Interaction of *Bordetella pertussis* Adenylate Cyclase with CD11b/CD18

ROLE OF TOXIN ACYLATION AND IDENTIFICATION OF THE MAIN INTEGRIN INTERACTION DOMAIN*  

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Adenylate cyclase toxin (CyaA) is one of the major virulence factors produced by *Bordetella pertussis*, the whooping cough agent. CyaA belongs to the repeat in toxin protein family and requires a post-translational fatty acylation to form cation-selective channels in target cell membranes and to penetrate into cytosol. We have demonstrated recently (2) that CyaA uses the αMP2 integrin (CD11b/CD18) as a specific cellular receptor. Here we show that the acylation of CyaA is required for a productive and tight interaction of the toxin with cells expressing CD11b. In addition, we demonstrate that the catalytic domain is not required for binding of CyaA to CD11b and that the main integrin interacting domain of CyaA is located in its glycine/aspartate-rich repeat region. These data decipher, for the first time, the interaction of CyaA with CD11b-positive cells and open new prospects for understanding the interaction of *Bordetella pertussis* with innate and adaptive immune systems.

*Bordetella pertussis* is the etiological agent of whooping cough, a highly contagious childhood respiratory disease, characterized by bronchopneumonia and paroxysmal coughing interrupted by inspiratory whoops. Among the variety of toxins produced by *B. pertussis*, the adenylate cyclase (CyaA)† is a crucial factor in the virulence strategy of the bacteria during the early phases of respiratory tract colonization (1, 2). The toxin allows the pathogen to escape host immune surveillance, mainly by intoxicating neutrophils and macrophages causing phagocyte impotence and inducing macrophage apoptosis (3–7). The role of CyaA in the pathogenesis of *B. pertussis* was clearly demonstrated in the mouse respiratory model. Indeed, genetically modified *B. pertussis* strains deficient in the expression of CyaA were impaired in their ability to induce pulmonary lesions and to cause lethal infection (8, 9). On the other hand, CyaA was shown to induce protective immunity against *B. pertussis* lung colonization in a mouse model (10–12).

CyaA is a 1706-amino acid-long polypeptide consisting of four functional domains: the adenylate cyclase activity (AC) domain (residues 1–400), the hydrophobic channel-forming domain (residues 500–700), the calcium-binding glycine/aspartate-rich repeat domain (residues 1000–1600), and the C-terminal domain harboring a secretion signal (residues 1600–1706). CyaA is able to invade eukaryotic cells and translocate its catalytic domain into the cytoplasm where, upon activation by endogenous calmodulin, it catalyzes the conversion of ATP into cAMP (13). The accumulation of cAMP in the cell cytosol is considered to be responsible for the toxic effect of this toxin (14). The main consequences of this intoxication are cell apoptosis and the alteration of phagocytic abilities and superoxide production (3, 7, 15–17).

CyaA requires calcium to acquire a translocation-specific conformation that allows the delivery of the catalytic domain into the cell cytosol (18, 19). CyaA is first produced as an inactive prototoxin, pro-CyaA, and it is converted to the active toxin upon post-translational fatty acyl modification that is catalyzed by the accessory protein acyltransferase, the product of the cyaC gene. Acylation of pro-CyaA consists of covalent palmitoylation at the ε-amino groups of two lysine residues, Lys-983 and Lys-860, located within conserved RTX acylation sites (20, 21), located within conserved RTX acylation sites (20, 21). Although acylation of Lys-860 seems not to be necessary for CyaA activity, acylation of Lys-983 was shown to be crucial (22). The covalent post-translational fatty acylation is required for translocation of the catalytic AC domain of the toxin through target cell membranes, as well as for the formation of hemolytic cation-selective CyaA channels.

