Atomic force microscopy measurements reveal multiple bonds between *Helicobacter pylori* blood group antigen binding adhesin and Lewis b ligand

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The strength of binding between the *Helicobacter pylori* blood group antigen-binding adhesin (BabA) and its cognate glycan receptor, the Lewis b blood group antigen (Leb), was measured by means of atomic force microscopy. High-resolution measurements of rupture forces between single receptor–ligand pairs were performed between the purified BabA and immobilized Leb structures on self-assembled monolayers. Dynamic force spectroscopy revealed two similar but statistically different bond populations. These findings suggest that the BabA may form different adhesive attachments to the gastric mucosa in ways that enhance the efficiency and stability of bacterial adhesion.

1. Introduction

The oncogenic pathway of gastric cancer is mainly associated with persistent *Helicobacter pylori* (*H. pylori*) infection that causes a negative spiral of events, including chronic inflammation, gastric mucosal atrophy and may ultimately result in dysplasia and cancer [1,2]. *H. pylori* is a bacterium highly adapted to the acidic gastric environment. Persistent infection of the gastric mucosa is required to induce chronic inflammation [3]. For long-term infection, *H. pylori* have a large family of outer membrane proteins, of which some are adhesins [4]. In particular, the blood group antigen-binding adhesin (BabA) has a high affinity (5 × 10⁻¹¹ M⁻¹) for the Lewis b (Leb) determinant, which is a fucosylated blood group antigen expressed in the human gastrointestinal epithelium [5,6]. Individuals infected with strains that express BabA are considered to have higher risk for duodenal ulcer and gastric cancer, i.e. overt disease [7–9].

The discovery of *H. pylori* opened up a new avenue for efficient therapy against gastric disease [10,11]. However, there is an increasing prevalence of *H. pylori* resistance to common antibiotics [12–14]. Once the adhesion of *H. pylori* to the gastric mucosa is crucial for the establishment of infection, the development of anti-adhesive therapies that block or diminish adhesion is of particular relevance.

Detailed studies of the binding of a ligand to its cognate receptor provide us with a better understanding of the molecular details regarding the local binding landscape that could aid the design of new potential drug candidates or alternative therapies. We previously developed bioengineered surfaces to investigate the Leb and other immobilized glycan structures, i.e. presented in solid phase as receptor mimetics for *H. pylori*. We reported that the immobilized glycans maintained their active conformations and promoted specific bacterial attachment [15].

To further characterize the adhesive properties of these immobilized ligands, we conducted force spectroscopy experiments, which quantify the strengths and unbinding rates of single-molecular bonds in the range of piconewton (10⁻¹² N) forces [16,17]. Atomic force microscopy (AFM) measurements have revealed...
subnanometre properties of biomolecular interactions in diverse scenarios ranging from molecular recognition to cell adhesion [18–21].

In dynamic force spectroscopy measurements, a biomolecular complex is subjected to a steadily increasing force until the bond breaks. This technique is based on simple sample architecture: the ligands of interest are attached to the AFM tip, and the corresponding receptors are attached to the surface of the substrate (figure 1).

The ligand-modified tip is then brought into contact with the sparsely distributed receptors immobilized on the surface, and this allows for bond formation. When the tip is pulled away from the surface, the spring deflects under the steadily increasing force on the receptor–ligand bond, until the bond ruptures. The rupture force depends on the loading rate, such that the most probable force (MPF) to rupture a simple bond defined by a single activation barrier will increase with the logarithm of the loading rate [22]. Analyses of the dependence of the most probable rupture force on the logarithm of the loading rate—referred to as force spectroscopy—can be used to characterize the dissociation of receptor–ligand pairs. Examples include selectins and glycoproteins, cadherins, neural cell adhesion protein and many others [19,23–26].

Importantly, such single bond strength measurements quantify non-equilibrium adhesive properties that could govern attachment under, for example, fluid shear stress. They reveal differences in dissociation rates or the existence of multiple binding interactions that could contribute to equilibrium binding in solution. Force spectroscopy complements solution-binding measurements, which reflect equilibrium binding free energies in the absence of force, such as the affinities reported by Fei et al. [27].

