Acylation of Lysine 860 Allows Tight Binding and Cytotoxicity of *Bordetella* Adenylate Cyclase on CD11b-Expressing Cells†

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ABSTRACT: The *Bordetella* adenylate cyclase toxin-hemolysin (CyaA, ACT, or AC-Hly) forms cation-selective membrane channels and delivers into the cytosol of target cells an adenylate cyclase domain (AC) that catalyzes uncontrolled conversion of cellular ATP to cAMP. Both toxin activities were previously shown to depend on post-translational activation of proCyaA to CyaA by covalent palmitoylation of the internal Lys^{983} residue (K983). CyaA, however, harbors a second RTX acylation site at residue Lys^{860} (K860), and the role of K860 acylation in toxin activity is unclear. We produced in *E. coli* the CyaA-K860R and CyaA-K983R toxin variants having the Lys^{860} and Lys^{983} acylation sites individually ablated by arginine substitutions. When examined for capacity to form membrane channels and to penetrate sheep erythrocytes, the CyaA-K860R acylated on Lys^{983} was about 1 order of magnitude more active than CyaA-K983R acylated on Lys^{860}, although, in comparison to intact CyaA, both monoacylated constructs exhibited markedly reduced activities in erythrocytes. Channels formed in lipid bilayers by CyaA-K983R were importantly less selective for cations than channels formed by CyaA-K860R, intact CyaA, or proCyaA, showing that, independent of its acylation status, the Lys^{983} residue may play a role in toxin structures that determine the distribution of charged residues at the entry or inside of the CyaA channel. While necessary for activity on erythrocytes, acylation of Lys^{983} was also sufficient for the full activity of CyaA on $CD11b^+$ J774A.1 monocytes. In turn, acylation of Lys^{860} alone did not permit toxin activity on erythrocytes, while it fully supported the high-affinity binding of CyaA-K983R to the toxin receptor CD11b/CD18 and conferred on CyaA-K983R a reduced but substantial capacity to penetrate and kill the $CD11b^+$ cells. This is the first evidence that acylation of Lys^{860} may play a role in the biological activity of CyaA, even if redundant to the acylation of Lys^{983}.

The whooping cough agent, *Bordetella pertussis*, secretes a repeat in toxin family protein (RTX)[1] adenylate cyclase toxin-hemolysin (CyaA, ACT, or AC-Hly) that is a key virulence factor of the bacterium and a promising antigen-delivery vector (*1*−*5*). CyaA is a 1706-residue-long protein consisting of a cell-invasive amino-terminal adenylate cyclase (AC) domain of ∼400 residues and of a channel-forming RTX part of ∼1300 residues (*6*−*8*). The primary toxin targets appear to be the myeloid phagocytic cells, such as macrophages, neutrophiles, and dendritic cells, which express the $\alpha_M\beta_2$ integrin CD11b/CD18 (CR3 or Mac-1) that binds CyaA with high affinity (*9*). Unlike other enzymatically active toxins that rely on endocytosis for cell penetration, CyaA appears to be delivering its catalytic adenylate cyclase domain into target cells directly across their cytoplasmic membrane (*10*−*12*). After membrane translocation, the AC enzyme is activated by binding cytoplasmic calmodulin and catalyzes uncontrolled conversion of ATP to cAMP (*13*). Intoxication by cAMP than results in rapid impairment of cellular microbicidal capacities, such as phagocytosis, chemotaxis, and oxidative burst activities, and induces macrophage death by apoptosis (*14*−*17*). Besides myeloid CD11b-expressing cells, the toxin can also bind and intoxicate with cAMP, at reduced but readily detectable levels, a broad variety of other eukaryotic cells, including mammalian erythrocytes on which CyaA can exert its hemolytic activity

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[1] Abbreviations: CyaA, acylated adenylate-cyclase toxin; CyaAΔ, truncated acylated CyaA with deletion of residues 1008−1706; pro-CyaA, nonacylated adenylate cyclase toxin; ACP, acyl-carrier protein; CyaC, protein toxin acyltransferase activating CyaA; RBC, red blood cell or erythrocyte; RTX, repeat in toxin family protein; HlyA, the RTX α-hemolysin of *E. coli*; *Bp*-CyaA, native CyaA produced by *B. pertussis*; r-*Bp*-CyaA, recombinant CyaA overproduced by *B. pertussis* 18323/pBN; *r-Ec*-CyaA, recombinant CyaA toxin produced in *E. coli*.

