Identification of a Region That Assists Membrane Insertion and Translocation of the Catalytic Domain of Bordetella pertussis CyaA Toxin*§

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Background: Translocation of the CyaA toxin across plasma membrane is still poorly understood.

Results: The region 375–485 is involved in membrane destabilization in vitro and required for cell intoxication.

Conclusions: The region 375–485 is crucial for membrane insertion and translocation of the catalytic domain of CyaA.

Significance: These results provide new insights on the early stages of the cell intoxication process.

The adenylyl cyclase (CyaA) toxin, one of the virulence factors secreted by Bordetella pertussis, the pathogenic bacteria responsible for whooping cough, plays a critical role in the early stages of respiratory tract colonization by this bacterium. The CyaA toxin is able to invade eukaryotic cells by translocating its N-terminal catalytic domain directly across the plasma membrane of the target cells, where, activated by endogenous calmodulin, it produces supraphysiological levels of cAMP. How the catalytic domain is transferred from the hydrophilic extracellular medium into the hydrophobic environment of the membrane and then to the cell cytoplasm remains an unsolved question. In this report, we have characterized the membrane-interacting properties of the CyaA catalytic domain. We showed that a protein covering the catalytic domain (AC384, encompassing residues 1–384 of CyaA) displayed no membrane association propensity. However, a longer polypeptide (AC489), encompassing residues 1–489 of CyaA, exhibited the intrinsic property to bind to membranes and to induce lipid bilayer destabilization. We further showed that deletion of residues 375–485 within CyaA totally abrogated the toxin’s ability to increase intracellular CAMP in target cells. These results indicate that, whereas the calmodulin dependent enzymatic domain is restricted to the amino-terminal residues 1–384 of CyaA, the membrane-interacting, translocation-competent domain extends up to residue 489. This thus suggests an important role of the region adjacent to the catalytic domain of CyaA in promoting its interaction with and its translocation across the plasma membrane of target cells.

Bordetella pertussis, the causative agent of whooping cough, produces a number of virulence factors. Among them, the adenylyl cyclase (CyaA) toxin, plays a critical role in the early stages of respiratory tract colonization by the pathogenic bacteria (1). CyaA is able to invade CD11b-expressing phagocytic cells, such as neutrophiles and macrophages, where, upon activation by calmodulin, it produces supraphysiological levels of cAMP that ultimately alter the phagocytic functions of these target cells (2–4).

CyaA is a 1706-residue-long bifunctional protein organized in a modular fashion; the ATP-cyclizing, calmodulin-activated, catalytic domain (AC) is located in the 400-amino-proximal residues, whereas the C-terminal 1306 residues are responsible for the hemolytic phenotype of B. pertussis (5, 6). Both activities can function independently as adenylyl cyclase and hemolysin, respectively. Several domains can be further defined in the C-terminal “hemolysin” moiety: (i) a hydrophobic region (located from residue 525 to 715) that contains several hydrophobic segments predicted to adopt α-helical structures and to insert into membranes in order to create the cation-selective pores responsible for the hemolytic activity (7, 8); (ii) a region (from residue 715 to 1000) containing two lysine residues, Lys-860 and Lys-983, that are selectively acylated by CyaC, a specific acyltransferase of B. pertussis (this post-translational modification is essential to convert the pro-CyaA polypeptide into an invasive toxin) (9, 10); and (iii) a region at the C-terminal end of the molecule (from residue 1000 to 1706) involved in toxin binding to its specific cellular receptor, the CD11b/CD18 integrin.

The abbreviations used are: CyaA, adenylyl cyclase; AC, adenylyl cyclase domain; RD, receptor binding domain; LUV, large unilamellar vesicle; PC, phosphatidycholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PE, phosphoethanolamine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; ANS, 8-anilino-1-naphthalene sulfonic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, p-xylene-bis-pyridinium bromide; AUC, analytical ultracentrifugation; SEC-TDA, size exclusion chromatography coupled on-line to a triple detector array; DPI, dual polarization interferometry; NR, neutron reflectometry; SMW, silicon-matched water; TEV, tobacco etch virus.

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‡This article contains supplemental data, Figs. S1–S6, and Tables S1 and S2.

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§The abbreviations used are: CyaA, adenylyl cyclase; AC, adenylyl cyclase domain; RD, receptor binding domain; LUV, large unilamellar vesicle; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PE, phosphoethanolamine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; ANS, 8-anilino-1-naphthalene sulfonic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, p-xylene-bis-pyridinium bromide; AUC, analytical ultracentrifugation; SEC-TDA, size exclusion chromatography coupled on-line to a triple detector array; DPI, dual polarization interferometry; NR, neutron reflectometry; SMW, silicon-matched water; TEV, tobacco etch virus.
Membrane Insertion of CyaA Toxin Catalytic Domain

Among the numerous aspects that remained to be clarified in this proposed model, a fundamental assumption is that the catalytic domain has to be transiently inserted into the cell membrane to allow calcium influx. To explore this aspect, we have investigated the membrane-interacting properties of the CyaA catalytic domain. Our results indicate that a protein covering the entire catalytic domain, AC384 (comprising residues 1–384 of CyaA) did not exhibit membrane association capability. We then characterized a longer polypeptide, AC489, encompassing residues 1–489 of CyaA, and we showed that AC489 was able to interact with lipid membranes and induce lipid bilayer permeabilization. We further showed that deletion of residues 375–485 within CyaA completely abolished the intoxication process of eukaryotic cells. Our results thus indicate a critical role of the region adjacent to the catalytic domain of CyaA. This is in agreement with the prior study of Gray et al. (29), who showed that the translocation of the catalytic domain could be blocked by a monoclonal antibody that recognizes an epitope located between residues 373 and 399. We propose that the membrane binding and destabilization properties of the N-terminal 489 residues of CyaA are directly involved in the early stages of the translocation process across the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Soy phosphatidylcholine (soy PC, 840054C), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 850457C), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, 850725C), cholesterol (700000P), and 1-palmitoyl-2-(6,7-dibromo)stearoyl-sn-glycero-3-phosphocholine (850480C) were from Avanti Polar Lipids (Alabaster, AL). 8-Anilino-1-naphthalene sulfonic acid (ANS) (A-47), ANTS (A-350), DPX (X-1525), and N-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (N-360) were purchased from Molecular Probes (Eugene, OR). 3D1 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All experiments were done in 20 mM Hepes, 50 mM NaCl, 2 mM CaCl₂, pH 7.4 (buffer A) at 25 °C, and lipid vesicles were prepared in 20 mM Hepes, 150 mM NaCl, pH 7.4 (buffer B) unless stated otherwise.

