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Letter

AFM Identifies a Protein Complex Involved in Pathogen Adhesion Which Ruptures at Three Nanonewtons

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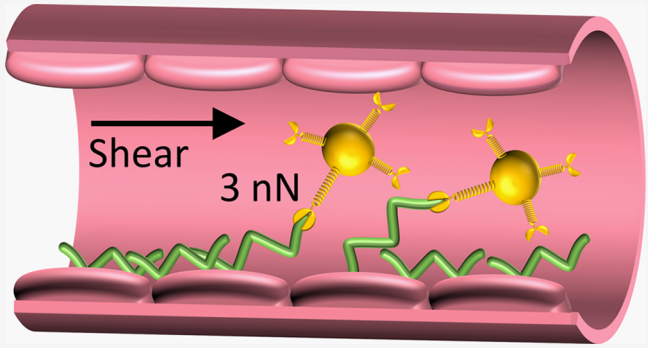
ABSTRACT: Staphylococci bind to the blood protein von Willebrand Factor (vWF), thereby causing endovascular infections. Whether and how this interaction occurs with the medically important pathogen *Staphylococcus epidermidis* is unknown. Using single-molecule experiments, we demonstrate that the *S. epidermidis* protein Aap binds vWF *via* an ultrastrong force, ∼3 nN, the strongest noncovalent biological bond ever reported, and we show that this interaction is activated by tensile loading, suggesting a catch-bond behavior. Aap−vWF binding involves exclusively the A1 domain of vWF but requires both the A and B domains of Aap, as revealed by inhibition assays using specific monoclonal antibodies. Collectively, our results point to a mechanism where force-induced unfolding of the B repeats activates the A domain of Aap, shifting it from a weak- to a strong-binding state, which then engages into an ultrastrong interaction with vWF A1. This shear-dependent function of Aap offers promise for innovative antistaphylococcal therapies.



KEYWORDS: *AFM, single-molecules, ultrastrong bond, Staphylococcus, Aap, vWF, physical stress, mechanoregulation, catch-bond*

The von Willebrand factor (vWF) is a mechanosensitive multimeric glycoprotein that is an essential component of the blood and of the endothelial basement membrane. Each mature vWF monomer exhibits a modular architecture with distinct domains dedicated to specific functions (Figure 1a).[1,2] From the N- to the C-terminus, A1 and A3 domains bind to constituents of the extracellular matrix of the subendothelium such as fibrillar collagens[3,4] and platelet glycoprotein Ibα (GpIb),[5−7] and the C4 domain contains an RGD motif that binds to the platelet integrin $\alpha_{IIb}\beta_3$.[8] Large multimers of vWF are stocked in endothelial cells, from which they are secreted into the blood.[9] Upon secretion, globular ultralarge vWF becomes extended under flow and is cleaved into smaller multimers.[10] vWF responds to mechanical forces, such as shear stress in flowing blood, which is critical for the protein biological functions.[11−13] The adhesive properties of vWF are promoted by a switch toward an extended conformation under flow.[4,11]

Staphylococci can recruit circulating vWF[14] and bind to immobilized vWF on activated endothelial cells in blood flow. This process interferes with the physiological functions of vWF, including platelet recruitment and coagulation, and causes various endovascular infections. vWF-binding by *Staphylococcus aureus* under shear flow has been widely investigated. Two mechanisms have been identified, one involving the bacterial cell surface protein A (SpA),[15−17] the other mediated by the secreted staphylococcal vWF-binding protein (vWFbp)[16,18] and cell surface protein clumping factor A (ClfA).[1,19,20] By contrast, there is just one report suggesting vWF-binding by *S. epidermidis*, the most commonly isolated infectious coagulase-negative staphylococci from contaminated prosthetic medical devices.[21] Such contaminations allow these bacteria to migrate and access the bloodstream thus potentially leading to bacteremia and sepsis.[22−24] Currently, the specific bacterial components at play in the binding of *S. epidermidis* to vWF and their binding mechanisms are completely unknown.