Here, single bond rupture forces were measured between the purified BabA protein from *H. pylori* and Leb immobilized on self-assembled monolayers (SAMs). Analyses of dynamic force spectra with an atomic force microscope revealed two distinct adhesive states. The new results suggest translational applications and therapeutic use of immobilized glycan receptors to reduce or eliminate adhesion of the more virulent and disease associated BabA expressing *H. pylori* strains.

2. Material and methods

2.1. Leb immobilization onto biotin-self-assembled monolayers

The biotinylated Leb glycan was immobilized onto immobilized neutravidin on biotinylated SAMs as described. This configuration was used because it follows a published protocol that was fully optimized to promote *H. pylori* recognition of and binding to these thus immobilized ligands [15]. The SAMs were assembled onto gold substrates (0.5 × 0.5 cm²), which were prepared as described elsewhere [28]. Before use, the gold substrates were cleaned with a fresh 'piranha' solution (seven parts concentrated sulphuric acid (95% (v/v); BDH Prolabo) and three parts of hydrogen peroxide (30% v/v; Merck)) for 5 min (caution: this solution reacts violently with many organic materials and should be handled with great care), thoroughly rinsed with Milli-Q water (18.2 MΩ cm resistivity at 25 °C) and absolute ethanol (99.9% (v/v); Merck) in an ultrasound bath, and then dried with a gentle nitrogen stream.

1-Mercapto-11-undecyltetra(ethylene glycol) (SH-(CH₂)₁₁-O-(CH₂-CH₂-O)₄-H; EG₄–thiol; 99%, Assemblon) and biotin-terminated tri(ethylene glycol) undecanethiol (SH-(CH₂)₁₀-CO-NH-(CH₂)₃–O-(CH₂CH₂O)₂-(CH₂)₃–NH–biotin; biotin–EG₃–thiol, 99%, SensoPath Technologies) were prepared as pure solution at...
2 mM in absolute ethanol. Biotin–SAMs were prepared by immersing the gold substrates in solutions containing 2.5% biotin–thiol (97.5 mol% ECD–thiol) with a 0.1 mM total final concentration, as previously described [15]. Incubation was performed at room temperature over 20 h. After the incubation, the SAMs were rinsed three times with fresh, absolute ethanol and dried with a gentle nitrogen stream. Neutravidin was used as a protein bridge to immobilize the biotinylated Leb. Neutravidin (Invitrogen, 0.1 mg ml\(^{-1}\) in phosphate-buffered saline (PBS)) was immobilized by incubation with 2.5 mol% biotin SAMs for 1 h in PBS buffer. After rinsing with PBS, neutravidin–SAMs were incubated for another hour with a biotinylated Leb\(^b\) (Fucol-2-Galβ1-3-Fucol-1-4-GlcNac-O(CH\(_2\))\(_6\)NH-CO(CH\(_2\))\(_3\)NH–biotin; Lectivity) solution (0.1 mg ml\(^{-1}\) in PBS) under the same conditions. Afterwards, the surfaces were thoroughly rinsed with PBS and dried with a gentle nitrogen stream. Surfaces with immobilized Leb\(^b\) were used immediately. These surfaces were previously characterized [15].

2.2. Atomic force microscopy tip modification and surface chemistry

The AFM tips were modified as described previously, with only slight changes [29]. AFM tips (SiN\(_4\), V-shaped, MLCT from Veeco Probes) were immersed in chloroform for 10 min. Afterwards, the cantilevers were dried with nitrogen and placed in ‘piranha’ solution for 30 min. After the ‘piranha’ treatment, the cantilevers were rinsed with Milli-Q water, followed by drying with nitrogen. Cantilevers were then coated with a gold film using the thermal evaporation method. First, a chromium layer of approximately 30 Å was deposited at a rate of approximately 0.1 Å s\(^{-1}\), and then a gold layer of approximately 600 Å was evaporated onto the chromium layer at a rate of approximately 1.0 Å s\(^{-1}\). A monolayer of 1,8-octanedithiol (Sigma-Aldrich) and 6-mercapto-1-hexanol (Sigma-Aldrich) was self-assembled onto the gold-coated cantilever by incubation in a mixture (10 mM) of these two thiols for 20 h. Changing the thiol ratios enabled control of the BabA density on the cantilever surface. Ratios from 1 : 10 up to 1 : 40 (1,8-octanedithiol : 6-mercapto-1-hexanol) were evaluated in order to identify the surface composition that resulted in optimum binding frequency for force spectroscopy measurements. The monolayers were then activated with poly (ethylene glycol)-methyl binding frequency for force spectroscopy measurements. In order to identify the surface composition that resulted in optimum binding frequency for force spectroscopy measurements, we tested different ratios of PEG and BabA.

