Structural and Functional Characterization of an Essential RTX Subdomain of *Bordetella pertussis* Adenylate Cyclase Toxin*

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The adenylate cyclase toxin (CyaA) is one of the major virulence factors of *Bordetella pertussis*, the causative agent of whooping cough. CyaA is able to invade eukaryotic cells by a unique mechanism that consists in a calcium-dependent, direct translocation of the CyaA catalytic domain across the plasma membrane of the target cells. CyaA possesses a series of glycine- and aspartate-rich nonapeptide repeats residues 1006–1613 of the prototype GG(N/D)DXX(L/I/F)X (where X represents any amino acid) that are characteristic of the RTX (repeat in toxin) family of bacterial cytotoxins. These repeats are arranged in a tandem fashion and may fold into a characteristic parallel \( \beta \)-helix or \( \beta \)-roll motif that constitutes a novel type of calcium binding structure, as revealed by the three-dimensional structure of the *Pseudomonas aeruginosa* alkaline protease. Here we have characterized the structure-function relationships of various fragments from the CyaA RTX subdomain. Our results indicate that the RTX functional unit includes both the tandem repeated nonapeptide motifs and the adjacent polypeptide segments, which are essential for the folding and calcium responsiveness of the RTX module. Upon calcium binding to the RTX repeats, a conformational rearrangement of the adjacent non-RTX sequences may act as a critical molecular switch to trigger the CyaA entry into target cells.

The adenylate cyclase toxin (CyaA) is one of the major virulence factors of *Bordetella pertussis*, the causative agent of whooping cough (1–3). The 1706 residue-long CyaA is a bi-functional protein endowed with both catalytic (adenylate cyclase) and hemolytic activities (2, 4, 5). Synthesized as an inactive precursor, it is converted to the active toxin by a post translational palmitoylation of two internal lysine residues (Lys\(^{860}\) and Lys\(^{983}\)) (6, 7). This active CyaA toxin is then able to deliver its catalytic domain directly across the plasma membrane of a variety of eukaryotic cells and disrupts their physiological functions by uncontrolled synthesis of cAMP (5, 8–11), leading to the cell death by apoptosis (12–14). CyaA is constructed in a modular fashion; the calmodulin-activated catalytic domain is located in the 400-amino-proximal residues, whereas the C-terminal moiety (residues 400–1706) is endowed with hemolytic activity (4, 5, 15, 16), which results from its ability to form cation-selective channels in membranes (17, 18). It also mediates the binding and internalization of the toxin into eukaryotic cells (5, 11, 19). The hemolytic and the RTX domains display structural characteristics that link CyaA to the RTX (repeat in toxin) family of bacterial toxins (20, 21). Indeed, it contains a pore-forming domain (from residues 500–700) with four hydrophobic segments (17, 18, 22, 23), the target site for the post-translational palmitoylation (7, 24), 30–40 copies of a characteristic glycine- and aspartate-rich nonapeptide repeats residues 1006–1613 of the prototype GG(N/D)DXX(L/I/F)X (where X represents any amino acid, and U represents any large hydrophobic residue such as Ile, Leu, Val, Phe, Tyr), representing the main calcium-binding sites of the protein (25) (see Fig. 1), and a non-processed C-terminal secretion signal (4, 26). The crystal structure of the *Pseudomonas aeruginosa* alkaline protease that has six of these consensuses RTX repeats revealed that these sequences constitute a new kind of calcium binding structure, called a parallel \( \beta \)-helix or parallel \( \beta \)-roll motif (27, 28).

The main originality of the CyaA toxin stems from its unique mechanism of penetration into eukaryotic cells, as its catalytic domain appears to be directly translocated through the plasma membrane of the target cells (5, 9–11, 29). Recently, it has been demonstrated that CyaA binds specifically to target cells through the \( \alpha_5\beta_3 \) integrin receptor (CD11b/CD18) (30, 31), which is expressed on a restricted subset of leukocytes including neutrophils, macrophages, and dendritic cells. Expression of this receptor most likely accounts for the high sensitivity of these cells to CyaA (13, 30).

CyaA is a calcium-binding protein that undergoes conformational changes upon binding of calcium (9, 19, 25). The entry/translocation of CyaA into target cells is strictly dependent upon the presence of calcium ions in the millimolar range, and the RTX domain is supposed to be directly involved in this process as it harbors the main low affinity Ca\(^{2+}\)-binding sites (9, 19, 25). How binding of calcium ions to the RTX motifs might trigger the translocation of CyaA into cells remains totally unknown. The RTX domain of CyaA is organized in five successive blocks (Ile to Val) of about 8 nonapeptide RTX motifs (Fig. 1) separated by linkers of variable length (from 23 to 49 residues). Previous results from Iwaki et al. (32) have suggested that the last 217 C-terminal residues of CyaA that encompasses the block V (residues 1490–1706) might constitute a functional subdomain; a truncated CyaA lacking the last 217 C-terminal residues was unable to intoxicate target cells but...
could be partially complemented by other CyaA fragments harboring at least these last 217 C-terminal residues (32). Subsequently, Bejerano et al. (33) corroborated these data by showing that a purified polypeptide comprising these 217 C-terminal residues of CyaA could fully restore toxic activity of an inactive truncated CyaA lacking the 76 C-terminal amino acids.

To gain insight into the potential function of the RTX calcium binding motif/domain, we have constructed different fragments derived from the RTX domain and characterized the functional (ability to complement in vivo an inactive truncated CyaA), biophysical (fluorescence, circular dichroism spectroscopy, and channel-forming activity in lipid bilayers), and biochemical (calcium binding) properties in vivo and in vitro. Our results suggest a model for the folding of the RTX modules in which the functional calcium binding structure extends beyond the RTX glycine/aspartate-rich motifs to include adjacent polypeptide sequences that might directly participate in the stabilization of the parallel $\beta$-helix fold.