Protein Preparation and Activity Assays—The adenylate cyclase catalytic domain (AC384) corresponds to residues 1–384 of the B. pertussis CyaA toxin followed by a glycine and a lysine residue (30). The longer form of the catalytic domain (AC489) corresponds to residues 1–489 of CyaA. These proteins were expressed in Escherichia coli using the expression plasmids pTRAC384GK (described in Ref. 30) or pTRAC489, respectively. This latter was constructed by subcloning between the BstBI and EcoRI sites of pTRAC384GK, a PCR-amplified DNA fragment encompassing amino acid residues 372–489 of CyaA (primer sequences available upon request).

The E. coli strain BLR (Novagen) was transformed either with plasmid pTRAC384GK or with plasmid pTRAC489. Cells were grown at 30 °C in a 1.6–liter fermentor in middle density medium containing 100 µg ml⁻¹ ampicillin until the A₆₀₀ reaches 5, and expression of both proteins was triggered by shifting the growth temperature to 37 °C. After 5 h of growth at 37 °C, cells were harvested by centrifugation, resuspended in 20 mM Hepes, pH 7.4, and disrupted in a French press apparatus. The lysates were then ultracentrifuged, supernatants were discarded, and pellets were resuspended in 20 mM Hepes, 8 mM urea, pH 7.4, and agitated for 2–3 h (or overnight) at 4 °C. After 10 min of centrifugation at 13,000 × g, the supernatants (“urea extract”), containing the urea-solubilized AC384 or AC489, were collected. AC384 was purified by two sequential chromatographic steps on DEAE-Sepharose followed by a Sephacryl S300 column as described previously (30, 31).

The urea extract containing AC489 was first loaded onto a DEAE-Sepharose column equilibrated in 20 mM Hepes, 8 mM
Membrane Insertion of CyaA Toxin Catalytic Domain

urea, pH 7.4. In these conditions, AC489 bound to the resin and was eluted by 20 mM Hepes, 8 mM urea, 0.5 mM NaCl, pH 7.4, after extensive washing with 20 mM Hepes, 8 mM urea, 10 mM NaCl, pH 7.4. The eluate was then diluted 5 times in 20 mM Hepes, 0.1 mM CaCl$_2$, pH 7.4, and applied to a calmodulin-agarose column equilibrated in 20 mM Hepes, 2 mM CaCl$_2$, pH 7.4. AC489 was retained on the resin, and, after washing with 20 mM Hepes, 2 mM CaCl$_2$, 0.5 mM NaCl, pH 7.4, and 20 mM Hepes, CaCl$_2$, 0.1 mM, pH 7.4, the protein could be eluted in 20 mM Hepes, 8 mM urea, pH 7.4. Finally, the protein was loaded onto a Sephacryl S300 column and eluted as a refolded soluble form in 20 mM Hepes, 50 mM NaCl, pH 7.4.

Protein batches were more than 95% pure, as determined from SDS-PAGE analysis and N-terminal sequencing. The integrity and identity of the samples were confirmed by measurement of the absolute molecular mass by surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS model PCS 4000, Ciphergen). The mass spectra showed a single peak of 41,580 ± 300 g mol$^{-1}$ and 52200 ± 300 g mol$^{-1}$ for AC384 and AC489, respectively, which corresponded to the expected molecular masses. Molar e values of 28,880 m$^{-1}$ cm$^{-1}$ and 35,870 m$^{-1}$ cm$^{-1}$ were computed from the sequence of AC384 and AC489, respectively. Enzymatic activity was measured as described previously (32).

AC489TEV corresponds to a variant of AC489 protein in which the TEV protease recognition site GTTENLYFQGA was inserted between Ser373 and Lys374 (see supplemental Fig. S6A). AC489TEV was produced in E. coli strain BLR transformed with plasmid pTRAC489-TEV, which was constructed by insertion of a synthetic oligonucleotide (sequence available upon request) into the unique BstBI site of pTRAC489. The recombinant protein was purified as AC489. The protein was digested with 0.1 mg of TEV protease (kind gift from N. Wolff) per mg of AC489TEV at room temperature for 2 h in 20 mM Hepes, 50 mM NaCl, pH 7.4 (see Fig. 6). Cleavage efficiency was checked by SDS-PAGE analysis.

The CyaA toxin was produced in E. coli strain BLR transformed with plasmid pCACT3 (expressing the LacI repressor) and purified by DEAE-Septarose and phenyl-Septarose chromatographies as described previously (33). The CyaA$_{375-485}$ variant, which lacks residues 375–485, was produced similarly using plasmid pCACT-Δ375–485. This latter was constructed by subcloning, between the BstBI and XhoI sites of pCACT3, a PCR-amplified, DNA fragment encoding CyaA codons 489–1006 (details of construction available upon request). The CyaA$_{375-485}$ protein was purified as CyaA. Both proteins were more than 90% pure as determined from SDS-PAGE analysis (supplemental Fig. S1).

The hemolytic and cytotoxic activities (i.e. ability to raise cAMP inside target cells) of the toxins were determined on sheep erythrocytes as described previously (33, 34) except that cAMP was measured with the “HitHunter® cAMP XS+ assay” kit (DiscoverRx, Birmingham, UK) following the manufacturer’s instructions (for details, see the supplemental data).

