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Identification of a Carbohydrate Recognition Domain in Filamentous Hemagglutinin from *Bordetella pertussis*

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The adherence of *Bordetella pertussis* to ciliated cells and macrophages is critical to colonization and infection of the respiratory tract. Adherence to both types of cells involves the recognition of eukaryotic carbohydrates by the bacterial adhesin filamentous hemagglutinin (Fha). The carbohydrate recognition domain (CRD) of Fha is considered an important antigen for subcomponent vaccines to maximize the generation of antiadherence antibodies capable of protecting against colonization. For identification of the CRD of Fha, a bank of eight monoclonal antibodies (MAbs) that mapped to four contiguous regions were tested for their ability to block Fha binding to lactosylceramide or to block bacterial binding to ciliated cells. Only MAb 12.5A9, which maps to amino acid residues 1141 to 1279, blocked both Fha binding to lactosylceramide and bacterial binding to ciliated cells. An 18-kDa polypeptide corresponding to this region was expressed in *Escherichia coli*. Cell lysates containing this protein bound to lactosylceramide in a manner identical to that of native Fha. Mutant strains of *B. pertussis* that contained an in-frame deletion of the coding sequence for this region produced a truncated Fha that showed negligible cross-reactivity with MAb 12.5A9. In an adherence assay, these mutant strains failed to bind efficiently to either ciliated cells or macrophages. The numbers of adherent bacteria for these strains were reduced to the number obtained with a nonadherent strain. We conclude that the region defined by residues 1141 to 1279 of Fha constitutes a CRD critical for bacterial adherence and represents a potential candidate for a subcomponent vaccine.

Bordetella pertussis adheres specifically to ciliated cells by using two secreted proteins, filamentous hemagglutinin (Fha) and pertussis toxin (Ptx) (20), which are expressed only by virulent cells. These two proteins serve as bifunctional ligands bridging the bacterial surface to glycoconjugates on the ciliary membrane. Although both adhesins have multiple binding affinities, it is the ability to bind lactosamines that is involved in the adherence of bacteria to their natural targets, ciliated cells (19). Interruption of this interaction in animal models by introduction into the trachea of the receptor analog lactose results in abortion of colonization of the respiratory tract (13). On the basis of available evidence, this property would most likely be attributable to the carbohydrate binding domains of Fha and Ptx. The carbohydrate recognition domains (CRDs) of Ptx have been localized to subunits S2 and S3, with the recognition of ciliary lactosamines being a property of S2 (12).

Fha is a 220-kDa protein with an affinity for lactosylceramides when assayed in direct binding studies with extracts of ciliated cells or standard glycolipids (19). Lactosamines or antilactosamine antibodies inhibit the adherence of *B. pertussis* to human ciliated cells in vitro and virtually eliminate the colonization of rabbit lungs by virulent *B. pertussis* (13, 19). Other affinities, such as those to sulfatides, have been shown to be involved in Fha-mediated bacterial adherence to tissue culture cells (1) but are of unknown importance in binding to respiratory cells or leukocytes.

The ability to inhibit the adherence of *B. pertussis* to ciliated cells distinguishes antisera from children recovering from pertussis from antisera from those receiving a whole-cell vaccine (21). An optimal subcomponent vaccine should also inhibit colonization. In an effort to identify a candidate for such a vaccine, we identified the CRD of Fha that binds lactosamines and mediates the adherence of whole bacteria to ciliated cells and macrophages.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. pertussis* strains used in this study are summarized in Table 1. The *Escherichia coli* strains used were DH5α, which is F^- ϕ80d*lacZ* Δ(*lacZYA*ΔM15)*U169 recA1 endA1 hsdR17* ($r_K^-m_K^+$) *supE44* λ^- *thy-1 gyrA relA1* (Bethesda Research Laboratories); SM10, which is *thi thr leu* su_{III} RP4-2-Tc::Mu (14); and K38 containing pGP1-2 (18).