**EXPERIMENTAL PROCEDURES**

**Construction of the CyaA-derived Proteins**—DNA manipulations were performed according to standard protocols (34) in the Escherichia coli XL1-Blue strain (Stratagene, Amsterdam, The Netherlands) as host cells. The plasmids pT7CACT1, coding for the acylated wild type CyaA, and pACT/1–1006, coding for CyaA/1–1006, the RTX domain of CyaA, have already been described (35–37). The plasmid pTRCyaA/1–1006, in which the sequence coding for the last block of repeated sequences (between the SmaI and BspE1 sites) has been replaced by an appropriate synthetic double-stranded oligonucleotides encoding a termination sequence. The plasmid pTRCyaA/1–1006 encoding the acylated truncated protein (CyaA1–1006) is a derivative of pTRCAG (37), in which the molecule C-terminal DNA sequence (encompassing the RTX last block of repeated sequences and the secretion signal, located between the SmaI and BamH1 sites) was deleted and replaced by an appropriate synthetic double-stranded oligonucleotide encoding an amino acid termination sequence. The plasmid pTRCyaA/1–1006 encoding the last block of repeated sequences and its N- and C-terminal-flanking regions (CyaA1–1006) is a derivative of pACT/1–1006 (17), constructed in two steps. First, the DNA sequence coding for the N-terminal part of the CyaA RTX domain (between the NdeI and SmaI sites) was deleted and replaced by an appropriate synthetic double-stranded oligonucleotide encoding the amino acids Met-Leu-Glu-Gly; then the C-terminal DNA sequence (located between the BspE1 and BamH1 sites) was replaced by a synthetic double-stranded oligonucleotide encoding a hexahistidine tag and a termination signal (Pro-Asp-His-His-His-His-His-Stop). The plasmid pTRCyaA/1–1006, in which the sequence coding for the last block of repeated sequences (between the SmaI and BspE1 sites) has been replaced by an appropriate synthetic double-stranded oligonucleotides encoding a termination sequence. The plasmid pTRCyaA/1–1006 encoding the acylated truncated protein (CyaA1–1006) is a derivative of pTRCAG (37), in which the molecule C-terminal DNA sequence (encompassing the RTX last block of repeated sequences and the secretion signal, located between the SmaI and BamH1 sites) was deleted and replaced by an appropriate synthetic double-stranded oligonucleotide encoding an amino acid termination sequence. The plasmid pTRCyaA/1–1006 encoding the last block of repeated sequences and its N- and C-terminal-flanking regions (CyaA1–1006) is a derivative of pACT/1–1006 (17), constructed in two steps. First, the DNA sequence coding for the N-terminal part of the CyaA RTX domain (between the NdeI and SmaI sites) was deleted and replaced by an appropriate synthetic double-stranded oligonucleotide encoding the amino acids Met-Leu-Glu-Gly; then the C-terminal DNA sequence (located between the BspE1 and BamH1 sites) was replaced by a synthetic double-stranded oligonucleotide encoding a hexahistidine tag and a termination signal (Pro-Asp-His-His-His-His-His-Stop). The plasmid pTRCyaA/1–1006, in which the sequence coding for the last block of repeated sequences (between the SmaI and BspE1 sites) has been replaced by an appropriate synthetic double-stranded oligonucleotides encoding a termination sequence.

![FIGURE 1. Alignment of the RTX repeated sequences of CyaA. The potential RTX nonapeptide repeats localized within the last 700 residues of CyaA are aligned according to the RTX consensus GGXGD/3X(X)UX (U is any large hydrophobic residues), where X represents any amino acid. Highlighted in bold are the RTX sequences that match at least four of the five positions of the core consensus (underlined). Non-RTX sequences that interspersed the five separate blocks of successive RTX motifs (numbered I–V) are displayed on the left. The arrowheads indicated the position of the trypsin cleavage sites identified in limited proteolysis experiments (see Fig. 9). Numbers correspond to the amino acid position within the wild type CyaA sequence.](attachment:alignment.png)

**RTX Domain of B. pertussis Adenylate Cyclase**

**Non-RTX sequences**

| Block I |
|---------|
| 1006 LEHQHII |
| 1014 GGAGDSIT |
| 1087 GGSDDLD |

| Block II |
|---------|
| 1138 GSRLNDRIA |
| 1210 GRGGDDL |
| 1247 GVDVYDNVRVNSV |

| Block III |
|----------|
| 1353 YSQTGHAHGIAAGRIGL |
| 1377 GAGVDDKGLG |
| 1489 FSFPGRGGLDAKGAVGLS |
| 1529 GAGVDV |
| 1612 IGAGDIDT |
| 1706 INAGADQLPFAQGNDL |

**RTX consensus:**

| 1006 LEHQHII |
| 1014 GGAGDSIT |
| 1087 GGSDDLD |
| 1138 GSRLNDRIA |
| 1210 GRGGDDL |
| 1247 GVDVYDNVRVNSV |
| 1353 YSQTGHAHGIAAGRIGL |
| 1377 GAGVDDKGLG |
| 1489 FSFPGRGGLDAKGAVGLS |
| 1529 GAGVDV |
| 1612 IGAGDIDT |
| 1706 INAGADQLPFAQGNDL |

**Block IV**

| 1353 YSQTGHAHGIAAGRIGL |
| 1377 GAGVDDKGLG |
| 1489 FSFPGRGGLDAKGAVGLS |
| 1529 GAGVDV |
| 1612 IGAGDIDT |

**Block V**

| 1353 YSQTGHAHGIAAGRIGL |
| 1377 GAGVDDKGLG |
| 1489 FSFPGRGGLDAKGAVGLS |
| 1529 GAGVDV |
| 1612 IGAGDIDT |

**FIGURE 1. Alignment of the RTX repeated sequences of CyaA.** The potential RTX nonapeptide repeats localized within the last 700 residues of CyaA are aligned according to the RTX consensus GGXGD/3X(X)UX (U is any large hydrophobic residues), where X represents any amino acid. Highlighted in bold are the RTX sequences that match at least four of the five positions of the core consensus (underlined). Non-RTX sequences that interspersed the five separate blocks of successive RTX motifs (numbered I–V) are displayed on the left. The arrowheads indicated the position of the trypsin cleavage sites identified in limited proteolysis experiments (see Fig. 9). Numbers correspond to the amino acid position within the wild type CyaA sequence.
GCCGCCGCCGATTC-3', including in-frame NdeI and BspE1 sites, respectively). The fragment obtained was then inserted into the NdeI and BspE1 sites of pTRCyaAΔ1–1489Δ1682–1706 (see above), leading to the addition of an in-frame His tag in the C-terminal end of the CyaA1528–1612 protein.

