the FEBS Journal

Staphylococcus aureus protein A binding to von Willebrand factor A1 domain is mediated by conserved IgG binding regions

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Keywords
Immunoglobulin G; protein A; *Staphylococcus aureus*; von Willebrand factor

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(Received 3 July 2006, revised 25 August 2006, accepted 29 August 2006)

doi:10.1111/j.1742-4658.2006.05482.x

Protein A (Spa) is a surface-associated protein of *Staphylococcus aureus* best known for its ability to bind to the Fc region of IgG. Spa also binds strongly to the Fab region of the immunoglobulins bearing V_H3 heavy chains and to von Willebrand factor (vWF). Previous studies have suggested that the protein A–vWF interaction is important in *S. aureus* adherence to platelets under conditions of shear stress. We demonstrate that Spa expression is sufficient for adherence of bacteria to immobilized vWF under low fluid shear. The full length recombinant Ig-binding region of protein A, Spa-EDABC, fused to glutathione-*S*-transferase (GST), bound recombinant vWF in a dose-dependent and saturable fashion with half maximal binding of about 30 nM in immunosorbent assays. Full length-Spa did not bind recombinant vWF A3 domain but displayed binding to recombinant vWF domains A1 and D′-D3 (half maximal binding at 100 nM and 250 nM, respectively). Each recombinant protein A Ig-binding domain bound to the A1 domain in a similar manner to the full length-Spa molecule (half maximal binding 100 nM). Amino acid substitutions were introduced in the GST-SpaD protein at sites known to be involved in IgG Fc or in V_H3 Fab binding. Mutants altered in residues that recognized IgG Fc but not those that recognized V_H3 Fab had reduced binding to vWF A1 and D′-D3. This indicated that both vWF regions recognized a region on helices I and II that overlapped the IgG Fc binding site.

Staphylococcus aureus is a commensal of moist squamous epithelial surfaces, primarily in the anterior nares, where it permanently colonizes 20% of the population and transiently colonizes another 60% [1]. It can cause superficial skin infections such as abscesses and impetigo, and more serious invasive infections such as osteomyelitis, septic arthritis and endocarditis. Indeed, *S. aureus* is the leading cause of infective endocarditis where the bacterium can initiate infection of previously undamaged heart valves [2].

Staphylococcus aureus has the ability to express a number of cell wall anchored surface proteins that bind to plasma proteins or to components of the extracellular matrix. This facilitates evasion of immune responses, colonization of damaged tissue and adhesion to host cells and to platelets [3–5]. The interaction

Abbreviations
FL, full length; GST, glutathione-*S*-transferase; Spa, *Staphylococcus aureus* protein A; vWF, von Willebrand factor.

between *S. aureus* and platelets is a critical step in *S. aureus*-induced endocarditis. It has been demonstrated previously that the plasma proteins fibrinogen and fibronectin can act as bridges between bacterial cells and the platelet intergrin GPIIb/IIIa on resting platelets, through interactions with staphylococcal surface proteins clumping factor A and fibronectin binding proteins [6–9]. In each case, antibodies specific to the surface protein are also required to form a bridge to the platelet FcγRIIa receptor. This can lead to infective vegetations in the vascular endothelium.

Protein A (Spa) is a major surface protein of *S. aureus*. It comprises four or five homologous repeat domains of 56–61 residues followed by a polymorphic variable repeat region X_r and a conserved region X_c, which includes a cell wall attachment sequence [10,11]. Structural analysis of a single Spa domain revealed that it is composed of a three-helical bundle. The solved structures of Spa domains in complex with IgG Fc and with a V_H3-derived IgM Fab demonstrate how different parts of the Spa repeat are involved in the two interactions. Indeed, it is possible for a single Spa domain to bind each ligand simultaneously [12–14].

Spa is known to bind human von Willebrand factor (vWF), a protein that is essential for haemostasis, with an affinity of 15 nM as measured by surface plasmon resonance using full length recombinant protein A and vWF that had been purified from plasma. This interaction was shown to occur in the presence of physiological IgG concentrations [15]. Heritable defects in vWF result in von Willebrand's disease, a common bleeding disorder, symptoms of which can mirror severe haemophilia. The main function of vWF is to capture platelets by binding to the platelet receptor GPIb-α and immobilize them at the site of damage to a blood vessel and to stimulate the formation of a blood clot. The vWF protein consists of four types of repeat domain A, B, C and D Domains are arranged in the sequence D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK in the mature protein (reviewed in [16]). The crystal structure of the recombinant A1 domain in complex with platelet glycoprotein GpIb-α has been solved [17–19]. Binding of circulating vWF to the ligands such as collagen in exposed subendothelial matrix of damaged blood vessels under high shear stress stimulates a conformational change which promotes immobilized vWF binding to GpIb-α on platelets [20–22]. Circulating platelets are captured and activated, stimulating the formation of a thrombus [23,24].

The ability of *S. aureus* to bind vWF could contribute to the adherence of the bacterium to platelets or to damaged blood vessels. By studying a Spa-deficient mutant of *S. aureus* it was shown that the Spa–vWF interaction is necessary for efficient recruitment of *S. aureus* by platelets under high shear stress in whole blood [25]. Also fluid shear adhesion experiments suggested that vWF binding to Spa can promote adherence of circulating *S. aureus* cells to immobilized collagen [26]. In this study we set out to analyze the interaction between Spa and vWF. We demonstrate, using *Lactococcus lactis* as a surrogate host for protein expression, that Spa is sufficient for adherence of bacteria to immobilized vWF under low shear conditions. We used recombinant Spa and vWF truncates to identify and characterize the domain(s) in each protein that is involved in binding, and refined the vWF binding domain in protein A by site-directed mutagenesis.

