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A novel von Willebrand factor binding protein expressed by Staphylococcus aureus

Joakim Bjerketorp,1 Martin Nilsson,1 Åsa Ljungh,2 Jan-Ingmar Flock,3 Karin Jacobsson1 and Lars Frykberg1

When a shotgun phage-display library of Staphylococcus aureus Newman was affinity selected (panned) against recombinant von Willebrand factor (vWf), a novel von Willebrand factor binding protein (vWbp) was found. Experimental data indicate that the interaction between vWbp and vWf is very specific and mediated by a region of 26 aa residues in the C-terminal part of vWbp. vWbp has an N-terminal secretory signal sequence but no cell wall anchoring motif, suggesting a soluble extracellular location. Mature vWbp could be purified from the culture supernatant and the identity of the protein was confirmed by N-terminal sequencing. vWbp migrates with an apparent molecular mass of 66 kDa and the deduced protein consists of 482 aa. The gene encoding vWbp, named vwb, was present in all S. aureus strains investigated.

Keywords: receptin, virulence factor, phage display

INTRODUCTION

Staphylococcus aureus is a pathogen responsible for a wide variety of diseases in humans and animals, including endocarditis, osteomyelitis, wound infections and mastitis. To interact successfully with the host, the bacterium has a large repertoire of components such as extracellular enzymes and toxins, as well as cell-wall-bound and secreted proteins that bind to proteins in host serum and extracellular matrix (Lowy, 1998). Proteins of microbial origin, cell-bound or soluble, which bind to mammalian proteins are generally considered as putative virulence factors and several such proteins from S. aureus have been molecularly characterized (Smeltzer, 2000). It should be noted that the outcome of virulence studies seems to be dependent on the animal model employed (Coulter et al., 1998; Darouiche et al., 1997). Nevertheless, studies of virulence in various animal models, with isogenic S. aureus mutants lacking a specific gene, have in several cases established the importance of a certain bacterial protein binding to a host protein, for some types of S. aureus infections. For example, the fibrinogen-binding proteins Efb (Palma et al., 1996) and ClfA (Moreillon et al., 1995), the collagen-binding protein Cna (Hienz et al., 1996; Patti et al., 1994; Rhem et al., 2000) and the IgG-binding protein A (Gemmell et al., 1997; Patel et al., 1987) are commonly considered as virulence factors. Further, a recent study showed that protein A also binds von Willebrand factor (Hartleib et al., 2000). Interestingly, in a vascular disease (Kawasaki disease) with symptoms and epidemiology overlapping with staphylococcal toxic shock syndrome, high protein A production was observed in the S. aureus strains isolated. This feature of the bacteria could possibly contribute to the pathological picture of this disease, with disturbed host haemostasis and an increased risk of vascular thrombosis (Wann et al., 1999).

The mature form of von Willebrand factor (vWF) is a large multifunctional glycoprotein, consisting of 2050 aa arranged in four different types of repeats (A through D). vWF exists as homodimers about 540 kDa in size, or multimers of different sizes up to 20000 kDa. Analysis by SDS-PAGE of plasma-derived vWF with reduced disulfide bonds reveals a predominant band with mobility corresponding to an apparent molecular mass of 225 kDa. The molecular mass of the vWF subunit, based

Abbreviations: HSA, human serum albumin; HRP, horseradish peroxidase; RT, room temperature; vWF, von Willebrand factor; vWbp, von Willebrand factor binding protein.

The GenBank accession number for the sequence reported in this paper is AY032850.
on its chemical composition, is approximately 270 kDa. vWF is synthesized exclusively by endothelial cells and megakaryocytes (Ruggeri, 1999). The endothelial cells generate a plasma pool of vWF with a concentration of \( \sim 10 \mu g \) ml\(^{-1} \) as well as an intracellularly stored supply of vWF in Weibel–Palade bodies. Megakaryocytes are responsible for vWF stored within the \( \alpha \)-granule of platelets. By supporting platelet adhesion and aggregation to exposed subendothelium in damaged blood vessels, vWF is an essential component in the maintenance of haemostasis, especially under conditions of rapid blood flow. vWF mediates platelet adhesion through two distinct platelet receptors: the glycoprotein (GP) Ib in the GP Ib–\( \alpha \)-IX complex and the GP IIb–IIIa (also called integrin \( \alpha_{\text{IIb}} \beta_3 \)). Furthermore, vWF transpots and stabilizes the coagulation factor VIII. vWF contains an Arg-Gly-Asp (RGD) motif also recognized by the endothelial integrin \( \alpha_{\text{IIb}} \beta_3 \) and vWF can in addition bind to various subendothelial components, such as collagens (type I, III and VI) and heparin-like glucosaminoglycans (Ruggeri, 1999; Ruggeri & Ware, 1993). Malfunctional vWF, or a reduced amount of this glycoprotein, leads to transportation of various subendothelial components, such as collagens, which is the most common inherited bleeding disorder (Mohlke et al., 1999).