Circular Dichroism, Analytical Ultracentrifugation, and Size Exclusion Chromatography Coupled to Tetra Detector—Circular dichroism (CD), analytical ultracentrifugation (AUC), and size exclusion chromatography coupled to a tetra detector array (SEC-TDA) were performed as described elsewhere (31, 35–38).

Preparation of Lipid Vesicles—Large unilamellar vesicles (LUVs) were prepared at a lipid concentration of 10 mM from PC (soy PC or POPC), DOPE, and cholesterol at a molar ratio of 6:3:0 or 6:3:1 in buffer B (20 mM Hepes, 150 mM NaCl, pH 7.4) by reverse phase evaporation and sequential filtration through 0.8-, 0.4-, and 0.2-μm polycarbonate filters as previously described by Chenal et al. (39). The suspensions of LUVs were monodisperse with mean hydrodynamic diameters of 150 nm, as checked by dynamic light scattering on a NanoZS instrument (Malvern) used as described elsewhere (38).

ANS Binding—Measurements were performed on an FP-6200 spectrophotometer (Jasco) in a Peltier-thermostated cell holder, using a 1-cm path length quartz cell (101.QS from Hellma). A bandwidth of 5 nm was used for the excitation and emission beams. Protein and ANS concentrations were 1 and 2.5 μM, respectively. The excitation wavelength was fixed at 360 nm, and emission spectra were recorded at 25 °C from 400 to 600 nm at a scan rate of 125 nm min$^{-1}$. All spectra represent the average of three emission spectra that were corrected for blank measurements.

Dual Polarization Interferometry—All dual polarization interferometry (DPI) measurements were performed on an AnaLight® 4D instrument (Farfield Group Ltd., Manchester, UK) using a 632.8-nm HeNe laser and a Harvard Apparatus PHD2000 pump to control the flow of the running buffer over the dual waveguide polarization surface. The pump was fitted with two 50-ml glass Hamilton gas-tight syringes for the perfusion of degassed buffer. Unmodified AnaChip® (a silicon oxynitride surface) was pre-prepared using UV ozone (ProCleaner, BIOForce Nanosciences (Ames, IA)), ex situ, for a cleaning time of 15 min at 20 °C. The chip was allowed to equilibrate under ambient conditions in its polypropylene container for 18 h before being mounted into the chip holder and loaded in the instrument.

Buffer B (thoroughly degassed using an Aries 101 degasser, Farfield Group Ltd.) was loaded into separate syringes and perfused over each of the corresponding two measurement channel surfaces at 50 μl min$^{-1}$. The dimensions of the channels were as follows: surface (S) = 15 mm$^2$ (15 × 1 mm); height (h) = 133 μm; and, consequently, volume (Vc = 2 μl). A calibration procedure was performed to define the thickness and refractive index of the waveguide chip and to determine the refractive index of the buffer flowing over the chip surface. The calibration steps were conducted using 80% ethanol (w/w) and HPLC grade water. All experiments were performed at 20 °C.

LUVs made of soy PC/DOPE/cholesterol (6:3:1) in buffer B were manually extruded at 20 °C through a 100-nm pore polycarbonate membrane (Avestin Inc. Lipofast extruder, 1-ml volume) for a total of 11 passes. The final loading concentration of LUVs used was 0.1 mg ml$^{-1}$, produced by diluting the extruded LUVs with buffer B supplemented with 2 mM CaCl$_2$. LUVs were injected over the dual waveguide buffer B surface at 25 μl min$^{-1}$ for 8 min, and the buffer was allowed to perfuse over the surface until a steady state response was reached. AC384 and AC489 proteins were filtered using a MILLES®-GV 0.22-μm filter unit, (Millipore). Stable lipid bilayers in their liquid crystalline state
were exposed to AC384 or AC489 proteins at 0.006, 0.019, 0.055, 0.16, and 0.5 μM concentrations. AC384 and AC489 proteins were injected at a flow rate of 50 μl min⁻¹ for 3 min, followed by buffer injections. From the transverse magnetic phase responses, the mass density of lipids ρₐ and proteins ρᵢ were deduced.

**Thermodynamics of AC489 Interaction with Membranes**—The partition coefficient (Kₓ), which is defined as the ratio of protein concentrations in the membrane and buffer phases respectively, was given by Equation 1,

\[
K_x = \frac{P_L / [L]}{P_W / [W]} = \frac{P_L / [W]}{P_W / [L]} \quad \text{(Eq. 1)}
\]

where \(P_W\) and \(P_L\) represent the amount of soluble protein (mol of protein in water phase) and amount of membrane-bound protein (mol of protein in lipid phase), respectively; [W] is the molar concentration of water; and [L] is the molar concentration of lipid. Given the relatively low number of molecules bound to the membrane, the molar concentration of protein in the aqueous phase was assumed to be equal to the injected protein concentration (Cᵢ).

The concentration of water is 55.5 M. The concentration of lipids, [L], is calculated from the experimental mass density data, \(\rho_L\), in the total channel volume,

\[
[L] = \frac{S \times \rho_L}{V \times M_L} \quad \text{(Eq. 2)}
\]

where \(S = 0.15\) cm², \(V_c = 2 \times 10^{-6}\) L, \(\rho_L\) in g cm⁻², and \(M_L\) the averaged molecular mass of lipids (\(M_L = 700\) g mol⁻¹). The amount of protein in solution is calculated as \(P_W = C_i \times V\), and the amount of membrane-bound protein is calculated as \(P_L = (\rho_L \times S) / M_p\), where \(M_p\) is the molecular mass of the protein.

Once the partition coefficient is calculated, the free energy (\(\Delta G\)) of transfer of the protein from water to membrane was calculated according to Equation 3,

\[
\Delta G = -RT \times \ln(K_x) \quad \text{(Eq. 3)}
\]

where \(\Delta G\) represents the free energy, \(R\) is the gas constant, \(T\) is the temperature, and \(K_x\) is the partition coefficient.