Results

Perfusion studies

To determine whether expression of protein A on the cell surface is sufficient for adhesion of bacteria to immobilized vWF under flow, *L. lactis* or *L. lactis* Spa^+ were perfused over glass slides coated with recombinant human vWF. Cells were perfused for 300 s, and adherent bacteria visualized by videomicroscopy. Adherent bacteria were observed only in the case of the *L. lactis* expressing protein A. Binding occurred at low shear rates (50 s^{-1}) but not at high shear rates (Fig. 1A). Quantitative analysis was performed by counting adherent cells from at least three separate fields at 60-s time intervals (Fig. 1B). This supports previous work suggesting that Spa on the surface of *S. aureus* is necessary for efficient attachment of bacteria to a vWF–collagen complex at low shear rates [26] by demonstrating that it is sufficient for this process.

Immobilized *L. lactis* or *L. lactis* Spa^+ were tested for their ability to recruit platelets in whole blood under various conditions of shear. None of the shear rates tested (50–1500 s^{-1}) supported platelet adhesion by *L. lactis* or *L. lactis* Spa^+ (data not shown).

Identification of regions within von Willebrand factor that bind to protein A

It was shown previously that vWF purified from human plasma bound to full length recombinant protein A with an apparent K_d of 15 nM measured by surface plasmon resonance [15]. Here ELISA-type binding assays were employed to study the interaction between recombinant protein A and recombinant vWF. Firstly, full length (FL) recombinant vWF and the extracellular repeat region of Spa fused to glutathione-*S*-trans-

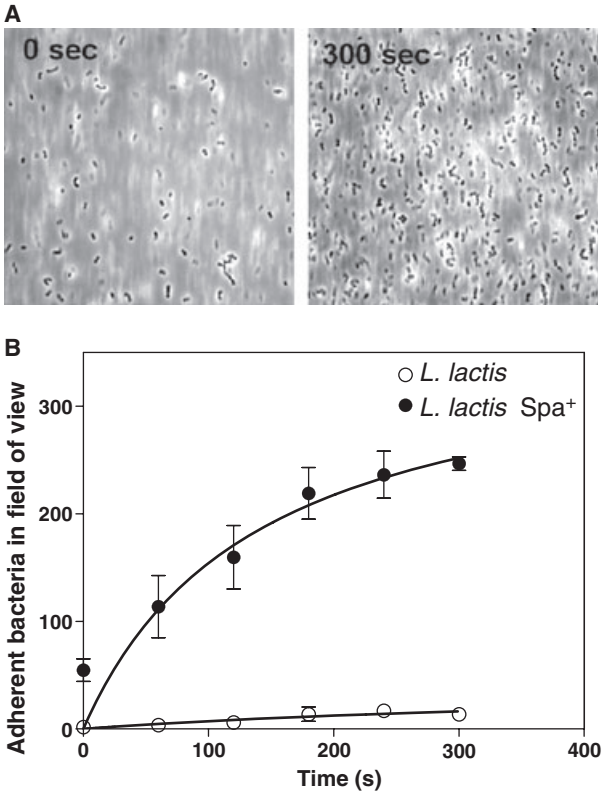


Fig. 1. Perfusion of *L. lactis* expressing Spa over vWF-coated slides. *L. lactis* or *L. lactis* expressing Spa were perfused over glass slides coated with full length vWF (100 μg·mL^{-1}). Live imaging of adherent cells by videomicroscopy was performed as described in Experimental procedures. (A) Images of *L. lactis* Spa+ after 0 and 300 s of perfusion over vWF. (B) Adherent bacterial cells were counted from three independent fields of view at 60 second intervals for both *L. lactis* and *L. lactis* Spa+.

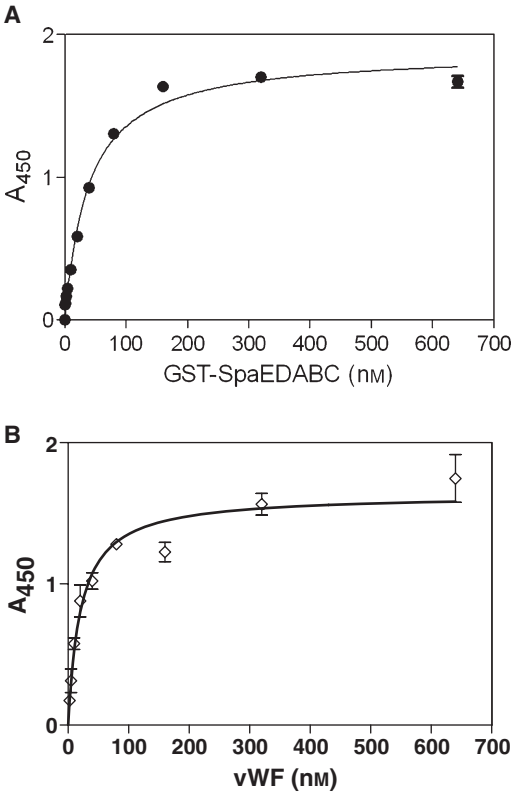


Fig. 2. Interaction of FL-vWF to GST-SpaEDABC. (A) Microtitre wells were coated with vWF (10 μg·mL^{-1}) and incubated with increasing concentrations of GST-SpaEDABC. HRP-conjugated chicken anti-GST IgY was used to detect bound GST-SpaEDABC. In the reverse assay, binding of soluble vWF to immobilized SpaEDABC (10 μg·mL^{-1}) was detected using HRP-anti(6×-His) monoclonal IgG1. (B) Values are the means ± standard deviation of triplicate wells. The experiment was performed three times in triplicate with similar results.

ferase (GST-SpaEDABC) were used. When FL-vWF was immobilized onto ELISA plates, binding of soluble GST-SpaEDABC occurred in a dose-dependent and saturable fashion with half maximal binding at ≈30 nM (Fig. 2A). Similar data were obtained when soluble FL-vWF was tested in binding experiments with immobilized GST-SpaEDABC (Fig. 2B). This showed that the Spa bound via its N-terminal EDABC domains to vWF specifically and with high affinity, and that recombinant human vWF behaved in a similar fashion to plasma-derived protein used previously.

