Characterization of the Elastin Binding Domain in the Cell-surface 25-kDa Elastin-binding Protein of *Staphylococcus aureus* (EbpS)*

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Our previous studies have established that a cell-surface 25-kDa elastin-binding protein of *Staphylococcus aureus* (EbpS) mediates binding of this pathogen to the extracellular matrix protein elastin. Results from binding assays examining the activity of various EbpS fragments suggested that the elastin recognition domain is contained within the first 59 amino acids. In this report, we have used functional analyses with synthetic peptides and recombinant truncated forms of EbpS to localize the elastin binding domain to a 21-amino acid region contained within residues 14–34 of EbpS. Further evidence for the importance of this domain was obtained by demonstrating that the inhibitory activity of anti-EbpS antibodies on staphylococcal elastin binding was neutralized when these antibodies were pre-absorbed with a truncated recombinant EbpS construct containing residues 1–34. Overlapping synthetic peptides corresponding to EbpS residues 14–36 were then generated and tested for elastin binding activity to define further the elastin binding domain, and results from these studies showed that sequences spanning amino acids Gln14–Asp23, Asp 17–Asp23, and Thr18–Glu34 inhibit binding of *Staphylococcus aureus* to elastin. Our analyses indicate that the hexameric sequence Thr18-Asn-Ser-His-Gln-Asp23 is the minimal sequence common to all active synthetic peptides, proteolytic fragments, and recombinant constructs of EbpS. Furthermore, substitution of Asp 23 with Asn abrogated the blocking activity of the synthetic peptides, demonstrating the requirement for a charged amino acid at this location. The composite data indicate that staphylococcal elastin binding is mediated by a discrete domain defined by short peptide sequences in the amino-terminal extracellular region of EbpS.

Cell-extracellular matrix (ECM)1 interactions are necessary events in various biological processes including embryonic development, inflammation, tumor cell metastasis, homeostasis, and microbial infections (1–3). Molecular interactions between ECM components and corresponding cell-surface receptors in struct cells to differentiate, migrate, adhere, or proliferate in directing these biological processes. Although there are exceptions, mammalian cells typically use the integrins, an α/β heterodimeric receptor complex, to interact with the ECM (4, 5).

Bacterial pathogens also interact with the host matrix through specific cell-surface ECM-binding molecules categorized collectively as adhesins or microbial surface components recognizing adhesive matrix molecules (3, 6). The Gram-positive bacterial pathogen *Staphylococcus aureus* has been found to interact with many ECM macromolecules such as collagen (7, 8), fibronectin (9), laminin (10), proteoglycans (11), fibrinogen (12), and elastin (13). Staphylococcal ECM adhesins do not have endogenous bacterial ligands and in general are thought to be used to assist in colonization of host tissues. For example, collagen (14) and fibronectin (15) adhesin mutants show a reduced capacity to cause disease in *in vivo* models but are otherwise phenotypically normal.

At the molecular level, all characterized staphylococcal adhesins function as monomers. Available evidence suggests that ligand-binding sites in staphylococcal ECM adhesins are contained within small regions of the extracellular domain. The ligand-binding site in the staphylococcal fibronectin adhesin, for example, has been mapped to a repetitive 38-amino acid motif, and synthetic peptides containing this sequence have been found to possess direct binding activity and to inhibit bacterial binding to fibronectin (16, 17). Similarly, a synthetic 25-amino acid peptide corresponding to the region between Asp209 and Tyr237 of the collagen adhesin has been shown to inhibit binding of type II collagen to *S. aureus* (18), suggesting that this short peptide sequence alone can mediate staphylococcal binding to collagen.