**Specular Neutron Reflectometry**—Neutron reflection was performed on free supported lipid bilayers using the Figaro reflectometer at the Institut Laue-Langevin Grenoble. The bilayers were produced by a combination of Langmuir-Blodgett and Schaefer techniques, onto polished silicon ingots measuring 80 × 50 × 20 mm and coated with a polymerized self-assembled phosphatidylcholine monolayer prepared by the technique outlined in Ref. 40. The block was then placed into the well of a Nima Langmuir trough, before filling with 18-megaohm Milli-Q water. A mixture of hydrophobic lipids POPC, DOPE, and cholesterol at a molar ratio of 6:3:1 were then spread from a solution of chloroform at 1 mg ml⁻¹ onto the water surface and compressed to 30 mM⁻¹ before vertical withdrawal of the block to cause deposition of a single LB layer, at an extraction rate of 5 mm min⁻¹. For the Langmuir-Schaefer transfers, a polytetrafluoroethylene neutron cell was placed in the trough before it was filled. After filling the trough, the monolayer was spread and compressed to the required pressure, and the substrate was mounted horizontally on the dipper such that its polished face was parallel to the water surface. The angle between the substrate and the horizontal was first coarsely adjusted using a small spirit level and then fine tuned by placing the substrate very close (~2–3 mm) to the water surface and adjusting the tilt until the substrate and its reflection appeared parallel to the naked eye. The substrate was then pushed through the interface at a speed of 3 mm min⁻¹ and then sealed in a custom-built PEEK neutron cell while still underwater and not opened during the measurements. Care was taken at all times not to introduce air bubbles into the cell during transfer of liquids. The outer casing of the cell was fabricated from stainless steel and sealed with a rubber O-ring and clamped shut. The cell was connected to an HPLC pump using polytetrafluoroethylene screw fittings (Omnifit) for exchanging buffers and contrasts as well as the different proteins. All exchanges were carried out at 1 ml min⁻¹. The temperature of the cell was adjusted by flushing temperature-controlled water from a heating water bath through the top and bottom clamping plates and held at 25 °C.

Measurements were made through the silicon block to the interface in the conventional neutron reflection liquid/solid configuration at angles of 0.6 and 3.8°. The collimation of the incident beam was adjusted to give a constant illumination of 6 × 3 cm. Varying subphase contrasts of D₂O, H₂O, and water matched to the scattering length density of silicon (silicon-matched water; SMW) allowed deconvolution of the reflectivity profiles. A typical run time at 3 subphase contrasts was 6 h. Reflectivity profiles were measured for the bilayer sample alone and then with AC384 or AC489 at 500 nm. Analysis was performed using the freely available software RasCAL, which uses a Parratt formulation to fit a model consisting of roughened layer structures to the reflectivity, with the best fit to the data being constrained by the multiple contrasts.

**LUV Permeabilization Assay**—Leakage of LUV content was assayed with ANTS (fluorescent probe) and DPX (quencher) entrapped in vesicles (41). LUVs (10 mM lipids) were prepared from a mixture of POPC, DOPE, and cholesterol (6:3:0 or 6:3:1) containing 20 mM ANTS and 60 mM DPX. Non-trapped probes were removed by desalting the LUV samples through a G-25 Sephadex column eluted with buffer B. For permeabilization assays, the LUVs (0.2 mM lipid final concentration) were incubated at 25 °C in buffer A under constant stirring. ANTS fluorescence was recorded continuously (excitation, 360 nm; emission, 510 nm; bandwidths, 5 nm) to set the base line. Then AC384, AC489, RD, or CyaA proteins were added to the sample, and fluorescence was recorded similarly up to 1 h.

Exponential functions were fitted to the experimental data (DPI and ANTS fluorescence) to extract amplitude and observed rate constant (k_obs) using the equation, \(y = A(1 - e^{-kt}) + b\). Accordingly, \(t_{50} = \ln2/k_{obs}\).
Membrane Insertion of CyaA Toxin Catalytic Domain

RESULTS

Biochemical and Biophysical Characterizations of AC384 and AC489 in Solution—The two proteins AC384 and AC489, encompassing residues 1–384 and 1–489 of CyaA, respectively, were overexpressed in E. coli as inclusion bodies. After solubilization in 20 mM Hepes, 8 mM urea, pH 7.4, the proteins were purified to near homogeneity (see supplemental Fig. S1 and “Experimental Procedures” for details) and readily refolded upon urea removal. Both purified AC proteins were able to interact with calmodulin with high affinity ($K_D^{(AC384)} = 0.06$ nM and $K_D^{(AC489)} = 0.20$ nM, as determined from concentrations giving half-maximal activation) and exhibited a high catalytic turnover ($>2000$ s$^{-1}$) in the presence of the activator. AC384 and AC489 were further characterized in solution to check their solubility, stability, folding, and hydrodynamic properties using a combination of biophysical approaches as described previously (31, 35–38). AUC and SEC-TDA showed that both proteins were fully soluble, homogeneous, and monomeric in the tested conditions (20 mM Hepes, pH 7.4, 50 mM NaCl at 25 °C) (supplemental Table S1). CD spectra in the far-UV range indicated that both proteins were folded (supplemental Fig. S2) and exhibited a secondary structure content matching with that predicted in silico (e.g. Self-Optimized Prediction Method with Alignment, SOPMA). Finally, analysis of the hydrodynamic properties by AUC and SEC-TDA revealed that both proteins displayed similar hydrodynamic properties (supplemental Table S1). Altogether, these data indicate that the two purified proteins were active, soluble, and monomeric.

We then investigated whether AC384 and AC489 exposed hydrophobic surfaces to the solvent by examining their ability to bind the apolar fluorescent probe, ANS (42). As shown in supplemental Fig. S3, upon the addition of AC384, the maximum emission wavelength of ANS was blue-shifted from 525 to 502 nm, and its fluorescence intensity increased 2-fold. Upon the addition of AC489, the maximum emission wavelength of ANS was blue-shifted from 525 to 498 nm, and its fluorescence intensity was about 3 times higher. These results therefore indicate that both AC proteins exposed similar hydrophobic regions to the solvent.