In order to identify the binding site(s) for Spa in vWF, the D′-D3, A1 and A3 truncates of vWF were tested for their ability to bind to immobilized GST-SpaEDABC. The A1 domain of vWF bound to GST-SpaEDABC dose-dependently and saturably with half maximal binding at 100 nm. Binding was also detected for the vWF D′-D3 domain but with a lower affinity (half maximal binding at 250 nm). In contrast, the A3 domain of vWF did not bind to GST-SpaEDABC (Fig. 3A). This shows that vWF contains a high affinity Spa binding domain in domain A1 and a lower affinity site in domain D′-D3. To demonstrate specificity of these interactions, vWF A1 and D′-D3 were tested for binding to immobilized GST-SpaEDABC in the presence of increasing concentrations of soluble GST-SpaEDABC. The soluble Spa inhibited binding in a dose-dependent and saturable manner, providing further evidence of the specificity of the interaction (Fig. 3B).

Individual domains of protein A bind von Willebrand factor

Each of the homologous domains E, D, A, B and C of protein A can bind individually to the Fc region of IgG and to the Fab region of IgM that of the V_H3

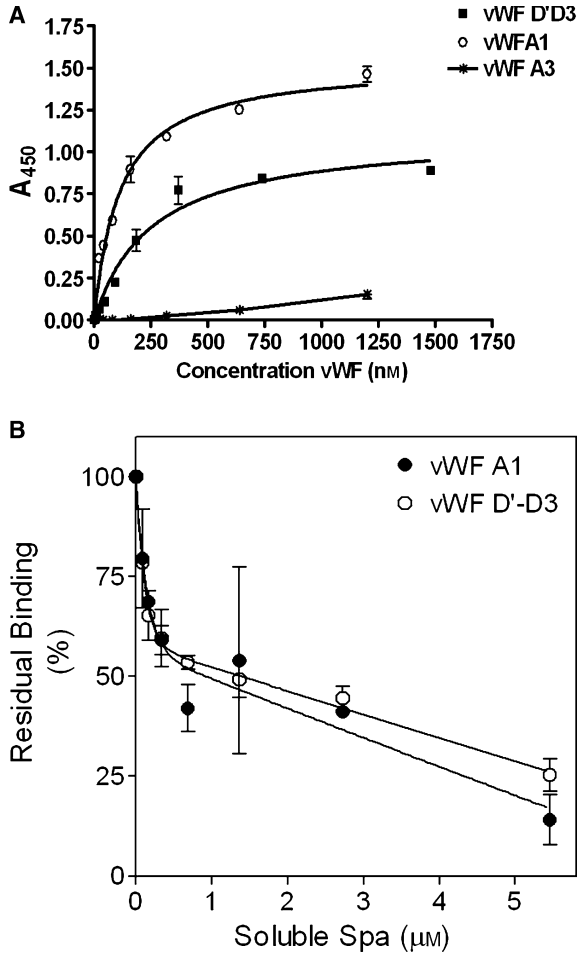


Fig. 3. Binding of GST-SpaEDABC to recombinant vWF truncates. (A) Microtitre plates were coated overnight with GST-SpaEDABC (10 $\mu g \cdot mL^{-1}$) and incubated with increasing concentrations of vWF D′-D3, A1 or A3. Bound vWF constructs were detected with HRP-anti(His) monoclonal IgG1. Half maximal binding for vWF A1 and D′-D3 truncates was observed at 100 nm and 250 nm, respectively. (B) vWF A1 (100 nm) or D′-D3 (250 nm) were tested for binding to immobilized Spa in the presence of increasing concentrations of soluble Spa. Percentage binding relative to vWF binding in the absence of soluble Spa was calculated. Values are the means ± SD of three separate experiments.

subclass [11,27]. In order to investigate if each domain can bind to vWF, GST-SpaE, GST-SpaD, GST-SpaA, GST-SpaB and GST-SpaC fusions were tested for their ability to bind to the vWF A1 domain by western ligand blotting. When 250 ng of GST-Spa constructs were probed with 250 nm vWF A1, binding was detected for each of the Spa constructs except for GST-SpaE (Fig. 4A). Weak binding was detected only when larger amounts of GST-SpaE were tested (Fig. 4B).

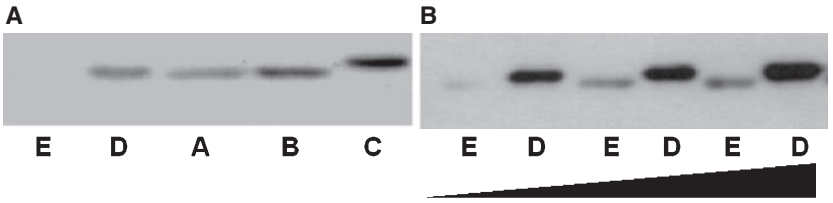
Binding of Spa domains to vWF was also investigated with ELISA-type binding assays. Each GST-Spa truncate was immobilized and tested for its ability to bind soluble vWF A1 and D′-D3 proteins. The GST-Spa fusions bound to vWF A1 dose-dependently and saturably with similar affinity (half maximal binding at $\approx$100 nM) (Fig. 5C). Each GST-Spa fusion also bound to the D′-D3 domain with similar affinities (not shown). It is noteworthy that GST-SpaE behaved in a similar fashion to the other GST-Spa constructs. The apparent lower affinity of the E domain for vWF A1 in ligand blotting could be explained by slower or improper renaturation of the protein after transfer from the SDS/PAGE gel to the poly(vinylidene difluoride) membrane. Alternatively, reduced binding may be due to the detrimental effect of blotting procedure on folding of the SpaE domain. It is also worth noting that the E domain of Spa has the greatest amino acid sequence differences from the other Spa domains.

