Prediction of Glycolipid-Binding Domains from the Amino Acid Sequence of Lipid Raft-Associated Proteins: Application to HpaA, a Protein Involved in the Adhesion of *Helicobacter pylori* to Gastrointestinal Cells

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ABSTRACT: Protein−glycolipid interactions mediate the attachment of various pathogens to the host cell surface as well as the association of numerous cellular proteins with lipid rafts. Thus, it is of primary importance to identify the protein domains involved in glycolipid recognition. Using structure similarity searches, we could identify a common glycolipid-binding domain in the three-dimensional structure of several proteins known to interact with lipid rafts. Yet the three-dimensional structure of most raft-targeted proteins is still unknown. In the present study, we have identified a glycolipid-binding domain in the amino acid sequence of a bacterial adhesin (*Helicobacter pylori* adhesin A, HpaA). The prediction was based on the major properties of the glycolipid-binding domains previously characterized by structural searches. A short (15-mer) synthetic peptide corresponding to this putative glycolipid-binding domain was synthesized, and we studied its interaction with glycolipid monolayers at the air−water interface. The synthetic HpaA peptide recognized LacCer but not Gb3. This glycolipid specificity was in line with that of the whole bacterium. Molecular modeling studies gave some insights into this high selectivity of interaction. It also suggested that Phe147 in HpaA played a key role in LacCer recognition, through sugar−aromatic CH−π stacking interactions with the hydrophobic side of the galactose ring of LacCer. Correspondingly, the replacement of Phe147 with Ala strongly affected LacCer recognition, whereas substitution with Trp did not. Our method could be used to identify glycolipid-binding domains in microbial and cellular proteins interacting with lipid shells, rafts, and other specialized membrane microdomains.

The first step of bacterial infection is the attachment to the host cell surface. It is generally admitted that many bacteria, as well as their toxins, interact specifically with discrete regions of the plasma membrane that are rich in cholesterol, sphingomyelin, and glycosphingolipids (*1*). These membrane microdomains, usually referred to as lipid rafts, are attractive to a wide range of pathogens including viruses, bacteria, parasites, and even prions (*2*). However, the molecular mechanisms involved in raft−pathogen interactions are still poorly understood. In particular, the characterization of structural motifs ensuring protein binding to glycolipid receptors is of primary importance. Recently, structure similarity searches allowed us to identify a common glycolipid-binding domain in HIV-1 surface envelope glycoprotein gp120, Alzheimer's β-amyloid peptide, and the cellular isoform of the prion protein (PrP^c) (*3*). This motif consists of a hairpin structure containing a water-exposed aromatic residue. A similar glycolipid-binding domain was then detected in CLN3, a protein involved in the transport of glycolipids to lipid rafts (*4*), in the bile salt-dependent intestinal lipase (*5*), and in the bacterial Shiga-like toxin (*6*). As many bacteria attach to host cells through binding of their adhesins to cell surface glycolipids (*7*), it is rational to search for glycolipid-binding domains in bacterial adhesins. The aim of the present study was to identify a domain of this kind in the adhesin of *Helicobacter pylori (H. pylori*),[1] a bacterium which is one of the most common pathogens in humans (*8*).

H. pylori is the primary cause of chronic gastritis and plays a pivotal role in the development of peptic ulcer disease (*9*). Moreover, persistent infection with *H. pylori* is a risk factor for the development of adenocarcinoma and lymphoma of the stomach (*10*). *H. pylori* interacts with selected glycolipids such as lactosylceramide (Galβ1−4Glcβ1−Cer; LacCer), while it totally ignores other glycolipid species such as globotriaosylceramide (Galα1−4Galβ1−4Glcβ1−Cer; Gb3) (*11*). The *H. pylori* adhesin A (HpaA) has been shown to mediate the binding of the bacteria to sialic acid-containing host molecules expressed on the surface of gastrointestinal cells (*12*). Correspondingly, *H. pylori* adherence could be inhibited by 3′-sialyllactose (NeuAcα2−3Galβ1−4Glc; 3′SL), a natural sialylated oligosaccharide, as well as by synthetic multivalent sialylated compounds (*13*). Except in specific long-passaged bacterial strains (*14*), HpaA is associated with the outer surface of the bacteria. As HpaA is highly conserved among *H. pylori* isolates, it is considered as a potential vaccine antigen (*15*). Given the prominent role

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[1] Abbreviations: $\Delta\pi$, variation of surface pressure; $\Delta\pi_{max}$, maximal surface pressure increase; HpaA, *Helicobacter pylori* adhesin A; *H. pylori*, *Helicobacter pylori*; Gb3, globotriaosylceramide; LacCer, lactosylceramide; π_i, initial surface pressure; 3′SL, 3′-sialyllactose.