In this investigation a shotgun phage-display library was made from chromosomal DNA of \( S. \) aureus strain Newman. The library was affinity selected (panned) against recombinant vWF, which resulted in the finding of a novel von Willebrand factor binding protein (vWbp).

**METHODS**

**Proteins and reagents.** Human fibronectin, chicken IgG, human IgG, human serum albumin (HSA) and casein were purchased from Sigma, and human fibrinogen from IMCO. Human vitronectin was purified as described previously (Yatohgo et al., 1988). Recombinant vWF was a kind gift from Professor F. Dorner, Vienna, Austria. Recombinant vWF has been shown to mediate platelet aggregation and to promote binding of collagen and coagulation factor VIII with activity comparable to human-plasma-derived vWF (Fischer et al., 1997). Goat anti-vWF antibodies were purchased from Kordia; antibodies against recombinant vWF (see below) developed in chicken were obtained from Immunogen AB; mouse anti-E-tag antibodies and horseradish peroxidase (HRP)-labelled anti-mouse antibodies were from Amersham Biosciences. HRP-conjugated anti-chicken antibodies and HRP–anti-goat antibodies were from Sigma. Restriction enzymes and DNA manipulation enzymes were from MBI Fermentas or Amersham Biosciences. Oligonucleotides were from Invitrogen.

**Bacterial strains, growth conditions and helper phage.** *Escherichia coli* TG1 (Sambrook et al., 1989) was used for construction of the phage library and production of phage stocks. *E. coli* BL21 (DE3) (Novagen) was used for expression of recombinant vWbp. *E. coli* was grown in Luria–Bertani (LB) broth or on LA plates (LB with 1 % agar) supplemented with 50 \( \mu g \) ampicillin ml\(^{-1} \) (LA-amp) when appropriate. Phage R408 (Promega) was used as helper phage for production of phage stocks. The *S. aureus* strains used were Newman, 8325-4, Wood 46 and five different human clinical isolates kindly provided by Dr B. Christensson, University of Lund, Sweden. Clinical isolates of *Staphylococcus epidermidis* were used as negative controls. *S. aureus* Newman A\( \Delta \)Eap, an isogenic mutant strain of *S. aureus* Newman in which the gene for staphylococcal extracellular adherence protein (Eap) has been deleted (J.-I. Flock, unpublished), was used for purification of vWbp. *Staphylococci* were grown in Tryptic Soya Broth (TSB, Oxoid).

**Construction of an *S. aureus* shotgun phage-display library.** A shotgun phage-display library was constructed from *S. aureus* strain Newman DNA as described previously (Jacobsson & Frykberg, 1995, 1996). In short, DNA fragments of approximately 0.5–5 kb, obtained by sonication, were ligated into the pg8SAET phagemid vector (Jacobsson & Frykberg, 1999). After transformation into *E. coli* TG1 and infection with helper phage R408, the final library consisted of 10\(^{8} \) individual clones and had a titre of 1.5 \( \times \) 10\(^{9} \) c.f.u. ml\(^{-1} \).

