washing three times with PBS-Tween 20 and twice with PBS. The substrate (100 μl) was then added (25 mg of o-phenylenediamine dihydrochloride and 5 μl of H₂O₂)

TABLE 1

| Data collection, phasing, and refinement statistics | Edge | Se-protein Peak | Remote | Native |
|---|---|---|---|---|
| Wavelength (Å) | 0.97931 | 0.97917 | 0.95370 | 1.0000 |
| Resolution (Å) | 1.80 | 1.80 | 1.80 | 1.75 |
| Unique reflections<sup>a</sup> | 9229 (678) | 9379 (846) | 9376 (902) | 23,008 (2248) |
| Redundancy<sup>b</sup> | 14.3 (3.1) | 15.8 (7.7) | 15.9 (8.3) | 5.1 (4.4) |
| Completeness<sup>c,d</sup> (%) | 97.0 (72.7) | 99.1 (91.7) | 99.8 (98.4) | 99.0 (98.9) |
| R<sub>merge</sub><sup>e</sup> (%) | 7.2 (80.1) | 6.9 (67.0) | 7.8 (51.2) | 7.9 (36.5) |
| J/σ<sup>f</sup><sup>g</sup> | 26.7 (0.8) | 32.4 (1.7) | 27.5 (2.0) | 16.3 (2.8) |

<sup>a</sup> Value for highest resolution shells: Se-protein 1.86–1.80 Å and native 1.81–1.75 Å are given in parentheses.

<sup>b</sup> R<sub>merge</sub> = ∑|I<sub>i</sub>−(I<sub>i</sub>/N)|/∑I<sub>i</sub>, intensity.

<sup>c</sup> Anomalous phasing power is F<sub>Ano</sub>/E; F<sub>Ano</sub> is the mean anomalous component of the heavy atom, and E is the r.m.s. lack-of-closure error.

<sup>d</sup> ND, not determined.

<sup>e</sup> r = ∑|F<sub>obs</sub>−F<sub>calc</sub>|/∑F<sub>obs</sub> where R<sub>merge</sub> is calculated from a randomly chosen 5% of reflections omitted from refinement, and R<sub>merge</sub> is calculated for the remaining 95% of reflections included in the refinement.

<sup>f</sup> Data cutoff for refinement: J/σ ≥ 2.

Crystal Structure of ALE-1-targeting Domain
in 10 ml of sodium phosphate-citrate buffer). The enzymatic reaction proceeded for 15 min at room temperature and was stopped by the addition of 100 μl of 2 N H2SO4. The optical density was measured at 492 nm on a Titertek Multiscan Spectrophotometer.

RESULTS

ALE-1-targeting Domain Belongs to Bacterial Homologue of SH3 (SH3b)—An asymmetric unit consists of two copies (molecules A and B) of the ALE-1-targeting domain, a.k.a. 92AA. At the N termini of these two molecules is a partial sequence of FLAG-tag, which is involved in intermolecular interactions (see “Discussion”). These two molecules are very similar, indicated by an overall root-mean-square deviation (r.m.s.d.) of 0.43 Å for all main chain atoms (residues 271–362), with the maximal deviations localized in some residues at the N and C termini: Asn274 (1.04 Å), Lys275 (1.41 Å), Tyr276 (1.08 Å), and Lys362 (1.25 Å). In this report, the discussion will refer to molecule B.

The ALE-1-targeting domain consists of eight β-strands (Fig. 2A). Two anti-parallel multiple-stranded β-sheets pack at approximately right angles: β5–β7 and the N terminus of β2 in sheet I; β3–β4, β8, and the C terminus of β2 in sheet II. The targeting domain has been classified as SH3b, a prokaryotic homologue of the eukaryotic SH3. Although eukaryotic SH3 and prokaryotic SH3b share little sequence identity (<20%), their tertiary structures are strikingly similar as indicated by DALI (23). SH3 domains are modular proteins that mediate protein-protein interactions in signal transduction cascades and membrane-cytoskeleton structures through recognition of proline-rich (PXXP) ligands; the surface of SH3 domains bears a large and relatively flat hydrophobic region to accommodate the special conformation of the proline-rich substrate (reviewed in Ref. 24).

