Conformational inactivation induces immunogenicity of the receptor-binding pocket of a bacterial adhesin

Dagmara I. Kisiela[a,1], Victoria B. Rodriguez[b,1], Veronika Tchesnokova[a], Hovhannes Avagyan[a], Pavel Aprikian[a], Yan Liu[c], Xue-Ru Wu[c], Wendy E. Thomas[b], and Evgeni V. Sokurenko[a,2]

Departments of [a]Microbiology and [b]Bioengineering, University of Washington, Seattle, WA 98195; and [c]Department of Urology, New York University School of Medicine, New York, NY 10016

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Inhibiting antibodies targeting receptor-binding pockets in proteins is a major focus in the development of vaccines and in antibody-based therapeutic strategies. Here, by using a common mannose-specific fimbrial adhesin of *Escherichia coli*, FimH, we demonstrate that locking the adhesin in a low-binding conformation induces the production of binding pocket-specific, adhesion-inhibiting antibodies. A di-sulfide bridge was introduced into the conformationally dynamic FimH lectin domain, away from the mannose-binding pocket but rendering it defective with regard to mannose binding. Unlike the native, functionally active lectin domain, the functionally defective domain was potent in inducing inhibitory monoclonal antibodies that blocked FimH-mediated bacterial adhesion to epithelial cells and urinary bladder infection in mice. Inhibition of adhesion involved direct competition between the antibodies and mannose for the binding pocket. Binding pocket-specific inhibitory antibodies also were abundant in polyclonal immune serum raised against the functionally defective lectin domain. The monoclonal antibodies elicited against the binding-defective protein bound to the high-affinity conformation of the adhesin more avidly than to the low-affinity form. However, both soluble mannose and blood plasma more strongly inhibited antibody recognition of the high-affinity FimH conformation than the low-affinity form. We propose that in the functionally active conformation the binding-pocket epitopes are shielded from targeted antibody development by ligand masking and that strong immunogenicity of the binding pocket is unblocked when the adhesive domain is in the nonbinding conformation.

FimH adhesin | antiadhesive antibodies

Eliciting antibodies that specifically block the function of target proteins is one of the main desiderata in the development of protective vaccines and therapeutic antibodies. The ability of microbial pathogens to attach specifically to host cells determines their tissue tropism, intracellular invasion, biofilm formation, and other qualities. Attachment is mediated by surface proteins called "adhesins," and antibodies specifically induced against the binding-pocket epitopes of viral and bacterial adhesins have been found to be highly efficient and broadly neutralizing (1–4). However, eliciting an immune response directly targeting the receptor-binding pocket can be challenging. Masking of the binding epitopes via interaction with natural receptors/ligands previously has been shown to prevent antigen recognition by specific antibodies (5). Here, we show that the mannose-binding site of the type 1 fimbrial adhesin of *Escherichia coli*, FimH, becomes highly immunogenic and induces adhesion-blocking antibodies only upon its functional inactivation by conformational locking.

FimH is a mannose-binding adhesin on the tip of fibrillar structures called "type 1 fimbriae" that are expressed by the majority of *E. coli* strains and also other Gram-negative bacilli (6, 7). FimH-mediated adhesion has been shown experimentally to be critical for uropathogenic *E. coli* strains to colonize and establish infection of the bladder (8–11). FimH is incorporated into the fimbrial tip through its C-terminal pilin domain, whereas the N-terminal lectin domain (LD) bears the mannose-binding pocket (12). In the fimbrial FimH (i.e., FimH in the context of the fimbrial structure), the LD switches from an inactive to an active conformation as the result of the drag force on a bacterium that is binding in flow. The inactive conformation displays a very low affinity to monomannose (K_d ~300 μM), whereas the active conformation binds with high affinity (K_d <1.2 μM), with the differences in affinity to tri/oligo-mannose structures being less dramatic (13). The key step of conformational switching is the separation of the LD from the pilin domain by tensile force leading to a shift of the mannose-binding pocket from a wide open (low-affinity or "inactive") into a tightly closed (high-affinity or "active") conformation (14). Because ligand binding to the open pocket reciprocally induces a conformational change across the LD that propagates to the interdomain interface located on the opposite site of the mannose-binding pocket (resulting in domain separation), the regulation is considered to be allosteric (13, 15). In contrast to fimbrial FimH, purified LD in the absence of the pilin domain assumes the active mannose-binding conformation. The active form of LD (as a purified domain or as FimH in complex with its chaperone, FimC) has been tested extensively as a candidate vaccine against uropathogenic *E. coli* (16–19). Despite a strong immune response against the antigens, induction of antibodies against the mannose-binding pocket could not be demonstrated, and a successful protective vaccine for human use has not been developed, leaving open the question of how the immune response to a FimH-based vaccine could be improved. Moreover, our recent study has shown that antibodies against functionally active

Significance

Antibodies targeting the receptor-binding pocket of viral and bacterial adhesins are highly protective against infection. However, functional site epitopes in the antigens are not always highly immunogenic, possibly because of the binding epitope-masking effects of natural ligand–receptor interactions. By using the mannose-specific fimbrial adhesin of *Escherichia coli*, FimH, we demonstrate that locking the adhesin in a low-binding conformation abrogates its capability to interact with mannose but, at the same time, facilitates the immune response against binding-pocket epitopes and production of adhesion-inhibitory antibodies. We believe our findings provide insight into strategies to elicit neutralizing and functional antibodies against a broad spectrum of receptor-binding proteins.