CyaA can penetrate at least to some extent a wide range of cell types, including the mammalian erythrocytes lacking membrane trafficking (18, 23, 24). However, CyaA toxicity effects such as the abrogation of phagocytic capacity and the induction of apoptosis were mainly elucidated on immune cells, namely neutrophils and macrophages (3, 7). In addition, CyaA was shown to display specific intoxication toward alveolar macrophages and to play a key role in mouse respiratory infection by *B. pertussis* (4). We have demonstrated recently (25) that CyaA binds specifically to target cells through the αMP2 integrin (CD11b/CD18) receptor. This binding was saturable and completely inhibited by anti-CD11b monoclonal antibodies. CyaA displayed a selective cytotoxicity toward CD11b+...
cells showing that its interaction with CD11b is required for the translocation of the catalytic domain and the subsequent increase of intracellular cAMP concentration and cell death. Moreover, sensitivity of CHO cells to CyaA cytotoxicity was dramatically increased upon expression of the CD11b/CD18 heterodimer. Furthermore, Ca^{2+} ions that are required for translocation of the catalytic domain into cells were also strictly necessary for CyaA interaction with CD11b (25). The importance of CD11b for interaction of CyaA with cells was further emphasized in a system where CyaA is expressed in a vector to deliver foreign antigens into antigen-presenting cells, further demonstrated in a system where CyaA is used as a histocompatibility class I peptide complexes correlating with CD11b for interaction of CyaA with cells was shown that its interaction with CD11b is required for cell invasion (34) in the Escherichia coli strain XL1-Blue (Stratagene, Amsterdam, Netherlands) as host cells. The plasmids coding for a non-acylated wild type pro-CyaA (pACT7), acylated wild type CyaA (pACT7-1), and wild type pro-CyaA (pACT7-373) encoding the hemolytic moiety (CyaA-(373–1706)) on the adsorption at 278 nm using a molecular extinction coefficient of 141 mM ° cm^{-1} was determined. The generated hybridomas were screened for the production of CyaA-specific monoclonal antibodies (mAb) by enzyme-linked immunoassay. Highly productive hybridomas were then selected and cloned by single-cell limiting dilutions and subsequently used to make ascites in BALB/c nude mice to generate large amounts of anti-CyaA mAb. The mAb were purified from ascites using T-Get™ purification kit (Pierce) according to the manufacturer's instructions. The antibody concentration was measured with Bio-Rad protein assay. Two of the monoclonal antibodies were used in this study: antibody 5G12 that reacts with an epitope localized within amino acids 1–190, and antibody 6D7 that reacts with an epitope localized within amino acids 1006–1706 (data not shown).

**Cells and Culture**—Chinese hamster ovary cells transfected with human CD11b/CD18 (CHO-CD11b cells), human CD11c/CD18 (CHO-CD11c cells), or transfected with the vector alone (CHO cells) were a gift kind of D. Golenbock (Boston University School of Medicine, Boston) and were cultured in the presence of neomycin as described previously (41).

**Antibodies**—Monoclonal antibodies specific for human CD11b (ICRF44, mouse IgG1, κ) and human CD11c (B-Ly6, mouse IgG1, κ) were purchased from Pharmingen.

**Binding Assays**—The assays were performed as described previously (25). Briefly, 2 × 10^5 cells were incubated with the indicated concentrations of CyaA molecules in Dulbecco’s modified Eagle’s medium containing 4.5 mg/ml glucose (Invitrogen), without serum, in 96-well culture plates for 30 min on ice. After washing, anti-CyaA catalytic domain mAb (5G12) or anti-CyaA repeat domain mAb (6D7) was added at 25 μg/ml. In some experiments, cells were preincubated with the indicated concentrations of CyaA molecules for 30 min on ice. Then CyaA-biotin (30 nm), anti-CD11b mAb (2 μg/ml), or anti-CD11c mAb (2 μg/ml) (Pharmingen) were added separately in the continuous presence of the toxins.

After washing and removing supernatant, cells were stained with goat anti-mouse IgG-PE (Caltag, Le Perray en Yvelines, France) or with streptavidin-PE (Pharmingen) at 1:300 dilution. The last wash was performed by adding known cAMP concentration. Aggregated and dead cells were subtracted by gatings based on propidium iodide exclusion. The binding data were deduced from the mean fluorescence intensity (MFI) and expressed as ΔMFI = (MFI value of cells incubated with CyaA) – (MFI value of cells incubated without CyaA) as percentage of binding = (sample binding)/maximum binding) × 100. The maximum binding corresponds to (MFI value of cells incubated with CyaA or anti-CyaA in the absence of competitor) – (MFI value of cells incubated with medium alone). The sample binding corresponds to (MFI value of cells incubated with CyaA or anti-CD11b in the presence of competitor) – (MFI value of cells incubated with medium alone).