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(*18*). This is due to the capacity of CyaA to form small cation-selective channels in cellular membranes and cause colloid-osmotic cell lysis (*10*, *19*, *20*). However, the physiological relevance of the toxin and/or signaling activities of CyaA on nonmyeloid cells, such as respiratory epithelial cells and tissues, remains unexplored.

Both the cytotoxic and hemolytic (channel-forming) activities of CyaA strictly depend on binding of approximately 40 calcium atoms per the glycine- and aspartate-rich RTX domain of CyaA, which harbors the typical nonapeptide repeats of a consensus sequence X-(L/I/V)-X-G-G-X-G-X-D forming the calcium-binding sites (*8*, *21*, *22*). Similar to other RTX cytotoxins, another key requirement for CyaA activity is the post-translational activation by covalent fatty acylation. The toxin is produced as an inactive proCyaA protoxin and was shown to be activated to mature CyaA by covalent amide-linked palmitoylation of the ϵ-amino group of lysine residue 983 (*23*). This reaction is catalyzed by a coexpressed accessory protein acyltransferase, CyaC, using the acyl-acyl carrier protein (acyl-ACP) as the donor of fatty acids (*23*−*26*). Acylation appears, indeed, to be required for toxin activity of CyaA on any type of cells, and it was recently shown to be essential also for tight interaction of CyaA with its CD11b/CD18 integrin receptor (*27*).

The extent of CyaA fatty acylation *in vivo* appears to depend on the producing strain. Initially, the *Bp*-CyaA extracted from a Tohama I-type *B. pertussis* 338 was found to be monoacylated by a single palmitoylation at only the Lys^{983} residue (*23*). The CyaA sequence, however, comprises two characteristic acylation sites conserved in RTX cytolysin sequences, suggesting that CyaA could be acylated on two lysine residues, Lys^{860} (K860) and Lys^{983} (K983), similarly to the homologous RTX α-hemolysin, HlyA, of uropathogenic *Escherichia coli* (*28*, *29*). The recombinant r-*Ec*-CyaA toxin coexpressed with CyaC in *E. coli* K12 was, indeed, found to bear a second acylation on the Lys^{860} residue (*30*). This was initially thought to account for the reduced specific hemolytic activity of r-*Ec*-CyaA, compared to *Bp*-CyaA (*30*). However, the recombinant r-*Bp*-CyaA protein overproduced by a *B. pertussis* 18323/pBN strain was also found to be acylated on both Lys^{860} and Lys^{983} residues. The reduced specific hemolytic activity of r-*Ec*-CyaA was, hence, attributed to its modification by mainly the unsaturated palmitoleil (*cis* Δ9 C16:1) fatty-acyl groups, while only saturated C16:0 palmitate residues were found to be attached to *Bp*-CyaA (*31*). It could further be shown, by using monoacylated r-*Ec*-CyaA variants activated by mutant CyaC acyltransferases, that acylation of the Lys^{983} residue was necessary and sufficient for conferring on CyaA the capacity to penetrate sheep erythrocytes, while acylation of CyaA at the Lys^{860} residue alone did not contribute to CyaA activity on these surrogate target cells (*32*). In contrast, it could be concluded that the Lys^{860} residue plays an acylation-independent structural role in translocation of CyaA across the erythrocyte membrane, because a conservative substitution of Lys^{860} by an arginine residue (K860R) provoked an important drop of toxin capacity to penetrate erythrocytes (*33*).