**Panning of the *S. aureus* phage-display library.** Microwells (Maxisorp, Nunc) were coated with 50 \( \mu g \) vWF in 200 \( \mu l \) coating buffer (0.05 M NaHCO\(_{3}\), pH 9.5) and incubated at room temperature (RT) with shaking for 1 h. The wells were then washed three times with phosphate-buffered saline containing 0.05 % Tween 20 (PBS-T). Two hundred microlitres of the phagemid library were then added to the vWF-coated wells, together with casein at a final concentration of 100 \( \mu g \) ml\(^{-1} \). Panning was carried out at RT with shaking for 4 h. After washing extensively with PBS-T, bound phages were eluted with 200 \( \mu l \) elution buffer (0.05 M sodium citrate, 0.15 M NaCl, pH 2.0) at RT for 2 min. After neutralization of this solution with 25 \( \mu l \) 2 M Tris/HCl, pH 8.7, 0.001–50 \( \mu l \) volumes of the eluate were added to 25 \( \mu l \) stationary-phase *E. coli* TG1, together with LB broth to a final volume of 200 \( \mu l \). The infection was allowed to proceed for 20–30 min at RT before the suspension was spread on LA-amp plates for determination of the number of c.f.u. in the eluate. The plates were incubated overnight at 37 \( \circ \)C, then 150 colonies were transferred to two identical plates for screening and sequencing. The rest of the colonies were collected and infected with 10 \( \mu l \) helper phage R408 (10\(^{11} \) p.f.u. ml\(^{-1} \)) for production of enriched phage stocks. The infected bacteria were mixed with 5 ml 0.5 % soft agar, poured on an LA-amp plate and incubated at 37 \( \circ \)C overnight. The phages produced were recovered and the resulting phage stocks used for subsequent repinnings, which were carried out as for the panning described above, except that repinnings were accomplished in 2 h.

**Screening and sequencing of phagemid clones.** After each round of panning, 150 colonies were picked in an identical pattern to two LA-amp plates, transferred to nitrocellulose filters (Schleicher & Schuell) and subsequently screened for expression of the phagemid expression tag (E-tag) with the anti-E-tag antibody. Phagemid DNA from positive clones was prepared with a Qiagen Miniprep kit (Qiagen) according to the manufacturer’s instructions. The inserted DNA fragments were sequenced using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Biosciences) and the samples analysed using the ABI 377 DNA Sequencer (Perkin Elmer) according to the manufacturer’s instructions. NTI Vector software (Informax) was used for handling the sequences obtained.

**Cloning of the vwb gene.** The *vwb* gene was PCR-cloned from *S. aureus* strain Newman using Pwo DNA polymerase (Roche Diagnostics) and the primers 5′-GAATTCTCATATGATT-

CATGAAAGCG-3′ (upstream) and 5′-GAATTCCGG-

ATGCATTATTGTGC-3′ (downstream). The primers were designed based on *S. aureus* sequence data available at...
TIGR (http://www.tigr.org/). The resulting PCR product was cloned into pUC18 for subsequent sequencing.

Binding specificity of phagemid particles of NvWb32. A phage stock of clone NvWb32 (Fig. 1) was prepared by infecting 500 µl of E. coli TG1 cells harbouring the phagemid with 10 µl helper phage R408 as above. After propagation in soft agar on an LA-amp plate, the phagemid particles were recovered as described above. The phage stock generated (2 × 10¹⁰ c.f.u. ml⁻¹) was used in an experiment to analyse the binding specificity of the phagemid particles, and also in an inhibition experiment. In the binding specificity experiment 10⁵ c.f.u. of the phage stock was panned against uncoated microwells and microwells coated with 2 µg of fibrinogen, fibronectin, vitronectin, vWf, IgG, HSA or casein. After 2 h of panning at RT the wells were washed, and the c.f.u. ml⁻¹ of the eluate determined as described above. In the inhibition experiment 5 × 10⁴ c.f.u. ml⁻¹ of the phage stock was mixed with chicken antibodies, either unspecific or specific against vWbp, at a final concentration of 0.08–50 µg ml⁻¹. After 1 h pre-incubation at RT the mixtures were transferred to vWf-coated microtitre wells (1 µg vWf per well) and incubated for 2 h, followed by washing and determination of the c.f.u. ml⁻¹ of the eluate as above.

Recombinant vWbp (rWbp-part, rWbp-mat) and purification of antibodies against rWbp-part. A part of the vwbp gene, encoding amino acids 124–392 of the mature vWbp, was amplified using Taq polymerase and the PCR primers 5’TATATACCATGCTAACCCTGAATTGAAAGACTT-3’ (upstream) and 5’TATATATTGCCGTGTTGATTGAA-3’ (downstream). The PCR product was cleaved with NcoI, treated with T4 polynucleotide kinase, ligated into a NcoI-restricted vector and transformed into E. coli BL21 (DE3) for expression and Bl21 (DE3) for expression and purification of recombinant protein using the IMPACT T7 expression system (New England Biolabs) with a self-cleavable affinity tag. This was done according to the manufacturer’s instructions, yielding essentially pure protein (rWbp-part) which was used for generation of antibodies in chicken by Edman sequencing by Bo Ek, Department of Plant Biology, Swedish University of Agricultural Sciences (SLU).