**Cyclic AMP Assay**—Cyclic AMP accumulated in cells exposed to the CyaA toxin was performed essentially as described previously (25). Briefly, 5 × 10^5 cells were incubated with the indicated concentrations of CyaA in Dulbecco’s modified Eagle’s medium + glucose for 20 min at 37 °C. After washing, cyclic AMP accumulated in cell cytosol was released by lysis with 0.1% HCl and boiling for 5 min at 120 °C. After neutralization with 0.1 N NaOH, the samples were then added to microtiter plates previously coated with a CAMP-bovine serum albumin conjugate and incubated with an appropriate dilution of the anti-CyaA antibody. After washing, anti-CAMP antibodies were revealed with anti-rabbit antibodies coupled to alkaline phosphatase. The CAMP content of each sample was determined from comparison with a standard curve obtained by adding known cAMP concentration.
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RESULTS

CyaA Specifically Binds to CD11b⁺ Cells and Inhibits CyaA-biotin and Anti-CD11b Monoclonal Antibody Binding to These Cells—To analyze further the role of acylation in interaction of the toxin with CD11b⁺ cells, we tested the ability of non-acylated pro-CyaA to compete with CyaA for binding to CHO-CD11b cells. As shown in Fig. 2B, when compared with the acylated CyaA, the non-acylated pro-CyaA exhibited a substantially reduced capacity to compete with biotinylated CyaA for binding to CD11b⁺ cells. To determine whether the lack of inhibition was due to an inefficient interaction with CD11b, we evaluated the capacity of pro-CyaA to block anti-CD11b binding to CHO-CD11b cells. Indeed, as compared with CyaA, pro-CyaA was unable to inhibit binding of the anti-CD11b mAb to the CHO-CD11b cells (Fig. 2C).

Because supraphysiological production of cAMP and cell intoxication are the consequences of CyaA interaction with CD11b⁺ cells, we then analyzed, using the CHO-CD11b cells, if these toxin functions are dependent on CyaA acylation. As expected, the pro-CyaA did not induce any cAMP increase in CHO-CD11b cells (Fig. 3A) and had no significant cytotoxic effect on these cells, as compared with the acylated CyaA (Fig. 3B). Taken together, these results clearly demonstrate that acylation of CyaA is necessary for a functional interaction of the toxin with CD11b⁺ cells and that the binding of pro-CyaA to CD11b is non-productive and does not trigger cytotoxic effects on CD11b-expressing cells.

The Catalytic Domain Is Not Required for CyaA Interaction with CD11b—Functionally, CyaA is composed of two main domains. As shown in Fig. 1A, using this assay, CyaA binding was specifically detected on CD11b⁺ cells. In the competition assay, different CyaA molecules (mutants or fragments) were tested for their ability to compete with binding of CyaA-biotin or anti-CD11b mAb to CD11b⁺ cells. Here the CHO-CD11b cells were incubated with CyaA at different concentrations for 30 min on ice. Then CyaA-biotin (30 nM) or anti-CD11b mAb (2 µg/ml) were added in the continuous presence of CyaA, and their binding to the cells was evaluated by FACS. As shown in Fig. 1B, CyaA efficiently inhibited both CyaA-biotin and anti-CD11b binding to CHO-CD11b cells in a dose-dependent manner. This inhibitory effect was specific for CD11b since CyaA was unable to compete with another ligand (anti-CD11c mAb) for its specific receptor (CD11c) expressed by CHO cells (Fig. 1B).