SH3 domains resemble a β-barrel, consisting of five β-strands (βa to βe). SH3 loops are conventionally defined as follows: the RT loop, located between strands βa and βb; the n-Src loop, between βb and βc; and the distal loop, between βc and βd. The RT loop contains conserved residues involved in defining ligand specificity and binding. Structurally, the n-Src loop flanks one end of the substrate binding groove. Between strands βd and βe is a 3_10 helix, a common feature of SH3s. The ALE-1-targeting domain and SH3 domains share all of the β strands (Fig. 2B): βa and β2, βb and β5, βc and β6, βd and β7, βe and β8. The positional r.m.s.d. for 52 equivalent α-carbons between the ALE-1-targeting domain and the Crk SH3 (1CKA.pdb) (25) is 2.4 Å. For convenience, we named the loop regions of the targeting domain after their SH3 counterparts.

The targeting domain (271–362) is larger than typical SH3 domains by 30 amino acids and has unique features. The N-terminal 20 residues...

FIGURE 2. Structural comparison of targeting domain with Crk SH3 domain. A, ALE-1-targeting domain (Chain B), with N-terminal FLAG-tag. Strands are numbered sequentially, and the loop regions are named after their SH3 counterparts. The main chain trace of the FLAG-tag from Chain A is shown as red stick model. B, stereo view of the superposition of main chain atoms of the targeting domain (purple) and Crk-SH3 (cyan). The β-stands of ALE-1 are numbered sequentially; and those of Crk are in alphabetical order. Equivalent β-stands between these two structures are labeled according to their designations in the targeting domain and Crk-SH3, respectively. The PXXP substrate of Crk is shown in stick model with surface rendering. The steric incompatibility can be visualized; the RT loop and n-Src loop of the targeting domain clash with the rendered PXXP. Molecular graphic images were produced using the UCSF Chimera package (35) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institutes of Health Grant P41 RR-01081).
Crystal Structure of ALE-1-targeting Domain

The strong affinity of the ALE-1-targeting domain to S. aureus cells led us to further study the binding mechanism. It has been known that the C-terminal domain of lysostaphin plays an important role in its binding to the S. aureus cell wall; however, the interacting component on the cell wall has not been identified (9). Poxtton et al. (27) reported C-teichoic acid as the cell wall component of *pneumococci*. C-teichoic acid is susceptible to hydrogen fluoride (HF) treatment; however, HF treatment of S. aureus cells did not attenuate the binding of the targeting domain (data not shown). Therefore, the possibility of C-teichoic acid as the target molecule could be excluded. We examined the binding between the targeting domain and S. aureus cells heat-pretreated without and with SDS (heat cells and SDS-heat cells, respectively) and trypsinized SDS-heat cells. These sequential treatments were to increasingly linearize PG structure. Binding of the targeting domain was greatly enhanced by these treatments of PG (Fig. 5), suggesting that the C-terminal domain of ALE-1 acts as a cell wall-targeting domain.

Targeting Domain Specifically Recognizes Interpeptide Bridge—The strong affinity of the ALE-1-targeting domain to S. aureus cells led us to further study the binding mechanism. It has been known that the C-terminal domain of lysostaphin plays an important role in its binding to the S. aureus cell wall; however, the interacting component on the cell wall has not been identified (9). Poxtton et al. (27) reported C-teichoic acid as the cell wall component of *pneumococci*. C-teichoic acid is susceptible to hydrogen fluoride (HF) treatment; however, HF treatment of S. aureus cells did not attenuate the binding of the targeting domain (data not shown). Therefore, the possibility of C-teichoic acid as the target molecule could be excluded. We examined the binding between the targeting domain and S. aureus cells heat-pretreated without and with SDS (heat cells and SDS-heat cells, respectively) and trypsinized SDS-heat cells. These sequential treatments were to increasingly linearize PG structure. Binding of the targeting domain was greatly enhanced by these treatments of PG (Fig. 5), suggesting that the C-terminal domain of ALE-1 acts as a cell wall-targeting domain.

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We and others (16, 28) have demonstrated that ALE-1 and lysostaphin selectively lyse S. aureus, which is ascribed to the specificity of glycylglycine endopeptidases; they cleave glycylglycine bonds in the pentaglycine interpeptide bridge of S. aureus. The C-terminal domain of lysostaphin binds to S. aureus cells but not S. simulans cells, the lysostaphin producing strain, implicating that not only does the enzyme activity establish the selective lysis, but the substrate specificity of the C-terminal targeting domain also confers this selectivity (9). When the ALE-1-targeting domain was incubated with various concentrations of S. aureus PG, the increase of bound targeting domain was dose-dependent (data not shown). To determine the binding specificity of the ALE-1-targeting domain, we purified several bacterial PGs containing diverse interpeptide motifs and tested the binding of the targeting domain to these PGs by ELISA (Fig. 6). The targeting domain binding was significantly lower in PGs of *Streptococcus mutans*, *Bacillus megaterium*, *Micrococcus lysodeikticus*, and *Lactobacillus plantarum* than in S. aureus 209P. The PG structures of these organisms differ from that of S. aureus 209P in their interpeptide bridge moieties: interpeptide bridges of S. mutans and M. lysodeikticus are L-Ala-L-Ala and L-Ala-D-
Glu/Gly-L-Lys-D-Ala respectively, whereas *B. megaterium* and *L. plantarum* do not have interpeptide bridges. Clearly, among these species, the ALE-1-targeting domain discriminatingly binds to *S. aureus* PG, which also intrigued us into further investigating the binding in the context of the composition as well as the length of the interpeptide bridge.