S. epidermidis has a small repertoire of cell surface proteins, among which the accumulation associated protein (Aap), an ortholog of the *S. aureus* protein SasG, which plays a significant role in pathogenesis by promoting cell aggregation during biofilm formation.[25] Aap is a long, flexible protein consisting of a N-terminal A-repeat region (11 partially conserved 16-residue repeats) and a 222 amino-acid L-type lectin region (collectively known as A domain), followed by a B domain containing 5 to 17 conserved repeats of 120-amino acid sequences and a proline/glycine-rich region (Figure 1a). The B region promotes self-

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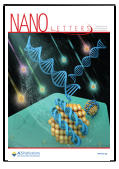

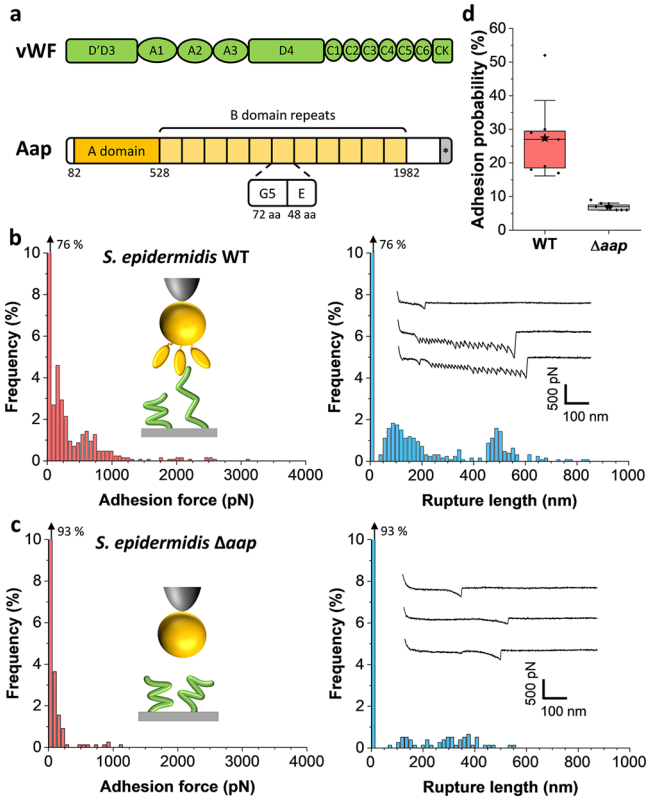

Figure 1. Aap mediates bacterial adhesion to vWF-coated surfaces. (a) Schematic representation of the vWF (top) and Aap (bottom) proteins. Aap expressed by *S. epidermidis* CSF41498 strain consists of an A region (including a lectin-like domain and a variable number of 16 aa repeats, dark yellow), a B-repeat region (light yellow) containing 11 tandem E-G5 domains (48 aa and 72 aa, respectively), a collagen-like proline/glycine-rich region and a cell wall anchoring motif (*) (LPDTG). vWF is a multimeric glycoprotein made of a variable number of 2,050 aa monomers. Each monomer adopts a modular architecture with diverse domains dedicated to specific cellular fonctions. (b) Unbinding force (left) and rupture length (right) histograms obtained by recording force−distance curves in PBS between *S. epidermidis* WT cells and vWF-substrates (total of n = 1,281 curves from 3 independent cells; for more cells see Figure S1). (c) Force data obtained under the same conditions for Δ*aap* cells (n = 768; 3 cells). (d) Box plots comparing the adhesion probability of multiple WT (n = 8) and mutant (n = 8) cells probed by SCFS against vWF-coated surfaces. All curves were obtained with an interaction time of 1,000 ms and a retraction velocity of 1,000 nm s^{-1}. In panels b and c, right insets present representative retraction force profiles.

assembly to form rope-like intercellular filaments and functional amyloid fibers in the biofilm.[26−28] The A region is involved in specific binding to host surfaces, e.g., human nasal epithelial cells[29] and human corneocytes.[30]

