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Adhesion of *Bordetella pertussis* to eukaryotic cells requires a time-dependent export and maturation of filamentous hemagglutinin

(bacterial pathogenesis/Arg-Gly-Asp-containing domain/adhesion/protein export/pertussis toxin)

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ABSTRACT **_Bordetella pertussis_, the human pathogen of whooping cough, when grown at 22°C is nonvirulent and unable to bind eukaryotic cells. In response to a temperature shift to 37°C, the bacterium acquires the ability to bind eukaryotic cells in a time-dependent fashion. By studying _in vitro_ the temperature-induced transition, from the nonvirulent to the virulent state, we found that binding to CHO cells is mediated by the Arg-Gly-Asp-containing domain of filamentous hemagglutinin (FHA), a protein with multiple binding specificities. This protein is synthesized as a 367-kDa polypeptide within 10 min after temperature shift, but requires 2 hr before it is detected on the bacterial cell surface and starts to bind CHO cells. Mutations affecting the cell surface export of FHA abolish bacterial adhesion to CHO cells, while mutations in the outer membrane protein pertactin strongly reduce binding. This suggests that multiple chaperon proteins are required for a correct function of FHA. Finally, several hours after maximum binding efficiency is achieved, the N-terminal 220-kDa portion of FHA that contains the binding regions is cleaved off, possibly to release the bacteria from the bound cells and facilitate spreading. The different forms of FHA may play different roles during bacterial infection.**

Bordetella pertussis colonizes the respiratory tract by specific adhesion to the ciliated cells and alveolar macrophages. During infection it releases a number of toxins that cause the systemic symptoms of the disease. Bacterial adhesion and host intoxication require factors such as pertussis toxin (PTX), adenylate cyclase, filamentous hemagglutinin (FHA), pertactin (PRN; also called 69K), and fimbriae (1, 2). In response to environmental stimuli, the expression of these proteins, and of many others, is coordinately regulated at the transcriptional level by the *bvg* locus (3–8). Virulence factors are synthesized at 37°C, while their expression is repressed at 25°C or upon addition of $MgSO_4$ or nicotinic acid to the culture medium (2, 3, 9). FHA, PRN, PTX, and fimbriae have been implicated in bacterial adhesion (10–18); adenylate cyclase and PTX are mostly involved in the evasion of the host defenses and intoxication (1, 19). FHA is an extracellular protein that is presumably synthesized as a precursor of 367-kDa of which only the 220-kDa N-terminal region is present in the mature protein (11, 20). However, the 367-kDa polypeptide predicted from the nucleotide sequence of the *fhaB* gene has never been detected. Export of FHA requires the product of genes mapping downstream from the *fhaB* gene, which are predicted to code proteins homologous to chaperon proteins (21, 22). Purified FHA possesses at least three defined domains of protein-binding activity: (*i*) a domain that is specific for carbohydrate-binding activity and recognizes galactose-containing glycoconjugates on both cilia and macrophages (23), (*ii*) an Arg-Gly-Asp-containing domain (RGD domain) that promotes adherence to the macrophage integrin CR3 and possibly to other integrins, (24, 25), and (*iii*) a heparin-binding activity (26). The above properties are reminiscent of eukaryotic extracellular matrix proteins.

PTX is a major virulence factor released in the extracellular environment, intoxicates most tissues, induces metabolic changes in the host, and is the only toxin known to bind whole bacteria to eukaryotic cells (27, 28). Subunits S2 and S3 of PTX contain two different binding activities. S2 recognizes preferentially glycoconjugates on ciliated cells and S3 recognizes macrophages (29). PRN is an outer membrane protein of 61 kDa processed from a 92-kDa precursor (14). It has been shown that eukaryotic cells bind purified PRN *in vitro* by an RGD domain (15, 30). However, a direct role of PRN in binding bacteria to eukaryotic cells has not been demonstrated.