**Binding of ALE-1-targeting Domain to PG Depends on the Composition and the Length of Interpeptide Bridge**—ALE-1- and lysostaphin-producing strains share a similar mechanism that protects them from autolysis by these detrimental enzymes. They carry the genes *epr* (29) and *lif* (30), respectively, which produce peptidyltransferases to incorporate i-serine residues into the interpeptide bridge. This process alters the composition of the interpeptide from pentaglycine (Gly5) to four glycines and one serine (Gly2-Ser-Gly2 or Gly4-Ser), thereby making the PG resistant to the lytic action of ALE-1 or lysostaphin (31). To address the effect of this substitution on the binding affinity, we compared the binding of the ALE-1-targeting domain to various staphylococcal PGs with genetically modified interpeptide bridges and their isogenic parents (Fig. 6). *S. capitis* EPK1 is an ALE-1 producing strain with the Gly2-Ser-Gly2 interpeptide bridges, whereas *S. capitis* EPK2 is its isogenic strain deleted of an *ale1/epr* plasmid to restore the Gly5 bridges (32). *S. aureus* TF5303 utilizes Gly5 in its interpeptide bridges, while the bridges of its isogenic strain TF5311 that expresses *epr* are Gly2-Ser-Gly2 (32). The targeting domain bound, at a very similar level, to the PGs of *S. aureus* 209P, *S. capitis* EPK2, and *S. aureus* TF5303, all of which have the Gly5 interpeptide bridges. Once Gly5 becomes Gly2-Ser-Gly2, binding is reduced considerably but not entirely abolished, as seen in *S. capitis* EPK1 and *S. aureus* TF5311. Evidently, even though these PGs all possess penta-amino acids as interpeptide bridges, the targeting domain shows a strong preference for Gly5.

FemA and FemB are peptidyltransferases involved in the addition of glycine to form the pentaglycine interpeptide (33). FemA specifically adds glycines 2 and 3, while FemB adds glycines 4 and 5 of the pentaglycine of *S. aureus* PG (34). The interpeptide of the *femB* mutant BB841 is triglycine and that of *femAB* mutant BB1221 is monoglycine. Compared with normal BB705 cells with the pentaglycine interpeptide, binding of the targeting domain was attenuated to 60% in BB841 and to 10% in BB1221 (Fig. 6), suggestive of the dependence of binding on the length of the interpeptide bridge.

**Truncation of N-terminal Strands Reduces Targeting Domain Binding to PG**—A number of bacterial species produce peptidoglycan hydrolyases that target specific types of substrate bacterial cells (16). The ALE-1-targeting domain shares significant homology with cell wall-targeting domains from various organisms (Fig. 7), suggesting that there may be common structural and functional elements within this protein family. Furthermore, the sequence alignment of targeting domains from some enzymes that selectively bind to *S. aureus* cells, including *S. aureus* autolysin, *S. aureus* phage Twort amidase, *S. aureus* phage PVL amidase, *S. simulans* lysostaphin, and *S. capitis* ALE-1, revealed additional strong conservation at the N-terminal strands (Fig. 7).
To verify the structural impact of the unique N-terminal strands of the ALE-1-targeting domain in recognizing \textit{S. aureus} PG, we truncated the first nine residues from the targeting domain and compared the specificity of the truncated form (83AA, residues 280–362) with that of the intact targeting domain (92AA). Truncation drastically reduced the targeting domain binding to \textit{S. aureus} PGs that has the Gly5 interpeptide (\textit{S. aureus} 209P, \textit{S. capitis} EPK2, \textit{S. aureus} TF5303, BB705, and BB270); however, the truncated form showed similar or even greater, but generally very low, affinity to PGs of other species, indicating that the truncation only affected its binding to \textit{S. aureus} PGs (Fig. 6). Furthermore, there was no significant difference in truncated protein binding to various PGs, regardless of interpeptide moieties. These results strongly suggest that the N-terminal nine residues of the targeting domain account for the specificity of \textit{S. aureus} by discriminating pentaglycine interpeptide bridges and the truncated protein can almost no longer bind to PGs.