Here, we sought to identify the *S. epidermidis* surface protein involved in vWF binding and to understand the mechanism involved in this interaction using atomic force microscopy (AFM)-based single-molecule experiments. The results show that Aap mediates *S. epidermidis* adhesion to vWF *via* an ultrastrong force. This extraordinary binding force exceeds the strength of all biomolecular complexes studied to date, including those from the prototypical adhesins SdrG, ClfA and ClfB engaged in strong dock, lock, and latch (DLL) interactions.[31−34] Strong Aap−vWF adhesion is activated by mechanical stress that induces a shift toward higher forces, both in terms of probability and strength, which is reminiscent of a catch-bond behavior.[35] The interaction requires both the A and B domains of Aap, on the one hand, and the vWF A1 domain, on the other hand. Monoclonal antibodies (mAbs) directed against either the A domain or the B repeats both efficiently inhibit the formation of the mechanostable Aap−vWF complex. These findings point to a force-regulated mechanism where mechanical unfolding of the B repeats triggers a conformational switch in the A domain which then specifically and strongly interacts with the vWF A1 domain. In future medical applications, inhibition of the Aap−vWF interaction by specific mAbs could help prevent the entry of *S. epidermidis* into the blood circulation and the development of vascular diseases.[36]

RESULTS

***S. epidermidis* Surface Protein Aap Mediates Bacterial Adhesion to vWF.** Unlike *S. aureus*, *S. epidermidis* has only a limited number of cell surface adhesins, among which the multifunctional Aap protein. To test whether Aap binds to vWF, we first studied the adhesion between individual *S. epidermidis* bacteria and vWF, by using single-cell force spectroscopy (SCFS; see Supporting Methods; Figure 1b). We used the *S. epidermidis* CSF41498 WT strain which expresses the Aap protein with 11 conserved B (G5-E) repeats of 120 residues each[37] and its corresponding mutant Δ*aap* strain lacking Aap (see Supporting Methods). Living cells were attached to colloidal probe cantilevers enabling us to record force−distance curves between single bacteria and vWF-coated substrates. Figure 1b presents the unbinding force and rupture length histograms of representative WT cells (for more cells, see Figure S1). A substantial number of curves exhibited adhesion events with forces of 260 ± 147 pN (mean ± s.d., from a total of n = 213 adhesive curves from 3 independent cells) and 833 ± 192 pN (n = 90). The adhesion probability dropped from 27% to 7% for the Δ*aap* mutant (n = 8 cells for both WT and mutant), indicating that Aap is a key adhesin involved in vWF-binding (Figure 1c,d).

The rupture lengths of these interactions showed a bimodal distribution peaking at 142 ± 76 nm (mean ± s.d.; n = 200 adhesive curves; 3 cells) and 492 ± 39 nm (n = 103). As the conserved B repeats of the related *S. aureus* SasG adhesin are known to sequentially unfold under force, we estimate that unfolding of the full B domain of Aap (1,320 amino acid residues for the CSF41498 strain) should give an extension of ∼475 nm which matches our ∼500 nm values. Further supporting B repeat unfolding, many (43%) adhesive curves displayed sawtooth patterns with equally spaced peaks resulting from the unfolding of repeated domains (Figure 1b, right inset). From these data, we estimate that vWF elongates only to a small extent of the total unfolding length.

Extreme Mechanostability of the Aap−vWF Complex. We then investigated the strength of the Aap−vWF interaction by means of single-molecule force spectroscopy (SMFS; see Supporting Methods), in which *S. epidermidis* cells were probed with AFM tips functionalized with vWF (Figure 2a,b; for more cells see Figure S2). We observed a broad distribution of adhesion forces with two prominent populations, i.e., moderate forces ranging from ∼300 to 400 pN and, most importantly, very strong forces, in the ∼2,500−3,500 pN range, sometimes reaching ∼4,000 pN. These strong forces were essentially lacking in SCFS data (Figure 1) likely because of the different geometries at play. In both SCFS and SMFS, vWF proteins were grafted through a nondirectional covalent immobilization strategy but due to the flat surface geometry in SCFS, vWF molecules tend to be attached to multiple sites, less flexible and