B. pertussis was grown on Bordet-Gengou agar supplemented with 10% sheep blood or in Stainer-Scholte medium (15). *E. coli* was grown in liquid or on solid Luria-Bertani medium. In some instances, ampicillin and kanamycin were used at concentrations of 100 and 40 μg/ml, respectively, for *E. coli*. When required, streptomycin, gentamicin, and kanamycin were used at concentrations of 300, 10, and 20 μg/ml, respectively, for *B. pertussis*. Conjugal transfer and allelic exchange of *B. pertussis* DNA fragments were performed as described elsewhere (16).

Expression vector construction. An expression vector was created to generate a polypeptide corresponding to amino acid residues 1141 to 1279 of Fha, a region that contains the putative CRD. From a plasmid-borne copy of *fhaB* (2, 8), a 410-bp fragment corresponding to this region was amplified by the polymerase chain reaction. The primers dAATTAA

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TABLE 1. *B. pertussis* strains

Strain (description)	Relevant characteristic(s)	Source or reference
BP536 (parental)	Str^r Nal^r	8
BP537 (*vir*)	Spontaneous avirulent derivative of BP536	8
BP-TOX6 (*ptx*)	Ptx^-	7
BPRU246 (*fhaB* with a deletion of the CRD; *ptx*)	FhaΔCRD[a]; Ptx^-; derivative of BP-TOX6	Current study
BPRU247 (*fhaB* with a deletion of the CRD)	FhaΔCRD; derivative of BP536	Current study

[a] FhaΔCRD, an internal in-frame deletion in *fhaB* produces a truncated Fha that lacks the CRD.

CATATGCTCGAGCATTCCACCATCGAG and dAATTA AGGATCCCTCGAGCACGCCCTGCTTGCC were used to generate a fragment compatible with the *Nde*I-*Bam*HI cloning sites of expression vector pET3a (9). The resulting plasmid, pSMP2, was transformed into *E. coli* K38 containing pGP1-2. Strains containing these two plasmids were used to express the *fhaB*-derived polypeptide.

Induced protein expression. To express the Fha-derived polypeptide generated from vector pSMP2, we used the T7 RNA polymerase system of Tabor and Richardson (18). In this system, the Fha-derived polypeptide is expressed from the T7 promoter, which is upstream from the coding sequence for the CRD. T7 RNA polymerase, under the control of the p_L promoter, is expressed in a temperature-dependent manner by *c*Its; both are present on plasmid pGP1-2. An *E. coli* strain containing pSMP2 and pGP1-2 was grown overnight at 30°C in Luria-Bertani medium. Bacteria were diluted into 10 ml of fresh medium and grown to an optical density at 650 nm of 0.45. Cultures were then placed at 42°C for 40 min. Cells were isolated by centrifugation, resuspended in 1/20 volume of 50 mM Tris-HCl (pH 7.6)–1 mM EDTA, and sonicated three times for 60 s each time to generate a cell lysate. Cellular debris was removed by centrifugation, and the supernatant, which contained expressed proteins, was characterized further. In some instances, the total cell pellet was resuspended in 2.5% sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and subjected to polyacrylamide gel electrophoresis.