Production and Purification of the CyaA-derived Proteins—Protocol for CyaA production has already been described elsewhere (25, 38, 39). All proteins were expressed in E. coli BLR strains (Novagen, Merck). Wild type CyaA and CyaA1–1490 were purified to greater than 95% homogeneity (as judged by SDS-gel analysis, Fig. 2B) from inclusion bodies by a two-step procedure including DEAE-Sepharose and phenyl-Sepharose chromatography as described (38, 39). CyaA1006–1706 and CyaA1006–1490 were purified by Ca²⁺-dependent phenyl-Sepharose chromatography as described previously (25). The cells were disrupted...
CyaA1528–1612 were directly purified from bacterial lysate (20 mM Hepes-Na, 50 mM NaCl, pH 7.5) on an immobilized nickel column (nickel nitrolotriacetic acid-agarose) according to the instructions of the manufacturer (Qiagen). The proteins eluted in the presence of 100 mM imidazole, pH 7.2, were then directly loaded (after dilution with 2 volumes of 20 mM Hepes-Na, pH 7.5) on a DEAE-Sepharose resin, equilibrated with 20 mM Hepes-Na, pH 7.5. After extensive washing with the same buffer, and dialyzed against 20 mM ammonium bicarbonate, pH 7.5. Protein concentrations were determined spectrophotometrically from the adsorption at 278 nm using a molecular extinction coefficient of 141 mM$^{-1}$ cm$^{-1}$ for the full length CyaA toxin, 132 mm$^{-1}$ cm$^{-1}$ for CyaA1490–1681, 67 mm$^{-1}$ cm$^{-1}$ for CyaA1006–1706, 52.5 mm$^{-1}$ cm$^{-1}$ for CyaA1006–1490, 25 mm$^{-1}$ cm$^{-1}$ for CyaA1490–1681, and 7.2 mm$^{-1}$ cm$^{-1}$ for CyaA1528–1612.

**Assay of Hemolytic and Invasive Activities**—The hemolytic and cell-invasive activities of the CyaA molecules and/or mixed CyaA fragments were characterized on sheep erythrocytes as described previously (25, 38). Purified proteins in 8 M urea, 20 mM Hepes-Na were directly diluted (at least 100-fold) into suspensions of sheep erythrocytes (5 × 10$^8$ cells/ml in 20 mM Hepes-Na, pH 7.5, 150 mM NaCl, 2 mM CaCl$_2$). Adenylate cyclase activity was measured as previously described (25, 38); one unit of enzymatic activity corresponds to 1 μmol of cAMP formed/min at 30 °C and pH 8.0.

**Membrane Experiments**—Black lipid bilayer membranes were formed as described previously (17, 23, 40). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole. The hole had a surface area of about 0.4 mm$^2$. Membranes were formed across the hole by painting onto a 1% solution of asolectin (lecithin type III from soybeans from Sigma) in n-decane. The 1 m KCl solutions (Merck) were buffered with 10 mM Hepes-KOH to a pH around 7. The temperature was kept at 20 °C throughout. The membrane current was measured with a pair of silver/silver chloride electrodes with salt bridges switched in series with a voltage source and an electrometer (Keithley 617). In the case of the channel recordings the electrometer was replaced by a homemade current amplifier. The amplified signal was monitored with a storage oscilloscope and recorded on a tape or a strip chart recorder.

**Fluorescence Spectroscopy**—The fluorescence spectra were recorded at 20 °C on a 2-ml sample using an LS-5B spectrofluorimeter (PerkinElmer Life Sciences). The excitation wavelength was selected at 292 nm with an excitation bandwidth of 5 nm, and the fluorescence emission spectra were recorded from 300 to 420 nm (emission bandwidth of 5 nm). CyaA1006–1706 and CyaA1490–1681 protein concentrations were 0.2 and 1.0 μM, respectively, in a 20 mM ammonium bicarbonate buffer, pH 8. The fluorescence emission spectra of the proteins were recorded at different calcium concentrations ranging from 0 to 2 mM. After each addition of CaCl$_2$, a 5-min equilibrium period was allowed before recording the spectrum. Finally, EGTA was added to a final concentration of 5 mM to record the fluorescence spectra of calcium-free proteins. The background fluorescence was recorded similarly in the absence of added proteins and subtracted from all spectra.

**Circular Dichroism Spectroscopy**—Circular dichroism spectra were recorded on a Jobin-Yvon CD62 micrograph at 20 °C with a 0.02-mm path length cylindrical suprasil quartz cell (Hellma). Protein samples were equilibrated in 20 mM ammonium bicarbonate, pH 8.0, by overnight dialysis at 4 °C, and spectra were determined at a protein concentration of 0.5 mg/ml in the presence or absence of 2 mM CaCl$_2$. Far UV region spectra represent the average of 5 successive scans between 180 and 260 nm with 0.5-nm steps using an integration time of 5 s between 180 and 200 nm and of 1 s between 200 to 260 nm. Base lines acquired on the 20 mM ammonium bicarbonate, pH 8, buffer (with or without the addition of 2 mM CaCl$_2$) under the same conditions were subtracted, and spectra were deconvoluted using the CDSSTR program from W. C. Johnson (41) included in the CDP software package.

**Analytical Ultracentrifugation**—CyaA RTX fragments were subjected to sedimentation equilibrium on a Beckman XL-A analytical ultracentrifuge equipped with standard double sector cells with 1.2-mm thick aluminum centerpieces. Protein samples were dialyzed extensively at 4 °C against 50 mM Hepes-Na, pH 7.5, 100 mM NaCl, 2 mM CaCl$_2$. Insoluble materials were removed by centrifugation at 14,000 × g for 15 min, and aliquots of CyaA1006–1706 (0.5 mg/ml; 6.95 μM) and CyaA1490–1681 (0.71 mg/ml; 31.9 μM) were then centrifuged at 20 °C at 20,000 and 30,000 rpm respectively, until perfect superposition of consecutive scans (about 20 h). The final equilibrium distributions were determined from absorption measurements at 280 nm. Theoretical partial specific volumes, calculated from the amino acid composition of CyaA1006–1706 and CyaA1490–1681 were 0.717 and 0.719 m$^3$ g$^{-1}$, respectively, and the solvent density of the Hepes/NaCl buffer was calculated to be 1.003 g ml$^{-1}$ at 20 °C. To calculate molecular weights, data were fitted using the Origin-based Optima XL-A data analysis software (Beckman). Different fitting models (single ideal component, two ideal components, and association of identical ideal components) for single data sets were systematically tested, and the best fit was retained on the basis of both the $x^2$ value and the lack of systematic deviation of the residuals.