In this work, we synchronized the expression of the virulence regulon by shifting cultures of *B. pertussis* from 22°C to 37°C and studied the time required by the bacterium to acquire specificity of adhesion to eukaryotic cells and the role exerted by FHA, PRN, and PTX. This approach may be applied to study the pathogenesis of bacteria that survive in environments with different temperatures.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *B. pertussis* strains used in this study are reported in Table 1. Bacteria were grown on Bordet Gengou agar (Difco) supplemented with 15% sheep blood. Liquid cultures were performed in water-bath rotary shakers in 100-ml flasks containing 25 ml of Stainer–Scholte modified medium (34) at 22°C for 12–13 days. The exponential phase of growth was maintained by a periodic 1:10 dilution of the cultures and then shifted to 37°C. Samples of cells and culture supernatants were collected at various intervals of time.

Immunoblot Analysis. To obtain cell lysates, periplasmic fractions, and supernatant samples, 15 ml of cultures were harvested and resuspended in 25% sucrose/50 mM Tris·HCl, pH 8, at an OD_{590} of 0.3. Of this suspension, 100 μl was used for hemagglutination and 20 μl was processed for SDS gel electrophoresis and immunoblot analysis. Western blots were processed by standard procedures and stained by the chemiluminescence detection system (Amersham). FHA was detected with a 1:2500 dilution of a polyclonal goat antiserum raised against the purified 220-kDa protein. The precursor form of FHA was identified by using an antiserum generated in mouse against a 56-kDa recombinant fragment encoded by

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Abbreviations: FHA, filamentous hemagglutinin; PTX, pertussis toxin; PRN, pertactin; RGD domain, Arg-Gly-Asp-containing domain.

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Table 1. *Bordetella* strains used in the study

Strain	Description	Ref.
BP-W28	Bvg$^+$ Sm^r	18
BP-536	BP338 Bvg$^+$ Sm^r	27
BP-347	BP338 *bvgS*::Tn*5* (Bvg$^-$) Sm^r	6, 27
BP-101*	BP536 Δ(2.4 kb *Bam*HI)*fhaB* RGD$^-$	10, 31
BP-102	BP536 Δ(3.4 *Bam*HI–*Bgl*II)*fhaB* FHA$^-$	10, 31
BP-353	BP338 *fimC*::Tn*5* defective in FHA export	22, 27, 32
BP-354	BP338 *fimC/D*::Tn*5* defective in FHA export	22, 27, 32
BP-PGH†	BPW28 (RGD → PGH)*fhaB* FHA$^+$	This study
BP-TOX6	BP536 Δ(3.4-kb *Bst*EII)*ptx* Km^r	24
BP-BBC42	BP536 *prn*::Km (PRN$^-$) Km^r FHA$^+$	33

*Expresses and secretes a shorter FHA that does not agglutinate erythrocytes.

†The RGD → PGH mutations, obtained by site-directed mutagenesis of nucleotides 1097–1099 of the *fhaB* gene, were transferred into *B. pertussis* chromosome by homologous recombination. Km^r, kanamycin resistance; Sm^r, streptomycin resistance.

nucleotides 9922–11666 of the *fhaB* gene which corresponds to the C-terminal portion of the *fhaB* open reading frame that is not present in the 220-kDa FHA protein (fragment H7 in ref. 11).

Adhesion Assays. Bacterial cultures grown at 22°C were diluted to an OD_{590} of 0.1 and grown at 22°C for an additional 12 hr with 3 mg of fluorescein isothiocyanate (Sigma) before the shift to 37°C. For each sample of the time-course experiment, 10^8 of fluoresceinated bacteria were collected, washed twice in PBS containing 0.1% gelatin, resuspended in the cell culture medium, and incubated for 30 min at 0°C or at 37°C (see the legends to Figs. 1 and 3) in the presence of 5×10^6 mammalian cells. Bacterial adhesion to eukaryotic cells was measured by flow cytometry. A FACStar (Beckton and Dickinson) with an excitation laser beam at 488 nm was used for the experiments. Forward-scatter laser and side-scatter laser (FSC/SSC) were used to gate eukaryotic cells and the fluorescence of the bacteria bound to the eukaryotic cells. Ten-thousand events were acquired in the list mode; the data were expressed as the percentage of cells emitting fluorescence.