In this work, we addressed the respective roles of acylation of Lys^{860} and Lys^{983} in the interaction of CyaA with cells expressing the CD11b/CD18 receptor and show that Lys^{983} also plays a structural role, controlling ion selectivity of CyaA channels. It is further shown for the first time that acylation of Lys^{860} might be of biological significance, because in the absence of acylation of Lys^{983}, it allows full receptor binding and partial toxin activity of CyaA on myeloid $CD11b^+$ cells.

EXPERIMENTAL PROCEDURES

Construction, Production, and Purification of the CyaA-Derived Proteins. Construction of CyaA-K860R was previously described (*33*). The CyaA-K983R and CyaA-K983V variants were constructed in an analogous way, by PCR mutagenesis using the primer pairs 5′-AACCGCCTACG-GAAGACGCACGGAGAAT and 5′-ATTCTCCGTGCGT-CTTCCGTAGGCGGTT and 5′-AACCGCCTACGGCG-TACGCAC GGAGAAT and 5′-ATTCTCCGTGCGTACGC-CGTAGGCGGTT, respectively. The absence of unrelated mutations in the constructs was verified by DNA sequencing. The plasmids for expression of the truncated CyaAΔ variants were obtained by replacement of the *Xho*I−*Sca*I fragments of the respective mutant pT7CACT derivatives by the *Kpn*I−*Sca*I fragment of pACTΔC1322 (*33*). This resulted in insertion of a TAA stop codon at the *Xho*I site and production of truncated CyaA proteins (CyaAΔ) having Asp^{1008} as the C-terminal residue.

The wild-type CyaA, proCyaA, and its monoacylated derivatives CyaA-K983R, CyaA-K983V, and CyaA-K860R, as well as their CyaAΔ variants, were produced in the presence or absence of the activating protein CyaC, using the *E. coli* strain XL1-Blue (Stratagene) transformed with the appropriate plasmid construct derived from pT7CACT1, as previously described (*33*). Bacteria were grown at 37 °C in 500 mL cultures in LB medium with ampicillin (150 μg/mL), and CyaA synthesis was induced by IPTG in mid-exponential phase. After a further 4 h of growth, the cells were collected and disrupted by ultrasound, the insoluble CyaA was extracted from cell debris with 8 M urea, 50 mM Tris-HCl at pH 8.0, and 0.2 mM $CaCl_2$, and the proteins were purified by ion-exchange chromatography on DEAE-Sepharose followed by hydrophobic chromatography on Phenyl-Sepharose (*37*). All experiments were repeated with proteins from at least two independent toxin preparations.

High-Resolution Two-Dimensional Gel Electrophoresis. Whole-cell extracts of clones expressing the truncated CyaAΔ proteins were prepared from exponentially growing cultures induced by IPTG (1 mM) for 3 h. Total protein samples were analyzed by two-dimensional (IEF/SDS−PAGE) gel electrophoresis as previously described (*32*−*34*), using 18 cm pH 5.0−6.0 Immobiline DryStrips (Amersham) for isoelectric focusing over 40 h at 119 400 Vh, followed by separation on 7.5% acrylamide SDS−PAGE slab gels (22 × 22 cm). This analysis was performed with the truncated CyaAΔ variants, because the theoretical pI values of the denatured and differently acylated full-length CyaA proteins differ by only 0.01 pH unit (4.27, 4.26, and 4.25 for the non-, mono-, and bi-acylated ACT, respectively) and the proteins could not reliably be resolved (*32*). The truncated CyaAΔ proteins, however, exhibit larger pI value differences (5.41, 5.35, and 5.29), allowing their unambiguous resolution. It has previously been demonstrated that deletion of the RTX domain does not affect the acylation status of the CyaAΔ

proteins, as compared to full-length proteins and validated by mass spectrometric characterization (*32*, *33*).