Membrane-Protein Interactions Studied by Dual Polarization Interferometry—Initial attempts to characterize the protein binding to LUVs by ultracentrifugation revealed that AC489 but not AC384 could bind to LUVs, although the overall partitioning of AC489 to the membrane fraction remained very low (see supplemental data and supplemental Fig. S4 for details), either because of a limited affinity of AC489 for LUVs or alternatively because of a fast kinetic of dissociation. We therefore explored the membrane binding process under steady-state conditions (i.e. without any fractionation steps) by using DPI, a relevant technique for the study of interactions of proteins with planar single lipid bilayers, which are spontaneously formed on the silicon oxynitride surface of the DPI analytical channels (43–46). DPI uses polarized light from a laser passing down stacked waveguides to monitor changes in the thickness and mass of immobilized molecules through changes in the resulting optical interference pattern. These changes are measured in real time and obtained when the light exits the waveguide structure. DPI can thus provide a direct analysis of protein binding to a planar lipid bilayer as well as information about structural changes within the lipid assembly.

A planar lipid bilayer membrane, made of soy PC/DOPE/cholesterol (6:3:1), was formed by lipid vesicle adsorption on the surface, and its structural parameters (thickness, mass, and birefringence) were characterized (supplemental Table S2). The membrane birefringence was determined to quantify the degree of alignment of the lipid molecules to the planar surface and the uniaxial packing and ordering of the membrane lipids. AC protein binding to this supported planar lipid bilayer was then monitored in real time, via the transverse magnetic and electric phase responses, which report the effective refractive index changes occurring at the surface of the sensor chip. Typical transverse magnetic phase shift data for the partition of AC384 and AC489 to the planar lipid bilayer are shown in Fig. 1, A and B, and the deduced mass changes are displayed in Fig. 1, C and D. No significant interaction of AC384 with the membranes was detected in these conditions (Fig. 1A). Conversely, the increase of the phase response indicated binding of AC489 to the lipid bilayer in a concentration-dependent manner. A rapid increase in membrane-bound AC489 was observed within the first few seconds after protein injection until a plateau was reached, which was followed by a biphasic dissociation upon rinsing with buffer (Fig. 1B). At the highest tested concentration of 0.5 μM protein in the bulk solution, up to 100 pg of AC489 were bound per mm$^2$ of lipid bilayer, whereas almost no deposition of AC384 occurred under these conditions (Fig. 1, C and D). It should be noted that there was no dramatic decrease of mass or thickness at any stage after the AC489 addition, suggesting that the overall structure of the lipid bilayer was not significantly modified upon protein binding. Taken together, these DPI results indicate that AC384 is not able to bind to the membranes, whereas the longer protein AC489 does partition into the lipid bilayer membranes.

The DPI data were further processed to determine the partition coefficient ($K_x$) and the binding kinetic parameters for each AC489 concentration tested. As shown in Fig. 1E, the partition coefficient ranged from 5 × 10$^{-5}$ to 3 × 10$^{-4}$, at the lowest and highest concentrations tested, respectively. The decrease of $K_x$ with increasing protein concentrations indicated that binding was anticooperative (47). From the partition coefficients, the free energy ($\Delta G$) of transfer of the protein from the buffer to the lipid phase was estimated to range between $-6$ and $-8$ kcal mol$^{-1}$ (Fig. 1E, inset) (see “Discussion”).

For each tested concentration, a single exponential function was then fitted to the time dependence mass increase to determine the apparent rate constants ($k_{obs}$) of binding (Fig. 1F). The $k_{obs}$ values increased as a function of the protein concentration in the liquid phase flowed in the DPI channels to reach a plateau corresponding to a maximum observed rate ($k_{max}$) of 0.17 s$^{-1}$, corresponding to a half-partition time of about 4 s. We also calculated the fractional surface occupancy of the membrane by the protein at saturating concentrations of AC489. From the maximal amount of membrane-bound protein of 100 pg mm$^{-2}$ of membrane surface, we estimated that at saturation, there was one AC489 molecule per 900 nm$^2$, which corresponds to a square of about 40 × 40 lipids (per membrane
leaflet, assuming that a lipid surface is about 0.7 nm²; see supplemental data).

Structural Insights from Specular Neutron Reflectometry—The binding of AC proteins to membrane was further characterized by specular neutron reflectometry (NR), which provides information about the layer composition, thickness, and interfacial roughness, perpendicular to the substrate surface of a protein/planar lipid bilayer complex (48–50). Hydrogen/deuterium (¹H/²H) contrast variation techniques were used to produce a set of specular NR profiles (48). The membrane used was a Langmuir Blodgett/Schaefer bilayer system (40) based on a mixture of hydrogenous lipids POPC/DOPE/cholesterol in the proportion 6:3:1. The planar supported lipid bilayers, in the absence or in the presence of proteins, were studied at two different water subphase contrasts by varying the D₂O/H₂O ratio, and their NR profiles are shown in Fig. 2. The interactions

FIGURE 1. Protein interaction with planar lipid bilayers followed by dual polarization interferometry. A and B, real-time transverse magnetic phase changes upon injections of 0.006, 0.019, 0.055, 0.16, and 0.5 μM AC384 (A) and AC489 (B) on a supported soy PC/DOPE/cholesterol (6:3:1) lipid bilayer. C and D, real-time mass density changes of the proteins, ρ, upon injections of 0.006 (dotted lines), 0.019 (dashed lines), 0.055 (continuous lines), 0.16 (heavy dashed lines), and 0.5 μM (heavy continuous lines) of AC384 (C) and AC489 (D). E, partition coefficient (Kx) as a function of the ratio of total protein versus total lipid concentrations (P/L). Inset, free energy (∆G) of protein partition from the buffer to the lipid phase as a function of P/L. F, observed binding constant rates (k_{obs}), deduced from fitting the real-time mass density changes ρ to a single exponential function, are reported as a function of AC489 protein concentrations.
of AC384 or AC489 were detected by the modifications observed in the NR profiles of the floating lipid bilayer. As shown in Fig. 2A, the NR profiles of the bilayers in the absence or in the presence of AC384 were identical, indicating that AC384 did not associate with membranes, in agreement with our data presented above. In contrast, the addition of AC489 to the bilayers induced significant changes of the NR profiles. This result was observed using two different contrast-matching conditions: SMW (Fig. 2B) and D2O (Fig. 2C). These results provided direct evidence that AC489 partitioned into the membranes. Moreover, the scattering length density of the bilayer was affected by the addition of AC489, showing that the protein was able to penetrate into the lipid bilayer (Fig. 2D). AC489 appeared to thicken and roughen the bilayer while increasing the scattering length density slightly, although from the resolution of the experiments, we could not precisely determine the depth of penetration of AC489. Taken together, these results indicated that AC489 not only bound membranes, as shown by DPI, but was able to insert into the membranes and modify the organization of the external lipid leaflet.