Construction of *B. pertussis* mutants. Several strains of *B. pertussis* that produced a truncated form of Fha that lacked the CRD were created in either a parental or a *ptx* background. Our strategy was to create an in-frame deletion in the chromosomal copy of the *fhaB* gene that would delete the coding region for the CRD and leave the downstream region intact. A 2.3-kb *Bam*HI-*Bam*HI fragment corresponding to nucleotides 2376 to 2873 from *fhaB* was subcloned into pSS1189, which was specifically designed for the allelic exchange of unmarked mutations by conjugal transfer in *B. pertussis* (16, 17). A unique 0.4-kb *Xho*I-*Xho*I fragment (nucleotides 3674 to 4088 [8]) that encompasses the coding region for the CRD was removed by restriction enzyme digestion. Upon religation, the vector was transformed into *E. coli*. A strain containing the resulting plasmid, pSMP4, lacked the 0.4-kb fragment and was chosen for conjugal transfer of the plasmid to both BP536 and BP-TOX6. Allelic exchange of the unmarked mutation with appropriate antibiotic selection was initially confirmed by screening of exconjugants for the loss of cross-reactivity with monoclonal antibody (MAb) 12.5A9, which is specific for the CRD. One exconjugant (BPRU247) from mating to BP536 and one (BPRU246) from mating to BP-TOX6 were used in further studies. These strains still cross-reacted with a polyclonal antibody to Fha, confirming the continued expression of Fha. Antibody binding to individual colonies was determined by standard colony lift procedures (10). For confirmation of the loss of the 0.4-kb fragment from *fhaB*, chromosomal DNA from these mutant strains was prepared and amplified by the polymerase chain reaction. By use of the forward and reverse primers (AATCATATGACGGTGGCG GCGAACTCGCTG and AATGGATCCCGTGTTGGTGAA ACTCGCCAC), which correspond to sites 50 bp 5′ and 3′ of the *Xho*I site, respectively, the expected 100-bp fragment was produced through amplification. Furthermore, this fragment contained only a single *Xho*I site. Amplification of this fragment with DNA from the parental strains produced the predicted 500-bp fragment (data not shown).

Protein-glycolipid binding assays. Lactosylceramide, sulfatides, and mixed gangliosides (Matreya, Inc., Pleasant Gap, Pa.) were separated by thin-layer chromatography as described previously (19). Total lipids were detected on a control plate with anisaldehyde. Duplicate plates were plasticized with polyisobutyl methacrylate in diethyl ether, incubated with 2% bovine serum albumin, rinsed, and incubated for 3 h with purified Fha (List Biologicals, Campbell, Calif.), concentrated Fha from bacterial culture supernatants derived from *B. pertussis*, or sonicates of *E. coli* expressing the putative CRD. Plates were rinsed with saline and incubated for 3 h with 1 μg of anti-Fha MAb 12.5A9, which is specific for the CRD, or MAb 12.6F8, which is the positive control antibody that binds to the carboxy-terminal region of Fha, per ml (2). Plates were rinsed again and incubated for 3 h with a 1:50 dilution of the peroxidase-conjugated anti-goat Fc fragment (Cappel Laboratories, Philadelphia, Pa.). Following another wash, plates were developed with a peroxidase substrate.

Bacterial adherence to mammalian cells. The adherence of *B. pertussis* to rabbit ciliated respiratory epithelial cells was measured by a published procedure (20). In brief, 10^9 bacteria were incubated with 10^5 ciliated cells for 3 h at 37°C on a roller drum. Nonadherent bacteria were removed by washing over a Nuclepore membrane filter. Adherent bacteria were visualized by a direct fluorescent-antibody technique, and the number of bacteria per ciliary tuft was quanitated for 25 cells. Results are expressed as the mean number of bacteria per tuft per assay, and each assay was performed in duplicate on at least three occasions. For some assays, anti-Fha MAbs (200 μg/ml) that map to distinct regions of Fha (Fig. 1) were added to the bacteria for 30 min prior to the addition of ciliated cells.

Human monocytes were purified from buffy coats on Percoll gradients and cultured in 12.5% human serum in Teflon beakers as described previously (22). Macrophages were harvested after 5 to 10 days and suspended at 1.0×10^6/ml in phosphate-buffered saline containing 3 mM glucose, 0.5 mg of human serum albumin (Armour Pharmaceutia, Kankakee, Ill.) per ml, and 0.3 U of aprotinin per ml.

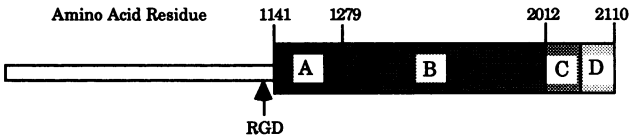

FIG. 1. Schematic representation of regions A to D of the Fha molecule (220 kDa) defined by the bank of MAbs listed in Table 2. This map is based on data from reference 2. The RGD (Arg-Gly-Asp) motif is involved in the binding of Fha to leukocyte integrin CR3 (7).