SpaD binding to vWF A1 is inhibited by IgG Fc regions but not a V_H3-Fab

Hartleib *et al.* reported inhibition of the Spa–vWF interaction by IgG [15]. This suggests a possible shared binding region on Spa for IgG Fc and vWF. However, the large IgG molecule might have sterically blocked vWF binding. In addition, the pooled IgG used may have contained antibodies against Spa [15]. To overcome these potential problems, here the Fc fragment of IgG was used for binding and inhibition studies along with a recombinant V_H3 heavy chain fragment JMSpa3-08, with a nonreactive V_H4-bearing IgM included as a control. Binding of Spa to Fc and V_H3-derived immunoglobulin occurs through distinct binding regions within the Spa domain and is non-competitive, as demonstrated by the structures of complexes and by sandwich ELISA assays [12,13]. Inhibition studies performed here revealed that the Fc fragment blocked vWF A1 binding in a dose-dependent and saturable manner while the V_H3 heavy chain fragment had no effect (Fig. 5), which strongly suggests that vWF A1 binds to the same region of Spa as the Fc fragment of IgG.

Amino acid substitutions of SpaD

GST-fusion proteins of Spa domain D bearing substitutions in residues known to be involved in Fc or V_H3-Fab binding [12,28] were generated, to identify the residues on Spa involved in binding to vWF A1. The D domain of Spa was chosen because its crystal structure has been solved. A 58 residue variant (GST-$SpaD_{58}$) of GST-SpaD, lacking the additional N-terminal residues unique to SpaD was also included in



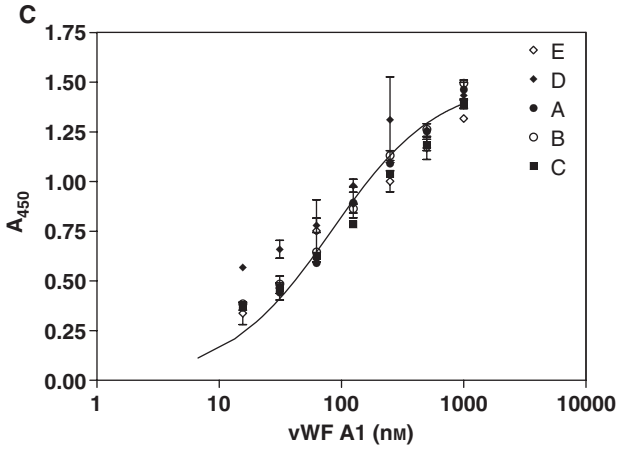


Fig. 4. Interaction of single GST-Spa domains with vWF A1 . (A) Individual GST-Spa domains (250 ng) were separated by SDS/PAGE, immobilized on poly(vinylidene difluoride) membrane and assayed for vWF A1 binding by ligand affinity blotting. (B) Increasing concentrations of domains GST-SpaE and GST-SpaD (500 ng, 1 μg, 2 μg) were assayed by for vWF A1 binding by ligand affinity blotting. (C) Interaction of GST-SpaE, GST-SpaD, GST-SpaA, GST-SpaB and GST-SpaC domains to soluble vWF A1 by ELISA-type assay. Values represent the mean of triplicate determinations. Binding curve represents average of all data values from GST-Spa truncates. Experiments were performed three times.

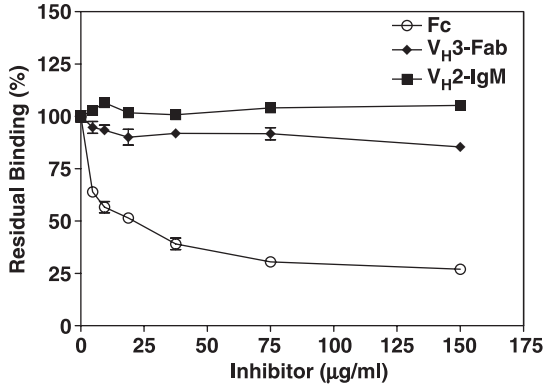


Fig. 5. Inhibition of Spa binding to vWF A1 by Fc and V_H3-Fab. Ninety-six-well plates coated with GST-SpaD (10 μg·mL^{-1}) were incubated with a mixture of vWF A1 (100 nM) and various concentrations of Fcα or V_H3-Fab. A V_H4-bearing IgM, which does not bind Spa, were also included. Bound vWF was monitored using a HRP-conjugated anti-Hisx6 IgG1. Percentage inhibition was calculated relative to vWF bound in the absence of inhibitor. The experiment was performed three times with similar results.

this study. A number of residues were substituted in order to investigate a role in vWF binding (Fig. 6A). The substitutions generated were F5A, Q9A, Q10A, F13A, Y14A, L17A, N28A, I31A, K35A (Fc binding), G29A, F30A, S33A, D36A, D37A, Q40A E47A (V_H3-Fab binding), Q32A (proposed to have a minor contact in both interactions), N21A and R27A (conserved residues in all Spa repeats but not involved in Fc or V_H3-Fab binding). Variants were tested for binding to IgG and vWF A1 by ELISA-type assay. GST-SpaD_{58} bound with a similar affinity to wildtype GST-SpaD to both ligands (not shown). The substitution of N21, which is not involved in Fc or V_H3-Fab binding also showed no reduction in vWF binding. Residues Q10, F13, Y14, L17, R27, N28, F30, I31 and K35 were shown to be important in vWF binding (Fig. 6B). When these residues were mapped on the solved structure of SpaD, they were seen to form a cluster between helices I and II. With the exceptions of R27 and F30, these residues coincide with those involved in IgG Fc binding (Fig. 6B) [28]. Interestingly, a similar binding profile was observed for GST-SpaD variants binding to the A1 and D′-D3 domains (data not shown). In contrast, residues known to be involved in the V_H3-Fab binding (G29, S33 and D36, D37, Q40, E47) did not demonstrate reduced vWF binding when substituted.