Lack of CyaA Acylation Affects Its Binding to CD11b⁺ Cells—Because it is well established that CyaA needs a post-translational palmitoylation to acquire its invasive activity and capacity to form hemolytic membrane channels, we tested whether the lack of acylation affects CyaA interaction with CD11b⁺ cells. In a binding assay, control CHO cells or CHO-CD11b cells were incubated with either CyaA or the non-acylated pro-CyaA. The binding was evaluated using a monoclonal antibody recognizing the catalytic domain of CyaA (AC). As shown in (Fig. 2A), at low concentrations, binding of both CyaA and pro-CyaA molecules to CD11b⁺ cells was rather comparable, with a slightly more efficient binding of the acylated CyaA. This could be due to its enhanced interaction with cell membrane, a better adapted conformation of CyaA for binding and/or higher affinity of the acylated CyaA for the CD11b receptor. Indeed, the pro-CyaA binding reached saturation at substantially higher protoxin concentrations, as compared with CyaA binding. The simplest explanation of this observation would be that pro-CyaA binds CD11b⁺ cells with lower affinity than CyaA. At high protoxin concentration, aggregates and/or oligomers of pro-CyaA would bind to the cells, and consequently, higher amounts of pro-CyaA would be detected. In contrast, very low binding of CyaA or pro-CyaA to control CHO cells was detected.

Acylation Stabilizes Interaction of CyaA with CD11b⁺ Cells—To analyze further the role of acylation in interaction of the toxin with CD11b⁺ cells, we tested the ability of non-acylated pro-CyaA to compete with CyaA for binding to CHO-CD11b cells. As shown in Fig. 2B, when compared with the acylated CyaA, the non-acylated pro-CyaA exhibited a substantially reduced capacity to compete with biotinylated CyaA for binding to CD11b⁺ cells. To determine whether the lack of inhibition was due to an inefficient interaction with CD11b, we evaluated the capacity of pro-CyaA to block anti-CD11b binding to CHO-CD11b cells. Indeed, as compared with CyaA, pro-CyaA was unable to inhibit binding of the anti-CD11b mAb to the CHO-CD11b cells (Fig. 2C).

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Acetylation of B. pertussis Adenylate Cyclase with CD11b

Binding of Recombinant CyaA to Erythrocytes—Toxin binding to sheep erythrocytes was assayed essentially as described previously (38). Toxins in 8 M urea, 20 mM Hepes-Na were directly diluted (at least 200-fold) into suspensions of sheep erythrocytes (5 × 10⁶/ml) in buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM CaCl₂) and incubated at 37 °C for 30 min. An aliquot was removed to determine the total adenylate cyclase activity added to each sample. The cell suspensions were chilled on ice and centrifuged 5 min at 3,000 rpm in a 4 °C. The pelleted cells were resuspended in buffer A; the suspension was transferred to fresh tubes to avoid carryover of toxin bound to tube walls, and cells were pelleted again by centrifugation and finally lysed with 0.1% Triton X-100. The enzymatic AC activities in the cell extracts corresponded to that of the toxin associated with the cellular membrane and was expressed as a percentage of total activity added to the cells. The adenylate cyclase activity was measured as described previously (40). One unit of adenylate cyclase activity corresponds to 1 µmol of cAMP formed in 1 min at 30 °C and pH 8.0.
domains harboring independent activities. The N-terminal domain harbors the adenylate cyclase activity (residues 1–400), whereas the C-terminal hemolysin moiety (residues 400–1706) is responsible for the delivery of the AC domain into target cells and the hemolytic activity of B. pertussis. To examine the role of these two functional domains in CyaA binding to CD11b/H11001 cells, we tested the ability of the AC catalytic domain (residues 1–384) and of the hemolysin moiety of the toxin (Hly, residues 373–1706) to compete for binding to CHO-CD11b cells with CyaA-biotin. As shown in Fig. 4A, the AC domain was unable to inhibit CyaA-biotin binding to CHO-CD11b cells, whereas the Hly moiety inhibited the binding of the anti-CD11b mAb as efficiently as the intact CyaA (Fig. 4B). Moreover, direct binding assays with the anti-CyaA mAb (5G12) specific for the AC domain did not reveal any significant association of the AC to the surface of CHO-CD11b cells, whereas binding of CyaA was readily detected (Fig. 5A). Direct binding of Hly to CHO-CD11b cells could not be detected with the 5G12 mAb that recognizes an epitope located within the first 200 amino acids of CyaA (data not shown). However, Hly binding was clearly demonstrated by using another anti-CyaA mAb (6D7), specific for the repeat domain (Fig. 5B). Only very weak binding of CyaA or Hly was detected with 5G12 or 6D7 mAbs on control CHO cells lacking CD11b expression (data not shown). Altogether, these results clearly demonstrate that the catalytic AC domain is not required for CyaA interaction with CD11b and that the CD11b interaction domain of CyaA is located in the Hly moiety of CyaA consisting of residues 373–1706.