ELISA. The amount of FHA exposed on the cell surface of *B. pertussis* was measured by the ELISA assay on bacteria fixed with formalin as described (31). About 10^8 bacteria were collected, washed in phosphate-buffered saline (PBS), resuspended in 0.5% formalin, and incubated 15 hr on a mixer at room temperature. Bacteria were collected, resuspended in 100 μl of PBS, dried down at 37°C for 15 hr, and washed with 1% bovine serum albumin (BSA) in PBS. Bacteria were then incubated with 100 μl of a 1:500 dilution of a polyclonal mouse serum anti-FHA for 45 min at 37°C, washed with PBS containing 1% BSA and incubated 30 min at 37°C with 100 μl of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse antiserum (Sigma). Plates were washed with PBS containing 1% BSA and developed (31).

RESULTS

Adhesion of *B. pertussis* Is Time-Dependent, Requires FHA, and Is Influenced by PRN and PTX. We have recently shown (35) that after temperature shift from 25°C to 37°C, *B. pertussis* transcribes first the *fhaB* gene (within 10 min) and then the *ptx* and *cya* (encoding adenylate cyclase) genes (2 hr later). To investigate how the differential synthesis of virulence factors may influence the ability of *B. pertussis* to bind eukaryotic cells, the wild-type strains BP-536 and BP-W28 and the avirulent mutant BP-347 were grown under modulating conditions (22°C), shifted to 37°C, and tested at different times for their ability to adhere to Chinese Hamster Ovary (CHO) cells. To measure adhesion, bacteria grown in the presence of fluorescein isothiocyanate were incubated for 30 min at 0°C with CHO cells and analyzed on a FACStar flow cytometer to determine the specific fluorescence intensity associated to CHO cells. Fig. 1*A* shows that wild-type strains BP-536 and BP-W28 grown at 22°C did not bind CHO cells. Adhesion initiated 2 hr after temperature shift to 37°C and increased linearly at 4, 6, and 8 hr. After this time, adhesion to CHO cells started to decrease. As expected, the *bvg*$^-$ mutant BP-347 did not bind CHO cells at any time after temperature shift. To establish the role of the known virulence factors in adhesion, mutants lacking the gene coding for PT (BP-TOX6), defective in the *fha* gene (BP-101 and BP-102) and defective in the *prn* gene (BP-BBC42) were tested in the adhesion assay as described above. Fig. 1*B* shows that the PTX$^-$ strain (BP-TOX6) bound CHO cells, however, at each point of the time-course experiment, the values of binding being ≈20% lower than those of the wild-type strain. The strain defective in the *prn* gene (BP-BBC42) did not show any adhesion for 6 hr after temperature shift. After this time some adhesion occurred but was 75% lower than that of the wild-type strain (Fig. 1*B*) and did not increase during the following 25 hr (data not shown). In marked contrast, the strains defective in the *fhaB* gene (BP-101 and BP-102) did not show adhesion at any time after temperature shift. To test whether export and/or assembly of FHA is necessary for adhesion, we tested strains BP-353 and BP-354 that contain the entire *fhaB* structural gene and are defective in the chaperon proteins required for FHA assembly and release into the culture medium (21, 22, 32). Fig. 1*C* shows that neither of these strains were able to adhere to CHO cells. Finally, to find out whether the FHA-mediated adhesion required the integrin-binding region of FHA or other binding