A novel vWf-binding protein in S. aureus

Inhibition of the binding between vWf and rvWbp-mat using specific antibodies. Microwells were coated for 1 h with 100 µl of a solution containing 1 µg rvWbp-mat ml⁻¹ and coating buffer (0.05 M NaHCO₃, pH 9.5) at RT with shaking. The wells were then washed three times with PBS-T, after which PBS with chicken antibodies, either unspecific or specific against vWbp, were added and incubated for 30 min, whereupon 125I-labelled vWf (IODO-BEADS Iodination Reagent Kit, Pierce) was added to the wells and the mixture was further incubated for 1 h. The final concentrations of the antibodies were 0.016–50 µg ml⁻¹. The wells were washed three times with PBS-T and bound 125I-vWf was detected with a gamma-radiation counter (Searle).

Purification and N-terminal sequencing of vWbp from S. aureus. vWbp was purified from an S. aureus strain Newman Δapl mutant (J.-I. Flock, unpublished). One hundred millilitres of an exponential-phase culture (OD₆₀₀ 3.0) was pelleted; the supernatant was sterile-filtered and subsequently passed through a HiTrap column with immobilized anti-vWbp antibodies. The eluate was collected in 1 ml fractions; the fractions were TCA-precipitated, the precipitates were dried and each fraction was resuspended in 10 µl water. The N-terminal sequence of the purified vWbp was determined by Edman sequencing by Bo Ek, Department of Plant Biology, Swedish University of Agricultural Sciences (SLU).

SDS-PAGE and Western blot analysis. Protein samples were prepared for gel electrophoresis by mixing equal volumes of protein solution and 2 × sample buffer [1 × sample buffer is 62.5 mM Tris/HisCl pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue]. After boiling, the samples were analysed by SDS-PAGE using the PhastSystem (Amersham Biosciences) with PhastGel Gradient 4–15% or 8–25% gels and PhastGel SDS Buffer Strips. Proteins were blotted onto ECL nitrocellulose filters (Amersham Biosciences). The presence of vWbp was detected either with chicken anti-vWbp antibodies and HRP–anti-chicken antibodies or with vWf, goat anti-vWf antibodies and HRP–anti-goat antibodies. Bound antibodies were detected with 4-chloro-1-naphthol (Sigma). 125I-labelled vWf was also used to detect vWbp, and bound 125I-vWf was visualized with Kodak BioMax MS film.

Purification of vWf from human serum. Human serum (15 ml) was passed over a HiTrap column with immobilized rvWbp-part. The eluate was collected in 500 µl portions, which were TCA-precipitated and resuspended in 5 µl distilled water. vWf was detected with goat anti-vWf antibodies and HRP-labelled anti-goat antibodies in a Western blot experiment as described above.

Peptide sequencing by mass spectrometry. Protein bands were excised from a Coomassie-blue-stained SDS-PAGE gel and cleaved with trypsin by in-gel digestion. Peptide analysis
was performed by electrospray ionization mass spectrometry according to Wilm et al. (1996) on a Q-Tof mass spectrometer using the Masslynx software (Micromass, Manchester, UK). This was done by Håkan Larsson, Department of Plant Biology, Swedish University of Agricultural Sciences (SLU).

**Detection of vwb in S. aureus.** Chromosomal DNA from eight different S. aureus strains and five S. epidermidis strains was prepared by using the DNeasy Tissue kit from Qiagen, supplemented with lysozyme at a final concentration of 250 µg ml⁻¹ in the cell lysis step. DNA was cleaved with EcoRI, separated on a 0.7% agarose gel and blotted to a nylon filter using the VacciGene blotting system (Amersham Biosciences). After UV-fixation the filter was probed overnight with a DNA fragment covering almost the entire vwb gene. After washing, the filter was exposed to a Kodak BioMax MR film for 24 h at −70 °C.