Terasaki tissue culture plates (Miles Laboratories Inc., Naperville, Ill.) were coated with human serum albumin (1 mg/ml) by incubation for 60 min at 20°C (24). The wells were washed, 5 μl of macrophage suspension was added per well, and the cells were allowed to spread at 37°C for 45 min. Bacteria were suspended in phosphate-buffered saline, washed, adjusted to a density of 2×10^8 CFU/ml, and labeled with fluorescein as described elsewhere (23). The attachment of *B. pertussis* to macrophages was determined by adding 2×10^5 fluorescein-labeled bacteria per well and incubating the mixture at 37°C for 30 min. The attachment of bacteria was scored by determining the mean number of bacteria on 100 macrophages by fluorescence microscopy. All assays were performed in triplicate on at least two occasions.

Hemagglutination assay. The hemagglutinating activity of culture supernatant fluids and bacterial cells was assayed with 0.7% goose erythrocytes as described previously (11).

RESULTS

MAb mapping of the CRD of Fha. A previous study described a bank of eight MAbs that mapped to four contiguous regions of Fha designated A, B, C, and D (Fig. 1) (2). In an attempt to identify the CRD of Fha, we tested these MAbs for their ability to block the binding of Fha to lactosylceramide and to inhibit the binding of *B. pertussis* to rabbit ciliated cells (Table 2). MAb 12.5A9, which maps to region A, blocked the binding of Fha to glycolipids and reduced the number of bacteria adherent to ciliated cells by 70%. These results suggested that the region of Fha corresponding to amino acid residues 1141 to 1279 contained a putative CRD that served as the adhesin between the bacteria and the ciliated cells.

TABLE 2. Effect of anti-Fha MAbs on Fha binding to glycolipids and bacterial adherence to ciliated cells

MAb	Region of Fha	Binding to glycolipids[a]	Bacterial adherence[b]
12.5A9	A	−	150 ± 22
13.6E2	A	−	320 ± 12
12.1F9	B	+	460 ± 51
12.2B11	C	+	590 ± 32
12.6F8	D	+	297 ± 38
12.2H5	H	+	610 ± 53
13.6H7	H	+	570 ± 47
13.2D10	H	+	540 ± 47
None		+	503 ± 16

[a] MAbs (100 μg) were premixed with Fha (50 μg) for 15 min. The mixture was overlaid onto thin-layer chromatography plates containing standard glycolipids, and the binding of Fha was detected with a goat anti-Fha polyclonal antibody (20) and a peroxidase-conjugated anti-goat antibody and by development with diaminobenzidine. Symbols: +, binding equivalent to native Fha binding; −, no binding. These MAbs mapped to distinct regions of Fha (Fig. 1) (2). MAbs in the H category block the hemagglutination of erythrocytes but have an undetermined epitope distinct from those of regions A to D.

[b] MAbs (100 μg) were premixed with BP536 (10^9 cells) for 30 min. The mixture was incubated with ciliated cells as described in Materials and Methods. Adherence is expressed as the mean ± standard deviation of the number of bacteria adherent to the ciliary tufts of 100 cells. Experiments were performed three times.