In a previous study, we showed that specific binding between *S. aureus* and elastin was mediated by a 25-kDa elastin-binding protein on the surface of *S. aureus* (EbpS) (13). Elastin binding activity was localized to the extracellular, amino-terminal end of EbpS within the first 59 amino acids (19). To better define the amino acids in EbpS responsible for the elastin binding activity, we have used overlapping synthetic peptides and truncated recombinant EbpS constructs in elastin binding assays. Our results demonstrate that the critical elastin recognition sequence within the amino-terminal domain resides between Gln14 and Glu34. Sequence comparison indicates that the minimal sequence shared by all active EbpS constructs is the hexamer Thr-Asn-Ser-His-Gln-Asp spanning residues 18–23.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, isopropyl-β-thiogalactoside, 5-bromo-4-chloro-3-indoly-β-D-galactoside, and *Hind*III-digested DNA markers were purchased from Promega (Madison, WI). Luria-Bertani medium and Luria-Bertani agar-medium capsules were from Bio 101 (La Jolla, CA). Tryptic soy broth (TSB) was obtained from Remel (Lenexa, KS). Na235I was from ICN (Costa Mesa, CA). Papain and protein A immobilized to
cross-linked agarose, Immunopure Sulfo-NHS-Biotinylation kit, and IODO-GEN were purchased from Pierce. QiAexpress vector kit type IV and the midi-prep plasmid purification kit were obtained from Qiagen (Chatsworth, CA). Nitrocellulose membrane and blotting paper were from Schleicher & Schuell. Affi-Gel 10 affinity support was from Bio-Rad (Hercules, CA). Staphylococcus aureus strain 12586 (Cowman, St. Louis, MO). The PROTEAN program (DNAStar, Madison, WI) was used to predict the secondary structure of EbpS constructs.

**RESULTS**

**Truncated Recombinant EbpS Constructs Bind to Elastin and Inhibit S. aureus Binding to Elastin**—Results from our previous studies examining elastin binding properties of various EbpS fragments (19), and recombinant constructs are summarized in Fig. 1. A cyanogen bromide fragment containing the first 125 amino acids of EbpS showed binding activity, whereas an EbpS degradation product lacking the first 59 amino acids, a cyanogen bromide fragment containing the carboxyl-terminal one-third of the protein, and a synthetic peptide corresponding to residues 1–13 did not interact with elastin. These results suggested that the elastin-binding site in EbpS is contained in amino acid residues 14–59 (shaded area in Fig. 1).

To identify the domain in EbpS critical for binding, two truncated recombinant constructs of EbpS (trEbpS-1 and trEbpS-2) were generated and tested for their ability to bind directly to elastin and to inhibit binding of *S. aureus* to elastin. trEbpS-1, with a predicted molecular mass of 12.8 kDa, contains residues 1–78 of EbpS, whereas trEbpS-2 spans residues 1–34 and has a predicted mass of 7.5 kDa. Characterization of the truncated constructs by mass spectrometry, peptide microsequencing, and immunoblotting with the anti-rEbpS antibody (Fig. 2B, lanes C and D) confirmed that correct truncated proteins have been expressed. Upon resuspension in a physiological buffer for analysis, trEbpS-1 appeared to have been degraded as evident from the two bands seen in Fig. 2. Another notable property of the two truncated EbpS proteins is that they migrated at higher than their predicted molecular mass when fractionated by SDS-PAGE (Fig. 2). This behavior has also been observed with full-length recombinant EbpS (rEbpS) (19). For reasons not fully understood, aberrant migration in SDS-PAGE appears to be a common characteristic of Gram-positive cell-surface proteins (12, 16, 21–24). To examine whether trEbpS-1 and -2 bind directly to elastin, the truncated proteins were biotinylated and reacted with tropoelastin that was transferred to nitrocellulose membranes. As shown in Fig. 3, both truncated constructs bound to the 67-kDa tropoelastin in the absence (lanes A and C), but not in the presence (lanes B and D), of competing soluble elastin peptides. The biotinylated proteins did not bind to bovine serum albumin under similar conditions (data not shown), supporting the specificity of the tropoelastin-binding interaction. A 45-kDa band
also reacted with the two biotinylated trEbpS constructs. This binding, however, was observed in all lanes of the Western ligand blot indicating that this interaction is nonspecific.