We have attempted to address the question as to how the ALE-1-targeting domain interacts with polyglycine by co-crystallization and computational docking simulation; however, both trials failed to yield any meaningful insights. Thus, upon the identification of these important structural elements, we selectively mutated several residues at various regions of the targeting domain and studied the PG binding of the resultant proteins (Fig. 8). The mutation sites include Asn\textsuperscript{274}, Tyr\textsuperscript{276}, and Tyr\textsuperscript{280} at the N-terminal strands; Ile\textsuperscript{293} and Arg\textsuperscript{296} near the projected PXBP recognition groove; and Trp\textsuperscript{358} at the C-terminal stand of the targeting domain and it is conserved among the \textit{S. aureus} recognition subset (Fig. 7). N274W hardly affected binding, whereas Y276A and Y280A mutants exhibited 2–3-fold reductions in binding. Some mutations at the PXBP groove of the ALE-1-targeting domain did not affect its binding (data not shown); however, binding of mutants I293W and R296A was decreased by at least 3-fold individually. W358A reduced the binding of the targeting domain to the \textit{S. aureus} PG by half, indicating that Trp\textsuperscript{358} may be partially involved in the binding.

**DISCUSSION**

It has been hypothesized that the C-terminal 92 residues of lysostaphin acts as a targeting domain that directs the interaction of lysostaphin with \textit{S. aureus} cell walls. Our studies on the C-terminal domain of ALE-1, a close homologue of lysostaphin, confirm that this domain interacts strongly and specifically with \textit{S. aureus} cell wall peptidoglycan. Given the extremely high sequence identity, the same conclusion may safely be drawn on the C-terminal domain of lysostaphin. Although a genuine substrate on the peptidoglycan as well as its interaction with the targeting domain has yet to be experimentally determined, the postulation that the targeting domain exclusively recognizes the pentaglycine interpeptide linkage is not entirely lacking evidence.

Based on the overall structural similarity between the eukaryotic SH3 and the ALE-1-targeting domain and the SH3-PXXP interaction, we investigated whether the targeting domain may interact with PXBP. The PXBP binding groove of Crk SH3 is broad (~600 Å²) and flat to accommodate the conformationally unusual substrate. Compared with SH3, the ALE-1-targeting domain shows significant changes at the loop

![FIGURE 6. Binding of native ALE-1-targeting domain (92AA) and its truncated form (83AA) to various peptidoglycans. Underneath the binding chart is the schematic representation of the structure of the interpeptide bridges of these PGs. Bound protein to PG was detected by an ELISA procedure and normalized against bound native form on 209P cells.](image-url)
regions, which greatly reduces the accessible surface for PXXP to 165 Å² and creates geometric incompatibility (Fig. 2B). Nevertheless, on the crystal structure, we observed that this groove is involved in molecular contact; FLAG-tag of molecule A interacts through hydrogen bonds and nonpolar residues, with a surface region of molecule B that consists of residues Thr291, Asp292, Ile294, Arg302, Gin306, Val309, His327, and Thr346 (Fig. 9, blue). This interaction pattern extends beyond the asymmetric unit; the FLAG-tag of molecule B interacts with molecule A of the adjacent asymmetric unit in a similar fashion. However, point mutants of this groove as well as the Trp/Pro pair show no effect on binding (data not shown). Therefore, it is likely that the interaction between the targeting domain and FLAG-tag is artificial and may not represent the true interaction of the targeting domain with cell wall PG components.

Among the cell wall-targeting domains, it appears that major structural elements are well maintained: five β strands (β3–β7) that form the core of the β-sheets, the C terminus of the β2 strand, and the β3-β4 tight-turn region, with several conserved residues scattered throughout these fragments of the sequence. A rather unique conservation pattern among these targeting domains has emerged, which includes Ser286, Phe287, Ile293, Arg296, Pro300, Gly301, Tyr301, Asp319, Gly320, Trp329, Tyr332, Gly337, Arg341, Leu342, and Val344 (Fig. 9, green). The conserved glycine residues are located at the turn regions of the protein, whereas the remaining residues are scattered on strands β4 through β7. Among them, polar and charged residues are involved in direct interactions with each other; Asp319 and Arg339 form a salt bridge to stabilize the tight turn between strands β3 and β4. Most of the hydrophobic and aromatic residues are involved in the construction of the β-sheets, providing the scaffold for the targeting domains. When these conserved residues are surface-rendered, a subset of them, including Pro300, Tyr318, Asp319, Val320, Tyr327, Gly337, and Arg341, create a continuous surface patch (Fig. 9, green) that is located on the opposite side of the FLAG-tag groove (Fig. 9, blue).