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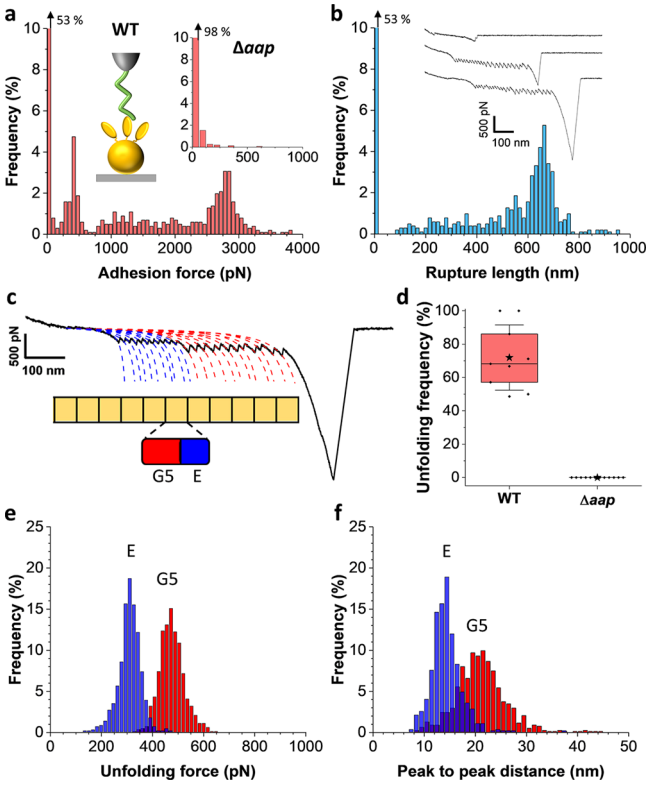

Figure 2. Binding strength and unfolding of single Aap adhesins. (a, b) Adhesion force (a) and rupture length (b) histograms with representative retraction force profiles (right inset) obtained by recording force−distance curves in PBS between a *S. epidermidis* WT cell and vWF-modifed AFM tips (n = 1,024 curves; for more cells see Figure S2). The left inset shows data obtained for the Δ*aap* strain (n = 1,516 curves; 3 cells). All curves were obtained with an interaction time of 1,000 ms and a retraction velocity of 1,000 nm s^{-1}. (c−f) Sequential unfolding of the E and G5 domains of Aap. (c) Typical sawtooth signature reflecting unfolding of protein repeats that were well-fitted with by the worm-like-chain (WLC) model (blue and red dashed lines). The number of peaks is consistent with the sequential unfolding of the E (blue) and G5 (red) subdomains of the B-repeat region of Aap. (d) Box plots highlighting the high frequency of unfolding events in WT cells (n = 9 cells), expressed as the ratio between the curves featuring a sawtooth pattern and the total number of adhesive curves. These events are lacking in mutant cells (n = 12). (e, f) Histograms of unfolding forces (e) and peak-to-peak distances (f) obtained by analyzing multiple unfolding patterns (n = ∼750 peaks, for both blue and red events, from two independent cells).

accessible than when grafted on a sharp tip in SMFS. These forces are completely unexpected and unusual as they largely exceed those of classical receptor−ligand bonds (<250 pN). Given our immobilization strategy, the specific adhesive events with low and moderate forces (<400 pN) might be due to different conformations of the vWF on the AFM tip that might not expose the proper sites for optimal binding to Aap. Importantly, adhesion was almost abrogated in Δ*aap* cells, with a probability shifted from 22% (n = 9 cells) to 3% (n = 12 cells) (Figure 2a, left inset), further confirming that the measured forces were specific to Aap.

Strikingly, WT cells, but not Δ*aap* cells, exhibited sequential, equally spaced force peaks, with a mean frequency of 72% (Figure 2c,d). Such sawtooth patterns are observed when stretching modular proteins[38] and reflect the unfolding of secondary structures. Most sawtooth profiles featured ∼11 low force peaks (312 ± 45 pN, n ∼ 750 peaks), followed by ∼11 high force peaks (475 ± 48 pN) (Figure 2e) and were well-fitted by the worm-like chain (WLC) model, thus in agreement with the unfolding of the 11 G5-E repeats of the Aap B region. The G5-E domains are mechanically strong as β-fold domains of modular proteins usually unfold at forces <300 pN.[38] This mechanostability results from tandemly arrayed mechanical clamps involving long stretches of hydrogen bonds and associated side-chain packing interactions along the β-strands.[39] The E repeats are less stable as their N-terminal clamps are shorter. The peak-to-peak distances were constant, i.e., 14 ± 3 and 21 ± 5 nm for the low and high force peaks, respectively (Figure 2f). Assuming that each residue contributes 0.36 nm to the contour length of a fully extended polypeptide chain and that the folded lengths of the E and G5 domains are 4.5 and 7.0 nm,[39] the measured peak interdistances match the expected 48 and 72 residues of single E and G5 domains. So, loading the Aap−vWF complex with relatively high force induces the sequential unfolding of the Aap G5-E domains. *In vivo*, we thus expect that Aap proteins on the bacterial cell surface will resist low to moderate shear conditions without unfolding.