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played by HpaA in *H. pylori* adhesion to host cells, it was sound to search for a potential glycolipid-binding domain able to recognize LacCer in the three-dimensional structure of this adhesin. Unfortunately, the structure of HpaA has not been resolved at the atomic level. Thus, we tried to identify the putative glycolipid-binding domain in the amino acid sequence of the protein deduced from the nucleotide sequence of its gene (*12*).

EXPERIMENTAL PROCEDURES

Synthetic Peptides. The synthetic peptides derived from HpaA (wild type, KKSEPGLLFSTGLDK; mutants, KKSEPGLLASTGLDK and KKSEPGLLWSTGLDK) and Shiga-like toxin 1 (wild type, KVGDKELFTNRWNLQ; mutant, KVGDKELATNRANLQ) were purchased from Eurogentec (Seraing, Belgium). The peptides were purified by high-performance liquid chromatography (purity >95%) and characterized by electrospray mass spectrometry (experimental M_r for wild-type HpaA and Shiga peptides were 1619.2 and 1847.2, respectively).

Surface Pressure Measurements. The surface pressure was measured with a fully automated microtensiometer (μTROUGH SX; Kibron Inc., Helsinki, Finland). All experiments were carried out in a controlled atmosphere at 20 ± 1 °C. Monomolecular films of the indicated glycolipids were spread on pure water subphases (volume of 800 μL) from hexane−chloroform−ethanol (11:5:4 v/v/v) as described previously (*3*). After spreading of the film, 5 min was allowed for solvent evaporation. To measure the interaction of the synthetic peptide with glycolipid monolayers, various concentrations of the ligand (0.5−10 μM) were injected in the subphase with a 10 μL Hamilton syringe, and pressure increases produced were recorded until the equilibrium was reached. The data were analyzed with the Filmware 2.5 program (Kibron Inc.). The accuracy of the system under our experimental conditions was ±0.25 mN·m^{-1} for surface pressure. The Origin software was used for statistical analysis and curve fitting.

Molecular Modeling. Molecular structures were visualized using the SWISS-PDB viewer (*16*) and the PyMol molecular garphics system (*17*). The amino acid sequence of HpaA was obtained trough the Swiss-Prot server (identification number Q48264). Molecular modeling of the HpaA peptide was performed with Hyperchem 7 (Cambridge Soft) and SWISS-PDB viewer on the basis of the structure of the HIV-1 gp120 V3 loop (Cambridge, MA). The molecular model of the toluene−galactose complex in water was obtained with Hyperchem 7.

RESULTS

First, we summarized the main properties of the glycolipid-binding domains identified so far by the combinatorial extension method (Supporting Information, Table 1) and checked if any sequence of HpaA could fulfill these criteria. The three-dimensional structure of a typical glycolipid-binding domain (HIV-1 gp120 V3 loop) is shown in Figure 1A. The motif consists of a hairpin structure containing (i) a solvent-exposed aromatic residue (Phe, Tyr, or Trp), (ii) several charged residues (Asp, Glu, Arg, or Lys) with some of them oriented toward the solvent, and (iii) a Gly and/or a Pro residue inducing the turn in the backbone Cα chain.