**Ca$^{2+}$ Binding Assay**—Binding of calcium to CyaA1006–1706, CyaA1490–1681, and CyaA1528–1612 was determined by ultrafiltration, as described previously using radioactive $^{45}$Ca as a tracer (42). Purified proteins were extensively dialyzed against 20 mM Hepes-Na, pH 7.5, 100 mM NaCl, and insoluble materials were removed by centrifugation at 14,000 × g for 15 min (42). For the binding assays, 0.2-ml samples of a 12–90 μM solution of dialyzed proteins were placed in the top compartment of Amicon Ultrafree-MC centrifugal filter devices (Millipore Corporation, Bedford, MA; molecular mass cut-off of 5000 Da). Ten μl of a 5 mM CaCl$_2$ solution containing about 500 cpm of $^{45}$Ca/nmol of calcium were added to the protein and thoroughly mixed by vortexing. Ten μl of this solution were taken for counting of total radioactivity in a scintillation counter. The mixtures were then centrifuged at room temperature in a tabletop centrifuge for about 30 s until 10 μl were filtrated through. The ultra-filtrate was added back to the top compartment (i.e. protein solution) and again mixed and centrifuged as above (this second centrifugation was done to alleviate any dilution due to the dead-volume of the filtration membrane). Radioactivity of a 10-μl sample of the filtrate, which contained the free calcium, was determined by scintillation counting. Then 10 μl of the radioactive CaCl$_2$ solution as well as 10 μl of the protein solution were added to the top compartment, and the whole centrifugation procedure described above was repeated. At each step the ratio radioactivity in 10 μl of filtrate/radioactivity in 10 μl of top compartment is equal to free calcium/total added calcium. This allows calculating the number of calcium ions bound per protein molecule as a function of free calcium.
Limited Proteolysis—CyaA\textsubscript{1006–1706} or CyaA\textsubscript{1490–1681} was diluted to 1.0 mg/ml in 20 mM Hepes-Na, pH 7.5, in the presence of either 10 mM EDTA or 10 mM CaCl\textsubscript{2}. The solutions were equilibrated at 37 °C, and then trypsin was added to a final concentration of 1.0 g/ml. After 5, 10, and 30 min of incubation at 37 °C, aliquots of 20 μl (20 μg of protein) were taken out from the reaction mixture, and proteolysis was stopped by the addition of 500 μM 4-(2-aminoethyl) benzene sulfonyl fluoride and SDS-gel loading buffer. The samples were stored at −20 °C until analysis by gel electrophoresis. N-terminal sequencing of the proteolytic fragments (separated by SDS-PAGE, electrotransferred to polyvinylidene difluoride membrane, and stained with Coomassie Blue) was performed on an Applied Biosystems ABI 494 sequencer at the Plate-forme de microsec´enage of Institut Pasteur. Kinetic analyzes of the proteolytic cleavage of the CyaA\textsubscript{1006–1706} or CyaA\textsubscript{1490–1681} were carried out by recording the decay of the protein intrinsic fluorescence. CyaA\textsubscript{1006–1706} or CyaA\textsubscript{1490–1681} was diluted to 0.5 μM and 2 μM, respectively, in 20 mM Hepes-Na, pH 7.5, containing either 10 mM EDTA or 10 mM CaCl\textsubscript{2}. The fluorescence emission intensities were recorded at 320 nm (the excitation wavelength was 292 nm with a 5-nm band pass) on a Jasco FP-750 fluorimeter thermostatted at 37 °C under constant agitation. Proteolysis was initiated by the addition of 3 nM (final concentration) TPCK-treated trypsin, and the kinetics were recorded for 2000 s.

### RESULTS

#### Functional Complementation of the Truncated CyaA\textsubscript{1–1490} by Different RTX Fragments

Several fragments derived from the CyaA RTX domain (Fig. 2A) were expressed in E. coli and purified to near homogeneity as judged from SDS-PAGE analysis (Fig. 2B). CyaA\textsubscript{1006–1706} and CyaA\textsubscript{1006–1490} were purified by Ca\textsuperscript{2+}-dependent phenyl-Sepharose chromatography. A hexahistidine tag was genetically fused to the C terminus of the two shorter variants to permit their purification on a nickel column. These polypeptides were first tested for their ability to functionally complement an inactive CyaA variant, CyaA\textsubscript{1–1490}, deleted from its last 217 C-terminal residues. This truncated toxin had neither hemolytic nor cytotoxic activities (Fig. 3) as previously reported (32, 33). Similarly, the RTX-derived polypeptides were not hemolytic (data not shown) and obviously not cytotoxic as they have no adenylate cyclase catalytic domain. As shown in Fig. 3, the full-length RTX domain (CyaA\textsubscript{1006–1706}) was able to partially restore the hemolytic and cytotoxic activities of the truncated CyaA\textsubscript{1–1490}, whereas the truncated RTX...
domain, lacking the last block (block V) of repeated sequences, CyaA\textsuperscript{1–1490}, had no complementing activity. The last 217 C-terminal residues of CyaA, which contain the block V of RTX motifs with N- and C-terminal-flanking regions (CyaA\textsuperscript{1490–1681}), was also able to restore a significant hemolytic activity and a low but detectable cytotoxic activity of CyaA\textsuperscript{1–1490}. The partial complementation of CyaA\textsuperscript{1–1490} by CyaA\textsuperscript{1490–1681} suggests that this latter fragment had a lower affinity for CyaA\textsuperscript{11490} than the full-length RTX domain. In contrast, the polypeptide fragment, CyaA\textsuperscript{1528–1632}, which corresponds precisely to the nine RTX nonapeptide motifs of block V (that is, without the N- and C-terminal-flanking regions), was unable to restore the hemolytic and cytotoxic activities of CyaA\textsuperscript{1–1490}. This indicates that the polypeptide sequences flanking the RTX block V have an essential functional role in restoring the biological activities to CyaA\textsuperscript{1–1490} (Fig. 3), in agreement with previous report of Bejerano et al. (33).

