Staphylococcus aureus causing osteomyelitis binds to a nonapeptide sequence in bone sialoprotein

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Bone sialoprotein is a glycoprotein of the bone and dentine extracellular matrix. This protein consists of 320 amino acids, of which 25% are glutamic and aspartic acid residues. Sialic acid, containing oligosaccharides and tyrosine sulphate residues, supplies additional polyanionic properties. Staphylococcal cells, isolated from patients suffering from infection of bone tissue, bind the bone-derived sialoprotein, an interaction which is

specifically inhibited by the recombinant bone sialoprotein core protein. We have previously shown that the 150 N-terminal amino acid residues of bone sialoprotein are responsible for the binding to staphylococcal cells. By using recombinant deleted variants of bone sialoprotein and synthetic peptides, we have now localized the staphylococcal binding site to less than 10 residues within the N-terminal part of the protein.

INTRODUCTION

Bone matrix contains mostly hydroxyapatite, whereas the organic matrix is dominated by collagen type I. Bone sialoprotein (BSP), which is found only in bone and dentine [1,2], is one of the non-collagenous proteins of bone tissue. Using immunogold-staining techniques, this protein has been shown to be present mostly in the osteoid [3], the newly forming bone close to the cartilage. BSP has a molecular mass of 59 kDa with a high content of O-glycosidically linked sialic-acid-rich oligosaccharides. The protein has been cloned and sequenced [4], and the molecular mass of the protein backbone is 33.6 kDa. The C-terminal part of the molecule contains an RGD sequence with eukaryotic cell-binding properties [5]. Furthermore, BSP contains extended sequences of acidic amino acid residues in the form of one stretch of 10 residues and several shorter sequences of glutamic acid [4].

BSP has been shown to be selectively bound by staphylococcal cells isolated from patients suffering from osteomyelitis and/or septic arthritis [6]; this interaction takes place in both the coagulase-positive and coagulase-negative staphylococci [6,7] and is specifically inhibited by the recombinant BSP core protein [8]. Staphylococcal cells have been found to bind BSP with a dissociation constant of about 10 nM [8]. BSP is cleaved by CNBr into two fragments of similar size. The N-terminal part of the BSP molecule inhibits efficiently the binding of native BSP to staphylococcal cells [8], whereas the RGD-containing C-terminal part of the molecule lacks an affinity for staphylococcal cells. Thus the BSP molecule could anchor the staphylococci to the bone tissue, by connecting the bacteria either to the cells or to the hydroxyapatite of bone tissue. Young persons have been found to be more prone to hematogenously spread osteomyelitis and arthritis [9]. Because the newly forming bone, the osteoid, is particularly rich in BSP, it is intriguing to speculate that the binding of BSP by the staphylococcal cells may be of importance in the specific localization of these bacteria in bone tissue and joints. A cell-surface protein, responsible for the specific interaction with BSP, has been isolated from *Staphylococcus aureus*

cells, and has a M_r of 97 000 [10]. A collagen adhesin on staphylococcal cell walls has been shown to be of importance in the development of septic arthritis in an experimental model for such infections in mice [11]. BSP-binding capacity was found in all staphylococci producing septic arthritis in this same model [12], thus indicating the possible significance of this interaction in the development of the disease.

EXPERIMENTAL PROCEDURES

Chemicals

BSA fraction V, ovalbumin grade V, ampicillin and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tryptone soya broth, yeast extract, tryptone and agar base were purchased from Oxoid Ltd. (Basingstoke, U.K.). Glutathione-Sepharose and Percoll were purchased from Pharmacia Biotech. (Uppsala, Sweden). Isopropyl β -thiogalactoside (IPTG) was purchased from Eastman Kodak Co. (New Haven, CT., U.S.A.). Bovine BSP was purified as previously described [1]. ³⁵S-Pro-mixTM was purchased from Amersham (U.K.). Iodo-beads were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), and [¹²⁵I]iodine was purchased from Amersham (Canada). BSP was radiolabelled to a specific radioactivity of (2–4) × 10⁷ c.p.m./ μ g.