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[1]D.I.K. and V.B.R. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: evs@u.washington.edu.

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purified LD do not inhibit but instead actually enhance bacterial adhesion by stabilizing fimbrial FimH in the active conformation (20), raising questions about the utility of active LD or FimH as the optimal vaccine candidate.

In the current study, the FimH LD was mutationally locked in an inactive conformation and was used for the induction of a panel of mAbs whose epitope specificity and functional properties were compared with those of different native forms of FimH and FimH-expressing bacteria.

Results

Purified FimH LD with the Double Mutation V27C + L34C Is Functionally Impaired. Because in purified form the native LD (LD^{nat}) of FimH is naturally locked in an active high-affinity conformation, we substituted cysteines for residues V27 and L34 in an attempt to lock the LD in an alternative inactive conformation through the formation of a di-sulfide bridge between C27 and C34, presumably stabilizing it in a low-affinity state (14). The mutant LD (LD^{mut}) indeed exhibited a significantly reduced mannose-binding capability relative to LD^{nat} when both His-tagged purified proteins were immobilized on a plastic surface and probed with soluble HRP containing mannose-rich N-linked oligosaccharides (Fig. 1). The HRP binding was compared in the absence and presence of 1% α-methyl-D-mannopyranoside (αmm, hereinafter also termed mannose), a strong inhibitor of mannose-dependent bacterial adhesion (Fig. 1). Although the exact conformation of purified LD^{mut} is unknown, residues 27 and 34 are positioned well away from the mannose-interacting loops (see below) and thus do not alter the primary structure of the binding-pocket epitopes. Therefore, the loss of function is indirect and conformational in nature.

Functionally Inactive Adhesive Domain Elicits Binding-Inhibitory Antibodies. The functionally inactive LD^{mut} was used as an antigen to generate mouse mAbs. A total of 14 positive hybridomas were selected through screening for antigen recognition and then were compared directly with a set of previously obtained mAbs raised against functionally active LD^{nat}. As shown in Fig. 2*A*, all antibodies raised against LD^{mut} recognized LD^{nat} at a level comparable to or slightly lower than antibodies raised against LD^{nat}. When LD^{nat} was exposed to antibody and then was probed

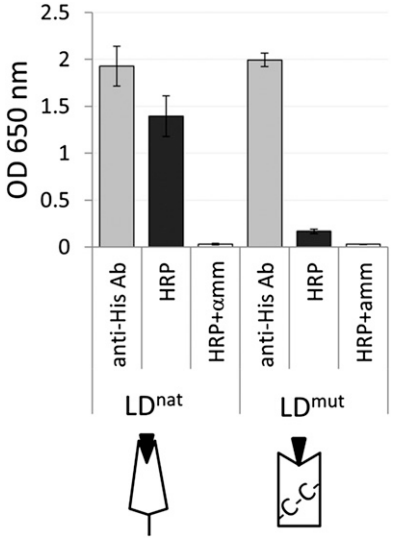

Fig. 1. Mannose-binding properties of purified native and V27C/L34C mutant LDs of FimH. Purified LD^{nat} and LD^{mut} were immobilized in wells in microtiter plates and were probed with anti-His antibody or soluble HRP in the presence and absence of 1% αmm, a soluble inhibitor of mannose-dependent binding. Data are shown as means ± SD.

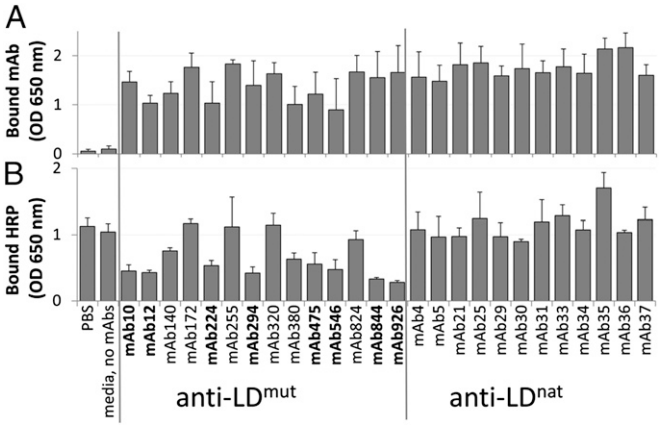

Fig. 2. Binding and inhibitory potency of mAbs raised against V27C/L34C mutant and native LDs. (*A*) Recognition of LD^{nat} by 1:100 diluted mAbs elicited against LD^{mut} and native LD^{nat} as evaluated by ELISA. (*B*) Potency of mAbs to block binding of soluble HRP to plastic-immobilized LD^{nat}. Inhibitory antibodies are shown in bold. In both panels, data are shown as means ± SD.