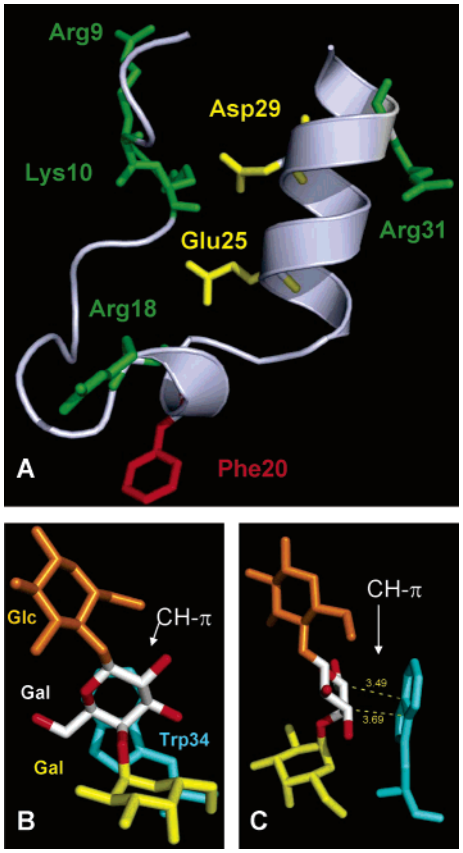

FIGURE 1: Three-dimensional structure of the glycolipid-binding domain and its interaction with GSL. (A) HIV-1 gp120 V3 loop (PDB entry 1CE4). The presence of at least one aromatic residue, which has to be fully exposed to the solvent, is required (here Phe20, in red). Basic amino acid residues are in green and acid ones in yellow. (B, C) Shiga-like toxin complexed with Gb3 (PDB entry 1CQF). The stacking interaction is driven by the proximity of the aliphatic protons of the sugar ring, which carry a net positive partial charge, and the π-electron cloud of the aromatic ring (here Trp34 of Shiga-like toxin, in cyan). It can thus be considered as a limit case of hydrogen bonding, in which the acceptor group is the electron cloud of the aromatic ring and the donor group is a C−H of the sugar ring. For this reason, it is usually referred to as CH−π interaction. The glycone part of Gb3 consists of two Gal residues (in yellow and white/red) and one Glc residue (in orange). Distances (dotted yellow lines) are expressed in Å.

The charged amino acid residues (in green and yellow) may interact with the charged polar head of membrane lipids (in particular, sphingomyelin and gangliosides in lipid rafts). The presence of at least one aromatic residue (Phe, Tyr, and/or Trp), which has to be fully exposed to the solvent, is required (here Phe20, in red). Indeed, a six-carbon sugar ring belonging to the polar head of the glycolipid receptor provides a complementary surface for this aromatic side chain. The stacking interaction is driven by the proximity of the aliphatic protons of the sugar ring, which carry a net positive partial charge, and the π-electron cloud of the aromatic ring (Figure 1B,C). It can thus be considered as a limit case of hydrogen bonding, in which the acceptor group is the electron cloud of the aromatic ring and the donor group is a C−H of the sugar ring. For this reason, it is usually referred to as CH−π interaction (*18*).

On the basis of these key features, secondary structure predictions together with the search for a specific cluster of amino acid residues (Gly, Pro, Asp/Glu/Arg/Lys, Phe/Tyr/Trp) may reveal the presence of a glycolipid-binding motif

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1
MKTNGHFKDFAWKKCLLGTSVVALLVGCSPHIIETNEVALKLNY

HPASEKVQALDEKILLLKPAFQYSDNIAKEYENKFKNQTTLKVE

EILQNQGYKVINVDSSDKDDFSFAQKKEGYLAVAMIGEIVLRPD
      139               153
PKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFVKVTILEPMSGES

LDSFTMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAI
                                          260
KSALNKIFASIMQEMDKKLTQRNLESYQKDAKELKNKRNR
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FIGURE 2: Amino acid sequence of the *H. pylori* adhesin HpaA. The putative glycolipid-binding domain is indicated by a frame.

in a protein whose structure has not been elucidated, as is the case for most bacterial adhesins. Aromatic residues are generally buried in the hydrophobic core of proteins. The presence of charged amino acids within the motif may significantly increase the probability of aromatic side chains to be expressed on the surface of the protein. Indeed, the conservation of Trp and Phe residues on the protein surface indicates a highly likely binding site, as shown by a systematic study on the biochemical nature of protein binding sites (*19*). Applying this algorithm to the sequence of HpaA allowed identification of one motif (amino acids 139−153) which fulfilled all of these criteria (Figure 2): an aromatic Phe residue (F147), a turn in the chain (induced by P143 and G144), and charged amino acids in the vicinity of the aromatic F147 (K139, K140, E142, D152, K153).