The CyaA Domain Interacting with CD11b Is Located within the CyaA Repeat Region—To identify the region of CyaA that interacts with CD11b, we expressed and purified various fragments of the Hly moiety of CyaA (comprising residues 373–1490, 700–1706, 700–1490, or 1006–1490, respectively) and tested them in the competition assay. However, none of these truncated polypeptides was able to compete in a significant manner with the binding of CyaA-biotin to CHO-CD11b cells. This suggested that the isolated fragments of the Hly moiety adopt an altered conformation. Therefore, a mutational approach was used to locate the CD11b binding domain of CyaA. Seventeen different modified CyaA molecules were constructed by insertion of the FLAG epitope of the amino acid sequence, DYKDDDDK at various defined positions throughout the toxin polypeptide, as outlined under “Experimental Procedures.”
hypothesized that insertion of a heterologous and highly charged peptide within the CD11b binding region(s) might potentially affect its structure and disrupt selectively the capacity of the toxin to interact with cells expressing CD11b, while having little impact on the capacity of the toxin to associate with cells lacking this integrin, such as erythrocytes. The 17 FLAG-tagged CyaA molecules were therefore expressed, purified close to homogeneity, and tested for the capacity to compete with binding of CyaA-biotin to CHO-CD11b cells. It should be noted that in two constructs, CyaA/H9004–510–515/FLAG and CyaA/H9004–1245–1273/FLAG, the FLAG epitope was used to replace the deleted amino acid residues 510–515 and 1245–1273 of CyaA, respectively. As shown in Fig. 6A, insertion of the FLAG epitope at three different sites located between residues 1166 and 1281 completely abrogated the capacity of the CyaA1166/FLAG, CyaA/H9004–1245–1273/FLAG, and CyaA1281/FLAG constructs to compete with CyaA-biotin for CD11b binding, when tested at 30 nM concentrations. In contrast, all other FLAG-tagged recombinant CyaAs were able to compete with CyaA-biotin for binding to CD11b/H11001 cells, although with some variable efficiencies (but exceeding 50% of the competitor activity of intact CyaA). Noticeably, three recombinant CyaA constructs with the FLAG epitope inserted close to the C-terminal end of the protein, at positions 1416, 1623, and 1648, respectively, and the CyaA construct with the FLAG inserted at position 926 (i.e. between the two CyaA acylation sites) were also partially impaired in their capacity to compete for CyaA-biotin binding to CD11b-expressing cells.

It was important to ascertain whether the loss of interaction of CyaA1166/FLAG, CyaA/H9004–1245–1273/FLAG, and CyaA1281/FLAG, with CHO/CD11b cells resulted specifically from the disruption by the FLAG insertions of an integrin binding domain of the toxin or whether it was due to a generalized collapse of the toxin structure and loss of also the other CyaA activities. Therefore, the ability of these recombinant CyaAs to bind to erythrocytes was tested. Although erythrocytes lack the CD11b/CD18 receptor, they bind substantial amounts of CyaA and have been previously widely used as a surrogate target cell model for analysis of the capacity of CyaA to directly interact with and penetrate across the cytoplasmic membrane of cells. As shown in Fig. 6B and compared with the intact CyaA, the erythrocyte binding capacity of most CyaA/FLAG molecules was affected upon insertion of the FLAG peptide at the given positions. However, besides the CyaA926/FLAG construct, which was totally unable to bind erythrocytes, all other CyaA/FLAG molecules still bound to red blood cell membrane more or less efficiently. It is plausible to assume that the complete loss of the erythrocyte binding activity of the CyaA926/FLAG construct was due to disruption of the structure required for fatty acylation of the adjacent lysine 983, which is absolutely required for the capacity of the toxin to interact with erythrocytes (21).