2.3. Force measurements and data analysis

The bond rupture forces were measured with a molecular force probe (MFP-1D, Asylum Research) using the Icor Pro software (WaveMetrics) for data acquisition and piezo control. The optical lever sensitivity was first calibrated by pressing the tip against a hard surface to obtain the tip deflection in nanometres. The cantilever spring constants, calibrated using the thermal method, were 0.011–0.025 N m\(^{-1}\) [30]. Force measurements were as described elsewhere [31], with modifications to what concerns the working buffer, which was constituted by PBS (pH = 7.4), due to the fact that H. pylori uses its flagellar motility to cross the mucus layer that covers and protects the gastric cells and reach the gastric epithelium, where the pH is more neutral [32]. Once there, H. pylori is able to adhere to the surface of the gastric epithelial cells, namely by the Leb–BabA interaction.

Briefly, we used a steady force ramp to rupture the bonds. In the measurements, the cantilever was brought into contact with the surface with an impingement force less than 30 pN, and then retracted at a constant velocity. Three to four thousand force curves were recorded at each loading rate used. The ‘nominal loading rate’ is the spring constant multiplied by the cantilever velocity, but the ‘effective loading rate’ is the actual loading rate on the bond, determined from the elastic stretch region of the flexible PEG tethers. The slope of the latter curve just prior to bond rupture determines the effective spring constant (k_{ad}). Thus, the effective loading rate at rupture is k_{ad} × v, where v is the cantilever velocity. The nominal loading rates used were 250, 1550, 3550, 5550 and 10,500 pN s\(^{-1}\). The surface chemistry, and hence the protein density on the tip, was adjusted, in order to achieve binding frequencies of 10–30%, such that not more than 30 of 100 touches to the surface generated an adhesion event. This increases the likelihood that the detected binding events represent single bonds. Force–extension curves were analysed with a custom written program. For each force–extension profile displaying a single rupture event, the rupture force and the effective loading rate were both determined. Histograms of the rupture forces measured at each loading rate were fitted to a probability distribution described by equation (2.2), in order to determine the most probable rupture force at a particular loading rate [31]. Further details regarding the data analyses are in the electronic supplementary material.

2.4. Bond kinetics

We analysed the dependence of the BabA–Leb\(^b\) bond rupture using the model of Evans & Ritchie [22]. According to Bell [33], the bond dissociation rate increases exponentially with an applied force as

\[ k_{off} = k_{ad} \times e^{f/f_0}, \]

where \( k_{ad} \) is the intrinsic dissociation rate of the unstrained bond. The so-called thermal force is \( f_0 = kT/x_p \), where \( x_p \) is the projection of the transition state along the force vector.

When the applied force increases linearly with time, the distribution of rupture forces \( p(f) \) at a given loading rate \( r_l \) is given by [22]

\[ p(f) = \frac{k_{all}}{r_l} \times \exp \left[ \frac{f - k_{all} \times f_0}{r_l} \right] \left( e^{f/r_l} - 1 \right). \]

The MPF is the maximum in the force distribution determined at a given pulling rate, and is related to the loading rate \( r_l \) by [22]

\[ f_{mp} = f_0 \times \ln(r_l) - f_0 \times \ln(k_{all} \times f_0). \]

For a simple bond confined by a single activation barrier, \( f_{mp} \) is predicted to increase linearly with \( \ln(r_l) \), and \( f_{mp} \) and \( k_{all} \) are obtained from MPF versus \( \ln(r_l) \) plots.