The N-terminal strands of the targeting domain add an important feature to its SH3-like fold, where an additional yet strong conservation pattern emerges among targeting domains that selectively bind to S. aureus cell walls (Fig. 7, red). These residues, together with the surface patch described above, constitute a deep and narrow groove (Fig. 9, red and green) that can potentially accommodate an extended penta- or hexapolypeptide with very small side chains by forming β-sheet-type interactions with strands β1 and β3 (β1-X-β3). The shape of the groove reveals its preference for glycine, thus even the substitution of glycine
polypeptide peptide alone (<5) may fit into this groove, in the case of PG, the glycan chains of the shortened polypeptide bridge will be brought into a clashing proximity, which impairs this otherwise favorable interaction. The combination of shortened length and size-increasing substitution further weakens this interaction, and the removal of the interpeptide completely eliminates the recognition. Ingeniously, the lysostaphin- and ALE-1-producing strains utilize this specificity to develop a self-protection mechanism to evade the otherwise suicidal production of these enzymes. Evidently, the specific amino acid composition and the length of the interpeptide bridge play central roles in ALE-1-targeting domain binding; substitution of glycerine by serine at position 3 or 5 significantly impairs the gross binding of the targeting domain to staphylococcal PGs, while the length of the interpeptide bridges, i.e. pentaglycine, confers the maximum binding of the targeting domain to PGs.

Selective mutations on different regions of the targeting domain disclose the degree of involvement in the substrate binding of these regions. The N-terminal strands are crucial for the recognition of pentaglycerine; therefore mutations could be potentially detrimental to this important function. Indeed Y276A and Y280A displayed appreciably decreased substrate binding, whereas N274W retained similar binding capacity as wild type. In the crystal structure, the side chain of Asn274 faces outwards from the recognition groove, but the side chains of Tyr276 and Tyr280 are an integral part of the groove (Fig. 9). Several residues are conserved in the cell wall-targeting SH3b domains, including Ile293 and Arg296, which are located near the FLAG-tag groove (Fig. 9, blue). Surface mutations at this groove did not affect its binding, confirming that it is not utilized by ALE-1; whereas binding of the I293W and R296A mutants suffered at least 3-fold decrease. In wild type, the side chain of Ile293 is buried inside the protein. The bulky side chain of the tryptophan residue in mutant I293W may disrupt the local or overall structure to the degree where binding to its substrate is nearly eliminated. As described previously, Arg296 plays an important role in forming the scaffold of the SH3b structure; hence, the R296A mutation may also adversely change the structure and affect its binding. Last, any potential function of the C-terminal region created by C terminus of strand β2, strand β8, and the long coil between strands β7 and β8 (Fig. 9) should not be overlooked, albeit there is no apparent conservation pattern among species. This region and the tentative Gly5 recognition subset may not be in direct contact with the pentaglycerine chain. It should be noted that Trp358 is conserved among the S. aureus recognition subset. Intriguingly, mutant W358A reduced the binding of the targeting domain to the S. aureus PG by half, indicating that this region may be partially involved in the substrate binding, possibly in contact with some other common feature of PG, such as the glycan chains.

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REFERENCES
1. Schindler, C. A., and Schuhardt, V. T. (1964) Proc. Natl. Acad. Sci. U. S. A. 51, 414–421
2. Browder, H. P., Zygmunt, W. A., Young, J. R., and Tavormina, P. A. (1965) Biochem. Biophys. Res. Commun. 19, 383–389
3. Sloan, G. L., Robinson, J. M., and Kloos, W. E. (1982) Int. J. Syst. Bacteriol. 32, 170–174
4. Schaffner, W., Melly, M. A., Hash, J. H., and Koenig, M. G. (1967) Yale J. Biol. Med. 39, 215–229
5. Schaffner, W., Melly, M. A., and Koenig, M. G. (1967) Yale J. Biol. Med. 39, 230–244
6. Climo, M. W., Patron, R. L., Goldstein, B. P., and Archer, G. L. (1998) Antimicrob.