**RESULTS AND DISCUSSION**

**Panning against vWF reveals a novel vWF-binding protein**

When the S. aureus library was panned repeatedly against vWF, a strong enrichment of binding phagemid particles occurred. This was manifested both as a large increase in the number of eluted phagemid particles and as an increase in the percentage of clones positive for E-tag expression in consecutive pannings. In the first panning 2 × 10⁵ phagemid particles were recovered from the well and in the second and the third panning the number of phagemid particles was 5 × 10⁶ and 2 × 10⁸, respectively. Accordingly, the frequency of E-tag-positive clones increased from 8%, to 70% and to 94% in the consecutive pannings. Expression of the E-tag reveals that a designed frameshift in the phagemid vector is corrected by the inserted DNA (Jacobsson & Frykberg, 1999). Thus, an enrichment of E-tag-positive clones suggests that an interaction between phagemid particles and ligand has occurred. This, together with the increased number of bound phagemid particles, strongly indicates a specific interaction between the ligand and the displayed polypeptides. After the second and third panning, 32 E-tag-positive clones were isolated and the inserts of the phagemids sequenced. Six of these clones did not harbour any gene of obvious interest and were considered background clones. However, the remaining 26 clones all contained overlapping inserts derived from the same gene (Fig. 1), suggesting that a gene encoding a vWF-binding protein had been identified. The gene was named vwb (von Willebrand factor binding). When compared to the sequence data from TIGR, the sequence obtained from the phage-display clones turned out to cover almost the entire vwb, except the last 44 nucleotides of the gene. The complete vwb gene was subsequently cloned from S. aureus strain Newman by PCR and the sequence was verified. From the alignment of the different phagemid clones, the vWF-binding region in the corresponding protein was mapped to 26 aa: TSTPTTYTEETTTQVPMPPTVERQTQQQI, which is equivalent to amino acids 333–358 in the deduced mature protein. The presence of the gene in different staphylococcal strains was tested by Southern blot hybridization with the whole vwb gene as a probe. The gene was present in all tested strains of S. aureus (Newman, 8325-4, Wood 46, and five different clinical isolates) but not in five S. epidermidis strains tested (data not shown).

**Sequence analysis of vwb**

The vwb gene is located between the clfA and emp genes. clfA encodes clumping factor A, the main fibrinogen-binding adhesin in S. aureus (McDevitt et al., 1994; Ni Eidhin et al., 1998) and emp encodes an extracellular-matrix-protein-binding protein (Emp), a cell-surface protein with multiple binding activities (Hussein et al., 2001). The clfA, vwb and emp genes are all in the same orientation according to the newly published complete genome sequence of two S. aureus strains, N315 and Mu50 (Kuroda et al., 2001). The same gene organization is also seen in the different S. aureus sequencing projects in progress at TIGR (http://www.tigr.org/), University of Oklahoma (http://www.genome.ou.edu/) and the Sanger Centre (http://www.sanger.ac.uk/). The gene vwb encodes a previously uncharacterized protein now named von Willebrand factor binding protein (VWbp). Kuroda et al. (2001) denote the gene product as a possible staphylococcal protease (function unknown). The amino acid sequences of the staphylococcal protease and VWPb from strain N315 shared 25% amino acid identity when a pairwise BLAST was performed at NCBI (http://www.ncbi.nlm.nih.gov/). Including the signal sequence, the deduced protein VWPb from strain Newman is composed of 508 aa starting with a leucine. The putative start codon TTG is preceded by a plausible ribosome-binding site (AGGAGA). Sequence analysis shows that the deduced mRNA transcript, after the translation stop codon, has a putative transcription terminator sequence with two stem–loop structures followed by a stretch of six U residues. It is not likely that vwb is co-transcribed with clfA, since the deduced clfA mRNA sequence also contains a putative transcription termination sequence. The sequence of vwb obtained from S. aureus strain Newman was found to be identical to the sequence available from TIGR (S. aureus strain COL) and one of the sequences from the Sanger Centre (a hyper-virulent community-acquired methicillin-sensitive S. aureus strain), was also found to be completely identical in all but one nucleotide, leading to the substitution of an isoleucine by a leucine. The vwb sequences from the published, complete genomes of S. aureus strains N315 (methicillin-resistant) and Mu50 (methicillin-resistant and vancomycin-resistant) are identical to each other but only 80% identical to the vwb sequence from strain Newman. The second sequence from the Sanger Centre (the epidemic methicillin-resistant S. aureus strain EMRSA-16), shares about 80% sequence identity at the nucleotide level compared to strain Newman and strain N315. The vwb sequence from the University of Oklahoma (strain 8325) is identical to vwb from strain Newman, except for one missing nucleotide. Also in one vwb sequence from the Sanger Centre (strain EMRSA-16) one nucleotide is missing but at a different position.
If correct, these frameshifts would lead to early disruptions of the \textit{vwb} ORF but it seems more likely that the missing nucleotide in \textit{vwb} in these two unfinished genomes is due to sequencing errors. Most of the differences between the \textit{vwb} genes from the different strains are found in the first half of the gene. Very few differences are found in the C-terminal half of the deduced protein sequences (data not shown), which includes the vWF-binding domain.