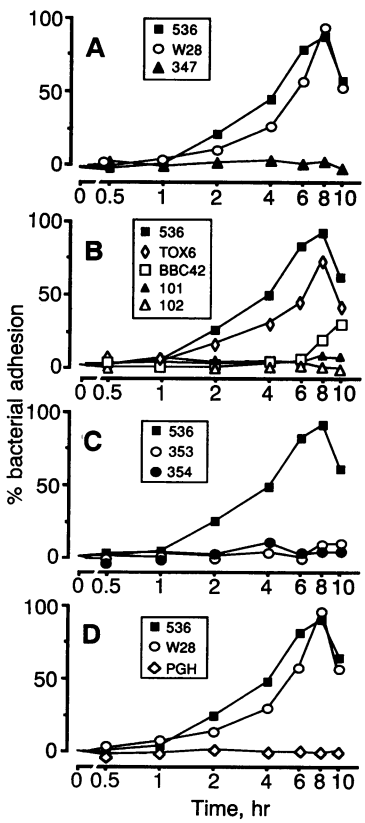

FIG. 1. Adhesion to CHO cells of wild-type and mutant *B. pertussis* strains grown at 22°C and at various times after temperature shift to 37°C; *A–D* are explained in *Results*. Binding was performed at 0°C. Similar results were obtained at 37°C. The time is reported as hours after temperature shift.

domains, we mutagenized two of the three amino acid residues involved in integrin binding, Arg-Gly-Asp → Pro-Gly-His (RGD → PGH). Fig. 1*D* shows that this mutant (BP-PGH) was unable to bind CHO cells. We conclude that adhesion of *B. pertussis* requires the RGD domain of FHA that is likely to bind cell surface integrins on CHO cells. However, we cannot rule out the possibility that binding to CHO cells is mediated by a process affected by the conformation around the RGD domain but not directly involving it. The absence of proteins involved in the export of FHA or the absence of PRN abolish or influence considerably the adhesion respectively, while the role of PTX is modest.

PRN Is Involved in Cell Surface Exposure of FHA. To investigate whether PRN influenced FHA activities that are different from binding CHO cells, we performed ELISA and hemagglutination assays on wild-type and mutant strains to study the presence and the function of FHA on the cell surface, respectively. Formalin-stabilized bacteria from a time-course experiment were used to coat microtiter plates, and the presence of cell surface-exposed FHA was detected by an ELISA technique with specific anti-FHA antibodies. In the wild-type strain, the presence of FHA on the cell surface was clearly detected 2 hr after temperature shift and increased thereafter (Fig. 2*A*). As expected, strains defective in *bvg* (BP-347) and in FHA export (BP-353) gave negative results. Surprisingly, the PRN-defective strain (BBC42) showed no significant FHA exposure for 4 hr. At 6 and 8 hr some surface exposure of FHA was detected; however, this was significantly lower than with the wild type. This behavior reminds one of that observed on the same strain in the CHO adhesion assay of Fig. 1*B*. Hemagglutination assays showed that the *bvg* and FHA-export mutants were unable to agglutinate erythrocytes (Fig. 2*B*). However, the PRN mutant and the mutant in the RGD domain of FHA, which were defective in CHO cell binding, showed normal levels of hemagglutinating activity. In conclusion, the absence of PRN influenced significantly cell surface exposure of FHA (detected by ELISA) and RGD-mediated adhesion but not hemagglutination activity.

PRN and RGD Mutant Strains Bind HEp2 Cells. To understand whether the results obtained on adhesion were extendable to other type of cells, we assayed bacterial adhesion in a number of cell lines. Among HEp2, Hela, WiDr, and CHO cells that are commonly used to test *B. pertussis* adhesion (10, 38, 39), the most efficient in binding were HEp2 and CHO cells that also showed a similar temporal pattern of adhesion (data not shown). Therefore, mutant and wild-type strains were tested for adhesion to HEp2 cells, and the results were compared with those obtained using CHO cells. Fig. 3 shows that BP-347 was negative all the time, confirming that HEp2 cells also require bvg-regulated factors for binding. Surprisingly, the PRN$^-$ and the RGD mutant strains showed a significant binding to HEp2 cells, although with lower efficiency than the binding of wild-type strains. We conclude that, while the PRN and the RGD domain of FHA are required for binding CHO cells, they are not essential for binding HEp2 cells. Therefore, it is likely that HEp2 cell adhesion is mediated by factors different from FHA and/or by FHA domains not involving the RGD site. Both the RGD domain and the carbohydrate-binding domain of FHA might be involved.