Tensile Loading Strengthens the Aap−vWF Interaction. To determine whether and how the Aap−vWF adhesion (rupture) force changes in response to mechanical tension, we performed dynamic force spectroscopy (DFS) analyses between vWF-modified AFM tips and WT cells (Figure 3), by varying the loading rate (*LR*, estimated from the force *vs* time curves, Figure 3c inset). Consistent with the Bell−Evans theory,[40] a log linear relationship was observed between G5 unfolding forces and the *LR* (Figure 3a,b), yielding a position of the energy barrier that separates the bound from the unbound state of x_u = 0.28 nm, and an off-rate constant at thermal equilibrium of k_{off}^0 = 5.4 ± 10^{-12} s^{-1}. The very low k_{off}^0 value illustrates the high mechanostability of the B repeats.

Notably, ultrastrong forces did not show such continuous increase, but a fuzzy distribution with data clouds arising from the different pulling speeds (Figure 3c). A dramatic switch was observed, from weak forces (121 ± 36 pN, n = 328 curves, 4 cells) at low *LR* (<10^4 pN/s), to ultrastrong forces (3,125 ± 215 pN, n = 584 curves) at high *LR* (>10^5 pN/s) (Figure 3d, Figure S3) This sharp transition both in terms of probability and strength in adhesion force is consistent with a catch-bond behavior,[41,42] in which mechanical loading strengthens molecular recognition bonds. Consequently, our data show (i) an increase in rupture force at higher *LR* concomitantly with (ii) a decrease in the density of events sustaining low forces (first population) and an increase in the density of events sustaining high forces (2nd population) at higher *LR*. Such a trend has been already described for some specific staphylococci adhesins that strongly bind their ligands through the DLL mechanism putatively involving a catch bond mechanism and for the FimH adhesin of *E. coli* binding mannose, the most thoroughly catch bond investigated so far. We thus hypothesize that the Aap−vWF complex, strengthened under mechanical load, might originate from a catch bond behavior.

Essential Roles of A and B Domains in vWF A1 Binding. To further dissect the mechanism controlling the extremely strong bond, we analyzed various protein fragments (see Supporting Methods). We found that the adhesion between vWF-tips and surfaces functionalized with fragments of either the A or B domains of Aap was very poor (Figure 4a,b). The adhesion probability (Figure 4f) was low (13%, n = 2,520 curves and 8%, n = 2,047), and high forces were never observed, thus

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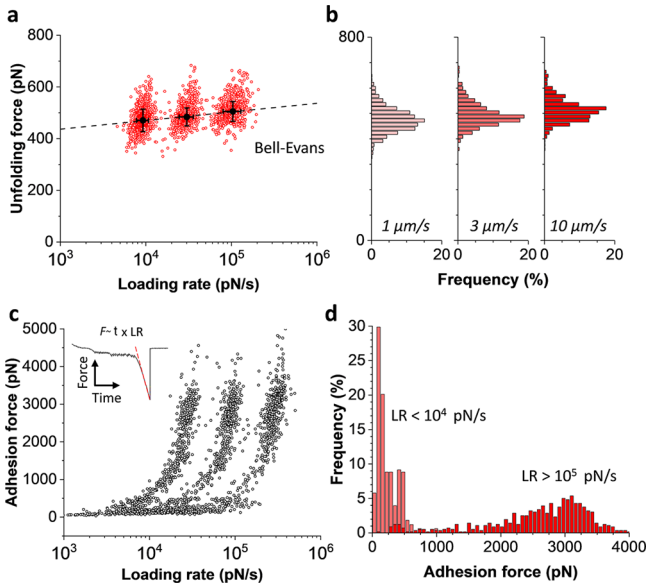