The observed clustering of several genes encoding putative virulence factors is another interesting thing to consider, since many chromosomal virulence determinants are often associated in so-called virulence blocks (Hacker \textit{et al.}, 1997). The \textit{clfA} gene is expressed in the exponential growth phase, but the expression is highest during postexponential growth (Ni Eidhin \textit{et al.}, 1998; Wolz \textit{et al.}, 1996). The conditions for expression of \textit{vwb} and \textit{emp} are not yet clarified at the mRNA level. At the protein level, Emp is detectable from mid-exponential growth and the concentration gradually increases up to stationary phase (Hussein \textit{et al.}, 2001). Preliminary data indicate that vWbp is expressed already early in the exponential phase, becomes more abundant during the exponential growth and decreases in the stationary growth phase (data not shown).

\textbf{Characterization of vWbp and its interaction with vWF}

vWbp has a functional secretory signal sequence, with positively charged lysine residues in the N-terminal region, a central hydrophobic region and a more polar C-terminal region containing a signal peptidase cleavage sequence with alanines at the $-3$ and $-1$ positions. However, vWbp has no cell-wall-anchoring sequence typical of surface-bound proteins in Gram-positive bacteria. Thus, the protein should be found in a soluble form outside the bacteria. To further investigate vWbp, the part of the \textit{vwb} gene encoding amino acids 124–392 of the mature vWbp was expressed in \textit{E. coli} and the recombinant protein was purified (rvWbp-part). Chickens were subsequently immunized with rvWbp-part and the resulting anti-vWbp antibodies were used to detect vWbp in the concentrated culture supernatant of \textit{S. aureus} strain Newman. In a Western blot, the anti-vWbp antibodies specifically recognized a protein of $\sim 66$ kDa. A protein of the same size was also detected by using vWf and anti-vWf-antibodies or by $^{125}$I-labelled vWf directly (Fig. 2).

To prove that the protein detected in the culture supernatant was indeed vWbp, attempts were made to purify the protein on immobilized anti-vWbp antibodies. Surprisingly, this resulted in isolation of the staphylococcal protein Map (Jonsson \textit{et al.}, 1995; McGavin \textit{et al.}, 1993)/Eap (Palma \textit{et al.}, 1999) as confirmed by N-terminal sequencing of the purified material. The N-terminals of mature Map and Eap are very similar, but not identical. Another \textit{S. aureus} protein, p70 (Fujigaki \textit{et al.}, 1998), is also similar to Eap and Map (Smeltzer, 2000). Since these proteins with similar characteristics were isolated from different strains of \textit{S. aureus} – Eap from strain Newman, Map from strain FDA 574, p70 from strain Wood 46 – they might be encoded by allelic variants of the same gene. Eap is known to bind to itself in addition to various host proteins. However, Eap does not bind chicken antibodies in a non-immunogenic manner, nor does it cross-react with the anti-vWbp antibodies (data not shown). From these results it is reasonable to assume that the isolation of Eap is not due to binding to the anti-vWbp antibodies, but instead is a result of a direct interaction between Eap and vWbp. Indeed, vWbp could be purified from the culture medium of an isogenic Eap-deletion mutant of \textit{S. aureus} strain Newman. The N-terminal sequence of secreted vWbp was determined by Edman N-terminal sequencing. The resulting sequence was VVSGEKNPYV, which fully corresponds to the N-terminal sequence of the anticipated mature vWbp consisting of 482 aa. The predicted molecular mass of the mature vWbp from the amino acid sequence (56.5 kDa) is smaller than the apparent molecular mass deduced from polyacrylamide gels ($\sim 66$ kDa). The same result was obtained under both reducing and non-reducing conditions. It is not uncommon that proteins have altered migratory properties in polyacrylamide gels, particularly the ones with unusual amino acid compositions. In vWbp, the percentages of acidic (DE) and basic (KR) amino acids are surprisingly high, but well balanced (DE 18 %, KR 19 %), giving the protein an estimated isolectric point very close to 7. This means that the net charge under physiological (host) conditions should be close to zero. Phagemid particles displaying amino acids 139–428 of the mature vWbp (clone NvWb32) were assayed for binding to vWf and to a number of other mammalian proteins. The results showed that NvWb32 bound at least 1000 times more efficiently to vWf than to any of the other proteins tested.