Discussion

Protein A is one of a number of surface proteins expressed on the surface of *S. aureus* that is known to interact with plasma proteins. Several studies have demonstrated a role for protein A in staphylococcal virulence. Protein A-defective (Spa$^-$) mutants have reduced virulence in murine models of septic arthritis, septicaemia and skin abscesses, most likely due to the antiphagocytic effect of Spa binding IgG Fc [29,30]. In a murine pneumonia model, Spa$^-$ mutants showed

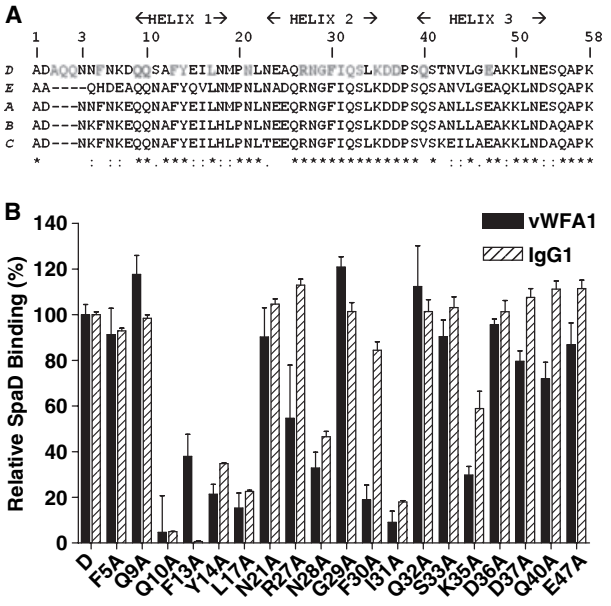


Fig. 6. Effect on ligand binding of SpaD substitutions. (A) Alignment of the five domains of protein A based on SpaD. *, Conserved residues; :, conserved substitutions in residues; ., semiconserved residues. Residues substituted or deleted by site-directed mutagenesis are highlighted grey. (B) ELISA plates were coated with GST-SpaD variants (10 $\mu g \cdot mL^{-1}$) followed by incubation with rabbit IgG or vWF A1 at concentrations corresponding to half maximal binding to wild-type GST-SpaD as previously determined by ELISA. Adherent protein was detected as described in Experimental procedures. Experiments were performed in triplicate on three separate occasions. Figures represent the mean ± SD from three independent experiments.

reduced virulence where the pro-inflammatory properties of Spa binding to tumor necrosis factor receptor-1 (TNFR-1) were strongly implicated [31]. Spa has been shown to trigger apoptosis of murine B cells that express antigen receptors with V_H3-Fab analogues, with greatest efficiency for marginal zone B cells that are involved in defense from blood borne infections. This likely contributes to immunosuppression during *S. aureus* infection [32].

Fluid shear experiments in solution have suggested a function for Spa in promoting bacterial adherence to platelets in whole blood under very high shear rates (5000 s^{-1}) [25], such as those found in stenotic vessels [23]. However, under physiologically relevant shear conditions we could not detect capture of platelets by immobilized bacteria. Spa can mediate adherence of staphylococci to immobilized collagen under flow in the presence of vWF [26]. Also it has been shown that Spa can mediate adherence of *S. aureus* to vWF-coated surfaces in conditions of low shear [15]. Here, by expression of protein A on the surface of a surrogate bacterial host *L. lactis* at a level that is comparable to that of *S. aureus* strain Newman [6] in the absence of other surface proteins of *S. aureus*, we demonstrated that Spa is sufficient to support bacterial adherence to immobilized vWF under low shear conditions (50 s^{-1}), representing normal venous shear rates [23]. This is in agreement with previous studies using *S. aureus* cells that indicated that Spa was necessary for efficient binding [15,26]. Perfusions of whole blood at low shear rates across immobilized *S. aureus* leads to platelet capture and thrombus formation that was shown to be triggered by clumping factor A (S Kerrigan, A Loughman, T Foster and D Cox, unpublished data). To determine if protein A contributed to this process, immobilized *L. lactis* expressing Spa was investigated for its ability to recruit platelets. However, under all conditions of shear tested, no platelet adherence was observed, suggesting protein A is not involved in thrombus formation by *S. aureus*. It is more likely that Spa promotes bacterial binding to immobilized vWF, either bound directly to exposed subendothelial tissue or to platelets that had been previously captured. This opens the possibility that vWF contributes to the recruitment of Spa-expressing bacteria into vWF-rich platelet thrombi.