Assay of AC Activity, Binding, and Cytotoxic and Hemolytic Activities on Sheep Erythrocytes. Adenylate cyclase enzymatic activity was measured in the presence of 1 μM calmodulin as described previously (*35*). One unit of AC activity corresponds to 1 μmol of cAMP formed/min at 30 °C and pH 8.0. Cytotoxic activity on sheep erythrocytes was determined as the capacity of various CyaAs to raise intracellular cAMP levels in sheep erythrocytes upon incubation of 5 μg/mL of the CyaA proteins for 30 min at 37 °C in HBSS (10 mM HEPES−Na at pH 7.4, 10 mM KCl, 140 mM NaCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM D-glucose). Hemolytic activity was measured by photometric determination (A_{541}) of the amount of hemoglobin released upon incubation of erythrocytes (5×10^8/mL), with 5 μg/mL of toxin for 210 min (*10*). Binding of CyaA to sheep erythrocytes was determined as previously described (*36*), using 5×10^8 cells/mL in 20 mM Tris at pH 8.0, 150 mM NaCl (TN buffer), and 2 mM $CaCl_2$ (TNC buffer). After 30 min of incubation at 37 °C, the unbound CyaA was removed by two cell washes in TN buffer at 6000*g* at 4 °C for 2 min, using fresh tubes at each step to avoid ACT carry over on tube walls. CyaA that was not integrated into the erythrocyte membrane was stripped-off by a final wash in 0.1 M Na_2CO_3 (pH 10.5), and the cells were lyzed in 50 mM Tris-HCl buffer at pH 8.0 and 0.2 mM $CaCl_2$ containing 0.1% Triton X-100 for determination of amounts of cell-associated AC enzyme. The activities of intact CyaA were taken as 100% activity. For the purpose of activity comparisons, it was verified that no saturation of binding to erythrocytes, hemolytic, and cell invasive activity (cAMP elevation) was reached for the intact CyaA or mutant toxins at the used protein concentration (5 μg/mL).

Binding of CyaA to CD11b/CD18-Expressing CHO Cells. Binding of CyaA proteins to $CD11b^+$ cells was determined as described previously (*27*). Briefly, 2×10^5 CHO-CD11b cells were incubated with the indicated concentrations of different CyaA proteins in DMEM medium without serum in 96-well culture plates for 30 min on ice. Competitor CyaA-biotin (a gift from D. Ladant) was added to 30 nM, and its binding to CHO-CD11b cells was measured by FACS. Results are expressed as the percent of CyaA-biotin binding = (CyaA-biotin bound in the presence of the competitor)/(maximal CyaA-biotin binding in the absence of the competitor CyaA) × 100%.

CyaA Toxin Activities on J774A.1 Cells. J774A.1 mouse macrophage-like cells were grown in RPMI media containing 10% fetal calf serum (FCS). Prior to assay, 10^5 cells were seeded per well of a 96-well plate and allowed to attach for 2 h. To avoid uncontrollable chelating of calcium ions that are required for CyaA activity, the phosphate-buffered (10 mM) RPMI medium was replaced by 150 μL/well of HEPES-buffered serum-free DMEM medium (1.9 mM Ca^{2+}), and the cells were allowed to rest in DMEM for 1 h at 37 °C in a humidified 5% CO_2 atmosphere. Toxin samples were prediluted from concentrated stocks to 100 times the final indicated concentration using 8 M urea, 50 mM Tris-HCl at pH 8.0, and 0.2 mM $CaCl_2$ buffer. Right before the addition to cells, the toxin samples were rapidly diluted further 25 times in prewarmed DMEM, to reduce the urea concentration to 0.4 M, and 50 μL aliquots of the diluted toxin samples were rapidly admixed with 150 μL of DMEM medium covering the cells, to yield the indicated toxin concentrations. This resulted in a final urea concentration of 80 mM. Appropriate blanks containing identical amounts of cells incubated with 80 mM urea in DMEM were systematically scored in all activity assays, and no effect whatsoever of the 80 mM urea concentration on cell viability and/or enzymatic activities was observed under the used conditions.

After 3 h of incubation with various toxins at the indicated concentration, lysis of the J774.1 cell was assessed by the lactate dehydrogenase (LDH) release assay, using the CytoTox 96 kit (Promega) according to the instructions of the manufacturer. Cell viability following exposure to the toxin was determined as the capacity of mitochondrial reductases to convert the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan, using the WST-1 assay kit (Roche) according to the protocol of the manufacturer.