FIGURE 8. Binding of the targeting domain (92AA) and its mutants to S. aureus 209P peptidoglycan. Bound protein to PG was detected by the ELISA procedure and normalized against wild type.

FIGURE 9. Molecular surface of the ALE-1 cell wall-targeting domain. Two orientations related by 180° rotation, shown by the stick models of C atoms, cover the important surface features. Highlighted in blue are the residues that are involved in the targeting domain and FLAG-tag interaction. Highlighted in yellow, at the bottom of the groove, are residues Trp329 and Pro343, both of which are buried with no accessible surface. In green are the conserved residues found in cell wall-targeting domains. In red are highly conserved residues at the first two N-terminal β strands, which are found in the targeting domains that specifically recognize S. aureus cell walls. Trp358, located near the C terminus, is highlighted in magenta. Color coding of the conserved residues is consistent with that of Fig. 7.
Crystal Structure of ALE-1-targeting Domain

Agents Chemother. 42, 1355–1360
7. Kerr, D. E., Plaut, K., Bramley, A. J., Williamson, C. M., Lax, A. J., Moore, K., Wells, K. D., and Wall, R. J. (2001) Nat. Biotechnol. 19, 66–70
8. Recsei, P. A., Gruss, A. D., and Novick, R. P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1127–1131
9. Baba, T., and Schneewind, O. (1996) EMBO J. 15, 4789–4797
10. Oshida, T., Sugai, M., Komatsuzawa, H., Hong, Y. M., Suginaka, H., and Tomasz, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 285–289
11. Baba, T., and Schneewind, O. (1998) EMBO J. 17, 4639–4646
12. Lai, A. C., Tran, S., and Simmonds, R. S. (2002) FEMS Microbiol. Lett. 215, 133–138
13. Whisstock, J. C., and Lesk, A. M. (1999) Trends Biochem. Sci. 24, 132–133
14. Ponting, C. P., Aravind, L., Schultz, J., Bork, P., and Koonin, E. V. (1999) J. Mol. Biol. 289, 729–745
15. Sugai, M., Fujiwara, T., Akiyama, T., Ohara, M., Komatsuzawa, H., Inoue, S., and Suginaka, H. (1997) J. Bacteriol. 179, 1193–1202
16. Otwinowski, Z., and Minor, W. (1997) Methods Enzymology 276, 307–326
17. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
18. Kleywegt, G. J., and Jones, T. A. (1996) Acta Crystallogr. Sect. D Biol. Crystallogr. Sect. D Biol. Crystallogr. 52, 826–828
19. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
20. Strand, E. A., Ehler, K., Labischinski, H., and Berger-Bachi, B. (1997) J. Bacteriol. 179, 9–16
21. Komatsuzawa, H., Suzuki, J., Sugai, M., Miyake, Y., and Suginaka, H. (1994) J. Antimicrob. Chemother. 34, 885–897
22. Holm, L., and Sander, C. (1996) Science 273, 595–603
23. Mayer, B. J. (2001) J. Cell Sci. 114, 1253–1263
24. Wu, X., Kruhagen, B., Feller, S. M., Zheng, J., Sali, A., Cowburn, D., Hanafusa, H., and Kuriyan, J. (1995) Structure (Camb.) 3, 215–226
25. Cesareni, G., Panni, S., Nardelli, G., and Castagnoli, L. (2002) FEBS Lett. 513, 38–44
26. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
27. Ehler, K., Tschierske, M., Morá, C., Schroder, W., and Berger-Bachi, B. (2000) J. Bacteriol. 182, 2635–2638
28. Sugai, M., Fujiwara, T., Komatsuzawa, H., and Suginaka, H. (1998) Gene (Amst.) 224, 67–75
29. Hegde, S. S., and Shrader, T. E. (2001) J. Biol. Chem. 276, 6998–7003
30. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) J. Comput. Chem. 25, 1605–1612
31. Ehrler, K., Tschierske, M., Morá, C., Schroder, W., and Berger-Bachi, B. (2000) J. Bacteriol. 182, 2635–2638
32. Sugai, M., Fujiwara, T., Komatsuzawa, H., and Suginaka, H. (1998) Gene (Amst.) 224, 67–75
33. Hegde, S. S., and Shrader, T. E. (2001) J. Biol. Chem. 276, 6998–7003
34. Ehlert, K., Schroder, W., and Labischinski, H. (1997) J. Bacteriol. 179, 7573–7576
35. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) J. Comput. Chem. 25, 1605–1612