3. Results

3.1. Optimizing immobilization conditions

To increase the probability of forming single receptor–ligand bonds (binding frequency of 10–30%), the optimal ratio between
1,8-octanethiol and 6-mercapto-1-hexanol was experimentally determined to be 1:20. This is important, because 1,8-octanethiol is used to form the bond to the linker and 6-mercapto-1-hexanol binds the protein, this being chemically diluted in a 6-mercapto-1-hexanol matrix, in order to avoid an excess of PEG linker and therefore BabA on the cantilever surface.

SAMs were first generated by a mixture composed of 97.5% EG4—thiol and 2.5% biotin—thiol. The 2.5% biotin SAMs formed were next used to immobilize biotinylated Leb glycan via a neutravidin bridge, which is a biotin-binding protein. The SAMs used in this study have been previously characterized and provide higher Leb immobilized on the surface [15]. The optimum conditions resulted in the desired binding frequency (10–30%), which generally ensures that bond rupture refers to single binding [30]. The range of concentrations tested was based on previous work [31], and the optimum concentrations experimentally determined for the BabA–Leb assays were 0.8 mg ml⁻¹ for the Leb glycan and 0.08 mg ml⁻¹ for the BabA protein.

3.2. Force spectroscopy measurements

In all experiments, the impingement force was kept at less than 30 pN to minimize non-specific binding. In control measurements between neutravidin—SAMs without Leb and Leb—SAMs assayed with a cantilever without bound BabA the non-specific binding frequency (number of adhesion events/number of tip–surface contacts) was less than 2–3%, compared with the binding frequency of 10–20% obtained when BabA protein was bound to the tip. Furthermore, the non-specific forces were low and randomly distributed.

In order to determine the number of bound states and their unstressed dissociation rates, the cumulative distribution of rupture forces at each loading rate was fitted with a multi-state binding model (see the electronic supplementary material). The MFP for each peak, as determined from the calculated maxima in the force distributions, was plotted against log₁₀ of the nominal loading rates.

Figure 2a–c shows the force histograms at the four loading rates tested. Visual inspection of these histograms reveals a broad peak that shifts to higher forces with increasing loading rate. This behaviour indicates specific bond formation between the tip and surface. However, the histograms are broader than predicted by the probability distribution for a single bond, and this could be due to the formation of more than one type of bond. This observation was further supported by the poor fit of the histogram to the probability distribution for single bond rupture (electronic supplementary material). Instead, the histograms are best described by a two-state model, in which two, independent bond rupture events contribute to the histogram. The best-fit model was justified using an F-test, which compares the goodness of fit of two models with different numbers of parameters [31].

Figure 2/ shows the resulting force spectra, or plots of the MFP versus log₁₀(fₜ) for each of the two peaks identified from the two-state binding model. From linear fits of the force spectra to equation (2.3), one obtains the thermal forces (fₜb), the parameter xₚ and the unstressed dissociation rate (kₚ) [31]. These fitted parameters are summarized in table 1.

In order to test whether the broad distribution of rupture events was instead owing to multiple, parallel tip–surface bonds rather than to the formation of two, different bond states, the data were also fitted to a model for N parallel, identical bonds, as described previously [34]. If the force was shared between N parallel bonds, then each bond would experience a force f/N, and the failure of each of the N bonds would be uncorrelated [34]. In this case, the force distribution (equation (2.2)) is approximated by replacing kₚ at and fₜb for a single bond by Nkₚ at and Nfₜb, respectively [25,34]. To determine whether the peak(s) at higher forces were owing to multivalent attachments, we used the bond parameters obtained for the low force peaks, in order to calculate probability distributions for N parallel, weak bonds, where N = 2, 3 or 4. However, the thus calculated probability distributions did not fit the second peak (not shown), and indicates that the two peaks in the distribution are due to two, independent bonds.