CyaA is able to form small cation-selective channels in lipid bilayers (17, 18, 23). Recent studies have shown that the addition of calcium above 0.6 – 0.8 mM causes a large increase of bilayer conductance without noticeably changing the unit conductance of the channels formed by CyaA (23). We examined the channel-forming ability of the truncated CyaA variant, CyaA\textsuperscript{1–1490}, in the absence or in the presence of the two RTX-derived polypeptides, CyaA\textsuperscript{1006–1706} or CyaA\textsuperscript{1490–1681}. The truncated form of CyaA formed channels that were indistinguishable from those formed by wild type CyaA, which means that they had a

**FIGURE 4.** Functional complementation of pore-forming activity of the inactive truncated CyaA\textsuperscript{1–1490} by CyaA\textsuperscript{1006–1706} and CyaA\textsuperscript{1490–1681}. The channel-forming activity of CyaA derivatives was assessed on membranes formed from asolectin/ n-decane. The aqueous phase contained 1 M KCl, pH 7. The temperature was 20 °C, and the applied voltage was 50 mV at the cis side. The panels show the original strip chart recordings of membrane current/conductance after the indicated addition to the cis side of the membrane, proteins, CaCl\textsubscript{2}, or EDTA as a calcium chelator. A, 440 ng/ml CyaA\textsuperscript{1–1490} was added to the cis side of the membrane (left arrow), which led to a small increase of membrane conductance. About 7 min later 1.8 mM CaCl\textsubscript{2} was added to the cis side (middle arrow), which did not influence the membrane conductance. After about 4 min, 1 \(\mu\)g/ml CyaA\textsuperscript{1006–1706} was added to the cis side of the membrane, resulting in a steep increase of membrane conductance. B, 440 ng/ml CyaA\textsuperscript{1–1490} was added to the cis side of the membrane about 6 min before the start of the recording, which led to a small increase of membrane conductance. About 11 min afterward 15 min after the start of the recording) 2 mM CaCl\textsubscript{2} was added to the cis side (left arrow) followed by the addition of 1.6 \(\mu\)g/ml CyaA\textsuperscript{1490–1681} 3 min later (middle arrow). Note that the CyaA\textsuperscript{1–1490}-mediated conductance only increased when the calcium concentration was increased to 4 mM (right arrow). C, 160 ng/ml wild-type CyaA was added to the cis side of the membrane about 10 min before the start of recording. About 14 min afterward (4 min after the start of recording), 1 mM CaCl\textsubscript{2} was added to the cis side (left arrow), resulting in a steep conductance increase. The addition of 4.5 mM EDTA to the cis side (right arrow) led to a transient decrease of the membrane conductance. The amplification of the signal was decreased 10 times toward the end of recording. Note that the addition of EDTA stopped any further CyaA-mediated conductance increase.
single channel conductance of about 50 picosiemens in 1 M KCl, 10 mM Hepes-KOH, pH 7 (data not shown). As shown in Fig. 4A, irrespective of the presence or the absence of calcium ions in the medium, CyaA1–1490 caused a small increase in conductance across the bilayer similar to that observed previously with the full-length wild-type CyaA in the absence of calcium or in the absence of the RTX domain (23). The full-length RTX domain, CyaA1006–1706 or CyaA1490–1681 did not affect the CyaA1–1490-mediated conductance when added in the absence of calcium or low calcium concentration (see Fig. 4B). However, when sufficient calcium was present in the medium, the addition of either CyaA1006–1706 (or of CyaA1490–1681) strongly stimulated the overall conductance of CyaA1–1490 (see Fig. 4, A and B). This result suggested that the RTX-derived polypeptides were able to mimic the effect of the missing RTX domain in terms of channel-forming activity in asolectin membranes.

Interestingly, the addition of the calcium-chelator EDTA only transiently blocked cation conductance of either the wild type CyaA or of the complemented pair CyaA1–1490/CyaA1006–1681 or CyaA1–1490/CyaA1490–1681. Fig. 4C shows an experiment of this type for wild-type CyaA. 160 ng/ml wild-type CyaA was added to the cis side of a black asolectin lipid bilayer. After about 10 min, 1 mM CaCl2 was also added to the cis side of the membrane to enhance channel formation (left arrow in Fig. 4C). After about 4 min, 4.5 mM EDTA was added to the cis side to reduce the calcium concentration below the critical concentration (right arrow in Fig. 4C). Subsequently, the membrane conductance showed some transient decrease presumably caused by the addition of EDTA to the cis side. Afterward, the CyaA-mediated conductance was constant and did not increase further (see Fig. 4C). This was also observed when the complemented pair CyaA1–1490/CyaA1006–1681 or CyaA1–1490/CyaA1490–1681 was used in the experiments. These results suggested that calcium binding to the RTX domain of CyaA is required mainly for the insertion of the toxin into the membrane but not for the maintenance of the CyaA-mediated conductance when the channels were already formed.

Altogether these data confirm that CyaA1490–1681 constitutes an autonomous domain capable of restoring partial hemolytic and cytotoxic activities to the inactive truncated CyaA lacking the corresponding polypeptide segment. Nevertheless, some differences concerning the calcium-induced activation of the CyaA-mediated conductance were observed. Whereas 0.6–0.8 mM calcium was sufficient to induce conductance increase of wild-type CyaA, the concentration for the complemented pair CyaA1–1490/CyaA1006–1681 was about 1.5 mM calcium, whereas about 4 mM calcium were needed to increase conductance in the case of CyaA1–1490/CyaA1490–1681 (see Fig. 4B).

**Fluorescence Spectroscopy of the CyaA RTX Fragments**—CyaA1006–1706 and CyaA1490–1681 contain seven and 2 tryptophan residues, respectively. The tryptophan emission spectra of both proteins were recorded in the presence and absence of Ca2+/H11001. The binding of Ca2+/H11001 induced a 2-fold increase in the fluorescence intensity of both proteins with no significant shift in the emission maximum wavelength (Fig. 5A). The dependence of the fluorescence emission intensity at 336 nm upon the added calcium concentrations is shown in Fig. 5B. In both cases the maximal change in fluorescence signal was reached at a calcium concentration of about 1 mM. Fast kinetic analysis of the fluorescence changes upon the addition (or removal) of calcium indicated that the binding (or dissociation) of calcium was a very fast process (data not shown); the apparent $k_{on}$ and $k_{off}$ constants were in fact too high to be accurately determined because a major fraction of the overall fluorescence changes occurred during the mixing time (4 ms) of stopped-flow apparatus. Altogether, these data show that the tryptophans of RTX Domain of B. pertussis Adenylate Cyclase
CyaA<sub>1006–1706</sub> and CyaA<sub>1490–1681</sub> are sensitive to conformational changes induced by Ca<sup>2+</sup> binding. Interestingly, the two tryptophan residues of CyaA<sub>1490–1681</sub> (residues at position 1621 and 1645 of wild type CyaA) are located outside the RTX nonapeptide motifs, in the C-terminal extension (see Fig. 1). Hence, the calcium-induced enhancement of the tryptophan fluorescence of CyaA<sub>1490–1681</sub> strongly suggests that the C-terminal peptide downstream of the block V undergoes a structural rearrangement upon calcium binding to the adjacent RTX motifs.