for mannose-specific binding capability, 8 of 14 LD^{mut}-raised antibodies caused at least 50% inhibition of LD^{nat} activity (Fig. 2*B*; inhibiting mAb numbers are shown in bold). In contrast, none of 12 LD^{nat}-raised antibodies inhibited the binding activity of LD^{nat}, a finding that is consistent with previous observations (20). The level of mannose-binding inhibition exerted by individual anti-LD^{mut} antibodies did not depend on their antigen-binding affinity, as shown in Fig. 2*A* and Fig. S1, indicating that the levels of mannose-binding inhibition of particular clones are the result of differences in epitope specificities.

From the LD^{mut}-elicited antibody clones that caused inhibition, we selected mAb475 as a representative for further investigation. We compared in detail its properties and those of mAb21 antibody, a representative LD^{nat}-raised antibody.

mAb475 Blocks Bacterial Adhesion in Vitro and Reduces Bladder Colonization in Mice. We assessed the inhibitory potency of mAb475 against bacterial cell attachment by testing the adhesion of the model uropathogenic *E. coli* strain UTI89 to the human bladder epithelial cell line T-24. As shown in Fig. 3*A*, bacterial adhesion was reduced dramatically (86 ± 2%) by mannose, indicating that the majority of *E. coli* UTI89 bacteria bind to uroepithelial cells in a FimH-dependent manner. A similar reduction in the number of cell-adherent bacteria (83 ± 2%) was detected in the presence of mAb475 (at a concentration of 50 μg/mL) that accounted for 96% inhibition of the mannose-dependent binding. In contrast, as reported previously (20), mAb21 significantly enhanced bacterial adhesion (220 ± 20%). In both antibody treatments, the effects were caused by specific FimH binding and not by bacterial aggregation, as monitored by microscopy.

We also evaluated the protective effect of mAbs in a mouse model of urinary bladder infection (Fig. 3*B*). Bacteria were pretreated with mAb475 or mAb21 and were inoculated into the bladder. After 24 h of infection, significantly fewer bacteria were recovered from the bladder when bacteria were pretreated with mAb475 (58% reduction, $P < 0.05$) than when bacteria were untreated. A slight reduction that did not achieve statistical significance (21%, $P = 0.227$) was observed with mAb21 pretreatment; thus the protective effect of mAb475 was significantly ($P < 0.05$) greater than that of mAb21 (Fig. 3*B*). Hence, the FimH inhibitory potency of mAb475 translates into the cell adhesion-blocking effect of the antibodies and the ability to protect against infection.

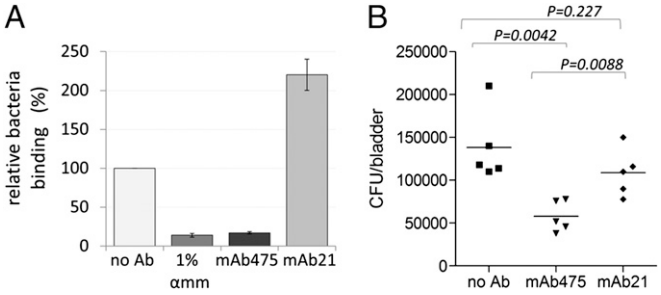

Fig. 3. Effect of mAb475 on *E. coli* UTI89 adhesion in vitro and bladder colonization in mice. (*A*) Binding of bacteria to a bladder epithelial cell line (T24). Before being added to T24 monolayers, bacteria were pretreated with cell culture medium (no antibody control), 1% αmm, mAb475, or mAb21 antibodies (50 μg/mL). Data are shown as mean ± SD. (*B*) Bacteria recovered from bladders 24 h after inoculation of mice. Bacteria were preincubated for 1 h with PBS, mAb475, and mAb21 and were transurethrally administered into mice bladders via catheter. Horizontal bars indicate the mean (n = 5 mice per group). P values for indicated datasets were determined by Student t test.