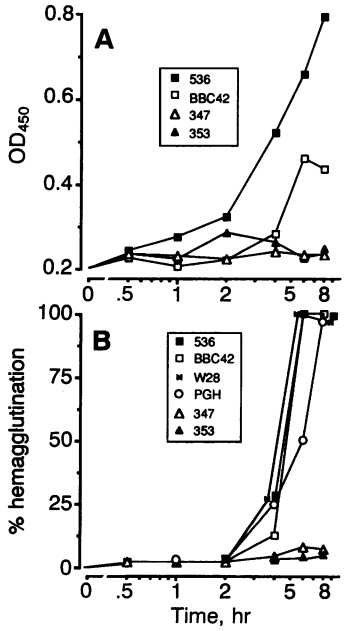

FIG. 2. (*A*) ELISA to determine the amount of FHA that is exposed on the surface of *B. pertussis*. (*B*) Hemagglutination of erythrocytes by 1:1 serial dilutions of bacterial whole-cell suspensions. The strain BP-TOX6 tested in the ELISA and hemagglutination assays gave results similar to those of the wild-type strain BP-356. The time is reported as hours after temperature shift.

Cell-Associated Precursor Form of FHA Mediates Adhesion to CHO Cells. By Western blot analysis, we investigated the time required for synthesis and releasing of FHA into the extracellular medium during the temperature-shift experiment. Fig. 4*A* shows that in agreement with the transcriptional data (35), FHA was not present in the cells grown at 22°C, was detectable 10 min after temperature shift, and increased during the first 30 min. Surprisingly, FHA was not detected in the supernatant for 10–14 hr (Fig. 4*B*). After this time, its level in the supernatant increased. The time lag observed between detection of FHA in the cells and its appearance in the supernatant, suggested that we investigate whether during this time the FHA protein was present as a full-length precursor form. To identify the precursor molecule of FHA, we raised antibodies against a recombinant polypeptide that mapped within the precursor and was predicted to be absent in the mature protein. This antibody was used in Western blot analysis of total cell lysates from a time-course experiment. From 30 min to 8 hr, this antiserum detected several bands, some of which had a molecular weight > 220 kDa (the size of purified FHA) (Fig. 4*C*). These bands decreased in size 10 hr after temperature shift, when the FHA started to be released into the supernatant and disappeared in the 25-hr sample. The same antiserum did not react with any protein present in the culture supernatant (data not shown). To better visualize the precursor and mature forms of FHA, selected samples of the time-course experiment analyzed in Fig. 4 *A* and *B* were loaded on a high-resolution polyacrylamide gel and analyzed by Western blotting by using an antibody against purified FHA. The cells taken at 1, 2, and 14 hr had several bands of molecular weight higher than 220 kDa (Fig. 5). The high molecular weight band was partially purified and shown to be recognized by antibodies against the 220-kDa mature form of the molecule and by antibodies against the carboxyl-terminal region of FHA not present in the mature protein (data not shown). On the contrary, supernatant samples showed only a band at 220 kDa. The cells taken at 14 hr, in addition to the high molecular weight bands, showed a major band comigrating with the FHA form present in the culture supernatant. In agreement with the nucleotide sequence prediction, we conclude that the highest molecular weight band found in whole cell lysates

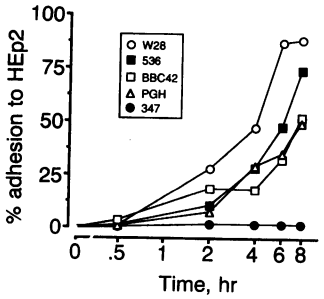

FIG. 3. Adhesion of wild-type and mutant bacterial strains to the HEp2 cell line. Binding was performed at 37°C. Time is in hours after temperature shift.