Figure 3. Influence of tensile loading on the mechanostability of the Aap−vWF complex. (a) Dynamic force spectrum (force as a function of loading rate *LR*, estimated from the *F vs* time curves) of the unfolding of single Aap G5 subdomains obtained between WT bacteria and vWF-tips at various retraction velocities (1, 3, and 10 μm s^{-1}). Data from 750 unfolding peaks from 2 cells are shown. The black dotted line stands for the Bell−Evans fit from which the energetic barrier and off-rate constant can be extracted: x_u = 0.28 nm and k_{off}^0 = 5.4 ± 10^{-12} s^{-1}. Unfolding peaks of the E domains could not be properly modeled due to their rather low resolution. (b) Distribution of G5 unfolding forces, further illustrating the slight increase in force with the retraction velocity. (c) Dynamic force spectrum of the unbinding force between single Aap adhesins and vWF-tips (n = 2,247 adhesive events; 4 cells), suggesting a force-enhanced Aap−vWF interaction, with a shift of low forces toward very high ones when increasing the *LR*. (d) Adhesion force histograms as a function of discrete ranges of loading rates, demonstrating a sharp transition between weak and ultrastrong forces when increasing tensile loading.

showing that both A and B domains are required for strong binding. When WT cells were treated with the A1 domain of vWF (Figure 4c), almost complete inhibition was observed (adhesion probability decreasing from 22% to 2%) (Figure 4c,f), meaning A1 is the primary, and probably the only, region of vWF involved in Aap binding.

To support the essential roles of the two Aap domains, WT cells were treated with mAbs directed against either the A or the B domains (mAbs$_A$ and mAbs$_B$, Figure 4d−f). Adhesion was abrogated with both treatments, and unfolding events were never observed, whereas forces were not altered when using an irrelevant mAb targeting integrin $\alpha_v\beta_3$. Hence, unfolding and high forces were specifically inhibited by mAbs$_A$ and mAbs$_B$, demonstrating that the ultrastrong interaction requires both the A and B domains of Aap.

DISCUSSION

We have identified a previously undescribed noncovalent biological interaction which is stronger than all receptor−ligand systems studied so far. The Aap−vWF interaction characterized here is activated by physical stress (Figure 4g), which explains how, *in vivo*, the pathogen can remain firmly attached to the host vessels while resisting the high shear of flowing blood (Figure 4h). The underlying mechanism involves the force-induced unfolding of the Aap B repeats which activates the Aap A domain to strongly bind to the vWF A1 region. Our work highlights the importance of protein nanomechanics in defining the adhesive functions of an important pathogen and offers promise for the use of mAbs as competitive inhibitors in future nanomedicine.

An exciting outcome of this study is that Aap binds vWF with an extremely strong force, ∼3 nN, by far larger than all classical molecular recognition systems. For years, it was believed that the streptavidin−biotin pair had the highest mechanostability (100−250 pN). Recently, it has been shown that stronger biological interactions do exist in nature,[33,34] which include the cohesin−dockerin complex of cellulolytic bacteria (1 nN)[43] and the prototypical DLL interaction (2 nN).[31,32] So, in the past few years, the DLL interaction has been considered as the strongest receptor−ligand bond. The Aap−vWF interaction discovered here outperforms the DLL system, featuring an even more extreme strength. That the complex sustains such forces without breaking the polypeptide backbones is likely to result from forces being directed along pathways nonparallel to the pulling direction.[44]

Another remarkable feature is that the Aap−vWF system is strongly mechanoregulated. When tensile loading is applied slowly, the interactions rupture at low forces, whereas when tension is loaded fastly, not only the interactions survive until higher forces but also a higher density of interactions does rupture at such extreme nN forces. This transition toward a stronger adhesion occurring with higher probability at increasing physiologically relevant loading rates suggests a catch-bond,[41,42] i.e., a bond that reinforces under tension, e.g., through allosteric regulation, and provides a molecular explanation as to how the pathogen can resist high shear stress of flowing blood during adhesion.