On the basis of this prediction, a 15-mer synthetic peptide corresponding to the proposed glycolipid-binding motif (139-KKSEPGLLFSTGLDK-153) has been synthesized. Its ability to interact with LacCer, one of the main glycolipid receptors for *H. pylori*, was investigated using the Langmuir film balance technique (*20*). LacCer was prepared as a monolayer at the air−water interface, and the synthetic HpaA peptide was injected in the aqueous phase. As shown in Figure 3a, the peptide induced a dramatic increase of the surface pressure ($\Delta\pi$) of the LacCer monolayer during the first 2 h of incubation, followed by a stabilization during the third hour. In contrast, the synthetic HpaA peptide did not interact with a monolayer of the glycosphingolipid Gb3, a control glycolipid which is not recognized by the whole bacterium (*11*). Therefore, it appeared that the interaction of the HpaA peptide with glycolipids is highly specific and corresponds to the specificity of interaction of *H. pylori* with its glycolipid receptors. Because the initial surface pressure of the monomolecular film of LacCer may affect the insertion of the peptide, we studied the interaction with different films prepared at various values of the initial surface pressure (π_i). In these expriments, it is expected that the ability of the peptide to interact with the glycolipid monolayer gradually decreases as π_i increases. This is because, at high surface pressures, the densely packed glycolipids do not allow the insertion of the peptide in the monolayer. As shown in Figure 3b, the interaction between the HpaA peptide and LacCer monolayers clearly followed this rule, which argues in favor of a specific interaction between the peptide and the glycolipid (*21*). The specificity of the LacCer−peptide interaction was further demonstrated by the lack of interaction of the peptide with Gb3 at all surface pressures tested.

The biological activity of Gb3 was checked by probing a Gb3 monolayer with a synthetic peptide derived from the glycolipid-binding domain of Shiga-like toxin 1. This toxin is known to interact with Gb3 (*6*). A synthetic Shiga peptide was specifically designed for these experiments (23-KVGD-KELFTNRWNLQ-37). This 15-mer peptide contained the key features of a glycolipid-binding domain, i.e., a hairpin structure with solvent-exposed aromatic acid residues (F30, W34). As shown in Figure 4a, the Shiga peptide induced a marked increase of the surface pressure of the Gb3 monolayer. One should note that the replacement of aromatic residues by Ala (F30A/W34A double mutant) strongly affected the interaction, consistent with structural studies (Figure 1B,C). The effect of the wild-type synthetic Shiga peptide regularly decreased when the experiment was performed at increasing values of the initial surface pressure (Figure 4b). These data demonstrated that the Gb3 monolayers were fully active. Thus, the lack of interaction of the HpaA peptide with Gb3 could not be attributed to an artifact linked to an impaired organization of Gb3 receptors in the monolayer prepared at the air−water interface. On the basis of these data, we could conclude that the synthetic HpaA peptide has the same specificity of interaction as the whole *H. pylori* bacterium.

Given the prominent role of aromatic residues in protein−sugar interactions (*6*), we synthesized a HpaA peptide in which the Phe147 residue was replaced with either Trp or Ala and analyzed the interaction of these mutant peptides with LacCer. When assayed at low concentrations (0.5 μM, i.e., the saturating concentration for the wild-type peptide), the F147A mutant did not interact with the LacCer monolayer, whereas both the wild type and the F147W induced a comparable increase of the surface pressure (data not shown).

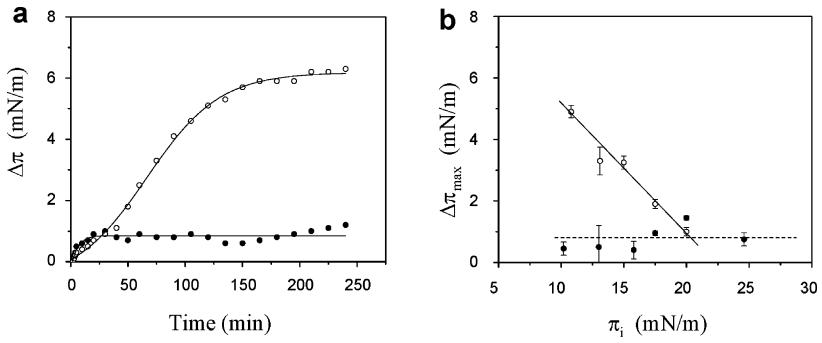

FIGURE 3: Interaction of the synthetic glycolipid-binding domain derived from HpaA with LacCer and Gb3. (a) Kinetics of interaction of the HpaA peptide with a monolayer of LacCer (○) or Gb3 (●). (b) Interaction of the HpaA peptide with monolayers of LacCer (○) or Gb3 (●) prepared at various values of the initial surface pressure (π_i). Results are expressed as means ± SD of three separate experiments.