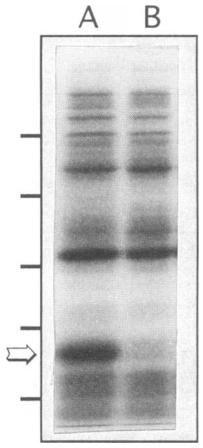

FIG. 2. Expression of the CRD of Fha in *E. coli*. An isogenic pair of *E. coli* strains containing pSMP4 (vector with an insert) (A) or pET3a (vector without an insert) (B) were induced by growth at 42°C to produce T7 RNA polymerase-dependent proteins. Bacteria were separated from media by centrifugation, resuspended in SDS sample buffer, and run on a 15% SDS-polyacrylamide gel. Total cell lysates (50 μg of protein) were applied to gel lanes, and the gel was stained with Coomassie blue. The arrow indicates the induced 18-kDa polypeptide; 18 kDa corresponds to the predicted molecular mass of the coding region inserted into the vector. Similar results were obtained with cultures grown with rifampin (data not shown). Molecular mass standards (97.4, 66.2, 45, 21, and 14.4 kDa bars from top to bottom) are indicated on the left.

Expression of the CRD of Fha in *E. coli*. If the region of Fha defined by MAb 12.5A9 were critical for binding to surface-exposed ciliary carbohydrates, then an 18-kDa polypeptide corresponding to this region may also have this property. The coding region for the CRD was obtained by the polymerase chain reaction from a plasmid-borne copy of *fhaB* and placed in the pET3a expression vector. Induction of an *E. coli* strain containing this vector produced an 18-kDa polypeptide (Fig. 2) that cross-reacted with polyclonal antiserum to Fha (data not shown).

Cell lysates of strains of *E. coli* with and without the expressed protein were overlaid on thin-layer chromatography plates containing glycolipid standards, and the binding pattern was compared with that of purified Fha. The lysate containing the 18-kDa polypeptide bound to lactosylceramide and sulfatides in a pattern similar to that of Fha (Fig. 3). This result suggests that the structural requirements for Fha to bind to these carbohydrates reside in this 18-kDa region of the protein.

A truncated Fha (FhaΔCRD) does not bind to carbohydrates. Strains (BPRU246 and BPRU247) of *B. pertussis* that produced Fha lacking the CRD (residues 1141 to 1279) were created by allelic exchange of an unmarked mutation. This truncated form of Fha (FhaΔCRD) was readily detected in the culture supernatants from these strains, and the amount of Fha associated with the parental strain was equivalent to the amounts of FhaΔCRD associated with the mutant strains

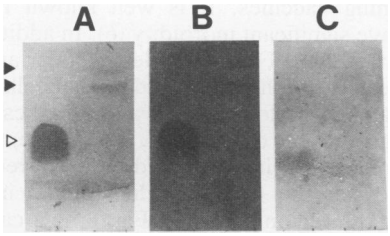

FIG. 3. Binding of the CRD of Fha to standard glycolipids. Three thin-layer chromatograms were made, with sulfatides in the left lane and lactosylceramide and gangliosides in the right lane. Chromatograms were overlaid as follows: A, Fha; B, *E. coli* lysate with the expressed putative CRD (depicted in lane A of Fig. 2); C, *E. coli* lysate without the expressed CRD (depicted in lane B of Fig. 2). Binding was detected with an anti-Fha polyclonal antibody and a peroxidase-conjugated second antibody and by development with a peroxidase substrate. Binding to lactosylceramide (solid arrows) and sulfatides (open arrow) was detected; no binding to gangliosides was found.

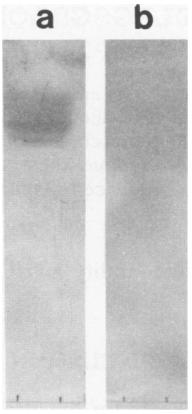

FIG. 5. Lack of binding of FhaΔCRD to lactosylceramide. Duplicate thin-layer chromatography plates containing lactosylceramide were overlaid with either partially purified Fha (50 μg of protein) from BP-TOX6 (a) or FhaΔCRD (50 μg of protein) from BPRU246 (b). Binding of the Fha molecules was detected with an anti-Fha polyclonal antiserum and a peroxidase-conjugated second antibody and by development with a peroxidase substrate.