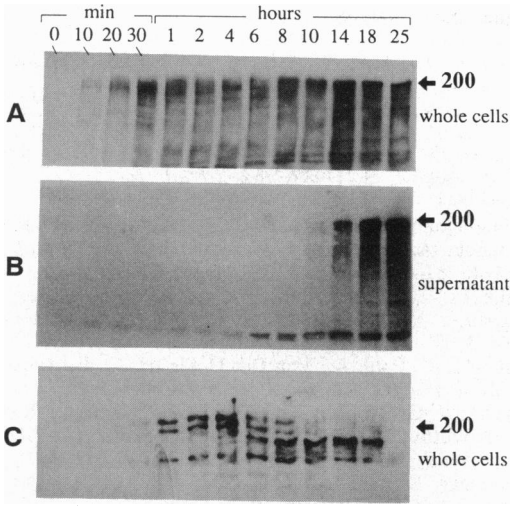

FIG. 4. Western blots to detect the presence of FHA in total cell lysates and culture supernatants at various times after 22°C to 37°C temperature shift. (*A*) Total cell lysates stained with the antibody against purified FHA. (*B*) Culture supernatants stained with the antibody against purified FHA. (*C*) Total cell lysates stained with an antibody raised against a recombinant fragment of FHA that is not present in the mature protein but is predicted to be present in the precursor form of the protein. Size is shown in kDa.

may represent the precursor form of FHA. These results provide evidence for the existence of a high molecular weight, cell-associated form of FHA. The observation that bacterial adhesion starts at 2 hr and reaches the maximum at 8 hr of the temperature-shift experiment, when only the precursor form of FHA is present, strongly suggests that this is the form that mediates adhesion to CHO cells. The secreted mature FHA product could act as a competitive inhibitor to eukaryotic cell adhesion, thereby explaining the decrease in bacterial adherence (see Fig. 1).

DISCUSSION

We have used temperature shift from 22°C to 37°C to obtain a synchronous induction of the synthesis, export, and maturation of the virulence factors of *B. pertussis*. These experiments that mimic the *in vivo* temperature shift encountered by pathogens when they infect homeothermic animals allowed us to obtain unique findings that had never been observed when growing the bacteria under standard laboratory conditions, suggesting that similar experiments may also be useful for other bacterial pathogens surviving at different temperatures (*Bordetella bronchiseptica, Yersinia, Escherichia coli, Shighella, Listeria, Salmonella, Pseudomonas, Vibrio cholera*). In *B. pertussis* that is isolated only from man and is not known to occur in environments at low temperature, the temperature regulation of virulence factors may be used to modulate the amount of virulence factors expressed in the upper and lower respiratory tract. Evidence in our laboratory indicates, in fact, that bacteria grown at 28°C, 32°C, and 37°C produce low, intermediate, and high levels of virulence factors, respectively (not shown).

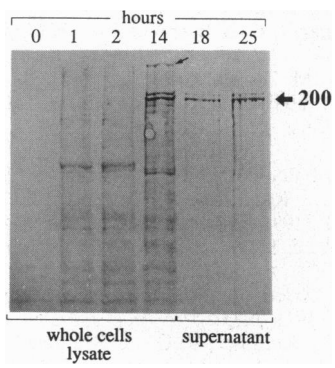

FIG. 5. Identification of a precursor form of FHA. Selected samples of Fig. 1 were run in an 8% polyacrylamide gel and the presence of FHA was detected by the antibody against the purified protein. At 1, 2, and 14 hr after temperature shift, the bacterial cells contain forms of FHA of molecular weight > 200 kDa. These forms are absent in the supernatant samples. The high molecular band indicated by the arrow in the whole-cell lysates is recognized also by the antibodies against the carboxyl-terminal region of FHA not present in the mature protein (not shown). Size is shown in kDa.