Effects of trEbpS proteins on elastin binding at the cellular level were tested by incubating *S. aureus* cells with radiolabeled elastin in the absence or presence of increasing amounts of either soluble full-length (rEbpS) or truncated forms of the adhesin. All three proteins inhibited binding of *S. aureus* cells to elastin in a concentration-dependent manner (Fig. 4). rEbpS and trEbpS-1 completely inhibited elastin binding at the highest concentration tested. trEbpS-2 was somewhat less effective as an inhibitor, with about 20% residual elastin binding activity at the highest inhibitor concentration.

Pre-absorption of the Anti-rEbpS Antibody with trEbpS-2 Neutralizes Its Inhibitory Effect—We have previously shown that Fab fragments of a polyclonal antibody raised against rEbpS inhibit binding of *S. aureus* to elastin (19), suggesting that a population of antibodies in the immune IgG recognizes a region in EbpS critical for elastin binding. To test this possibility, anti-rEbpS IgGs were absorbed to the trEbpS-2 construct coupled to Affi-Gel 10, and unbound IgGs were collected. Immunoblotting revealed that the unbound immunoglobulins (trEbpS-2 negative) retained the ability to interact with full-length rEbpS and trEbpS-1 (Fig. 2C, lanes B and C), although with reduced activity toward trEbpS-1. As expected, the trEbpS-2-negative immunoglobulin fraction that was not absorbed to the trEbpS-2 construct did not react with trEbpS-2 on Western blot (Fig. 2C, lane D).

Fab fragments from both the original and trEbpS-2-negative IgGs were generated by papain digestion and tested for their effects on staphylococcal elastin binding. Consistent with previous findings, Fab fragments from the original anti-rEbpS
IgGs abrogated binding of *S. aureus* to elastin (Fig. 5). In contrast, Fab fragments from the trEbpS-2-negative IgGs did not inhibit *S. aureus* binding to elastin at the highest concentration tested (500 μg/ml).

**Contiguous Synthetic EbpS Peptides Inhibit *S. aureus* Binding to Elastin**—The findings described above suggest that the elastin recognition domain in EbpS is contained within residues 14–34. To define more precisely the elastin binding domain, overlapping synthetic peptides within this region were generated (Fig. 6) and tested for their ability to inhibit staphylococcal elastin binding. We first searched for repetitive sequences in EbpS as a candidate elastin binding domain since several staphylococcal and streptococcal ECM adhesins have been shown to use repetitive domains for ligand recognition (16, 22). Although no identical repetitive sequences were identified, there are two related sequences, 21HQDHTEDVE29 and 37HQDTIENTE45, in the amino-terminal end of the molecule. The sequence 21HQDHTEDVE29 is within the putative amino-terminal elastin-binding site and is contained in all active EbpS constructs. The second sequence, 37HQDTIENTE45, is present only in full-length EbpS and trEbpS-1, which are the most efficient elastin-binding constructs of EbpS.

To determine whether the HQDHTEDVE sequence might participate in elastin binding, we generated two synthetic 17 amino acid peptides, P1 and P2, corresponding to residues 18–34 (Fig. 6). The P1 peptide was made according to the deduced sequence of EbpS. In the P2 peptide, Asp23, Glu26, and Glu29 were substituted with Asn, Pro, and Gln, respectively. The charged amino acids were targeted for substitution because staphylococcal elastin binding has been shown to involve electrostatic interactions (25). As shown in Fig. 6, the P1 peptide inhibited *S. aureus* binding to elastin in a concentration-dependent manner with an IC<sub>50</sub> of 0.4 mM, whereas the P2 peptide failed to inhibit elastin binding (IC<sub>50</sub> = >1.3 mM).

To define the elastin binding domain more completely, three overlapping 10-mers spanning amino acid residues 14–36 were generated (Fig. 6) and tested for their ability to inhibit *S. aureus* binding to elastin. Peptide P4 (residues 21–30) containing the HQDHTEDVE sequence and the P5 peptide (residues 27–36) only reduced elastin binding by approximately 35% at the highest concentration tested, whereas the P3 peptide (residues 14–23) inhibited binding of *S. aureus* to radiolabeled elastin by more than 95% in a concentration-dependent manner, indicating that the HQDHTEDVE sequence does not represent the elastin binding domain of EbpS. IC<sub>50</sub> values obtained from these experiments for P3, P4, and P5 were 0.7, >1.6, and >2.1 mM, respectively (Fig. 6).