Bacterial strains

S. aureus (strain O24) was originally isolated from a patient with acute osteomyelitis [9], and the *Staphylococcus epidermidis* strain 7686, isolated from a peritoneal catheter, was kindly donated by G. Pulverer, University of Cologne, Germany. The staphylococci were stored at –70 °C, subcultured on blood/agar and grown in Trypticase Soy broth before experiments were carried out. The staphylococci were then washed twice in cold PBS and suspended

Abbreviations used: BSP, bone sialoprotein; GST, glutathione S-transferase; IPTG, isopropyl β -thiogalactoside.

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in buffer [137 mM NaCl/5 mM KCl/0.7 mM MgSO₄/1.2 mM CaCl₂/10 mM Hepes (pH 7.4)/0.1 % ovalbumin] at a concentration of 10¹⁰ bacteria/ml.

Construction of BSP–glutathione S-transferase (GST) fusion proteins and their expression in bacteria

BSP–GST fusion proteins were produced in bacteria transformed with constructs encoding 3′- and 5′-deleted variants of BSP linked to GST. An *XmnI*/*HincII* fragment of BSP (nts 138–613 in [4]) was ligated to pBluescript KSII (Stratagene, La Jolla, U.S.A.) cleaved with *SmaI*/*HincII*. 3′-deletions were made by Bal 31 digestions of *HincII*-cleaved BSP/pBluescript KSII vector [13]. After exonuclease digestions, 3′-deleted BSP cDNAs were excised with *Bam*HI and ligated to *Bam*HI-/*SmaI*-cleaved pGEX-3X (Kabi–Pharmacia, Uppsala, Sweden). The extent of deletion was determined by DNA sequence analysis [4]. 5′-deletions were made by PCR amplification using BSP/pBluescript KSII as DNA template and the reverse T3 primer (ATTAACCCTCACTAAAG). The forward primers were: α (GGGATCCAGGATTCTGAAGAAAACGGG), β (GGGATCCGCTACTTTCTTTATAAGCAC) and γ (GGGATCCTCCAGTCCAGGGAGGCGAGC). The $\beta\gamma_{rev}$ construct was amplified using the β -primer and γ rev (GGAATTCCTGCCCTCGGACTGGAAC). PCR amplifications were performed in a thermal cycler using *Taq* DNA polymerase (Boehringer–Mannheim, Germany): 95 °C for 30 s, 55 °C for 45 s and 72 °C for 120 s, 30 cycles in total. The PCR products were subjected to DNA sequence analysis to confirm their correct sequence, digested with *Bam*HI/*HincII*, and ligated to *Bam*HI-/*SmaI*-cleaved pGEX-3X. The constructs were used to transfect *Escherichia coli* UT 5600 bacteria (Kabi–Pharmacia, Uppsala, Sweden). Overnight cultures of bacteria were diluted 1 : 100, grown for 2 h, and then induced by adding IPTG (to a final concentration of 0.1 mM). Bacteria were harvested 7 h later, and after washing twice in PBS the bacteria were lysed by mild sonication and then centrifuged. The supernatant was passed over a glutathione–Sepharose column and bound material was subsequently eluted with glutathione [14]. The eluted material was dialysed before use. The BSP–GST fusion proteins were analysed by SDS/PAGE to evaluate the differences in size of the fusion peptides. The fusion proteins migrated in SDS/PAGE as single components, with the expected differences in size (results not shown).

These fusion proteins were then either used as potential inhibitors of the binding of native ¹²⁵I-labelled BSP to staphylococcal cells, or labelled with ³⁵S for direct binding to the cells.

³⁵S-labelling of fusion protein

When labelled fusion protein was to be used, *E. coli* bacteria were cultured overnight in 2 × YT medium, centrifuged and resuspended in M9 medium [13]. After 3 h, ³⁵S-Pro-mix™ was added to a final concentration of 20 μ Ci/ml, as well as IPTG (to a final concentration of 0.1 mM); bacteria were then allowed to grow for another 8 h. Metabolically labelled fusion protein was then purified as described above, and the radioactivity associated with the fusion protein was determined.