Epitope of Inhibitory mAb475 Overlaps the Mannose-Binding Pocket. To investigate the mAb475 epitope, its binding was tested against a library of fimbria-incorporated FimH with various point mutations in the LD (Table S1). Most substitutions that abolished or significantly reduced antigen recognition by mAb475 (>50% relative to the wild type) were located on the top of the β-barrel fold (Fig. 4*A*), overlapping the mannose-binding pocket residues on three different loops (21, 22). These residues included Phe1, Asn46, Asp54, Gln133, and Asn135, and amino acid substitutions at each of these positions abolished mannose-specific binding of bacteria (Fig. 4*B* and Table S1). However, the overlap with mannose-interacting residues was partial, because mutants Ile52Ala and Asn136Ala, which caused a >50% decrease of mAb475 binding and are positioned close to the residues mentioned above, retained their ability to interact with mannose (Fig. 4*B* and Table S1). At the same time, mutation of two other residues (P111K and P85S), which reduced mannose binding >50% but were not close to the binding pocket, had no effect on mAb475 reactivity with the fimbriae.

There was no overlap between the putative mAb475 epitope and the mAb21 epitope comprising residues 26, 29, and 153–157 located at the bottom of the LD, away from the binding pocket (20); mutations in the mAb21 epitope had no inhibiting effect on mannose binding (Table S1).

Functional Competition and Mimicking Between mAb475 and Mannose. The overlap between mannose- and mAb475-binding residues prompted us to examine whether there is a direct competition between the ligand and antibody binding to LD. We tested the interaction of mAb475 with functionally active LD^{nat} in the absence and presence of mannose (Fig. 5*A*). Mannose inhibited mAb475 binding in a dose-dependent manner, with as little as 0.01% mannose abrogating more than 90% of mAb475 binding to purified LD^{nat}. In contrast, mannose had no effect on mAb21 interaction with LD^{nat}.

The direct competition between mAb475 and mannose for FimH binding suggested the possibility of a significant functional overlap. We examined the extent to which mAb475 binding mimics mannose in more detail. Because fimbrial FimH naturally shifts between low- and high-affinity conformations, we compared the abilities of mAb475 and a mannosylated substrate (HRP) to bind to purified type 1 fimbriae incorporating two alternative variants of FimH. One was $FimH^{K12}$ that represents natural FimH expressed by laboratory *E. coli* strain K12 (and uropathogenic strain J96) and has an LD that, under static conditions, is predominantly shifted toward the low-affinity conformation. The other fimbrial FimH variant was $FimH^{K12\_HA}$ that has a structurally altered pilin domain, resulting in an LD shifted toward the high-affinity conformation even under static conditions (13). In both FimH variants, the primary structure of LD is identical and corresponds to that of purified LD^{nat}. As expected, HRP binding to the low-affinity conformation of $FimH^{K12}$ was much weaker than to the high-affinity conformation of $FimH^{K12\_HA}$ (Fig. 5*B*). A similar pattern was observed for mAb475, which bound better to the high-affinity variant than to the low-affinity variant, although the difference was not as great as with mannose-specific HRP binding. Of note, the LD^{mut}-elicited noninhibitory mAb255 bound better to the low- than to the high-affinity FimH variant (Fig. 5*B*). Although it would be interesting to determine the epitope recognized by mAb255, so far our mutant library screening has not revealed the epitope recognized by mAb255. Another FimH-specific functional property of mannose is its ability to induce FimH in the low-affinity conformation to switch (in a reversible way) to the high-affinity conformation allosterically, as demonstrated by the ability of mAb21 (the active state-specific antibody) to recognize $FimH^{K12}$ in the presence but not in the absence of soluble mannose (20). As shown in Fig. 5*C*, pretreatment or simultaneous treatment of $FimH^{K12}$ fimbriae with mAb475 significantly increases mAb21 binding to fimbrial $FimH^{K12}$. This result revealed that mAb475 and the mannose ligand exert the same structural (activating) effect on the FimH conformation, confirming not only direct competition but also functional mimicking between the inhibitory antibody and natural ligand. Also, this result demonstrates that mAb475 and mAb21 do not interfere with each other's binding to FimH and, although mAb21 alone has FimH binding-enhancing properties, in combination with mAb475 the inhibitory effect of the latter prevails (Fig. S2).

Mannose-Binding Pocket Is a Universal Epitope for Inhibiting Antibodies. We tested whether other inhibitory mAbs that were elicited against LD^{mut} have structural and functional characteristics similar to those described for mAb475. All anti-LD^{mut} antibodies that inhibited the mannose-binding activity of immobilized LD^{nat} were able to block mannose-specific bacterial adhesion, with direct correlation between the two properties (Fig. S3 *A* and *C*). Also, all LD^{mut}-elicited antibodies recognized the natural conformation of fimbrial FimH (Fig. S3*B*). In contrast, different LD^{nat}-elicited mAbs that recognize four separate classes of LD^{nat} epitopes (20) bound fimbrial FimH equally well but failed to block bacterial adhesion (S3 *A* and *B*). Binding of inhibitory antibodies to fimbrial FimH was abolished in the presence of mannose (Fig. S4), suggesting that, like mAb475, they target epitopes located in the binding-pocket area. Indeed, testing