Interestingly, the CyaA/H9004–1245–1273/FLAG and CyaA1281/FLAG constructs, which were completely unable to bind to CD11b+ cells, exhibited a reduced but substantial capacity to bind to erythrocytes. Moreover, the CyaA1166/FLAG construct,
which did not bind to CHO-CD11b cells at all, exhibited a rather high erythrocyte binding capacity (50% of intact CyaA) that was fully comparable with the binding activity of most other CyaA/FLAG molecules that bound CD11b efficiently. These results exclude the possibility that at least in the case of the CyaA1166/FLAG construct the peptide insertions brought about a generalized collapse of CyaA structure that would be expected to cause loss of other toxin functions and of the membrane interaction capacity of the CyaA1166/FLAG molecule. These results strongly suggest that insertion of the FLAG epitope at position 1166 and probably also at position 1281 and between residues 1245 and 1273, respectively, ablated rather selectively the integrin binding function of the toxin by altering the structure of its main integrin binding domain. This conclusion is further strengthened by the observation that the CyaA1166/FLAG, CyaA1245–1273/FLAG, and CyaA1281/FLAG constructs were also capable of delivering their AC domains across the membrane into erythrocyte cytosol (invasive activity) with the CyaA1166/FLAG protein exhibiting about 50% and the other two constructs about 20% of cell invasiveness of intact CyaA (data not shown).

To characterize further the CyaA domain that interacts with CD11b, we performed a more thorough analysis of the ability of selected CyaA/FLAG constructs to compete with CyaA-biotin for binding to CHO-CD11b cells by characterizing the concentration dependence of their CD11b binding (Fig. 7A). As expected, the CyaA1166/FLAG, CyaA1245–1273/FLAG, and CyaA1281/FLAG proteins were unable to inhibit CyaA-biotin binding to CD11b cells, even at concentrations as high as 240 nM. However, all other CyaA/FLAG constructs inhibited the CyaA-biotin binding in a dose-dependent manner, similarly to intact CyaA although CyaA1416/FLAG exhibited a reduced capacity to compete with CyaA-biotin at low concentrations.

To corroborate these data, we also tested the ability of the CyaA-FLAG proteins to compete for the binding of anti-CD11b mAb to the integrin. As shown in Fig. 7B, in contrast to
all other CyaA/FLAG molecules tested, CyaA1166/FLAG, CyaA1245–1273/FLAG, and CyaA1281/FLAG were completely unable to compete with anti-CD11b monoclonal antibody binding to CHO-CD11b cells, showing that these molecules are selectively affected in their interaction with CD11b.

Altogether, these results indicate that the portion of the CyaA RTX repeat domain delimited by residues 1166 and 1281 is of crucial importance for interaction of CyaA with CD11b+ cells and therefore represents the main integrin binding domain of CyaA.

### DISCUSSION

The biological activity of the adenylate cyclase toxin (ACT or CyaA) is entirely dependent on the covalent post-translational fatty acylation. In the absence of palmitoylation of the conserved Lys-983 residue, CyaA is unable to bind productively the erythrocyte membrane and deliver its catalytic domain into erythrocyte cytosol, while also being unable to form the hemolytic CyaA membrane channels (20–22). CyaA was repeatedly shown to penetrate with detectable efficiency a large variety of eukaryotic cells. It was, however, demonstrated that its primary target cells are myeloid cells such as neutrophils and lung macrophages that are particularly sensitive to CyaA activity and are committed to apoptosis by toxin action (3, 6, 7). We have, indeed, recently shown that the CyaA toxin binds a specific cellular receptor, an αβ2 integrin (CD11b/CD18), which is exclusively expressed on immune cells such as neutrophils, macrophages, or dendritic cells and that expression of CD11b most likely accounts for the high sensitivity of these cells to CyaA (25). In the present study, we showed that CyaA acylation plays a major role in its interaction with CD11b+ cells. Indeed, although non-acylated pro-CyaA was able to bind CD11b+ cells as efficiently as CyaA, it was inefficient in competing with acylated CyaA or anti-CD11b mAb binding to CHO-CD11b+ cells, and it was completely unable to cause cell intoxication by raising intracellular cAMP levels. This suggests that while still interacting with CD11b, the interaction of pro-CyaA with CD11b is non-productive, and in particular the affinity and/or stability of the pro-CyaA-CD11b complex differs significantly from that involved in interaction of the mature CyaA with CD11b. Moreover, although pro-CyaA is still able to bind the CD11b receptor, this interaction does not allow membrane penetration of the protoxin. Hence, toxin acylation may be needed to confer a translocation-competent conformation on CyaA that is required for the transmembrane delivery of the catalytic domain to the cell cytosol where it can catalyze the conversion of ATP to cAMP.