For determination of intracellular cAMP levels, the cells were incubated with various concentrations of CyaA for 30 min in DMEM containing 100 μM IBMX (3-isobutyl-1-methylxanthin) as the phosphodiesterase inhibitor. The reaction was stopped by addition of 0.1% Tween-20 in 100 μM HCl, and the samples in 96-well plates were heated for 15 min at 100 °C to denature cellular proteins (cAMP is acid- and heat-resistant). The samples were neutralized by the addition of 150 μM unbuffered imidazol, and the cAMP concentration was determined by an antibody competition immunoassay as previously described (*37*). Briefly, microtiter ELISA plates (Nunc-Immuno, Maxisorp) were coated with a cAMP-BSA conjugate (a gift from D. Ladant) diluted to 5 μg/mL in 0.1 M Na_2CO_3 at pH 9.5. The plate wells were washed twice in 50 mM Tris-HCl, 0.15 M NaCl, and 0.1% Tween-20 at pH 8 (TBS-Tween), saturated for 3 h with 2% BSA in TBS (TBS-BSA) and washed 3 times with TBS-Tween. A total of 100 μL of the sample or the cAMP standard (Sigma) was directly added to the plate wells coated with cAMP-BSA and containing 100 μL of anti-cAMP rabbit antibodies (a gift from A. Ullmann) diluted at 1:3000 in 2% TBS-BSA. Upon incubation at 4 °C overnight, the plates were washed 4 times with TBS-Tween, and anti-rabbit-peroxidase conjugate (1:1000) was added in PBS-BSA. After incubation at 37 °C for 2 h, the wells were washed 4 times with TBS-Tween, and the peroxidase activity was determined using *o*-phenylenediamine (Sigma−Aldrich) as the substrate.

Planar Lipid Bilayer Measurements. Activities of the CyaA proteins on black lipid bilayer membranes were measured at 20 °C essentially as previously described (*38*), using a Teflon cell with two water-filled compartments connected by a small circular hole of an area of ~0.4 mm^2. Membranes were formed across the hole from a 1% solution of soybean asolectin (lecithin type IIIs; Sigma Chemical Co., St. Louis, MO) in *n*-decane. The electrical measurements were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a homemade current−voltage converter. The amplified signal was recorded on a strip chart or tape recorder. All salts were obtained from Merck (Darmstadt, Germany, analytical grade) and were buffered with 10 mM HEPES-KOH to pH 7.

For ion-selectivity measurements, the membranes were formed in a 100 mM KCl solution. The toxin was added to

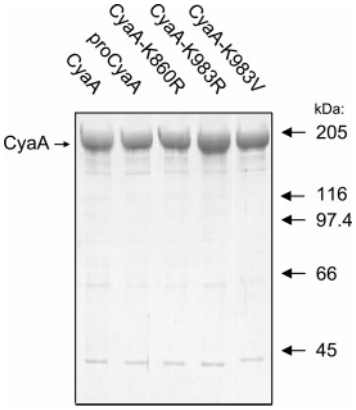

FIGURE 1: SDS−PAGE analysis of the purified CyaA proteins. The nonacylated proCyaA, the doubly acylated CyaA, and the monoacylated CyaA-K860R, CyaA-K983R, and CyaA-K983V proteins, respectively, were expressed in *E. coli* XL1 and purified by DEAE and Phenyl-Sepharose chromatography as previously described (*37*). A total of 2 μg of purified protein was separated on 7.5% SDS−PAGE gel and stained by Coomassie Blue.