This study identifies two binding regions on vWF, domains, D′-D3 and A1, with half maximal binding values of 250 nM and 100 nM, respectively (Fig. 3B). Specificity of the interactions is demonstrated by the ability of soluble protein A to compete for binding to immobilized Spa (Fig. 3B). The D′-D3 region of vWF is involved in binding and stabilizing blood coagulation Factor VIII. The A1 domain contains the binding site for the platelet receptor, GpIb. Spa binds to the D′-D3 and A1 domains with estimated half maximal binding values of 250 and 100 nM, respectively. vWF has estimated dissociation constants for coagulation Factor VIII , which it binds through the D′-D3 region, ranging from 200 to 400 pM [33,34]. However, it is reported that only one vWF subunit in fifty binds Factor VIII [34]. Therefore, it is possible that D′-D3 provides a more available, lower affinity site for Spa on vWF. The availability of binding sites on vWF is probably also limited by its globular shape [35,36]. The vWF A3 domain did not bind Spa (Fig. 3A).

Each individual protein A Ig-binding domain was tested for binding to vWF A1 by ligand affinity blotting. Binding was initially detected for domains D, A, B and C, but not domain E (Fig. 4A). While there is high homology between Spa domains, there are some differences (Fig. 6A). Indeed, SpaE is the most heterologous Spa domain. An interaction was observed when increased amounts of Spa E were used (Fig. 4B). When

single Spa domains were tested for binding to vWF A1 by solid phase binding, all individual domains bound with equal affinity (Fig. 4C), suggesting the reduced vWF binding observed may be due to the reported lower stability of domain E under denaturation-refolding conditions [37].

To help identify the region on Spa domain D responsible for vWF binding, the Fc region of human IgG and recombinant V_H3 heavy chain fragment JMSpa3-08 were tested for their ability to inhibit binding of SpaD to vWF. The Fc fragment, but not the V_H3-Fab inhibited vWF A1 binding to Spa in a dose-dependent and saturable manner (Fig. 5). This suggested the binding site for vWF on Spa domain D lies in a similar region to that of the Fc binding site. Site-directed mutagenesis of residues on Spa domain D confirmed that helices I and II contain the vWF binding site as well as that of Fc, with residues Q10, F13, Y14, L17, N28, I13 and K35 important for interactions with both ligands. This is probably due to specific contacts, as previous mutational studies in this region indicate that Spa maintains its native structure [38–40]. However, the binding site is not identical as the substitution of R27A reduced binding to vWF A1, but not IgG (Fig. 6B). This residue lies in helix II of Spa, and its side chain points between helices I and II, consistent with the inhibition studies (Fig. 7). With the exception of F30, substitution of residues that are involved in binding V_H3-Fab had no effect on binding to vWF A1 (Fig. 6B). This is consistent with the inability of recombinant V_H3-Fab to inhibit binding of Spa to vWF A1 (Fig. 5). The observed reduction in binding of the F30A variant may be due to structural changes in the Spa protein because the side chain of F30 contributes to the hydrophobic core of the protein A helical structure, lying between L44 and L51 of helix III (Figs 6A and 7). Indeed, an F30A substitution in Spa has previously been shown to have dramatically decreased structural stability, despite still maintaining its affinity for the Fc region of IgG [39–41]. This is not the case for vWF A1 . This may also explain the minor reduction in binding of variants D37A, Q40A and E47A, as helix III is involved stabilizing the three-helical structure of Spa [42]. Relative binding of the Spa substitutions to IgG (Fig. 6B) were in agreement with previous reports [28,41].

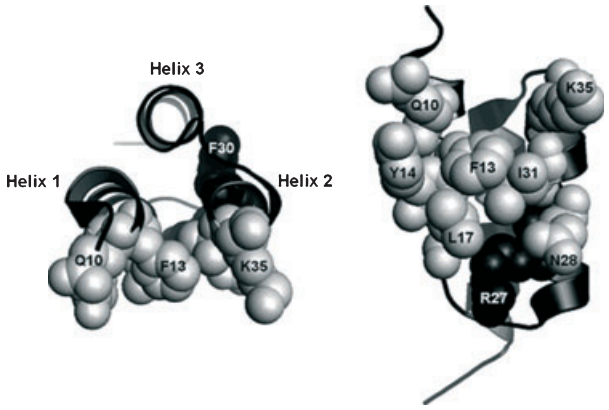


Fig. 7. The binding site for vWF A1 on SpaD. Ribbon diagram of SpaD from above (A) and the side (B) based on its solved structure (PDB ID: 1DEE), with residues shown to be important in vWF A1 binding highlighted in spacefill. Amino acids which also show an importance in binding IgG Fc are coloured grey, while those residues only affecting the vWF A1 interaction are coloured black. The binding site for both vWF A1 and IgG Fc is confined to the helix1–2 interface. Variant F30A shows a decrease in vWF A1 binding, but is known to be important in the structural integrity of the Spa three-helix bundle.

In conclusion, we have demonstrated a direct role for Spa in adherence to surfaces through its interaction with vWF under flow. Mutagenesis studies on Spa have defined the binding site on Spa for vWF as lying between helices I and II, the same region that is responsible for the Fc interaction. All five Spa domains are capable of binding vWF, which may reduce competition for binding sites by Fc and vWF. Full length Spa has a binding stoichiometry of two IgG molecules per molecule of Spa [43,44]. Indeed, vWF interactions occur in the presence of normal serum levels of IgG [15]. This region of Spa could be a potential therapeutic target; however, further studies to define the specific role *in vivo* of the interaction are required.

Experimental procedures

Bacterial strains and growth conditions

DNA cloning was performed in *Escherichia coli* XL1-Blue (Qiagen, Madison, WI, USA). TOPP3 (Stratagene, La Jolla, CA, USA) or M15(pREP4) (Qiagen) were used for expression of recombinant proteins. *E. coli* was routinely grown in Luria broth at 37 °C with shaking at 200 r.p.m. *L. lactis*::(pKS80) and *L. lactis* (pKS80::*spa*$^+$) were grown in M17 medium containing 0.5% (v/v) glucose. Ampicillin (100 μg·mL^{-1}), kanamycin (50 μg·mL^{-1}) and erythromycin (10 μg·mL^{-1}) were incorporated into the media where appropriate.