![Fig. 2. SDS-PAGE and Western blot of culture medium from an exponential-phase culture of \textit{S. aureus} strain Newman, concentrated 100-fold. Lanes: 1, Coomassie-blue-stained 8–25% SDS-PAGE gel; 2, Western blot treated with chicken antibodies against vWbp and HRP-labelled anti-chicken antibodies; 3, Western blot (of an identical gel) treated with vWf, goat anti-vWf antibodies and HRP-labelled anti-goat antibodies to detect the interaction; 4, autoradiograph of a Western blot treated with $^{125}$I-labelled vWf.](image-url)
Typical experiment are shown.

Table 1. Binding study with phagemid particles displaying the vWf-binding region (clone NvWb32)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Bound phagemid (c.f.u. ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWf</td>
<td>3 x 10⁷</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>4 x 10⁴</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>5 x 10³</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>1 x 10³</td>
</tr>
<tr>
<td>IgG</td>
<td>1 x 10³</td>
</tr>
<tr>
<td>HSA</td>
<td>1 x 10³</td>
</tr>
<tr>
<td>Casein</td>
<td>2 x 10³</td>
</tr>
<tr>
<td>None (uncoated well)</td>
<td>3 x 10³</td>
</tr>
</tbody>
</table>

The phagemid particles were panned against different mammalian proteins and the number of bound phagemid particles was determined as c.f.u. ml⁻¹. The results from one typical experiment are shown.

Fig. 3. Inhibition study with phagemid particles displaying the vWf-binding domain. The phagemid particles were panned against vWf in the presence of antibodies against vWbp (■) or unspecific antibodies (○) at different concentrations. The number of bound phagemid particles was determined as c.f.u. ml⁻¹. Values are mean ± SD from two experiments.

Fig. 4. Inhibition of binding of ¹²⁵I-labelled vWf to immobilized rvWbp-mat by chicken antibodies, either specific against vWbp (■) or unspecific (○). Values are mean ± SD from two experiments.

Possible consequences of the interaction between S. aureus and vWf

A recent publication demonstrated that protein A, besides its IgG-binding ability, also has affinity to vWf, and consequently protein A promotes adhesion of S. aureus to a vWf-coated surface (Hartleib et al., 2000). This means that S. aureus has at least two proteins recognizing vWf, one cell-surface-attached and one secreted as reported in this paper. To further complicate the picture, it has been reported that protein A in some strains exists in a soluble form, not bound to the surface of S. aureus (Lindmark et al., 1977). The function and importance of vWbp during S. aureus infections is not yet known. The frequency of S. aureus strains carrying the vwb gene is high (15/15 discussed in this paper), which indicates an important role for the encoded protein. Since the extracellular fibrinogen-binding protein, Efb, has been shown to contribute to virulence and to delay wound healing (Palma et al., 1996), it is tempting to speculate that vWbp also could have similar
functions, since both fibrinogen and vWF are crucial components in the events leading to the formation of blood clots. Actually, in experimental settings mimicking a vascular wound during rapid blood flow as in the normal arteriole, platelet adhesion and aggregation require vWF but not fibrinogen (Savage et al., 1996; Ikeda et al., 1991; Weiss et al., 1989). Therefore, the bacteria may benefit from also disturbing the platelet–vWF interaction in the wound healing process. Further, it is interesting to consider the putative interaction between Eap, a sticky protein secreted by Staphylococcus aureus, and vWbp. Eap can bind back to the bacteria, as well as to various components of the extracellular matrix, thus serving as an adhesive bridge between host cells and Staphylococcus aureus (Palma et al., 1999). If vWbp binds vWF at sites of vascular damage and Eap binds to vWbp, these interactions may function as a homing device and subsequently direct the bacteria to the wound where colonization can occur.

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