Sequence comparison of P3 and other active EbpS constructs revealed that the hexapeptide 18TNSHQD23 is the only sequence shared by all active constructs. However, the hexapeptide TNSHQD (P6) and its control TNSHQS (P7) did not inhibit staphylococcal elastin binding at any concentration tested (0.1–3.0 mM). These findings suggest that although the presence of the TNSHQD sequence is essential for elastin binding by EbpS, additional flanking amino acids are required for its activity. Furthermore, abrogation of activity in the P1 peptide by substitution of Asp23 to Asn, Glu26 to Pro, and Glu29 to Gln suggests that the carboxyl side chain of Asp23 is critical for elastin recognition if the 18TNSHQD hexapeptide indeed plays an important role in elastin recognition.

To test these hypotheses, we generated synthetic peptides P8 corresponding to the wild type EbpS sequence spanning residues 14–25, P9 with Asp17 in P8 substituted with Asn, P10 with Asp23 in P8 substituted with Asn, P11 with 1 amino acid added to TNSHQD in the amino-terminal direction, P12 as a scrambled peptide control of P9, and P13 with 2 residues added to TNSHQD in the carboxyl-terminal direction and tested for their ability to inhibit staphylococcal elastin binding (Fig. 6). Both peptides P8 and P9 inhibited staphylococcal elastin binding in a concentration-dependent manner with IC<sub>50</sub> values of 0.8 and 1.0 mM, respectively. However, the P10 peptide with Asn substituted for Asp23 failed to inhibit binding (IC<sub>50</sub> = >4.3 mM), demonstrating the importance of a charged amino acid at this location. The P11 peptide showed concentration-dependent inhibition of binding with an IC<sub>50</sub> of 0.9 mM, although both its scrambled control (P12) and P13 were inactive (Fig. 6). These results indicate that more than two flanking amino acids in the carboxyl-terminal direction are required to make the TNSHQD hexapeptide active, whereas addition of one residue in the amino-terminal direction is sufficient to render specific elastin binding activity to the hexamer TNSHQD.

**DISCUSSION**

ECM adhesins are important for bacterial colonization of and dissemination through host tissues. To understand better the mechanism of *S. aureus* adhesion to elastin, we sought to...
identify the elastin binding domain in EbpS, the cell-surface elastin-binding protein of *S. aureus*. By using overlapping EbpS fragments, polyclonal anti-EbpS antibodies, and recombinant constructs, we mapped the elastin binding domain in EbpS to the extracellular amino-terminal region of the molecule. Two truncated recombinant constructs spanning EbpS residues 1–34 and 1–78 inhibited staphylococcal elastin binding. When the polyclonal anti-EbpS antibody, previously shown to inhibit *S. aureus* binding to elastin, was absorbed to the trEbpS-2 protein spanning residues 1–34, the antibody lost its inhibitory activity. These findings further localized the elastin binding domain in EbpS to residues 14–34.

Eleven overlapping synthetic peptides spanning amino acids 14–36 were then used to define better the elastin binding domain in EbpS. Among these, only peptides corresponding to residues 14–23, 17–23, and 18–34 of EbpS specifically inhibited elastin binding by more than 95%. Our analyses revealed that the hexameric sequence Thr<sup>18</sup>-Asn-Ser-His-Gln-Asp<sup>23</sup> is the only sequence common to all active synthetic peptides, proteolytic fragments, and recombinant constructs of EbpS. Further evidence that this sequence is important for elastin binding was the loss of activity when Asp<sup>23</sup> was substituted with Asn in the synthetic peptide corresponding to residues 14–25 and 18–34. Interestingly, the synthetic hexamer TNSHQD by itself did not inhibit staphylococcal binding to elastin. These findings suggest that although the presence of the TNSHQD sequence is essential for EbpS activity, flanking amino acids in the amino- or carboxyl-terminal direction and the carboxyl side chain of Asp<sup>23</sup> are required for elastin recognition.