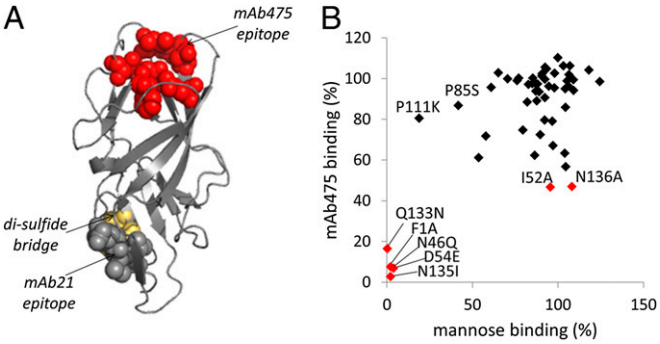

Fig. 4. Mapping of mAb475 epitope. (*A*) Ribbon representation of the crystal structure of the FimH LD (derived from Protein Data Bank ID code 3JWN) with residues critical for binding of mAb475 (red spheres) and mAb21 (gray spheres). The position of the introduced cysteine bridge residues (V27 and L34) is indicated by yellow spheres. (*B*) Scatter diagram showing relationship between mAb475 binding to different FimH mutants and the mutants' capability to bind mannose. Mutations reducing mAb475 binding greater than 50% are shown in red. Data are taken from Table S1.

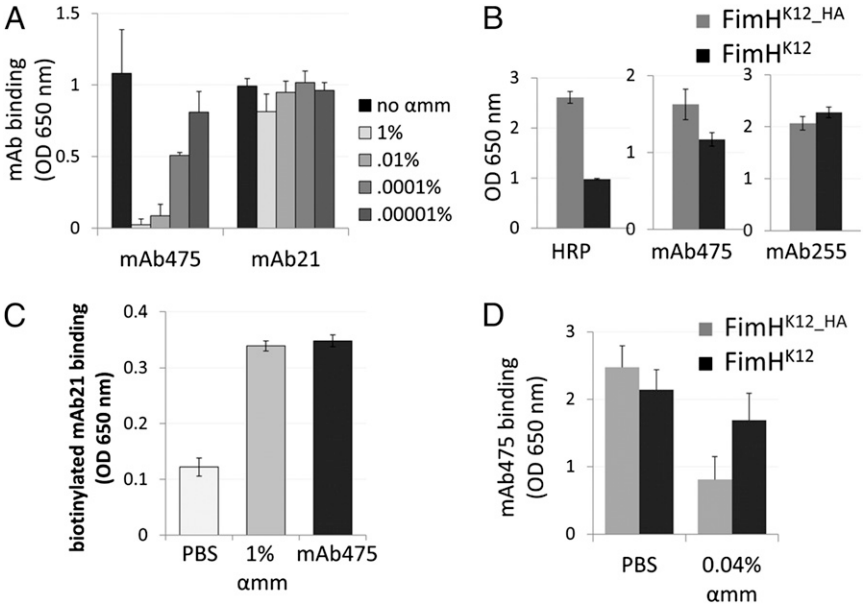

Fig. 5. Mechanism of epitope recognition by mAb475. (*A*) Binding of purified mAb475 and mAb21 (13 nM) to LD^{nat} in the absence and presence of serial dilutions of αmm as determined by ELISA. (*B*) Binding of HRP (0.5 mg/mL), inhibitory mAb475, and noninhibitory mAb255 to different structural variants of fimbrial FimH ($FimH^{K12\_HA}$ and $FimH^{K12}$). (*C*) Binding of biotinylated mAb21 to fimbrial $FimH^{K12}$ in the absence and presence of 1% αmm or 1:20 diluted mAb475. (*D*) Binding of mAb475 to different structural variants of fimbrial FimH ($FimH^{K12\_HA}$ and $FimH^{K12}$) in the absence and presence of 0.04% αmm as determined by ELISA. In all panels, data are shown as means ± SD.

antibody binding to the set of various binding-pocket mutants (Table S2) revealed that different inhibiting antibodies universally target the binding pocket of the adhesin. Importantly, despite similar epitope specificity, each of the inhibitory antibodies possessed different light-chain V regions as determined by nucleotide sequence analysis of antibody-producing hybridoma clones.

Accessibility of Mannose-Binding Pocket Epitope Is Masked by Blood Plasma Glycoproteins. The recognition of the functionally active conformation of FimH LD by a broad panel of inhibitory antibodies elicited by LD^{mut} indicates that the binding epitopes are fully preserved in the inactive conformation. However, the active conformation fails to elicit a binding pocket-specific response during immunization. It has been reported previously that natural receptor–ligand interactions could interfere with antibody recognition of the binding epitopes (5). Thus, we tested whether avid binding of mannose or mannose-like compounds could explain the failed antibody response against the binding epitopes of the functionally active adhesive domain of FimH.