Peptide synthesis

Peptides were synthesized in an Applied Biosystems 430 A peptide synthesizer, using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry and activation with 2-([¹H]benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). After cleavage from the resin, peptides were purified by reverse-phase chromatography on a Vydac 218YTP1022 column eluted with a

gradient of acetonitrile in 0.1 % trifluoroacetic acid/water. Fractions were collected after analysis by plasma-desorption mass spectrometry and then freeze-dried.

Binding assays

Binding of ¹²⁵I-labelled BSP to *S. aureus*

S. aureus (strain O24) was grown overnight at 37 °C in Trypticase Soy broth, washed twice in PBS, and suspended in buffer at a concentration of 10¹⁰ bacteria/ml. Staphylococci were mixed with ¹²⁵I-labelled BSP, and buffer or dilutions of the potential inhibitor, at concentrations ranging from 0.2–40 μ g/ml (corresponding to a molar excess of about 10–1000 compared with ¹²⁵I-BSP) in buffer containing 0.1 % ovalbumin, as described earlier [9]. After 90 min incubation end-over-end at 4 °C, 100 μ l of the mixture was layered on a Percoll gradient, centrifuged, and the supernatant discarded. Radioactivity associated with the pellet was compared with the amount of radioactivity in 100 μ l of incubation mixture. Inhibition of the binding of ¹²⁵I-labelled BSP by the fusion proteins, as well as by synthetic peptides, was compared with the binding of BSP without inhibitor.

Binding of ³⁵S-labelled fusion proteins to staphylococcal cells

S. aureus cells were grown and harvested as described above and suspended in buffer at a concentration of 10¹⁰ bacteria/ml. ³⁵S-labelled fusion protein was added at concentrations of (2–8) × 10⁴ c.p.m./10⁹ bacteria and (2–8) × 10⁵ c.p.m./10⁹ bacteria, and incubated as described above. Radioactivity associated with bacteria was measured in a β -counter and compared with the total amount of radioactivity added to the staphylococcal suspension.

RESULTS

Inhibition of BSP binding by BSP–GST constructs

The BSP–GST fusion constructs (Figure 1) were used to inhibit the binding of ¹²⁵I-BSP to staphylococcal cells. The 3′-deletion constructs A, B, C, D, D₁ and D₂ inhibited the binding of ¹²⁵I-BSP to staphylococcal cells with similar concentration dependencies, whereas the 3′-deletion constructs D₃ and D₄ did not affect the binding (Figure 2). Extracts of GST from *E. coli* UT 5600 transformed with expression plasmid without insert did not inhibit the binding of ¹²⁵I-BSP to staphylococcal cells (results not shown). These results suggest that the sequence between Thr-50 and Phe-60 is essential for staphylococcal binding; the location of this binding site was verified by using BSP constructs deleted from the N-terminus and denoted by α , β and γ . However, whereas constructs α and β inhibited the binding of ¹²⁵I-BSP to bacteria, construct γ did not show any inhibitory activity (Figure 3). The latter results indicate that the binding site is located between Arg-42 and Phe-60. On the basis of these results, we constructed a fusion protein, $\beta\gamma_{rev}$, which included amino acid residues Arg-42–Gly-64. In agreement with the results obtained with 3′- and 5′-deletion constructs, the $\beta\gamma_{rev}$ fusion protein inhibited the binding of ¹²⁵I-labelled BSP to *S. aureus* (Figure 3). In addition, multifunctional binding protein(s) have been found in lysates of staphylococcal cells (see below; also [14a]). Such proteins could influence the inhibitory activity of fusion proteins in the present study, and explain inhibitory activities up to approx. 50 %.