A three-state model was also tested, in order to determine whether the broad distribution in the tail of the histogram in figure 2, particularly at the higher pulling rates, is due to a third bound state. However, as the loading rate increased, the MFP of the putative third bond varied randomly with log(fₜ). This indicated that the tails at higher loading rates are owing to non-specific binding rather than owing to the formation of an additional bound state. For this reason, the force distributions were fitted by using the two-state model (electronic supplementary material, figure S2). Further details of the data analyses are given in the electronic supplementary material.

Table 1 shows the fitted values for molecular bonds involved in the BabA–Leb interaction.

4. Discussion

Helicobacter pylori adhesion to gastric epithelial cells is important for establishment of a lifelong persistent infection by the more virulent and adhesive H. pylori types. Bacterial adhesion induces alterations of gene expression both in H. pylori and in the gastric host cells [35,36] contributing to establishment of a chronic mucosal inflammation and development of gastric diseases [7,8,37].

Moreover, adhesion protects bacteria from host clearance mechanisms such as mucous shedding and peristaltic movements. Approximately 20% of H. pylori present in the gastric epithelium adhere to the surface of mucus epithelial cells [38]. BabA is an outer membrane protein adhesin, which mediates adherence between H. pylori and the ABO/Leb histo-blood group glycan type of antigen expressed on the surface of gastric epithelial cells [5,39,40].

In these AFM investigations of bonds between the BabA protein and its cognate Leb receptor, several factors should be considered. First, the molecular anchorage to the surfaces should be much stronger than the receptor–ligand bonds being studied. Second, the surface density of the molecules should be sufficiently low, in order to ensure single-molecule interactions. Third, tethering the molecules to flexible PEG spacers increased the likelihood that the binding site of covalently bound protein would be more accessible, rather than blocked by some immobilized orientations. Fourth, unspecific adhesion to the modified surfaces should be minimized in order to increase the signal to noise in the measured receptor–ligand bond rupture forces [21,41]. The density of the BabA protein on the AFM cantilever probe tip and the glycan Leb ligand on the substrate (SAMs) were experimentally adjusted to meet the expectation that the surface layers were neither non-specifically bound nor clustered. In addition, a PEG linker was used to tether the protein, to ensure orientational flexibility and to provide a non-fouling background to avoid non-specific tip–surface adhesion. Moreover, binding frequencies in the 20–30% range and analyses of the force histograms indicated that the rupture forces likely corresponded to single-molecule events.

The experimentally determined bond rupture data suggested that a two-state binding model best describes the force data, measured between purified ligand and adhesin. A previous report of BabA–Leb bond strengths, based on optical tweezers measurements with bacteria, identified a single slip bond [42].
There are important differences between the measurements and analyses used in the two studies. First, the prior optical tweezers study measured binding between a bead modified with Leb and a bacterium bound to an optically trapped bead. Measurements with cells can be complicated by the complexity of the cell surface and the potential for increased non-specific binding. Indeed, the force histogram was more rugged than reported here. To minimize non-specific binding, in the tweezers study, the bead–cell contact time was kept short. The reported histogram was also broad, and the range of forces was similar to that observed in these AFM measurements of Leb binding to the purified, immobilized adhesin. Different from the prior study, however, here we analysed the histograms in more detail using established methods [26], and found that the broad histograms could be attributed to a second bound state. The separate analyses of the two peaks contributing to the force histogram would necessarily give rise to differences in the estimated bond parameters in these two studies.

Interestingly, dissociation rates determined for the BabA–Leb complexes were $3.00 \pm 0.70$ and $0.60 \pm 0.10$ s$^{-1}$, respectively. Although the force histograms were similar, these rates are more than 100 times the rate estimated from optical laser tweezers data. The faster off-rates are in the same range as reported for the self-association of mucins [43] and for single L-selectin–carbohydrate bonds [23]. The values for $x_b$ for the weak and stronger BabA–Leb bonds are $0.26 \pm 0.03$ and $0.23 \pm 0.01$ nm, respectively. These are also within the range reported for other biomolecular interactions, including glycosaminoglycan interactions with cartilage aggrecan ($x_b \approx 0.31 \pm 0.08$ nm) [44] and individual mucin1–antibody bonds ($x_b \approx 0.28 \pm 0.02$ nm) [45]. Another example is the value of $x_b \approx 0.26$ nm determined for the mannuronan AlgE4 polysaccharide–protein bond [46]. These values are all lower than the 0.86 nm reported previously for BabA–Leb binding.