**LUV Permeabilization**—The capacity of the AC489 protein to interact with membranes, as demonstrated above, prompted us to examine whether it might be able to alter the membrane permeability of LUVs. The permeabilization of the vesicles, containing the co-entrapped probes ANTS and DPX, was monitored by following the fluorescence recovery of ANTS after release of the probes in the extravesicular milieu (Fig. 3). In all of these experiments, the total lipid concentrations were kept constant (0.2 mM lipids). A buffer injection (buffer A) was performed to check that efflux in the absence of added proteins remained in the noise level, and Triton X-100 (2 mM) was added to fully disrupt the vesicles and reveal the maximal fluorescent signal. The full-length CyaA toxin, which exhibits a pore-forming activity (51–54), was used as a positive control for LUV permeabilization. As shown in Fig. 3A, the purified acylated CyaA protein (20 μg ml⁻¹ (i.e. 0.11 μM)) indeed induced a fast and efficient release of the fluorophore/quencher pair from the LUVs. As a negative control, the CyaA RD domain (residues 1000–1706), which contains the C-terminal calcium-binding RTX motifs but lacks the hydrophobic segments, did not cause any release of the entrapped dyes from vesicles (not shown). Upon the addition of AC384 (20 μg ml⁻¹ (i.e. 0.5 μM)), no significant increase of fluorescence was noticed, indicating that the catalytic domain was not able to permeabilize the LUVs (Fig. 3A), in agreement with our above described results. Nevertheless, AC489 induced a significant release of dyes, although the maximal fluorescence intensity upon the addition of AC489 (20 μg ml⁻¹ (i.e. 0.4 μM)) only reached about 20% of the signal.
obtained with the whole CyaA, even after many h of incubation. Further addition of AC489 protein (up to 40 μg ml⁻¹) did not enhance the LUV permeabilization (not shown). Interestingly, when AC489 (at the same concentration) was mixed with an excess of calmodulin prior to the addition to the LUVs, the dye release was much less efficient (not shown), suggesting that the AC489-calmodulin complex had a much lower permeabilizing capability as compared with the free protein. Besides, the permeabilizing activity of AC489 was abrogated upon preincubation of the protein with the 3D1 monoclonal antibody, which recognizes an epitope at the C-terminal end of the catalytic domain (amino acids 373–399) (29) (supplemental Fig. S5).

We also explore the influence of the lipid composition and in particular the potential contribution of cholesterol onto the permeabilization properties of AC489. As shown in Fig. 3, the extent of vesicle leakage produced by AC489 or CyaA was similar or even slightly more efficient on LUVs made of PC/PE (6:3; Fig. 3B) as compared with LUVs made of PC/PE/cholesterol (6:3:1; Fig. 3A), indicating that cholesterol is not required for the AC489-induced permeabilization of LUVs. Taken together, these results showed that upon binding to LUVs, AC489 was able to alter the membrane permeability, whereas AC384 neither bound nor permeabilized the vesicles.

The membrane permeabilization by AC489 was further studied as a function of protein concentration (Fig. 4A). For each tested concentration, a single exponential function was fitted to the kinetic of permeabilization to determine the maximal amplitude of fluorescence and the observed rate constants (kobs). Fig. 4B shows that the fluorescence amplitude increased with the protein concentrations until reaching a plateau value, whereas the kobs values were roughly similar (~0.3 min⁻¹) at the different AC489 concentrations tested (Fig. 4B, inset).

It is noteworthy that this observed rate constant of dye release, kobs, corresponds to a half-permeabilization time of about 140 s, a much longer time scale as compared with the kinetics of AC489 binding to LUVs (t₁/₂ = 4 s). This suggests that the LUVs were not disrupted upon binding of AC489 but rather were gradually and slowly depleted of the intravesicular dyes.

We further investigated the AC489-induced membrane permeabilization as a function of the ionic strength. In all of the above-described experiments, the LUVs were prepared in a Hepes buffer containing 150 mM of NaCl and diluted for the permeabilization assay in a Hepes buffer containing 50 mM NaCl. Here, the permeabilization assays were carried out after diluting the LUVs in buffer containing various concentrations of NaCl while keeping the added AC489 protein and final lipid

FIGURE 3. Protein-induced membrane permeabilization. Time course of ANTS and DPX efflux from LUVs composed of POPC/DOPE/cholesterol at a molar ratio of 6:3:1 (A) or 6:3:0 (B) in the presence of CyaA, AC489, or AC384. Lipid and protein concentrations were 0.2 mM and 0.02 μg ml⁻¹, respectively (CyaA, 0.11 μM; AC489, 0.4 μM; AC384, 0.5 μM). A buffer injection was performed as a control (excitation, 360 nm; emission, 510 nm; bandwidths, 5 nm). The maximal fluorescence signal (in arbitrary units (a.u.)) obtained after the addition of Triton X-100 (2 mM) was 795 in the case of the POPC/DOPE/cholesterol preparation (A) and 980 in the case of the POPC/DOPE preparation (B).