Binding of ³⁵S-labelled fusion proteins to *S. aureus*

In addition to studying the inhibition of ¹²⁵I-BSP binding by fusion proteins, we also investigated the direct binding of

Figure 1 Sequence of BSP-fusion proteins and synthetic peptides

The 3'-deletions (A–D₄), 5'-deletions (α – γ) and $\beta\gamma_{rev}$, were produced in bacteria as GST-fusion proteins. Three residues at the beginning and at the end of each protein is shown. The dashed lines indicate identity with sequence in A. Amino acid residues are numbered from the initiation Met residue [4].

Figure 2 Binding of ¹²⁵I-labelled BSP to *S. aureus* O24 in the presence of potential inhibitors

Binding in buffer alone was set at 100%, and the inhibitory capacity of added fusion proteins was related to binding in buffer alone. The data are shown for fibronectin (▲), BSP (◇) and A–D₂ [(<); representing fusion proteins A, B, C, D, D₁ and D₂] which all show similar inhibition, and D₃ (■) and D₄ (◆), the different fusion proteins after 3'-deletions of BSP cDNA. Inhibitors were added to concentrations representing a 10–1000-fold molar excess compared with ¹²⁵I-BSP.

metabolically radiolabelled BSP–GST fusion proteins to staphylococcal cells. [³⁵S]Met-/Cys-labelled fusion proteins D₁ and D₂, as well as α and β , bound to bacteria, whereas D₃ and γ did not show any significant binding capacity (Table 1). Furthermore, metabolically radiolabelled $\beta\gamma_{rev}$ fusion protein bound to the staphylococcal cells (Table 1). These results correlate with those from the experiments where the BSP–GST fusion proteins were used as inhibitors of ¹²⁵I-BSP binding to staphylococcal cells (Figures 2 and 3). We concluded, therefore, that a stretch of 10–18 amino acid residues in the BSP molecule appeared to be

Figure 3 Binding of ¹²⁵I-BSP to *S. aureus* O24 cells in the presence of potential inhibitors

Binding in buffer alone was set at 100% and the 5'-deletions of BSP cDNA used were denoted α (□), β (○) and γ (■). The inhibitory activity of the construct $\beta\gamma_{rev}$ (Δ) is also shown. Highest concentration of inhibitors used were approx. 20–40 μg/ml.

involved in the binding to staphylococcal cells. In the inhibition assays the BSP–GST fusion proteins may have undesirable effects on the ¹²⁵I-BSP ligand. Possibly, interactions between BSP–GST fusion proteins and ¹²⁵I-BSP may cause conformational changes which lead to inhibition of ¹²⁵I-BSP binding. However, the direct binding of BSP–GST fusion proteins to staphylococcal cells strongly suggests that the fusion proteins exert their inhibitory activity by competing with the BSP-binding site on the staphylococcal cells.

Table 1 Binding of ^{35}S -labelled fusion proteins to *S. aureus* 024 cells

(A) Radioactivity associated with bacteria was compared with total radioactivity $[(2-8) \times 10^5 \text{ c.p.m.}]$ added to the samples, and expressed as % binding. (B) shows results with a 10-fold dilution of the labelled fusion protein added. (For details, see Experimental Procedures).

	^{35}S -fusion protein bound (%)	
	A	B
BSP	40.9 $\pm$ 4.7	38.2 $\pm$ 4.8
D ₁	47.9 $\pm$ 4.6	47.6 $\pm$ 4.8
D ₂	32.8 $\pm$ 5.3	30.9 $\pm$ 5.8
D ₃	6.3 $\pm$ 2.0	5.2 $\pm$ 1.9
α	27.6 $\pm$ 6.3	24.1 $\pm$ 5.2
β	46.9 $\pm$ 3.8	44.7 $\pm$ 3.6
γ	4.1 $\pm$ 1.8	4.8 $\pm$ 1.6
$\beta\gamma_{\text{rev.}}$	42.8 $\pm$ 9.13	9.9 $\pm$ 7.3

Figure 4 Inhibition of binding of ^{125}I -BSP to *S. aureus* 024 cells by synthetic peptides

The peptides YFYPLKRFVQGG (■), LKRFPVQGG (◆), GGQVPFRKL (○), KQRFGLGPV (△), KRFPVQGG (▣), RFPVQGG (◇), FPVQGG (●) and LKRF (▼) were added to the incubation mixture at indicated concentrations and incubated for 90 min. Binding is expressed as % of ^{125}I -BSP bound in the presence of buffer alone.