The question arises whether the range of mechanical tensions applied here is biologically relevant. *S. epidermidis* is mostly known as a commensal pathogen of healthy skin.[45] But its propensity to form biofilms[46] and thus to colonize and contaminate indwelling medical devices allows this species to cause bacteremia and sepsis[22,23] such as central line-associated and catheter-related bloodstream infections, notably reported for premature infants[22,24] but also prosthetic valve endocarditis.[47] In the bloodstream, bacteria can encounter a wide range of shear forces due to the dynamic flow conditions, that we have mimicked in our dynamic force spectroscopy investigations. In the veins, the shear rate is on the order of 10 s^{-1}, while in small arteries it can be larger than 2,000 s^{-1}. In atherosclerotic arteries extreme shear rates of 40,000 s^{-1} can be generated.[48] So the loading rates that *S. epidermidis* can experience under physiological conditions can exceed 10^5 pN/s.[49] As we observed Aap−vWF binding strengths as high as 4 nN at a loading rate of 10^4 pN/s, we expect that this interaction will occur *in vivo* and support tight bacterial attachment to the vascular endothelium (Figure 4h).

The unique mechanical properties of Aap play a critical role in the formation of the extremely strong interaction. The involvement of the two distant A and B domains points to an allosteric regulation process, where the activity of a protein like an enzyme is modulated by binding of a cofactor at a site physically distinct from the active site. Binding to the allosteric site generally leads to dynamic conformational changes in the protein. Excitingly, allostery is known to regulate the prototypical and widely investigated catch bond formed between *E. coli* FimH and mannose.[49,50] Basically, force-induced structural alterations in one part of the protein are linked to a shift from low- to high-affinity conformation of the ligand-

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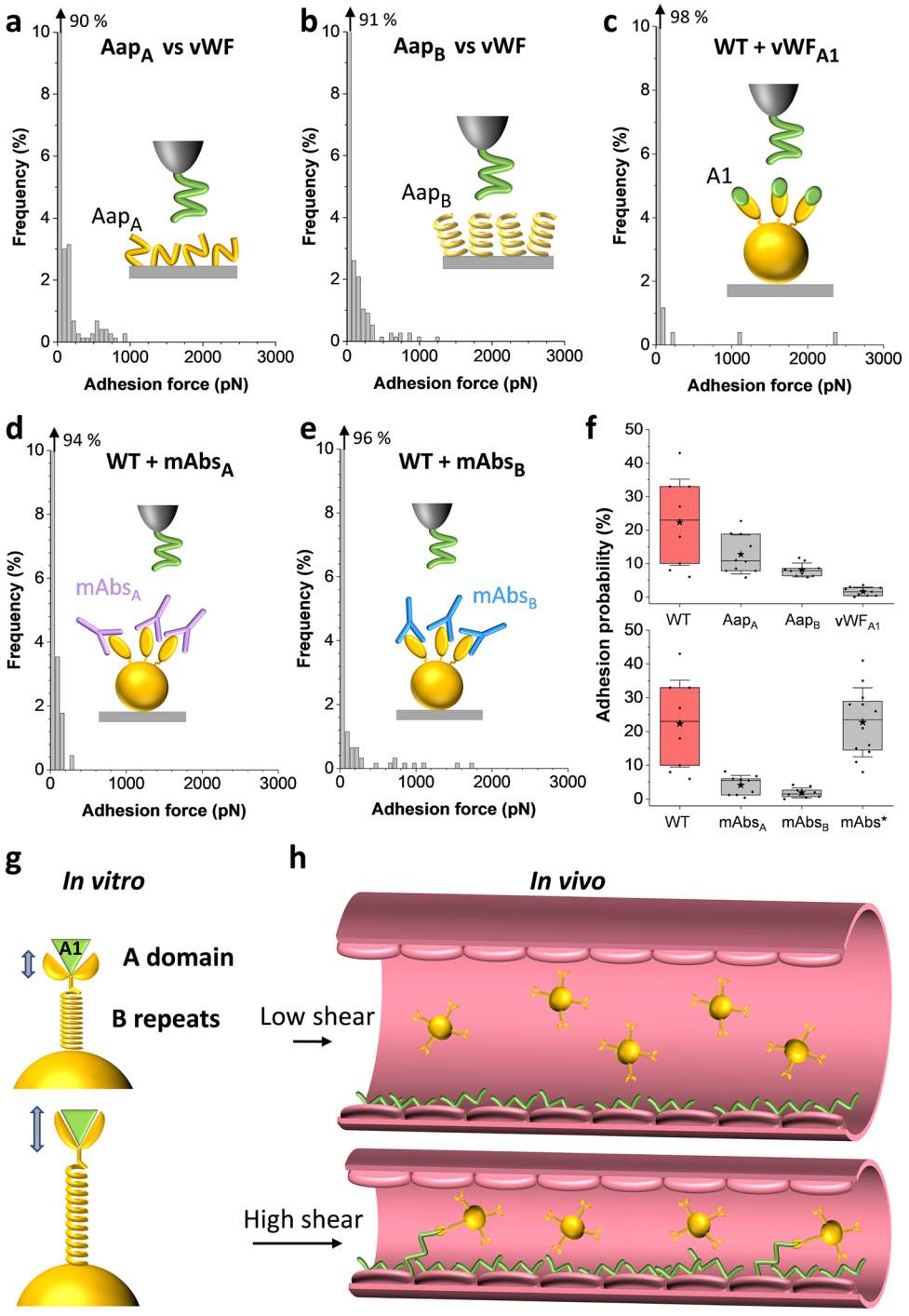