Functionally, CyaA can be divided in two main domains: one endowed with adenylate cyclase activity located between residues 1 and 400, and one accounting for hemolytic activity and located within residues 400–1706 (13). After toxin interaction with target cells, the catalytic domain can be directly translocated across the plasma membrane of erythrocytes. Our data show that although the catalytic domain plays a key role in the cytotoxic activity of CyaA by catalyzing conversion of ATP to cAMP, this domain is not required for binding of CyaA to its receptor. The results further show that the main CD11b-interaction domain of CyaA is located within the hemolysin moiety and more precisely in a portion of the glycine- and aspartate-rich RTX repeat region consisting of residues 1166–1281. Indeed, insertion of the FLAG epitope at positions 1166, 1245–1273, and 1281 of CyaA completely abolished the binding of the recombinant toxins to CD11b+ cells. Yet these proteins still exhibited a substantial binding activity (Fig. 6B) and cell intoxication activity (from 20 to 50% that of intact CyaA, data not shown) in the surrogate assay system on erythrocytes, where toxin activity does not depend on the interaction with CD11b.

This indicates that FLAG insertions at positions 1166, 1245, and 1281 did not impair the overall structure of CyaA or its acylation but rather selectively ablated the capacity of those constructs to interact with the CD11b+ cells. Altogether these results suggest that residues 1166–1281 of CyaA delineate an essential domain involved in toxin interaction with the αβ2 integrin (CD11b/CD18).

Interestingly, the CD11b binding capacity was somewhat reduced also for proteins CyaA1416/FLAG, CyaA/FLAG1623, and CyaA/FLAG1648, suggesting that an accessory CD11b-interacting domain of CyaA might be located toward the C-terminal end of the RTX repeat portion of the toxin or that the FLAG insertion in this region affected a conformation necessary for calcium binding required for CyaA interaction with CD11b.

The present results identify the region 1166–1287 as a major CD11b-binding motif of CyaA and offer an attractive explanation for our previous observation that binding of CyaA to CD11b was strictly calcium-dependent (25). As the RTX domain is involved in calcium binding and undergoes major structural rearrangement upon calcium binding (19), it is tempting to speculate that the CD11b-binding motif located in the region 1166–1287 might be exposed only in the calcium-bound conformation of RTX domain. Indeed, we proposed earlier that the polypeptide regions that connect the five different blocks of RTX repeated motifs in CyaA shifted from a disordered structure to an α-helical conformation upon calcium binding to the RTX motifs (19). The CD11b-binding motif identified here within the amino acid region 1166–1287 of CyaA is, indeed, located between the second and third block of RTX repeats of CyaA. One can hypothesize that the α-helical structure of this segment is involved in the formation of a docking site for CD11b.

CyaA has been used previously in several passive and active vaccination protocols in mouse models of protection against pertussis. Immunization with anti-CyaA-specific antibodies or with purified CyaA reduced the time course of the respiratory tract colonization by B. pertussis and protected the mice against a lethal intranasal infection (42, 43). Moreover, antibodies specific for CyaA were detected in the sera of human infants infected with B. pertussis (44, 45). Our results suggest that a CyaA molecule lacking the CyaA/CD11b interaction domain can be designed and considered for evaluation as a safe acellular vaccine for protection against B. pertussis infection. The catalytic activity of such CyaA molecules can be easily inactivated by dipeptide insertions within the ATP-binding site, located between residues 188 and 190 of CyaA (46). In combination with the deletion within the CD11b interaction domain, these alterations of CyaA could preserve the immune cells from potentially negative effects, such as signaling upon the integrin engagement by the toxoid and/or some functional interference due to competition of the CyaA toxoid for binding of other ligands to CD11b that also serves as the complement receptor CR3.

In conclusion, our present data provide important new insights into the role of acylation and of different domains of the adenylate cyclase of B. pertussis in its interaction with CD11b+ cells. This opens the way to the elucidation of subsequent biological activities triggered by interaction of the toxin with its receptor.

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