Inhibition of BSP binding by synthetic peptides

To further characterize the binding specificity of BSP, we synthesized peptides which encompassed amino acid residues Tyr-51–Gly-64 (Figure 1). We established that a synthetic peptide YFYPLKRFVQGG, corresponding to Tyr-51–Gly-64 in BSP, as well as a shorter peptide LKRFPVQGG (Leu-56–Gly-64), inhibited the binding of ^{125}I -BSP to staphylococcal cells (Figure 4). We found that synthetic peptides containing the same amino acids in reverse order, GGQVPFRKL, showed some inhibitory activity, whereas a randomly scrambled sequence of the same amino acids, KQRFGLGPV, did not significantly inhibit binding of ^{125}I -BSP to staphylococcal cells (Figure 4). By deleting one amino acid at a time from the N-terminal end of the LKRFPVQGG peptide, we found that the inhibitory activity depended on the presence of the LKR sequence. Thus a peptide containing only the LKRF sequence inhibited binding of ^{125}I -BSP to staphylococcal cells (Figure 4), whereas the fibronectin peptide, GRGDS, used as control, did not inhibit the binding (results not shown). When we compared inhibitory activity at a

certain concentration (50 $\mu\text{g/ml}$) for all peptides used, we found that the two peptides comprising the longest and most accurate order sequences, YFYPLKRFVQGG and LKRFPVQGG, showed the strongest inhibition (60–70 %) of ^{125}I -labelled BSP binding. The reverse peptide, GGQVPFRKL, as well as the two shorter peptides, KRFPVQGG and RFPVQGG, and the shortest peptide used in the study, LKRF, showed similar inhibitory activity (32 %), whereas the randomly scrambled peptide only inhibited BSP binding by 15 % (Figure 4). The peptide FPVQGG did not inhibit the BSP binding at all (Figure 4).

DISCUSSION

Bone sialoprotein is a bone glycoprotein with a high content of aspartic and glutamic acid residues constituting 25 % of the whole sequence. Such acidic amino acids may be involved in non-specific binding to basic components of bacteria. Staphylococci have earlier been shown to bind to the N-terminal half of BSP, which contains stretches of acidic amino acid residues [1,10,15]; however, the binding of radiolabelled BSP is not inhibited by osteopontin [10], another glycoprotein of bone tissue which also contains stretches of exclusively acidic amino acid residues. We have now determined that the staphylococcal binding site in BSP does not include any acidic amino acid residues. The fusion protein D₂ (Phe-22–Phe-60) does inhibit the binding of BSP and binds to staphylococcal cells. On the other hand, the construct D₃ (Phe-22–Ala-49) neither binds to nor inhibits the binding of BSP to bacteria. These results show that a sequence of ten amino acid residues, located between Thr-50 and Phe-60, is essential for the binding of BSP to staphylococcal cells; the GST-fusion proteins deleted from the N-terminus localize the binding site to this region in the BSP molecule. The fusion protein β containing BSP sequences between Arg-42 and Glu-161 inhibits the binding of BSP and binds to staphylococcal cells, whereas the construct γ (Pro-60–Glu-161) neither binds to bacteria nor inhibits this binding. The GST-fusion protein $\beta\gamma_{\text{rev.}}$ (Arg-42–Gly-64) both binds to and inhibits the binding of BSP to staphylococcal cells. The results from the inhibition studies, as well as the direct binding studies with fusion proteins, strongly suggest that the fusion proteins exert their effect by competing with ^{125}I -BSP for the same binding site on the staphylococcal cells. Using synthetic peptides, we confirmed the finding that a sequence including LKRFPVQGG (Leu-56–Gly-64) inhibited the binding of BSP to staphylococcal cells. The inhibitory effect of the reverse sequence peptide may indicate that the distances between charged and hydrophobic residues play a role in the inhibitory activity, supported also by the finding that a randomly scrambled peptide did not show any significant inhibitory activity. The short peptide LKRF also inhibited the binding of BSP to staphylococci, though somewhat less efficiently than the longer peptides, including the LKR sequence; the unrelated fibronectin peptide GRGDS did not inhibit binding. These results show that the binding of BSP to the recently identified 97 kDa staphylococcal cell-surface receptor [10] involves a stretch of about 10 amino acid residues, with basic and hydrophobic amino acids, and depends on the presence of an LKR sequence. The staphylococcal cells do appear to recognize a short stretch of amino acid residues in BSP but seem to be independent of the secondary structure, as determined by surrounding sequences. A comparison of the peptide YFYPLKRFVQGG, derived from the rat BSP sequence, with sequences in the Swiss Protein sequence bank did not reveal any significant homologies with other proteins, with the exception of pig and human BSP. The human (YFYPLK-RFPVQGS) and pig (YFYPLKRFVQSS) sequences are