The effect of various divalent cations on the fluorescence of CyaA<sub>1006–1706</sub> and CyaA<sub>1490–1681</sub> was also tested. As shown in Fig. 5C, the fluorescence intensities at 336 nm (λ<sub>max</sub>) of CyaA<sub>1006–1706</sub> and CyaA<sub>1490–1681</sub> were unaffected by the addition of 2 mM MgCl<sub>2</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub>, RbCl<sub>2</sub>, or CoCl<sub>2</sub>. Only SrCl<sub>2</sub> induced a significant enhancement of the fluorescence of these proteins. These data are in good agreement with electrophysiological studies that revealed that only SrCl<sub>2</sub> can trigger a very small increase of CyaA-mediated membrane conductance in lipid bilayers starting with about 3 mM, whereas Mg<sup>2+</sup>, Sr<sup>2+</sup>, and Ba<sup>2+</sup> cations could not (23). Yet Rhodes et al. (43) detected Mn<sup>2+</sup> binding to full-length CyaA by EPR spectroscopy and reported that Mn<sup>2+</sup> at high concentrations (above 10 mM) was able to confer some cytotoxic activity to CyaA. At variance with their results, in our conditions no hemolytic activity and also no channel formation in lipid bilayers could be detected in the presence of Mn<sup>2+</sup>.

Because CyaA<sub>1528–1612</sub> contained no tryptophan but 3 tyrosine residues (Fig. 1), we attempted to examine the effect of calcium on the tyrosine fluorescence properties of this polypeptide. The tyrosine fluorescence emission spectrum of CyaA<sub>1528–1612</sub> was unaffected by the presence or the absence of Ca<sup>2+</sup> (data not shown); therefore, no conclusion regarding calcium binding to CyaA<sub>1528–1612</sub> could be drawn.

Circular Dichroism of the CyaA RTX Fragments in the Far UV Region—To further characterize the structural changes induced by calcium binding, the secondary structure of the different fragments were studied by circular dichroism. The far UV spectra of the proteins, equilibrated in ammonium bicarbonate, were recorded in the presence or the absence of 2 mM CaCl<sub>2</sub>. As shown in Fig. 6, calcium binding triggered a large change in the CD spectra of CyaA<sub>1006–1706</sub> and CyaA<sub>1490–1681</sub> characterized by an increase of the positive band at 192 nm and a decrease of the negative band at 222 nm. In contrast, the CD spectrum of CyaA<sub>1528–1612</sub> was not affected by the addition of calcium (Fig. 6C).

When equilibrated in 6 M guanidinium hydrochloride, the spectrum of CyaA<sub>1490–1681</sub> had the characteristics of an unfolded polypeptide. The addition of calcium had no effect on the CD spectrum recorded in these conditions. Therefore, the changes of the CD spectra observed upon the addition of calcium to CyaA<sub>1490–1681</sub> and CyaA<sub>1006–1706</sub> equilibrated in ammonium bicarbonate buffer represent authentic calcium-induced conformational modifications of the secondary structures of the native polypeptide(s). The CD spectra of the different polypeptides were deconvoluted according to the CDSSTR procedure of Johnson (41), and the deduced compositions of secondary structures are shown in Table 1. This analysis shows that Ca<sup>2+</sup> binding to CyaA<sub>1006–1706</sub> is accompanied by an increase in the α-helical and β-strand contents of the protein, respectively, whereas calcium binding to CyaA<sub>1490–1681</sub> mainly induces an increase in its β-structure content. As expected, the secondary structure content of CyaA<sub>1528–1612</sub> was unchanged upon the addition of calcium. Very similar deconvolution results were obtained with the CONTINLL program included in the CDPro package.

From the above experiments we conclude that the full-length CyaA RTX domain as well as the C-terminal fragment, CyaA<sub>1490–1681</sub>, are both sensitive to calcium binding. Surprisingly, the short fragment,
CyaA\(^{1528-1612}\), corresponding to the 9 RTX repeat motifs of block V without the flanking regions, did not exhibit any changes in secondary structure upon the addition of calcium, although it should possess all the calcium-binding sites of CyaA\(^{1490-1681}\).

### Sedimentation Analysis of the CyaA RTX Fragments

To examine whether the structural changes in the CD signal of CyaA\(^{1006-1706}\) or CyaA\(^{1490-1681}\) upon calcium binding might result from an oligomerization process, diffusion-sedimentation equilibrium analysis were carried out. Representative sedimentation profiles for CyaA\(^{1006-1706}\) at 20,000 rpm and CyaA\(^{1490-1681}\) at 30,000 rpm (both at 20 °C) in the presence of 2 mM CaCl\(_2\) are shown in Fig. 7, A and B. The curves through the data are global fits of the ideal single-species model (see “Experimental Procedures”). The curve-fitting residuals are shown in the upper panels. The value of \(M_r\) deduced from this analysis was 75,230 \((\pm 1400)\) for CyaA\(^{1006-1706}\) and 21,260 \(\pm 1100\) for CyaA\(^{1490-1681}\).

### Ca\(^{2+}\) Binding Properties of the CyaA-RTX Fragments

Direct Ca\(^{2+}\) binding to the CyaA-RTX fragments was carried out by ultrafiltration using \(^{45}\)Ca\(^{2+}\) as tracer. As shown in Fig. 8, the full-length RTX domain of CyaA, CyaA\(^{1006-1706}\) bound about 30 Ca\(^{2+}\) ions with an affinity in the millimolar range. This stoichiometry is in good agreement with the number of canonical RTX sequences (as defined in Fig. 1) (15) found in the RTX domain of CyaA, suggesting that each of these motifs can bind one calcium ion. The additional less conserved RTX motifs might also bind calcium in certain conditions as was found in previous studies (25, 43). The CyaA\(^{1490-1681}\) fragment bound \(\sim 6-7\) calcium ions per molecule at the highest free calcium concentration tested. This value is in good agreement with the presence of 7 canonical RTX repeat motifs in this polypeptide and/or aggregation.