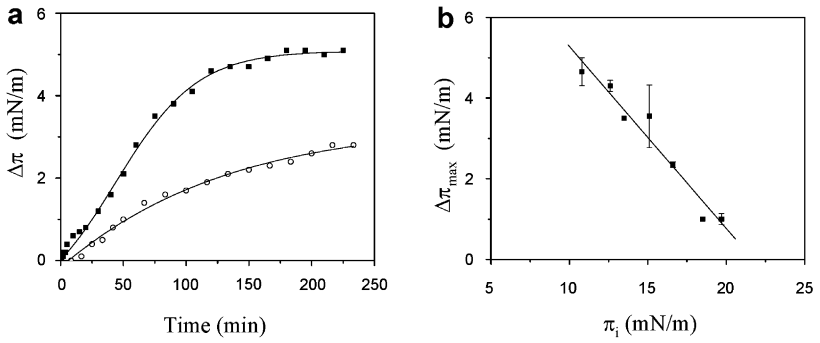

FIGURE 4: Interaction of the synthetic glycolipid-binding domain derived from Shiga toxin with Gb3. (a) Kinetics of interaction of the wild-type Shiga peptide (●) or the F30A/W34A double mutant (○) with a monolayer of Gb3. (b) Interaction of the wild-type Shiga peptide with monolayers of Gb3 prepared at various values of π_i. Results are expressed as means ± SD of three separate experiments.

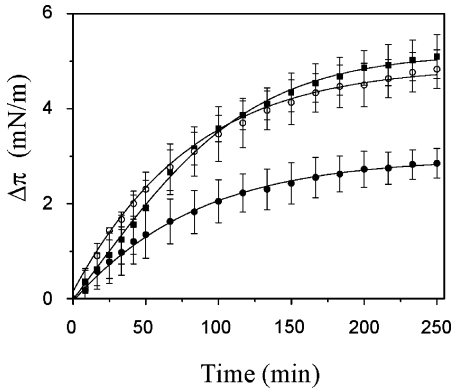

FIGURE 5: Importance of the aromatic residue 147 in the synthetic HpaA glycolipid-binding domain for LacCer recognition. The wild-type (■) or mutant peptides F147W (○) and F147A (●) were incubated in the aqueous phase underneath a LacCer monolayer. Final concentrations of the peptides were 10 μM. Changes in surface presion were continuously recorded as a function of time. The results are expressed as means ± SD of five separate experiments.

A series of experiments were then conducted at high peptide concentrations (10 μM, i.e., 20 times the saturating concentration of the wild-type peptide) in order to characterize the glycolipid-binding activity of these peptides under oversaturating conditions. As shown in Figure 5, the wild-type and F147W mutant induced a rapid increase of the surface pressure. The equilibrium was reached in both cases after 2 h of interaction: at this time, the maximal surface pressure increases ($\Delta\pi_{max}$) were 5.10 ± 0.46 and 4.83 ± 0.40 mN·m^{-1}, respectively, for the wild type and the F147W mutant. In comparison, the F147A mutant induced a slower and weaker increase of the surface pressure, with a $\Delta\pi_{max}$ of 2.85 ± 0.31 mN·m^{-1}. Overall, these data underscored the binding specificity of the synthetic glycolipid-binding domain predicted from the HpaA adhesin and emphasized the prominent role of the aromatic residue at position 147 for glycolipid recognition.

DISCUSSION

The putative glycolipid-binding domain of HpaA has been predicted from the amino acid sequence of the adhesin, which has been deduced from the nucleotide sequence of the adhesin gene. In this respect, the design and chemical synthesis of a functional glycolipid-binding domain exhibiting such a high selectivity for glycolipid receptors are remarkable. Yet the nature of the molecular mechanisms responsible for the specific interaction of the peptide with