when measured on Coomassie blue-stained polyacrylamide gels and in immunoblots from whole-cell lysates (Fig. 4). FhaΔCRD showed greatly reduced or absent reactivity with MAb 12.5A9 (Fig. 4). As expected, FhaΔCRD reacted with MAb 12.6F8, which is specific for region D. Indirect immunofluorescence labeling of avirulent bacterial strain BP537 exposed to Fha and FhaΔCRD indicated equivalent capacities of the two Fha molecules to bind to the bacterial cell surface. Both strains BP536 and BPRU247 retained the ability to hemagglutinate (60 and 80 units per 10^{10} cells), indicating that the surface localization and function of Fha and FhaΔCRD were similar. Taken together, these results suggest that deletion of the CRD did not affect the production, bacterial cell surface localization properties, or secretion of the truncated protein. However, in contrast to the parent molecule, FhaΔCRD failed to bind to lactosylceramide immobilized on thin-layer chromatography plates (Fig. 5).

***B. pertussis* mutants that produce FhaΔCRD fail to bind to macrophages and ciliated cells.** For confirmation that the loss of the CRD of Fha would interrupt the adherence of whole bacteria to eukaryotic target cells, BPRU246 and BPRU247, which produce FhaΔCRD, were tested for the ability to bind to rabbit or human macrophages and ciliated cells, respectively. Because *B. pertussis* can bind to macrophages via the CRDs of either Ptx or Fha (7, 13), the macrophage adherence assay was performed with Ptx^- bacteria to selectively evaluate Fha-dependent binding. Binding to the respective cell types for these mutants was reduced to the level of the avirulent control strain (Table 3). Since these mutant strains produced amounts of Fha equal to that produced by the parental strain, as assessed in cell extracts, and harbored the same amount of cell surface Fha, as assessed by fluorescence microscopy and hemagglutination assays, these results further suggest that the region deleted by the mutation contains the domain of Fha responsible for carbohydrate-dependent binding to both macrophages and ciliated cells. The fact that virtually all binding to macrophages was inhibited indicates that the deletion of the CRD may also affect the neighboring RGD domain, which accounts for

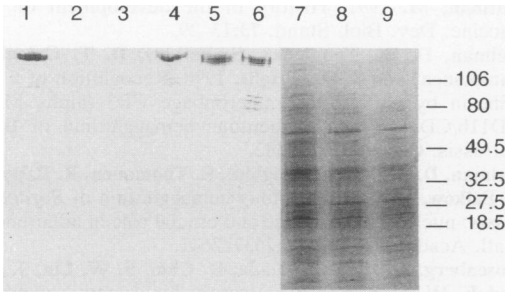

FIG. 4. Characteristics of FhaΔCRD. Whole-cell lysates of strains BPRU246 and BPRU247 producing FhaΔCRD were compared with a whole-cell lysate of wild-type parental strain BP536 for reactivity with anti-FHA MAbs. Lanes: 1, 4, and 7, BP536; 2, 5, and 8, BPRU246; 3, 6, and 9, BPRU247. Immunoblots were probed with MAb 12.5A9, which is specific for the CRD (lanes 1 to 3), or MAb 12.6F8, which recognizes the carboxy terminus of Fha (lanes 4 to 6). Fifty micrograms of total protein was loaded per lane and electrophoresed in triplicate gels. Coomassie blue-stained samples are depicted in lanes 7 to 9. Numbers at right are sizes in kilodaltons.

TABLE 3. Abrogation of bacterial adherence to ciliated cells and macrophages by deletion of the CRD of Fha

Cell type	Strain[a]	Adherence[b]
Ciliated cells	BP536	467 ± 56
	BPRU247	156 ± 23
	BP537	117 ± 13
Macrophages	BP-TOX6	343 ± 62
	BPRU246	51 ± 19
	BP537	36 ± 14

[a] Since both Fha and Ptx independently mediate bacterial adherence between bacteria and macrophages, while both adhesins together are required for adherence to ciliated cells, the adherence assays for these two different cell types were performed with two different bacterial genetic backgrounds. The adherence assays for ciliated cells were done with isogenic strains that were Ptx^+ (BP536 and BPRU247), while the adherence assays for macrophages were done with isogenic strains that were Ptx^- (BP-TOX6 and BPRU246). BP537 is a negative control and is $Fha^- Ptx^-$.