### Table 1

| Secondary structure composition | CyaA\(^{1006-1706}\) | CyaA\(^{1490-1681}\) | CyaA\(^{1528-1612}\) |
|--------------------------------|----------------|----------------|----------------|
| No Ca\(^{2+}\)                | 2 mM Ca\(^{2+}\) | No Ca\(^{2+}\) | 2 mM Ca\(^{2+}\) |
| α-Helix                      |                |                |                |
| %                            | 7              | 8              | 10             |
| β-Sheet                      |                |                |                |
| %                            | 24             | 20             | 23             |
| Turns                        |                |                |                |
| %                            | 12             | 15             | 14             |
| Other                        |                |                |                |
| %                            | 48             | 57             | 53             |
| Root mean square error       | 0.122          | 0.099          | 0.087          |

Data demonstrate that both CyaA\(^{1006-1706}\) and CyaA\(^{1490-1681}\) proteins are in a monomeric state in the presence of calcium, thus excluding that the calcium-induced changes in the secondary structure were due to protein oligomerization and/or aggregation.

### FIGURE 7.

Sedimentation equilibrium analysis of CyaA fragments. Representative data for CyaA\(^{1006-1706}\) (left panel) and CyaA\(^{1490-1681}\) (right panel) were obtained at 20 °C in buffer consisting of 20 mM Hepes-Na, pH 7.5, 100 mM NaCl, and 2 mM CaCl\(_2\), CyaA\(^{1006-1706}\) and CyaA\(^{1490-1681}\) were centrifuged at 20,000 and 30,000 rpm, respectively. The smooth curves are global fits of the ideal single-species model. The curve-fitting residuals are shown in the upper panels. The value of \(M_r\) deduced from this analysis was 75,230 \((\pm 1400)\) for CyaA\(^{1006-1706}\) and 21,260 \(\pm 1100\) for CyaA\(^{1490-1681}\).
the shorter polypeptide CyaA\textsuperscript{1528–1612} was unable to bind calcium in our experimental conditions, although it possesses the same RTX repeated sequences as CyaA\textsuperscript{1490–1681}. These data indicate that binding of calcium to these RTX motifs is critically dependent upon the presence of the adjacent polypeptide sequences that might directly participate in the stabilization of the parallel $\beta$-helix fold.

Limited Proteolysis of the CyaA-RTX Fragments — Limited proteolysis experiments were carried out to probe the conformational changes induced by calcium binding to the CyaA-RTX fragments. CyaA\textsuperscript{1006–1706} and CyaA\textsuperscript{1490–1681} were incubated in the presence of either calcium or EDTA, with trypsin (at a 1/1000 ratio) for various times, and analyzed by SDS-PAGE. As shown in Fig. 9, A and B, the two proteins in their calcium-free form were rapidly proteolyzed as compared with the calcium-bound polypeptides. Kinetic analysis of the proteolytic degradation, as monitored by the decay of intrinsic fluorescence of tryptophan residues (Fig. 9C), confirmed that the calcium-free forms of the CyaA fragments were much more susceptible to trypsin proteolysis than calcium-bound forms. Fig. 9D shows the N-terminal...
sequences of the main trypsin-resistant fragments derived from the calcium-bound CyaA1,006–1,706 (fragments a–d) or CyaA1,490–1,681 protein (fragment e). These data revealed that the main cleavage sites are located within the polypeptide segments that link the different RTX blocks (Fig. 1). The kinetics of appearance of these proteolytic fragments (Fig. 9A) suggest a hierarchical process for the proteolysis of calcium-bound CyaA1,006–1,706 in which the protein was first cleaved at Arg-1,367 into fragments a and b (Fig. 9). Fragment a was further cleaved at Arg-1,220 into fragments c1 and d, whereas fragment b was cleaved at Arg-1,491 to yield fragment c2. When the proteins were digested in the presence of EDTA, additional cleavage sites were found within the RTX blocks (data not shown). This suggests that, in the absence of calcium, these proteolytic cleavage sites were much more exposed to the protease than they are in the calcium-bound conformation. Altogether, these data indicate that, upon calcium-binding, both CyaA1,006–1,706 and CyaA1,490–1,681 adopt compact conformations as compared with the calcium-free proteins, thus shielding potential cleavage sites from the action of trypsin.

**DISCUSSION**

The RTX motif is a structural motif found in a number of cytolysins produced by Gram-negative bacteria as well as in several other potential bacterial pathogenic proteins (20, 21). The name RTX stems from a Gly- and Asp-rich nonapeptide repeat of canonical sequence GXXGXXDXUX, present in variable number, generally in a tandem fashion. This represents a specific type of Ca$^{2+}$ binding domain that is essential for the function of the proteins. Indeed most of the cytolysins are calcium-dependent, and it has been shown that purified *E. coli* α-hemolysin and *B. pertussis* CyaA can bind calcium in solution (25, 44–46). Three-dimensional structure analysis of the *P. aeruginosa* alkaline protease, which possesses six of these consensus RTX motifs, revealed that these repeated sequences constitute a new kind of calcium binding structure called a parallel β-helix or parallel β-roll (47). In this structure the first six residues of each motif form a turn that binds calcium, and the remaining three residues build a short β-strand. The arrangement of consecutive turns and β-strands builds up a right-handed helix of parallel β-strands, one turn of this helix consisting in two consecutive nine residues. Calcium is bound between two consecutive turns by the conserved aspartic acids. We previously showed that CyaA, which possesses about 30–40 of such RTX sequences, binds a large number of calcium ions in solutions with an affinity in the millimolar range, suggesting that each RTX motif can bind a calcium ion (25). A noticeable difference is that calcium ions appeared to be bound to the alkaline protease with a much higher affinity, as they could not be removed from the protein by calcium-chelators such as EDTA. Although the role of the RTX motifs in calcium binding is clearly demonstrated, the structural and functional consequences of calcium binding to these domains remain largely unknown.