**Figure 2.** Adhesion measurements between BabA and Leb. (a–e) Histograms of the rupture force distribution measured at the different tested loading rates. (f) The force spectra (most probable force versus log($r_f$) plots) of BabA–Leb bonds associated with the major peaks.

**Table 1.** Dissociation rates, thermal forces and lengths of the BabA–Leb bonds determined from linear fits to the force spectra. $f_b$, thermal force; $k_{off}$, dissociation rate; $x_b$, bond length.

<table>
<thead>
<tr>
<th>Bond Type</th>
<th>$f_b$ (pN)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$x_b$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>18.3 ± 2.3</td>
<td>0.6 ± 0.1</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>Weak</td>
<td>15.9 ± 1.7</td>
<td>3 ± 0.7</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>
As noted above, a possible reason for this difference may be due to the use of the one-state versus two-state model.

It is worth considering alternative reasons for the apparent difference, beyond the models used. First, one of the two bonds observed in the AFM measurements could form more slowly than the short dwell time used in the optical tweezers experiments, and may not have been as prevalent in the previously reported force histograms [43,47]. Alternatively, the recombinant, purified protein could be more conformationally heterogeneous than the membrane-bound form. In this case, the purified protein might adopt conformations with slightly different bond properties, whereas the membrane bound BabA population could be more uniform. Exploring these possibilities is beyond the scope of this work, but they are possibilities to be considered.

In a similar study regarding the Leb−BabA interaction, Younson et al. [48], using surface plasmon resonance, also proposed that the interaction of Leb and BabA is far more complicated than what could be described by a simple one-state binding model, suggesting a two-step conformational change model. This model is described by a first binding step, followed by a slower conformational change in order to form a relatively stable complex. However, structural analyses of changes in the BabA protein, to the best of our knowledge, have still not yet been performed in order to further deepen this hypothesis to translate our data. How the two bound states identified in this study contribute to H. pylori adhesion has yet to be determined. However, it is important to highlight that in the gastric environment, where shear stress at the gastric wall, constant cell renewal and mucus shedding make it difficult for bacteria to adhere, different bonds with different kinetics and strengths could play an important role in initiating and maintaining a chronic bacterial infection [49].

These results are also important for better identifying BabA−Leb properties that may contribute to the biology of infection. Multivalent interactions, for example, are well known to enhance the lifetime and apparent affinity of particle−cell or pathogen−cell interactions [45,50]. In addition, some E. coli exhibit catch bonds between adhesins on the pili and ligands on target cell surfaces [51]. Recent single bond rupture studies of cadherins demonstrated that the adhesive extracellular domains exhibit multiple bonds [52]. One of the cadherin bonds is a catch bond, and a second is a slip bond [53]. Together, these could enable cadherins to adjust cell adhesion to changes in the mechanical environment.

This new insight into the adhesion complex BabA−Leb also suggests that the BabA protein, for which the structure has not been determined, may possess different domains for bacterial binding to the host ABO/Leb binding sites in the gastric mucosa.

These findings, based on measurements with purified adhesin and ligand, further identified additional biophysical properties of the BabA−Leb interaction. The strong, specific binding mediated by BabA suggests that translational applications of synthetic Leb structures, as nanoadhesives for H. pylori binding is highly appropriate for the development of new therapeutic strategies for infection management.

5. Conclusions
The new results reveal new insights into the biophysical properties of BabA−Leb adhesion, and suggest that the H. pylori BabA protein can form different bonds with Leb glycans. By disclosing some of the molecular features of the H. pylori binding mechanisms for glycan structures expressed in the host gastric mucosa, the new results may further guide the development of alternative therapies for H. pylori eradication based on immobilization of glycan structures onto mucoadhesive biomaterials for clearance of H. pylori infection.

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