FIGURE 4. Effect of increasing AC489 concentrations on LUVs permeabilization. A, time course of ANTS and DPX efflux from LUVs composed of POPC/DOPE/cholesterol (6:3:1) in the presence of the indicated concentrations of AC489. For each concentration, the time dependence of dye release was fitted to a single exponential function. B, amplitude and observed constant rates (kobs) (inset) obtained from the fitting procedure.
concentrations constant (0.4 mM and 0.2 mM, respectively). Fig. 5A shows that the dye release was dependent on the NaCl concentrations in the diluting buffer. The fraction of dye leakage decreased with increasing salt concentrations, and no leakage was detected beyond 150 mM NaCl in the extravesicular buffer (and 150 mM NaCl inside the LUVs). The strong dependence of the AC489-mediated LUV permeabilization upon the extravesicular salt concentrations suggests that either the protein ability to bind to and permeabilize membranes was affected by the ionic strength or, alternatively, the dye release from the vesicles required a chemical gradient across the membrane.

To discriminate between these two possibilities, similar experiments were performed with LUVs prepared in buffer containing 300 mM NaCl and diluted for the permeabilization assays in buffer with various salt concentrations. Fig. 5B shows that in these conditions, the vesicle permeabilization elicited by AC489 was still highly efficient at an extravesicular NaCl concentration of 150 mM. However, it was gradually abolished when the salt concentration was increased until 300 mM. The amplitudes of the fluorescent signals obtained in these different experiments were plotted in Fig. 5C, as a function of the ratio of extravesicular versus intravesicular NaCl concentrations. These data clearly indicate that the AC489-induced membrane permeabilization was not dependent upon the absolute extravesicular NaCl concentration, but rather, the fluorophore release triggered by AC489 was strictly dependent upon the salt gradient across the LUV membrane. These results also suggest that the limited extent of dye leakage observed even at saturating AC489 concentrations might be the consequence of the progressive dissipation of the electrochemical gradient across the lipid vesicles.

Finally, to determine the contribution of the C-terminal domain in the membrane permeabilizing activity of AC489, we attempted to produce the isolated polypeptide AC(374–489) encompassing residues 374–489 of CyaA. Unfortunately, this polypeptide turned out to be toxic for the bacteria and could not be expressed in bacteria (data not shown). Alternatively, we constructed a variant of AC489 protein (AC489TEV) harboring the TEV protease cleavage site inserted between Ser373 and Lys374 (supplemental Fig. S6A). AC489TEV could be produced and purified as AC489 and was efficiently cleaved in vitro by the TEV protease into two fragments AC(1–373) and AC(374–489) (Fig. 6A). Importantly, although AC489TEV exhibited similar LUV permeabilizing activity as AC489, the cleaved protein...
displayed only a reduced dye release capacity as compared with the uncleaved protein (Fig. 6B). The AC(374–489) fragment was further purified by size exclusion chromatography (Superdex 200) in the presence of 6 M urea. However, after dialysis, the isolated AC(374–489) fragment was found to be largely aggregated (supplemental Fig. S6) and unable to permeabilize the membranes (not shown).

Taken together, these results indicate that the integrity of AC489 was required for optimal membrane permeabilization and suggest that, rather than working autonomously, the C-terminal domain cooperates with the catalytic moiety to alter the permeability of LUVs.

Deletion of Residues 375–485 of CyaA Abolishes Cell Intoxication Capability but Enhances Hemolytic Activity of the Toxin—To assess the potential role of residues 375–485 in the cytotoxic properties of CyaA, we produced a CyaA variant, CyaAΔ375–485, lacking residues 375–485, and characterized its hemolytic activity and its ability to intoxicate sheep erythrocytes. As shown in Table 1, deletion of residues 375–485 within CyaA totally abrogated the ability of the toxin to increase intracellular cAMP in target cells, whereas it strongly enhanced its hemolytic activity as compared with the wild type, full-length toxin. These latter results are in agreement with the prior report of Gray et al. (29), who showed that deletion of the entire N-terminal region in CyaA up to residue 489 also increased the hemolytic activity. It is also noteworthy that neither AC384 nor AC489 displayed any hemolytic or intoxication activities (Table 1). Altogether, our data clearly demonstrate that the region encompassing residues 375–485 is essential for the translocation of the catalytic domain of CyaA across the plasma membrane of target cells.

**DISCUSSION**

Among bacterial toxins, CyaA is remarkable because it is able to translocate its catalytic domain directly across the plasma membrane of the target cells from the extracellular milieu into the cytosol. How the CyaA AC domain crosses the hydrophobic environment of the membrane is a fascinating question from a biophysical perspective.

As a first step to explore this process, we attempted in the present study to determine whether the catalytic domain possesses an intrinsic propensity to associate with membranes and eventually to alter their permeability, a property that may facilitate the passage of the polypeptide through the plasma membrane. We first analyzed the putative membrane binding properties of a polypeptide corresponding to the core of the catalytic domain of CyaA (i.e. encompassing residues 1–384). Our results clearly indicate that this protein, AC384, was not able to associate with membranes and had no detectable effect on LUV permeability. These results prompted us to characterize a longer protein, AC489, encompassing the whole catalytic domain and extending up to CyaA residue 489, located before the first hydrophobic segment that is part of the membrane insertion domain of the toxin (residues 525–715). We anticipated that the CyaA region spanning residues 384–489 might be required for favoring a putative association of the adjacent catalytic domain with the membrane. Until now, this region has not been precisely characterized, although Gray et al. (29) previously suggested that it might be implicated somehow in the internalization process (see below).