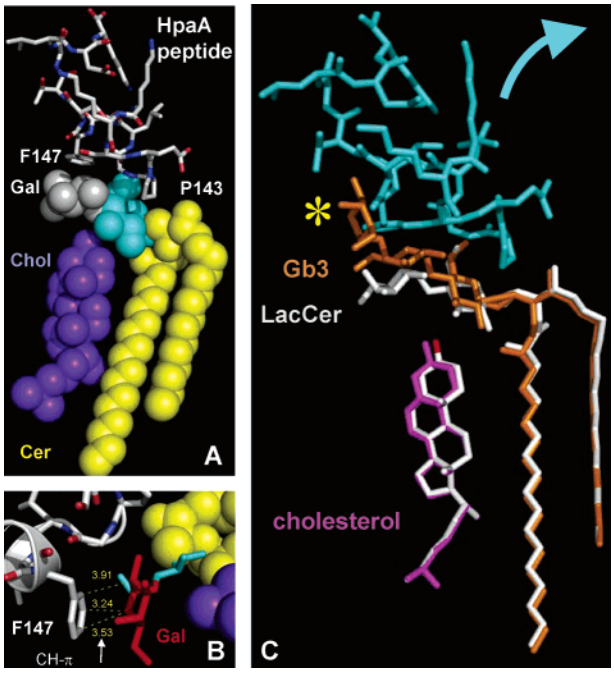

FIGURE 6: Models showing the interaction of the synthetic HpaA peptide (residues 143-PGLLFST-149) with LacCer (panels A and B) or LacCer superimposed with Gb3 (panel C). Panel A: Gb3 is represented with three colors: yellow (ceramide backbone), blue (glucose), and gray (galactose). Cholesterol is in purple. Panel B: Note the stacking CH−π interaction (<4 Å) between the galactose residue of LacCer (in red) and the aromatic Phe residue of the HpaA peptide (in white) (arrow). Panel C: LacCer is in white, Gb3 in orange, and the HpaA peptide in blue. The asterisk in yellow indicates the steric clash that renders impossible the interaction of the peptide with Gb3. The blue arrow shows the repulsion of the peptide when LacCer is replaced with Gb3. Note that the position of cholesterol is only slightly affected by the nature of the glycolipid (in white with LacCer, in purple with Gb3).

LacCer was intriguing, as Gb3 has just one more galactose residue than LacCer. In order to get insights into these mechanisms, we used a molecular modeling approach. The three-dimensional structure of the synthetic glycolipid-binding peptide was modeled onto the V3 domain of HIV-1 gp120 (our prototype glycolipid-binding domain) using the Protein Data Bank model 1CE4. The structural motif was then injected into the Hyperchem 7 program for energy minimization and then merged with LacCer (Figure 6A,B) or Gb3 (Figure 6C). A cholesterol molecule was also placed in the vicinity of each glycolipid to mimic the organization of lipid rafts in the plasma membrane (*22*). Molecular mechanics simulations were then performed with the MM+

field force as previously reported (*23*). As shown in Figure 6A, a very good fit between LacCer and the peptide was obtained. The Phe147 side chains stacked onto the hydrophobic side of the galactose ring of LacCer, which stabilized the complex through a series of CH−π stacking interactions. These interactions are frequently observed between sugars and lectins (*24*) and are the most common feature of glycolipid−protein interactions (*25*). They occur when the carbon−carbon distance between the sugar ring and the aromatic cycle is <4 Å (*18*, *26*). The Pro143 residue lies in a sphingolipid-specific cavity of LacCer, which is located in the ceramide backbone of the glycolipid (Figure 6A,B). The cholesterol molecule fitted with the surface of the hydrophobic part of LacCer, forming a complex that is consistent with the existence of packed sphingolipid−cholesterol domains (i.e., lipid rafts) in the external leaflet of the plasma membrane (*2*, *22*). When Gb3 was injected in place of LacCer, it became apparent that this glycolipid could not bind to the peptide as LacCer does, because of a steric hindrance caused by the third sugar of the glycone part of Gb3, i.e., galactose (Figure 6C). Moreover, no obvious fit could be obtained by molecular modeling of Gb3 and the synthetic HpaA peptide, explaining the lack of interaction between these partners in our monolayer assay.

Overall, this modeling study is in perfect agreement with the monolayer experiments performed with mutant peptides. Namely, the replacement of Phe by another aromatic residue (i.e., Trp in the F147W mutant HpaA peptide) had virtually no impact on LacCer recognition. Thus, it is clearly the aromatic surface and not the bona fide structure of the side chain at position 147 that is reponsible for glycolipid recognition. Indeed, in the absence of aromatic residues (i.e., the F147A mutant), the peptide showed only a weak interaction with the glycolipid, even at oversaturating concentrations (Figure 5). Similar data were obtained with a mutant Shiga peptide lacking its aromatic residues (i.e., the F30A/W34A mutant), which further confirmed the prominent role of sugar−aromatic interactions in glycolipid−protein complexes (*24*, *27*, *28*).