almost identical with the rat sequence within the motif LKRFP-VQGG, differing only in the last amino acid (G in the position of S) in human BSP, and the two last amino acids (GG in place of SS) in the pig sequence. The human sequence also contains a His residue in position 5 of the peptide sequence, instead of a Pro residue in the rat and pig sequences.

The binding of staphylococcal cells to fibronectin [16–25] and collagen [26,27] has been studied to some extent. One main binding site for fibronectin in *S. aureus* cells is located in a 27–30 kDa fragment present in the N-terminal part of the molecule. This site is distinct from the collagen-binding site, as well as from the eukaryotic cell binding sites [16–19], and is composed of five type I repeats rich in intramolecular disulphide bonds. A second fibronectin staphylococcal binding domain has been localized to a 120 kDa C-terminal fragment [22,23]. This binding site, which has been shown to involve type I repeats, is found only in one of the fibronectin chains, and is dependent on the intrachain disulphide bond located in the region between the heparin- and gelatin-binding domains [23]. It has been shown that staphylococci recognize not only several types of collagens but also isolated collagen type I α -chains and peptides generated by CNBr cleavage of α -chains [26,27]. Synthetic peptides (Pro-Gly-Pro)_n, mimicking the structure of collagen, inhibit the binding of collagen to staphylococcal cells [27], whereas (Pro-Ala-Gly)_n and polyproline are not inhibitors of collagen binding to staphylococci [27]. In protein-overlay experiments, staphylococcal cell lysates from different strains have been shown to contain cell-surface proteins with somewhat different *M_s* but with similar binding properties, i.e., showing affinity for a variety of different extracellular matrix components [14a]. Not only vitronectin and thrombospondin, but also fibrinogen and fibronectin, interact with these proteins and, to a lesser extent, BSP and collagen [14a]. It was possible to block this type of BSP interaction by ovalbumin in protein-overlay experiments; however, the interaction between the purified 97 kDa BSP-binding protein and BSP was not affected by the addition of ovalbumin, but was specifically inhibited by the addition of unlabelled BSP [10]. This type of multifunctional binding protein could influence the inhibitory activity of fusion proteins and peptides in the present study, and explain inhibitory activities of the order of 50 %.

We propose that, in order to increase their virulence, staphylococci have apparently evolved cell-surface receptors which recognize short stretches of amino acid residues in extracellular-matrix proteins. Staphylococcal resistance to antibiotics is an increasing clinical problem, and alternative methods for prevention and treatment of such infections are vital. The use of peptides to inhibit staphylococcal adhesion to tissues, or to a foreign implanted material, should be considered and tested as a new therapeutic method.

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