[b] Bacteria were incubated with cells as described in Materials and Methods. Adherence is expressed as the mean ± standard deviation of the number of bacteria adherent to 100 cells. Experiments were performed twice.

S2 30 LTVAELRGSGDLQEYLRHVTRG 51
Fha 1224 L VADG GPIVV EAGELVSHA 1242

FIG. 6. Comparison of the amino acid sequences of the lactosamine binding domain of S2 and the CRD of Fha. Letters indicate amino acids in the single-letter code. Boldfaced letters show positions of identity or conservative substitutions. Three gaps were introduced into the Fha sequence to facilitate alignment.

approximately half the ability of *B. pertussis* to bind to macrophages (7).

DISCUSSION

Fha is a critical adhesin for the attachment of *B. pertussis* to respiratory ciliated cells and alveolar macrophages. In both cases, Fha presents a CRD that recognizes lactosamines (19). The current study indicates that the region of Fha spanning residues 1141 to 1279 constitutes a CRD, on the basis of the following evidence. An antibody to this region blocks bacterial adherence to cilia. When cloned and expressed in *E. coli*, the polypeptide mapped by this antibody binds to lactosylceramide and sulfatides in a manner similar to that of the parent molecule. Conversely, FhaΔCRD (truncated Fha lacking the CRD) does not bind to these glycolipids, and strains of *B. pertussis* expressing FhaΔCRD fail to efficiently bind to ciliated cells or macrophages.

Binding to lactosylceramide is a property shared by Fha and the S2 subunit of Ptx (13). The lectin domain of S2 was recently localized to a short segment near the amino terminus (12). A comparison of the sequence of this S2 lectin domain with the sequence of the 139 amino acids of the CRD from Fha revealed a region of sequence similarity over a 20-amino-acid region (Fig. 6). This result suggests that the two proteins might use similar motifs to recognize their common carbohydrate target on respiratory epithelial cells and alveolar macrophages.

Fha has two other carbohydrate recognition properties that are not explained by the CRD defined in this study. It has been shown that an epitope near the N terminus and defined by MAb X3C blocks the hemagglutination of erythrocytes by Fha (5). This epitope also blocks the association of bacteria with Chinese hamster ovary cells in a sialic acid-dependent manner. The respiratory cells that might bear receptors for this domain are as yet unknown, so the role that this amino-terminal domain plays during natural infection remains speculative. The CRD described in our study differs in both the target cell specificity (ciliated respiratory cells and alveolar macrophages) and the preferred carbohydrate ligand (lactosamine). The distinction between the two domains is further underscored by the facts that the FhaΔCRD protein retains the ability to hemagglutinate and that antibodies tested in our study that block hemagglutination (Table 2, H) do not map to our CRD and do not block bacterial adherence to ciliated cells or macrophages.

The ability of antibodies to the CRD from Fha to block bacterial adherence to ciliated cells is crucial to the rational design of a subcomponent vaccine. The ability to generate antibodies that block adherence defines an immunogen that would be effective in protecting against colonization as well as the clinical disease whooping cough. Current vaccines do not eliminate colonization (6). A CRD-based vaccine would have several potential advantages over current whole-cell or Fha-containing vaccines. It is well known that pertussis vaccines have significant morbidity (6). In addition, vaccines containing the entire Fha molecule engender antibodies that are cross-reactive with human endothelial cells and C3bi (3, 4). Although the biological relevance of these cross-reactions remains to be determined, a vaccine that presents non-cross-reactive epitopes would be preferred. Therefore, we suggest that the CRD of Fha defined in this study, particularly the region shown in Fig. 6, is a candidate for a subcompent vaccine and will be tested in animal models of pertussis as a protective immunogen.

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