With the noticeable exception of glucosylceramide (a metabolic intermediate for more complex glycosphingolipids) and fucosylceramide (a tumor marker), most if not all membrane glycolipids contain at least one accessible galactose residue in their glycone part (*29*). Compared with D-glucose or other natural D-hexoses, D-galactose is unique in that the H atoms linked to the C3, C4, and C5 of the sugar ring form a "hydrophobic" surface. This hydrophobic side of galactose is particularly adapted to establish stacking CH−π interactions with aromatic cycles such as toluene. Molecular dynamics simulations suggested that toluene could interact with the hydrophobic side of galactose in aqueous solution (Figure 7). As the structure of toluene is close to the side chain of Phe, one could reasonably conclude that stacking CH−π interactions between galactose and surface-exposed aromatic side chains could significantly contribute to carbohydrate recognition by glycolipid-binding proteins. Nevertheless, it should be pointed out that, apart from stacking CH−π interactions, classical H-bonds are also frequently observed in sugar−protein interactions (*27*−*31*). In this respect, mutants Shiga and HpaA peptides in which the aromatic residues were substituted with Ala were still able to interact with their glycolipid receptors, although with a marked lower efficiency (Figures 4 and 5). Therefore, further studies with a panel of modified synthetic peptides will provide a more detailed analysis of the amino acid residues involved in HpaA−LacCer interaction. In particular, it will be of interest to evaluate the contribution of turn-inducing residues as well as charged residues of the glycolipid-binding domain.

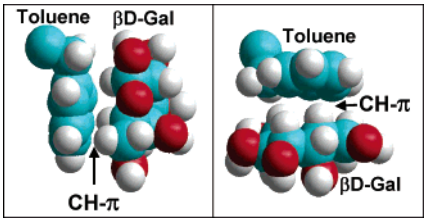

FIGURE 7: Molecular modeling of a toluene−galactose complex in water. The geometry of toluene and β-D-galactose (βD-Gal) was mimimized with the Polak−Ribiere algorithm. The molecules were then introduced in a periodic box of 18.70 Å^3 in the presence of 216 water molecules. Molecular dynamics simulations with the MM+ field force were then conducted for 10 ps with Hyperchem 7 software. Two distinct views of the complex are shown, without the surrounding water molecules.

The glycolipid-binding domain we have characterized in HpaA adhesin is contiguous to amino acids 134−139 (KRTIQK), which form a putative 3′SL binding motif (*32*). The latter sequence does not contain any aromatic residue but is rich in basic amino acids that could interact with the negatively charged sialic acid of 3′SL. We would like to emphasize that we do not suggest that the glycolipid-binding motif formed by residues 139−153 of HpaA controls the whole adhesion process of *H. pylori* to the surface of host cells. As a matter of fact, *H. pylori* expresses several adhesins and may interact with a wide variety of glycolipids and proteins (*33*, *34*).

As a matter of fact, the experimental approach developed here demonstrates that it is possible to predict the presence of a glycolipid-binding domain in a protein on the basis of its amino acid sequence. In the model of the lipid shell proposed by Anderson and Jacobson (*35*), proteins targeted to lipid rafts interact preferentially with cholesterol and sphingolipids, so that these proteins have an affinity for preexisting rafts. Therefore, the characterization of structural motifs involved in the interaction of proteins with raft lipids is required to validate the concept. Computational structure similarity searches resulted in the finding of a common glycolipid-binding domain in several unrelated proteins (*2*−*6*). The structural signature of the motif, namely, a surface-exposed aromatic residue and a turn, was sufficient to predict and synthesize a functional and highly selective glycolipid-binding domain from the sequence of a bacterial adhesin. As discussed above, such domains may also exist in various galactose-binding proteins such as lectins, although in this particular case there is no absolute requirement for a turn (*27*).

In conclusion, our strategy may allow the identification of specific glycolipid-binding motifs in the sequence in a wide range of pathogen and cellular proteins known to interact with glycolipid receptors. As structural data are lacking for most membrane proteins, our approach may also be used to characterize such glycolipid-binding domains in proteins that are encased in a lipid shell prior to their targeting

to lipid rafts. This is currently under investigation in our laboratory.

SUPPORTING INFORMATION AVAILABLE

One table listing the amino acid sequences of all glycolipid-binding domains that have been identified by the combinatorial extension method. This material is available free of charge via the Internet at http://pubs.acs.org.

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