Although mAb475 recognized the active high-affinity forms of FimH better than the low-affinity conformation (Fig. 5*D*), the pattern was reversed in the presence of soluble mannose (Fig. 5*D*), with the antibody binding to the high-affinity forms drastically reduced relative to the low-affinity forms. We tested whether host mannosylated glycoproteins, such as those present in blood plasma, might exert the same effect on mab475 epitope recognition at naturally occurring concentrations. We found that although blood plasma significantly abrogated epitope recognition in the high-affinity FimH (Fig. S5*A*), it had only a minor effect on mAb475 binding to the low-affinity FimH variant. Again, the recognition of the low-affinity FimH was significantly greater than that of the high-affinity FimH variant in the presence of plasma components. At the same time, blood plasma had no inhibitory effect on mAb21 recognition of the high-affinity FimH variant (Fig. S5*B*). Of note, plasma passed through a Con A-agarose chromatography column to remove mannosylated compounds showed significantly reduced inhibitory potency against mAb475 binding to fimbrial FimH. In contrast, plasma passed through glutathione-agarose preserved its inhibitory potency, as demonstrated in Fig. S6.

Polyclonal Anti-LD^{mut} but Not Anti-LD^{nat} Serum Contains mAb475-Like Inhibitory Antibodies. To determine if the results with mAbs selected by screening truly reflect differences in the overall nature of antibodies elicited by the two conformational forms of FimH, polyclonal sera raised against these two LDs (obtained from mice used for hybridoma production) were tested for the presence of mAb475-like antibodies. Both types of immune sera recognized FimH significantly better than preimmune antiserum (Fig. S7*A*). Because mannose competes directly with mAb475 for binding, the immune sera were compared for recognition of FimH in the presence of soluble mannose. As shown in Fig. S7*B*, mannose markedly inhibited anti-LD^{mut} but not anti-LD^{nat} polyclonal antibody binding. Thus, pocket-specific (i.e., mannose-competing) antibodies appear to be present in abundance in the anti-LD^{mut} serum but not the anti-LD^{nat} serum. We also tested the potency of these sera in blocking mannose-specific binding of FimH (Fig. S7*C*). Even at 1:50 dilution, more than 80% of HRP binding to FimH could be inhibited by anti-LD^{mut}, indicating that the adhesion-inhibitory activity shown above for mAbs is reflected by antibodies present in immune serum in physiological concentration. In striking contrast, the inhibitory activity of anti-LD^{nat} serum was not different from that of the preimmune serum and therefore likely results from the inhibitory effect of mannosylated serum proteins rather than specific anti-FimH antibodies (Fig. S7*C*). Taken together, these results suggest that anti-LD^{mut} but not anti-LD^{nat} serum contains abundant inhibitory antibodies elicited against the binding-pocket epitopes.

Discussion

The general concept of vaccination often assumes that the immune response against a particular antigen is the outcome of the immunogenic properties of the antigen itself. Failure to induce broadly neutralizing antibodies against pathogens is a major barrier in development of preventive vaccines, including a vaccine against HIV. In the present study we have demonstrated that direct competition of natural ligands with antibodies against binding-site epitopes masks these strategically important immunogenic determinants. However, the immunogenicity of the binding epitopes was enabled by the functional inactivation of the binding site.

Previous failure to induce binding pocket-specific, inhibitory antibodies against the FimH adhesin with the native functionally active adhesive domain prompted us to test the immunogenic properties of an alternative form of LD that is stabilized in the inactive state. The loss of function was induced without changing the primary structure of the binding-pocket loops, i.e., was conformational in nature. Unlike the native LD, the functionally defective domain was very potent in inducing mAbs that specifically

blocked the function of native FimH and, interestingly, bound the active domain better than the original antigen.

Production of anti-FimH antibodies has been described previously (16–18). Although immunization with purified LD or a FimH-FimC complex was shown to be protective against infection in animal models, the molecular mechanism of the protection was not elucidated, and it remains unknown to what extent, if any, specific function-blocking properties of the antibodies were involved. It is possible that the protective response resulted from a nonspecific bacterial aggregation by the anti-FimH antibodies or their general opsonic properties. Thus, it remains unclear whether the vaccine could be optimized to be successful in humans.