In this work we have characterized an essential subdomain from the RTX-repeated structure of CyaA, located at the C-terminal moiety of the toxin. This CyaA fragment encompasses the last block of RTX repeat motifs (block V) as well as flanking non-RTX sequences at both the N and C termini. Strikingly, we found that these flanking sequences were essential for calcium binding to the RTX repeat motifs. The CyaA1,490–1,681 fragment contains only the 9 RTX tandem repeats of block V, which was unable to bind calcium, and its secondary structure was not affected by the presence or absence of calcium. However, when the 9 RTX tandem repeats of block V were expressed with the N- and C-terminal-flanking sequences, the CyaA1,490–1,681 fragment was able to bind up to 6–7 calcium ions per polypeptide with an affinity in the millimolar range. Because the first two of the nine repeats of block V only poorly match the RTX consensus sequence (see Fig. 1), this result suggests that only the seven truly canonical RTX nonapeptide repeats could bind calcium in vitro. This is in good agreement also with the stoichiometry of about 30 calcium ions bound to the whole RTX domain (CyaA1,006–1,706) that contains 31 canonical RTX sequences as defined in Fig. 1. Whether the degenerated RTX motifs might also bind calcium and/or contribute to structural stabilization of the calcium-bound conformation of the CyaA repeat domain, in particular when the toxin is bound to the receptor and target cell membrane, remains to be clarified. Welch (48) has indeed proposed a model in which calcium binding to the non-canonical RTX repeats of HlyA (and other RTX cytolysins) could contribute to the transition from the water- to the lipid-soluble state of these proteins.

Fluorescence, circular dichroism spectroscopy, and limited proteolysis studies revealed that, upon binding of calcium, the CyaA1,490–1,681 protein undergoes significant conformational changes, as is the case for the whole RTX domain of CyaA. The increase in the β-sheet content of CyaA1,490–1,681 upon calcium binding may indicate a stabilization of the β-roll structure. Furthermore, the 2-fold enhancement of the tryptophan fluorescence emission intensity of the CyaA1,490–1,681 protein upon binding of calcium is particularly noteworthy, as the two tryptophan residues of the protein are located outside the RTX motifs within the C-terminal-flanking polypeptide segment (see Fig. 1). This indicates that these two residues (Trp-1,621 and Trp-1,645) experienced a different environment in the calcium-bound versus calcium-free forms of the CyaA1,490–1,681 protein. Hence, calcium binding to the RTX...
motifs of CyaA (1,290–1,681) is able to induce conformational changes in the adjacent C-terminal non-RTX polypeptide segment.

Altogether, these results suggest a model for the folding of this essential RTX motif in which the functional calcium binding structure extends beyond the RTX glycine/aspartate-rich motifs to include adjacent polypeptide segments that might directly participate in the stabilization of the parallel β-helix fold. Upon calcium binding to the RTX repeat motifs of CyaA (1,290–1,681), the N- and C-terminal polypeptide extensions could interact with the RTX β-roll structure to stabilize its calcium-bound conformation. Such a model is in line with the previous study of Lilie et al. (28), who showed that a synthetic 75-mer polypeptide containing exactly 8 consensus RTX motifs of the sequence GSS-GNDNLS did not possess any regular secondary structure even in the presence of high concentration of Ca2+ (100 mM). β-Structure could only be detected upon the addition of Ca2+ and polyethylene glycol, an unspecific secondary-structure stabilizing agent. Further studies showed that the acquisition of secondary structure was tightly associated with the polymerization of this model RTX polypeptide, suggesting that intermolecular interactions were essential to stabilize the β-roll fold of this synthetic protein. The properties of this synthetic minimal RTX peptide are somewhat similar to what was observed with the CyAA1,252–1,612 fragment, containing only the block V RTX repeats, in that they are both unable to respond to calcium. It is noteworthy that in the crystal structure of the P. aeruginosa alkaline protease, the RTX β-roll is tightly packed against the protease domain (47). This may indicate the RTX β-roll is generally unstable unless stabilizing contacts can be established with additional polypeptide segments.

The observation reported here on the CyA subdomains might be pertinent to other RTX cytolsins. We propose that the RTX functional unit consists of the tandem repeated nonapeptide motifs flanked by adjacent polypeptide segments that are essential for the calcium-induced folding of the protein. Calcium binding to the RTX nonapeptide repeats induces a conformational rearrangement of the adjacent sequences, acting as a molecular switch to trigger the biological activity of the proteins. Indeed, prior studies on different RTX cytolsins have highlighted the critical role of polypeptide segments adjacent to the RTX-repeated sequences. In the case of CyA, Bejerano et al. (33) have shown that a stretch of 15 amino acids called “block A,” located C-terminal to the last RTX repeats, is essential for the toxic activity of the protein since it is required for the toxin binding and insertion into the membrane. Hence, a conformational change in the CyA block A segment as a result of calcium binding to the adjacent RTX motifs might be required for the toxic activity of the protein. It is noteworthy than one of the 2 tryptophan residues from the CyA (1,290–1,681), Trp-1621, shown to be sensitive to calcium binding, belongs to a highly conserved trimino acid stretch (N/D/W/F/Y) in the core of the block A segment. Deletion and/or mutations of the homologous A block of the E. coli α-hemolysin, were similarly shown to abolish the hemolytic activity of HlyA (49). Besides, Cortajarena et al. (50) showed that the same region of HlyA is also a major determinant for the specific binding of HlyA to the red blood cell surface through interaction with glycoporphin. Other studies on related RTX toxins have suggested that the polypeptide segments at the C terminus of the RTX motifs might be involved in the binding of these toxins to their β2-integrin receptor on target cells (51). We also showed recently that a region essential for CyA binding to its cellular CD11b/CD18 (αMβ2 integrin) receptor encompasses a non-RTX segment between the RTX block II and block III motifs. Calcium-induced conformational rearrangements of this segment might explain the strict calcium dependence of the CyA/CD11b association (31).

One of the key questions that remains to be clarified is how calcium binding to the repeat region located at the C terminus of CyaA can modulate insertion of the molecule into the target cell membrane and formation of cation channels. Both events involve the hydrophobic segments located in the central region of the protein. This implies that the Ca2+-induced conformational changes of the RTX domain, as evidenced by CD and fluorescence spectroscopy, must be transmitted somehow to the hydrophobic region of CyA. In the case of E. coli α-hemolysin, Schindel et al. (52) have shown that Ca2+ binding to HlyA triggers fluorescence changes of a fluorescent probe introduced in the hydrophobic region of the protein. One attractive hypothesis, in line with the model proposed earlier by Welch (48), could be that binding of Ca2+ to the RTX nonapeptide repeats could trigger their transient association with distinct segment(s) of the protein, thus promoting the transition from the water-soluble to the membrane-associated state of the cytolsins. Validation of this model will await further characterization of such putative intramolecular interactions.

Acknowledgments—We thank A. Ullmann for stimulating discussions and critical reading of the manuscript, R. Nageotte for the analytical ultracentrifugation experiments, and J. D’Alayer for the microsequencing experiments.

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RTX Domain of B. pertussis Adenylate Cyclase

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