The adhesion of *B. pertussis* to eukaryotic cells is a complex phenomenon that involves multiple binding specificities of the FHA protein and possibly other adhesins. We have demonstrated that binding of *B. pertussis* to CHO cells requires only the RGD domain of FHA; agglutination of erythrocytes is independent from the RGD domain and is likely to involve the carbohydrate binding domain of FHA, while the binding to HEp2 cells is likely to involve both mechanisms and possibly also other *bvg*-regulated proteins. It should be noted that Leininger *et al.* (36) found (*i*) that 63% of binding to CHO cells is due to FHA and PRN and 37% is due to other molecules and (*ii*) that FHA-mediated binding is independent of the RGD domain. Since bacteria were grown at 37°C in a nonsynchronized induction, their results may not be in contrast with those described here. In our experiments, the maximum value of FHA accumulation in bacterial samples has been observed already at 30 min of temperature shift (Fig. 4*A*). However, binding to CHO and HEp2 cells was detected only 2 hr after temperature shift, and the maximum efficiency of binding was observed at 8 hr of induction (Fig. 1*A*). The time lag observed between the synthesis of FHA and the adhesion to eukaryotic cells suggests that FHA requires this time interval to be exported to the external surface of the bacterium and be properly folded and processed (Figs. 4 and 5). These molecular events require the expression of FHA-specific gene products (Figs. 1–3), which chaperon FHA (21, 22). Surprisingly, PRN was also necessary for the FHA-mediated adhesion to CHO cells. In fact, in the absence of PRN, the FHA was poorly exposed on the external surface of the bacterium (Fig. 2*A*), while the binding activity, mediated by the RGD domain of FHA, was delayed an additional 4 hr and reached a level that was 25% of the wild type. We have also found that PRN is synthesized after 2 hr of temperature shift (data not shown). This late synthesis of PRN may account for the time lag observed between the synthesis of FHA and the binding activity mediated by the RGD domain. Surprisingly, PRN did not influence FHA activities such as hemagglutination (Fig. 2*B*). Therefore, it is likely that a direct role of PRN in bacterial adhesion to eukaryotic cells is at the level of membrane structure modification or directly on the surface delivery and/or folding of the FHA protein. Furthermore, the delay in maximal bacterial adherence may also be due to the time required for formation of complexes between mature FHA and PRN or other bacterial surface molecules. However, the molecular mechanism by which PRN participates in the FHA-mediated, RGD domain-dependent adhesion is not known as yet. In this context, it has been described (37) that, in eukaryotic cells the RGD domain-mediated binding is influenced considerably by polypeptide regions located far away from the RGD site and sometimes also on separate polypeptide. Since PRN and FHA are both exported on the surface of the bacterium in some defined structures (30), we propose that these proteins interact with one another and that the binding to eukaryotic cells requires a protein–protein contact between PRN and FHA. If the two RGD domains of PRN were involved in this

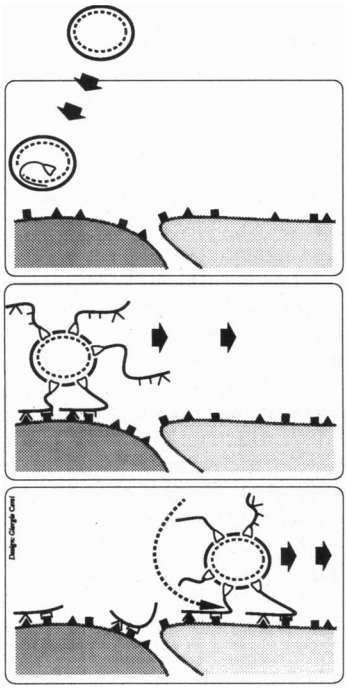

FIG. 6. Model showing the expression and the function of the different forms of FHA. After infection of a homeothermic animal, *B. pertussis* starts to synthesize FHA (*Top*), which 2 hr later is exported as a 367-kDa polypeptide to the surface and mediates adhesion to carbohydrate and integrin receptors of eukaryotic cells (*Middle*). Several hours later the 220-kDa extracellular portion of FHA is cleaved off, allowing the bacterium to detach from the bound cell and adhere to other cells (*Bottom*).

interaction, this could explain the inhibition of cell invasion shown with peptides containing the RGD domain of PRN (30). In conclusion, bacterial adhesion may be mediated by specific structures containing FHA and PRN.

A possible model of bacterial adhesion is presented in Fig. 6. After infection, the bacterium synthesizes FHA that would first display the carbohydrate binding region that assures binding to different type of cells, including cilia and macrophages. The expression of late genes (PRN, PTX, and others) selectively help the FHA-mediated adhesion, which is increased by the exposure of the RGD-binding domain. In the late stage of the infection the FHA is cleaved off to allow the bacteria to facilitate spreading. The released form of FHA may also play a role in attachment and detachment of the bacteria from the cells and also in colonization of the environment.

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