We show here that FimH-blocking mAbs successfully inhibited bacterial adhesion to epithelial cells and significantly decreased *E. coli* infection of the urinary bladder in mice, in contrast to the antibodies produced against the functionally active LD. That we also detected abundant inhibitory antibodies in the sera of animals from which mAbs were derived shows that a specific adhesion-blocking polyclonal immune response can be induced against FimH by using its binding-inactive but not its binding-active variant. Although the dysfunctional adhesin was obtained without affecting the primary structure of the binding site, it is possible that FimH inactivation by mutation of certain non-epitope amino acid residues inside the binding pocket also could lead to induction of inhibitory antibodies. Further studies are required to evaluate the protective potency of such antibodies induced in vivo and to determine whether and how the induction of binding-inhibitory antibodies could be optimized in FimH vaccine development. The inhibitory antibodies targeted FimH epitopes that overlapped with the mannose-binding pocket. Moreover, the antibodies competed directly with the ligand for binding. Not only did high concentrations of mannose abolish recognition of the binding-pocket epitopes by inhibitory mAbs, but the inhibitory mAbs also exhibited properties similar to those of mannose in interacting with low- and high-affinity conformations of FimH and in allosterically converting the low-affinity conformation into high-affinity conformation. These results suggest that, to a certain extent, antibodies mimic the action of a simple sugar, likely because interaction with the same amino acids on separate loops of the binding pocket leads to similar structural rearrangement in the pocket and consequently in the FimH protein in general. Along the same lines, an explanation for the interesting observation that the inhibitory antibodies bound more strongly to the high-affinity LD conformation might be that the epitope residues are in a more optimal configuration for stable antibody binding in the tighter pocket than in the open, low-affinity pocket conformation that potentially is more dynamic in nature. However, structural data are necessary to confirm this notion unambiguously.

Antibodies mimicking the physiological function of natural ligands have been described previously (23–25). Interestingly, they often are abundant in patients with autoimmune syndromes in which a humoral response is directed against receptor proteins, e.g., autoantibodies against the thyrotropin receptor in Graves' disease or insulin receptor-specific antibodies in insulin autoimmune syndrome (26, 27). By mimicking the functions of the ligands (thyroid hormone and insulin, respectively), these antibodies are directly involved in in the pathophysiology of hyperthyroidism and hypoglycemia. However, targeted elicitation of simple sugar-mimicking antibodies was not known and no such potential effects were described as part of a vaccine response.

The fact that inhibitory antibodies against conformationally inactivated LD recognize the native form of LD very well shows that the epitopes are well preserved and exposed for antibody interaction in the functionally active antigen and in naturally occurring FimH. Why then do the same epitopes appear to be very immunogenic in the inactive but not in the active LD? Unlike the natural fimbrial forms of FimH, in which the LD transiently shifts back and forth between high-affinity active and low-affinity inactive conformations, purified, pilin domain-free LD is locked in the active conformation (Fig. 6). We propose that the strong, high-affinity binding of natural mannosylated compounds to the locked active conformation of LD shields the epitopes from the initiation of the immune response much more effectively than when the LD is mutationally locked in the inactive conformation (Fig. 6). In the course of the immune response, antigen comes into contact with a variety of tissue fluids (e.g., plasma and lymph) that contain glycoproteins with terminal (nonreduced) mannose in their N-linked high-mannose or hybrid type oligosaccharides (e.g., orosomucoid, serum transferrin, and others). Indeed, we show that blood plasma contains mannosylated glycoproteins in sufficiently high concentration to block antibody recognition of the active LD but not of the inactive form. It is important to stress that induction of the adaptive immune response by B cells initially involves interactions between low-affinity antibody and B-cell receptor–antigen (27, 28). Thus, ligand masking of the epitopes is likely to be especially effective at the early stages of the immune response. At the same time, response to the inactive conformation should be initiated much more effectively and should lead to the maturation of B-cell lines producing high-affinity antibodies that become active against natural inactive or active conformational forms of FimH.

Although we do not show directly the stage at which a differential response is initiated against different LD forms, we believe that the scenario presented is plausible. Indeed, in vivo ligand–receptor interactions have been reported for HIV envelope glycoprotein gp120 and CD4 receptor from primate T-helper cells (29). However, a conformational change occurring in gp120 upon CD4 receptor binding, rather than direct masking of the antigen-binding epitopes, was shown to prevent a neutralizing immune response. It is possible that masking of the binding pockets by interaction with receptors or soluble receptor-like compounds also could be a natural process used by bacterial pathogens to avoid induction of neutralizing antibodies against FimH or other lectin-like or protein-binding adhesins.

Although the phenomenon described here of increased immunogenicity of binding epitopes applies first of all to proteins that undergo conformational changes, we believe that the general principle of the functional inactivation of the binding site potentially could be important for a broader spectrum of binding proteins, including those that do not undergo major conformational changes, as long as the structure of the binding epitope is not altered significantly by the binding-inactivating alterations. Functional inactivation appears to be vital for immunogenicity from a number of perspectives. It opens the possibility of improved development of antimicrobial vaccines by inducing strong immune responses against conformational epitopes of binding sites of the target proteins. Such an approach also can be used