The AC489 protein was produced in E. coli and purified to homogeneity, and its physico-chemical properties were characterized in solution. The protein was monomeric and folded and exhibited an enzymatic activity and an affinity for calmodulin that were very similar to that of AC384. The membrane binding properties of AC489 as well as of AC384 were then characterized by DPI and specular NR. Both methods provided direct evidence that AC489 partitioned into the membrane, whereas AC384 did not. The NR data also indicated that the AC489 protein not only interacted with the external leaflet of the bilayer but penetrated significantly into the lipid bilayer. The DPI studies revealed the rapid kinetics of binding and dissociation of the AC489 protein with the membrane bilayer. This allowed us to determine the partition coefficients and the free energy (ΔG) of transfer of the protein from the buffer to the lipid phase. Interestingly, the calculated ΔG values (between −8 and −6 kcal mol−1) were found to be typical of amphitropic proteins; such proteins can switch between soluble and membrane-bound states to regulate their functions (55, 56). The free energy of partitioning of amphitropic proteins is accordingly far lower than that of integral membrane proteins, allowing them to reversibly associate with membrane, depending upon environmental conditions and/or structural modifications. In fact, many bacterial toxins exhibit amphitropic behavior because they are usually secreted by their host as soluble entities that can diffuse to their eukaryotic target cells, where they will reversibly interact with membranes to invade the cell (22, 57, 58).

Finally, we evaluated whether the binding of AC489 to the membrane might trigger a destabilization of the lipid bilayer, leading to a transient permeabilization of LUVs loaded with entrapped dyes. No significant dye release was detected in the presence of AC384, whereas AC489 induced a dose-dependent leakage of lipid vesicle content. Notably, at the highest AC489 concentration tested, only a fraction of the total encapsulated dyes were released even after prolonged incubation. On the contrary, the same vesicles could be fully permeabilized by the cytolytic activity of the full-length CyaA. We found that the AC489-mediated dye release from LUV permeabilization was strictly dependent upon a chemical gradient across the bilayer. This may explain why the dye leakage induced by AC489 is partial, reaching a plateau when the gradient is dissipated.

Analysis of the kinetic parameters of AC489 membrane binding and membrane destabilization from DPI and permeabilization data, respectively, indicates that whereas the mem-

**TABLE 1**

| Membrane Insertion of CyaA Toxin Catalytic Domain |
|-------------------------------------------------|
| Hemolytic and intoxication activities of CyaA toxin and its variants |
| Red blood cells were resuspended in 20 mM Hepes, 150 mM NaCl, pH 7.5. All experiments were performed at 37 °C in the presence of 2 mM CaCl2. Hemolytic activity was measured by photometric determination of the amount of hemoglobin released (A541). Intoxication activity was determined by measuring intracellular cAMP accumulation. As a negative control, we checked that both activities were abolished in the presence of EDTA. |
| | CyaA | CyaAΔ375–485 | AC384 | AC489 |
| | Relative hemolytic activity | 100 | 425 | 6 | 6 |
| | Relative intoxication activity | <1 | <1 | <2 | <2 |
brane partitioning of AC489 occurs in a few seconds ($t_{1/2} = 4$ s), the time scale of LU\'s permeabilization was on the order of minutes (e.g. $t_{1/2}$ of 140 s). This suggests that the membrane insertion process may take place in at least two steps; after partitioning into the membranes in the first step, AC489 may undergo a conformational reorganization in the membrane, leading to a permeabilizing-competent state. This state could locally destabilize the membrane, allowing a flux of ions and/or small molecules across the lipid bilayer in the presence of a chemical gradient.

Altogether, our results indicate that AC489 exhibits the intrinsic property to bind to and permeabilize membranes, whereas the AC384 protein, corresponding to the catalytic domain, strictly speaking, does not. Hence, the C-terminal extension of AC489 is critical to confer to the protein the capacity of partitioning to the membranes and to locally destabilize the lipid bilayer. Unfortunately, the C-terminal domain of AC489 could not be isolated as a monomeric and soluble form (supplemental Fig. S6), and thus its intrinsic membrane binding and permeabilization capacities could not be assessed. However, other results suggested that the integrity of AC489 was required for optimal membrane permeabilization (Fig. 6).

From these data, we propose that the membrane destabilizing capacity of the AC489 region may be directly implicated in the translocation of the catalytic domain across the lipid bilayer. Indeed, by altering the integrity of the membrane, the cost of the translocation of the catalytic domain across a locally destabilized plasma membrane is reduced, as suggested previously for other bacterial toxins (49). Our present results also demonstrate that the region encompassing residues 384–489 of CyaA is critical for both membrane binding and membrane permeabilization activities of AC489. Furthermore, deletion of the corresponding residues within the full-length CyaA protein completely abolished the invasive capacity of the toxin, as demonstrated by the inability of the CyaA$_{375–485}$ variant to increase cAMP into target cells. A potential role for the region adjacent to the CyaA catalytic domain was already suspected from the previous study of Gray et al. (29), who showed that the translocation of the catalytic domain could be blocked by the 3D1 monoclonal antibody that recognizes an epitope located between residues 373 and 399. They also found that the deletion of the N-terminal region in CyaA up to residue 489 strongly enhanced the hemolytic activity of the toxin as compared with the wild type full-length protein or a deleted variant lacking the first 373 residues. They proposed that the region encompassing residues 373–489 may undergo a conformational change upon delivery of the catalytic domain that could affect the structure of CyaA responsible for hemolytic activity (29). Our results indeed demonstrate for the first time a precise biological role for this region of CyaA and indicate that, if the calmodulin-dependent enzymatic domain is restricted to the amino-terminal residues 1–384 of CyaA, the membrane-interacting, translocation-competent polypeptide domain in fact extents up to residue 489.

CONCLUSION

According to the current model of intoxication, after CyaA binding to its cell receptor through its C-terminal RD domain, the hydrophobic segments (residues 525–715) are supposed to insert into the plasma membrane to anchor the protein to the target cells. At that stage, the N-terminal region of CyaA, AC489, may be ideally positioned to interact with the membrane bilayer and to alter its permeability, as shown here. This would facilitate (i) the calcium influx required for the toxin relocalization into lipid rafts (21, 27, 28) and (ii) the translocation of the catalytic domain across the plasma membrane. Other factors, like binding to the intracellular activator calmodulin, may also favor the translocation and proper refolding of the catalytic domain inside the target cells.

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Note Added in Proof—While this article was in preparation another article was published on the same subject (59).

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