Figure 4. Dissecting the molecular mechanism of the ultrastrong Aap−vWF interaction. (a−c) Identification of the Aap and vWF binding domains. Histograms of adhesion forces (n = 256 curves for each condition) in PBS between vWF-tips and surfaces functionalized with A fragments (a) or B fragments (b) of Aap or a *S. epidermidis* WT cell preincubated with the vWF A1 domain (10 μg/mL) (c). (d, e) Inhibition by monoclonal antibodies (mAbs): histograms of adhesion forces between vWF-tips and *S. epidermidis* WT cells preincubated with 10 μg/mL mAbs directed either against the A domain (d) or B domain (e) of Aap. As a control, an irrelevant anti-integrin mAb was also tested (see mAb* in f). (f) Box plots comparing the differences in adhesion probability between native WT cells (n = 9 cells, 256 curves per cell), A and B fragments, and A1 and mAbs treated cells (n = 10 cells at least for each treatment). (g, h) Proposed model of allosteric regulation of the Aap−vWF interaction. (g) Force-induced structural alterations in one part of Aap, i.e., unfolding of the B repeats, are linked to a conformational shift from a weak to a very strong ligand-binding site located in another part of the protein, the N-terminal A domain. (h) *In vivo*, bacterial cells experience a wide range of shear stresses throughout the vasculature, with larger vessels having lower shear than smaller ones. While shear rates are low in the veins (top), they can be very high in arteries (bottom), enabling formation of ultrastrong Aap−vWF bonds.

binding site located in another part of the protein.[41] Our results support the notion that the Aap−vWF interaction is controlled by an allosteric regulated catch bond (Figure 4g,h). Under mechanical tension, unfolding of the Aap B repeats activates the A domain, shifting it from a weak- to a strong-binding state and therefore enabling it to engage in ultrastrong binding to A1. An

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alternative model in which unfolding of every single B repeat would expose a site that would bind to A1 is very unlikely because the B and A domains are distant from each other, so how would they all simultaneously bind to the small A1 domain? If this was to happen in series there should be large variations in the measured forces given the variability in the number of putative interacting sites. If all bonds were to rupture in parallel, there should be an enormous variability in the magnitiude of the ultrastrong force, again given the large and variable number of sites involved.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.1c02105.

Methods, Supplementary Figure 1 of single-cell force spectroscopy of the Aap−vWF interaction. Supplementary Figure 2 of single-molecule force spectroscopy of the Aap−vWF interaction, Supplementary Figure 3 of increasing the loading rate favors the probability of forming ultrastrong bonds (PDF)

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#C.C. and M.M.G. contributed equally.

Author Contributions

C.C., M.M.G., G.P., P.S., and Y.F.D. contributed to the design of experiments, data interpretation, and writing of the paper. C.C. and M.M.G. performed the experiments and collected the data.

Notes

The authors declare no competing financial interest.

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