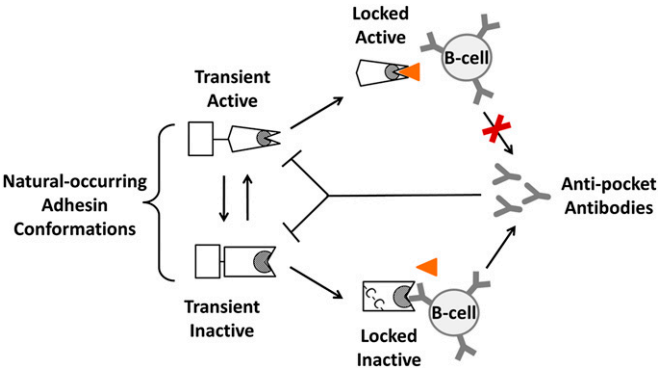

Fig. 6. Schematic representation of different conformational forms of the LD of FimH and their potency in inducing binding pocket-specific antibodies. The left rectangle represents the pilin domain, which is connected to the LD via a linker. The triangular indent on the LD represents the mannose-binding site, and the shaded portion represents the functional epitope. The orange triangle represents the mannose ligand.

in obtaining functional ligand-like antibodies for therapeutic or research purposes. Also, exposure of new epitopes that previously were hidden on self-antigens is one of the mechanisms underlying autoimmune diseases (30), although the mechanisms for unmasking self-epitopes are not fully understood. Our observations suggest that even a partial loss of the protein's ability to interact with its ligand (as the result of physiological dysfunction or a somatic mutation) could contribute to the uncovering of previously ligand-shielded self-epitopes. Understanding the mechanisms that lead to the exposure of self-epitopes is necessary to develop better therapies to treat and possibly prevent these diseases.

Materials and Methods

mAbs. The purified mutant LD (amino acids 1–160) was used to raise mouse mAbs by hybridoma technology by Covance Inc.. Hybridoma supernatants were screened for antigen recognition by ELISA using LD^{mut}-coated plates. The antigen-binding hybridoma clones were tested for IgG content by ELISA using HRP-conjugated anti-mouse IgG antibody (Bio-Rad). In all comparative experiments concentrations of IgG in hybridoma supernatants were adjusted to the same level.

HRP-Binding Assay. Microtiter plates (96 wells) were coated with purified LD (50 μg/mL) or fimbriae (0.2 mg/mL) in bicarbonate buffer at pH 9.6. The immobilized LD was incubated with 50 μg/mL HRP (unless otherwise stated) for 45 min at 37 °C. After extensive washing, a colorimetric reaction was developed by adding 3,3′,5,5′-tetramethylbenzidine (TMB) to the wells, and absorbance was read at 650 nm. For inhibition, HRP binding was tested in the presence of 1:20 dilutions of hybridoma supernatants, 1% (wt/vol) α-methyl-D-mannopyranoside (αmm, hereinafter also called mannose), or 1:50 dilutions of polyclonal antisera from mice used for hybridoma production. In the experiment in which the reciprocal effect of mAb21 and mAb475 was analyzed, HRP was added to immobilized fimbriae that previously had been incubated with 1:20 diluted mAb21 and 1:20 diluted mAb475 alone or that had been incubated with both of these antibodies sequentially for 1 h each. A control experiment testing linearity of OD values in the range 0.1–1 and 0.1–2.5 affirmed the proportional relationship between values in these two ranges of measurements.

ELISA. Binding of mAbs to immobilized fimbriae or LD was carried out in 96-well microtiter plates. Both fimbriae and LD were immobilized in 0.02 M $NaHCO_3$ buffer for 1 h at 37 °C and then were washed twice with PBS. Fimbriae were immobilized at 0.2 mg/mL and LD at 0.05 mg/mL, unless otherwise stated. Plates were quenched for 15 min with 0.2% (wt/vol) BSA in PBS. The immobilized ligands were incubated with various dilutions of hybridoma supernatants (1:100–1:5,000) or 1–13 nM purified mAbs or 1:450 diluted polyclonal antisera from mice used for hybridoma production or preimmune serum for 1 h at 37 °C. Bound antibodies were detected with HRP-conjugated goat anti-mouse antibodies (Bio-Rad). For detection of biotinylated antibodies, HRP-conjugated streptavidin (Sigma) was used. In some experiments, immobilized LDs were probed with anti–tetra-His antibody (Qiagen). The reaction was developed using TMB as described above. For inhibition, mAb binding was tested in the presence of different dilutions of αmm or physiological concentrations of human blood plasma collected from healthy volunteers. In some experiments, Con A-agarose– or glutathione-agarose purified plasma was tested as an inhibitor. For epitope mapping, the wells in microtiter plates were coated with purified fimbriae at 0.3 mg/mL and were incubated with 1:800 diluted mAbs for 1 h at 37 °C.

Statistical Analysis. Each experiment was performed multiple times, and mean values from independent experiments were used for calculating the SD.

Detailed descriptions of all other materials and methods are provided in *SI Materials and Methods*.

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