DNA constructions

Fragments of the region of *spa* encoding the extracellular immunoglobulin-binding domains were isolated and cloned as follows; plasmid pSPA7235 [45] containing the entire

Table 1. Oligonucleotides for single *spa* domain constructs. Restriction sites are indicated in bold type.

Name	Sequence (5′– to 3′)
Fw_*spaE*	CCG**GAATT**CATGCTGCGCAACACGATGAAG
Rv_*spaE*	CCG**CCATGG**TTATTTTGGTGCTTGAGAGTCA
Fw_*spaD*	CCG**GAATTC**AAGCTGATGCGCAACAAAATAAC
Fw_*spaA*	CCG**GAATTC**AAGCTGATAACAATTTCAACAAAG
Rv_*spaD/A*	CCG**CCATGG**TTATTTCGGTGCTTGAGATTCG
Fw_*spaB*	CCG**GAATTC**AAGCGGATAACAAATTCAACAAAG
Fw_*spaC*	CCG**GAATTC**AAGCTGACAACAAATTCAACAAAG
Rv_*spaB/C*	CCG**CCATGG**TTATTTTGGTGCTTGAGCATCAT

coding region of the *spa* gene of *S. aureus* 8325-4 was used as a template for amplification of the five- and single-domain repeats of *spa*. Oligonucleotides were designed to each individual domain to allow amplification of individual protein A repeats (Table 1). Cross reaction of primers due to high sequence homology of the repeats was overcome by digestion of the *spa* template with unique restriction endonucleases prior to single domain amplifications. Restriction sites were incorporated at the 5′ ends of the primers to facilitate directional cloning. PCR amplification was carried out in a DNA thermal cycler (Techne, Cambridge, UK) with *Pfu* DNA polymerase (Stratagene). Reactions were carried out with a 1 min denaturation step at 94 °C, a 1 min annealing step at 55 °C, and elongation at 72 °C for 1–2 min depending on the length of the desired PCR product. This standard cycle was repeated 30 times followed by incubation at 72 °C for 10 min. PCR products were purified using the High Pure™ PCR product purification kit (Roche, Basel, Switzerland), digested with the appropriate restriction endonucleases (Roche) for 1 h at 37 °C, and cloned into plasmid pGEX-KG, previously digested with these enzymes. This was then transformed into *E. coli* XL1-Blue, and positive transformants were sequenced.

Site-directed mutagenesis of a domain of *spa*

Mutations were introduced into domain D of protein A using a PCR-based mutagenesis strategy. Briefly, overlapping oligonucleotides carrying the desired mutation were combined with standard flanking primers to yield two overlapping mutant products. These were combined and amplified using the flanking primers alone to yield the mutant fusion product. In some cases, mutations were introduced using the Quikchange® method, according to manufacturer's instructions (Stratagene). The following amino acid substitutions were constructed: F5A, Q9A, Q10A, F13A, Y14A, L17A, N21A, R27A, N28A, G29A, F30A, I31A, Q32A, S33A, K35A, D36A, D37A, Q40A and E47A. A variant of Spa domain D lacking three additional codons unique to this domain was also created by PCR as previously described, generating a 58 residue variant [13]. Oligonucleotides used to introduce mutations are listed in Table 2. Amplimers were cloned directionally into pGEX-KG and expressed as described above.

Table 2. Oligonucleotides used for *spaD* variants. Reverse primer for all variants is the reverse complement of forward primer. Restriction sites are indicated in bold type; underlined bases indicate a changed codon.

Name	Forward Primer (5′– to 3′)
Fw_$spaD_{58}$	CCG**GAATT**CAAGCTGATAATAACTTCAACAAAG
F5A	GCAACAAAATAACGCGAACAAAGATC
Q9A	CTTCAACAAAGATGCACAAAGCGCC
Q10A	CAACAAAGATCAAGCAAGCGCCTTC
F13A	CAAAGCGCCGCGTATGAAATC
Y14A	GCGCCTTCGCGGAAATCTTG
L17A	CTATGAAATCGCGAACATGCC
N21A	GAACATGCCTGCGTTAAACGAAG
R27A	GAAGCGCAAGCTAACGGCTTC
N28A	CCAACGTGCGGGCTTCATTC
G29A	GCAACGTAACGCGTTCATTCA
F30A	GTAACGGCGCGATTCAAAGTC
I31A	GTAACGGCTTCGCGCAAAGTC
Q32A	GGCTTCATTGCGAGTCTTAAAG
S33A	GCTTCATTCAAGCGCTTAAAGAC
K35A	GTCTTGCGGACGACCCAAG
D36A	GTCTTAAAGCGGACCCAAGCC
D37A	CTTAAAGACGCGCCAAGCC
Q40A	GACGACCCAAGCGCAAGCACTAACG
E47A	CGTTTTAGGTGCAGCTAAAAAATTAAACG

Induction and purification of recombinant proteins

For expression of recombinant Spa truncates, pGEX-KG constructs were purified from *E. coli* XL1-Blue using the Wizard® plasmid purification kit (Promega Corp., Madison, WI, USA) and transformed into *E. coli* TOPP3. Overnight cultures were inoculated into fresh medium and grown to a D_{600} of 0.5. Isopropyl thio-β-D-galactoside was added to a concentration of 1.5 mM and the culture was grown for a further 3 h. Cells were harvested by centrifugation at 8200 ***g*** for 10 min in a Sorvall GS-3 rotor (Sorvall, Bishops Stortford, UK). The pellet was resuspended in NaCl/P_i containing protease inhibitor (Roche), lysozyme (200 μg·mL^{-1}) and DNase I (3 μg·mL^{-1}). Cells were lysed by repeated passage through a French Pressure Cell (SLM Instruments, Urbana, IL, USA). Cell debris was removed by centrifugation at 34 500 ***g*** for 30 min in a Sorvall SS-34 rotor and the supernatant was filtered through a 0.45 μm filter. Recombinant proteins expressed from pGEX-KG contained an N-terminal GST fusion of 26 kDa. The GST-fusion proteins were purified using a GSTrap™ column (Amersham, Uppsala, Sweden) and dialysed against NaCl/P_i. Recombinant five- and single-domain GST-fusion proteins had approximate molecular masses of 59 kDa and 32 kDa, respectively.