A survey of the active synthetic EbpS peptides showed that four (P1, P3, P8, and P11) are strongly acidic (pI ≤ 5.1). This finding raised the possibility of nonspecific inhibition in which any peptide with two or more strongly acidic amino acid residues, such as Asp and Glu, can interact with elastin. Our results, however, show that the highly negatively charged P5 and P12 peptides (pI = 3.7 and 3.9, respectively) are inactive, excluding the possibility that elastin binding is simply or solely a consequence of strong negative charge. More importantly, our results show that the elastin binding domain is sequence-specific in that the scrambled peptide P12, which is a control for P11, the shortest functional elastin binding sequence, is inactive.

The minimal requirements for elastin recognition by EbpS are surprisingly similar to what has been observed for the interaction between *S. aureus* and fibronectin. Fibronectin binding to *S. aureus* is mediated by a surface fibronectin-binding protein, and the fibronectin-binding site in this adhesin has been mapped to an extracellular 38-amino acid motif repeated three times and partially a fourth time (16). A similar mechanism has been found to be utilized by integrins and their matrix ligands in that the integrin-binding site in the ligands consists of short peptide sequences presented on flexible loops between β-strands (27). Furthermore, the affinities of the short integrin-binding peptides are considerably lower than those of intact ligands as with our short elastin-binding EbpS peptides. We are in the process of determining the crystal structure of EbpS to study directly whether the TNSHQD epitope is indeed stabilized by flanking α-helical domains and to define the elastin recognition sequence.

The properties of staphylococcal elastin and fibronectin recognition mechanisms appear to be opposite that of their corresponding mammalian receptors. Mammalian receptors bind to their respective ligands through the interaction of structural domains in the receptor and a short contiguous peptide sequence in the ligand. Structural domains formed by both the α and β integrin subunits (27), for example, interact with short peptide sequences such as RGD (28), LDV (29), REDV (30), and IDAPS (31). Similarly, the 67-kDa mammalian elastin-binding protein recognizes the hydrophobic GVAPG hexapeptide sequence in elastin (32). In contrast, staphylococcal elastin and fibronectin adhesins appear to interact with their ligands by fitting a small region of the adhesin stabilized by flanking residues into a structural binding pocket formed by the ligand. For example, the staphylococcal binding domain in fibronectin is a structural domain that requires all five type I modules in the amino-terminal region for binding (33).

The different strategy in receptor-ligand recognition used by *S. aureus* also raises the interesting possibility that other elastin-binding proteins may use a similar approach for interacting with elastin. Sequence comparisons, however, failed to detect the TNSHQD sequence in several known elastin-binding proteins, including pancreatic and neutrophil elastases (34–36), lysozamin (37), microfibril-associated glycoprotein (38), or lysozyme (39). Furthermore, none of these proteins showed significant homology to the extended elastin-binding EbpS sequence corresponding to residues 14–34. The exact sequence in elastin recognized by EbpS is still unknown, although previous studies with recombinant tropoelastin fragments have localized the recognition sequence to the amino-terminal one-third of the protein (13).

Similar to the ligand recognition mechanism of the staphylococcal fibronectin adhesin protein, the TNSHQD hexamer by itself could be inactive because it folds improperly, and flanking residues are required to form a conformation that is necessary for efficient activity. This might explain why affinities of active EbpS synthetic peptides for binding to elastin are considerably lower than those of larger elastin-binding EbpS constructs. Although the three-dimensional structure of EbpS is still unknown, the amino-terminal region of full-length EbpS is predicted to fold into amphipathic α-helices (Eisenberg method, PROTEAN program) except for regions including residues 14–23 which is where the TNSHQD sequence resides. These predictions may imply that the flanking residues in EbpS are required to stabilize the flexible binding domain containing the TNSHQD sequence for interaction with the ligand elastin. A similar mechanism has been found to be utilized by integrins and their matrix ligands in that the integrin-binding site in the ligands consists of short peptide sequences presented on flexible loops between β-strands (27).

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