Recombinant V_H3-Fab was produced in *E. coli* XL1-Blue from pComb3::JMSpA3-08 as described previously [46]. Murine V_H4 IgM was produced as described elsewhere [47].

Recombinant FL-vWF and truncates D′-D3, A1, and A3 were cloned and expressed as described previously, from baby hamster kidney cells, *Pichia pastoris* and *E. coli*, respectively [18,48,49]. Recombinant vWF A1 domain was also produced in *E. coli* M15 cells harbouring plasmid pREP4 (Qiagen) from plasmid pQE30-vWF-A1 as described previously [17,50]. All vWF truncates contained an N-terminal histidine tag.

Protein analysis and western ligand blotting assays

Recombinant proteins were analyzed by SDS/PAGE using gels containing 4.5–12.5% (w/v) acrylamide. Gels were stained using Coomassie™ brilliant blue (Amersham) or by silver staining [51]. In some cases, proteins were transferred electrophoretically to poly(vinylidene difluoride) western blotting membranes (Roche) by the wet system (Bio-Rad, Hercules, CA, USA) in Tris/HCl (0.02 M), glycine (0.15 M), and methanol (20% v/v). Membranes were incubated for 15 h at 4 °C in 5% blocking reagent (Marvel milk powder, Premier International Food, Spalding, UK). Membranes were washed and incubated with gentle agitation with vWF as previously described [15]. $F(ab')_2$ were isolated from polyclonal anti(vWF-HRP) (Dako, Glostrup, Denmark) by pepsin digestion (Pierce, Rockford, IL, USA) for detection of FL-vWF. vWF truncates were detected using HisProbe-HRP (Pierce). Membranes were developed using LumiGLO chemiluminescent substrate (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions.

Preparation of flow chamber slides

Purified vWF was used at a concentration of 100 $\mu g \cdot mL^{-1}$. A 500 μL solution was applied to glass slides (75 × 25 mm) and allowed to attach for 2 h at room temperature in a humidity chamber. Slides were washed three times in $NaCl/P_i$ buffer to remove any unbound protein. Finally, the slides were blocked with 1% BSA for a further 1 h at 37 °C.

Videomicoscopy

Overnight cultures of bacteria were washed twice in $NaCl/P_i$ and resuspended to a D_{600} of 1. Next, cells were perfused over immobilized vWF at various shear rates. A syringe pump (Harvard Biosciences, Holliston, MA, USA) was used to aspirate bacteria through the flow chamber. Bacterial adhesion was visualized using phase contrast microscopy (63X LD-Achroplan objective) through the flow chamber (GlycoTech, Rockville, MD, USA) mounted on a Zeiss Axiovert-200 epi-fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK). Images were captured every second up to 300 s by a liquid chilled Quantix-57 CCD camera (Photometrics Ltd, Tuscon, AZ, USA). Bacterial adhesion was analysed using METAMORPH (Universal Imaging Corp., Downingtown, PA, USA).

Recombinant protein binding assay

Microtitre plates (Sarstedt, Nümbrecht, Germany) were coated with Spa or vWF in carbonate buffer overnight at 4 °C. Wells were washed three times with Tween 20 (0.05% v/v) in $NaCl/P_i$ and blocked at 37 °C with 3% (w/v) BSA in $NaCl/P_i$ for 2 h at 37 °C. Wells were again washed, and varying concentrations of appropriate ligand in 3% BSA were added. Plates were incubated for 1 h at 37 °C. Unbound protein was removed by washing. Bound vWF was detected as before. Bound Spa was detected by HRP-chicken anti-GST IgY (Gallus Immunotech, Fergus, ON, Canada). After washing, 100 μL of a chromogenic substrate solution (1 $mg \cdot mL^{-1}$ tetramethylbenzidine and 0.006% H_2O_2 in 0.05 M phosphate citrate buffer pH 5.0) was added, and plates were developed for 10 min. The reaction was stopped by the addition of 2 M H_2SO_4 (50 μL per well), and plates were read at 450 nm. Data from ELISA-type assays was graphed and analysed using GRAPHPAD PRISM version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Inhibition studies

ELISA plates were coated and blocked as before. Wells were incubated with mixtures containing increasing concentrations of inhibitor and a standard concentration of ligand corresponding to its half maximal binding to the coated protein as determined by ELISA-type binding assays. Inhibition was performed using Spa and vWF truncates, and also the Fc portion of human IgG (Jackson Laboratories, Inc., Newmarket, UK) and a V_H3-Fab fragment, produced in *E. coli* from plasmid pComb3::JMSpA3-08 as described previously [46]. Wells were washed and bound protein was detected as before. Percentage inhibition was calculated from the percentage of bound protein detected in the absence of inhibitor.

Acknowledgements

TJF and DC would like to acknowledge funding from the Health Research Board of Ireland (Programme Grant RPO09/2002). GJS wishes to acknowledge support from the National Institute for Allergy and Infectious Disease (NIAID R01 AI40305).

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