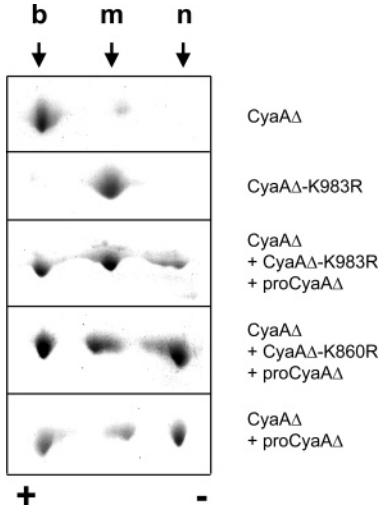

FIGURE 2: Analysis of CyaAΔ acylation by two-dimensional electrophoresis. A total of 60 μg of total protein extract of IPTG-induced cells producing the CyaAΔ proteins was prepared and analyzed by two-dimensional (IEF/SDS−PAGE) electrophoresis, as previously described (*32*, *33*). Binary or ternary mixtures of extracts were prepared by directly mixing equal volumes of extracts containing similar amounts of total cell proteins. The gels were stained with Coomassie Blue, and only the corresponding gel sections comprising the spots of CyaAΔ-derived proteins are shown. The relative positions of the spots reflect their relative position in respect to the anode (+) and cathode (−) in the original pH gradients after isoelectric focusing, depending on the pI shifts introduced by the positive-charge-neutralizing acylation of lysine residues 860 or 983. Note that arginines substituting the lysine residues cannot be acylated by CyaC and, hence, introduce a positive charge leading to increased pI of the mutant proteins in respect to doubly acylated CyaAΔ. The arrowheads labeled b, m, and n indicate the positions of the bi-, mono-, and nonacylated CyaAΔ forms, respectively.

both sides of the membrane, and the increase of membrane conductance because of insertion of CyaA channels was observed using an electrometer (Keithley 617). After incorporation of 10−100 channels into the membrane, the instrumentation was switched to the measurement of the 0 current potential and a KCl gradient was established by adding 3 M KCl solution to one side of the membrane. Analysis of zero-current membrane potential was performed using the Goldman−Hodgkin−Katz equation (*39*).

For activity measurements, toxins were added at indicated concentrations to both sides of the membrane bathing in 1 M KCl at pH 7 and 50 mV, and membrane conductance was taken 30 min after toxin addition, once the conductance increase in time became negligible (*19*).

RESULTS

Acylation of the Lysine 860 Residue Alone Is Not Sufficient for Toxin Activity on Erythrocytes. We aimed to determine whether acylation of the Lys^{860} residue (K860) contributes to toxin activities on target cells. Therefore, we constructed by site-directed mutagenesis a CyaA-K983R toxin variant carrying a conservative arginine substitution (K983R) of the Lys^{983} residue and characterized the activities of CyaA-K983R in parallel to the previously described CyaA-K860R protein carrying an arginine substitution of Lys^{860} (*32*). The proteins were expressed in the presence of the activating acyltranferase CyaC and purified close to homogeneity from *E. coli* cells (Figure 1). As documented in Figure 2, it could be confirmed by using the truncated CyaAΔ protein variants, lacking the RTX domain and thus well-resolved in a previously validated 2D electrophoretic assay (*32*, *33*), that substitution of Lys^{983} by an arginine residue introduced a positive charge at residue 983 that was not neutralized by fatty acylation. This yielded an increased pI value and a corresponding mobility shift of the CyaAΔ-K983R protein, which made it migrate at the same position as the CyaAΔ-K860R protein monoacylated on the Lys^{983} residue (Figure 2). These results further corroborate our previous observation that the Lys^{860} and Lys^{983} residues of CyaA are recognized and acylated by CyaC independently of each other (*32*, *33*).

Toxin activities of CyaA-K983R were first compared to that of intact CyaA and of the monoacylated CyaA-K860R protein, using sheep erythrocytes (RBCs) as model target cells lacking the CD11b/CD18 integrin receptor. As shown in Table 1, in contrast to the monoacylated CyaA-K860R protein, which still exhibited about 10% of the intact toxin capacity to raise the cAMP level in erythrocytes, the CyaA-K983R mutant was essentially unable to deliver its AC catalytic domain across the erythrocyte cytoplasmic membrane and to elevate the intracellular cAMP concentration above the background cAMP level of RBCs exposed to the nonacylated proCyaA. In parallel, also the activities of a CyaA-K983V mutant, with an uncharged valine residue substitution (K983V) of the Lys^{983}, were examined and found to be similarly reduced in respect to intact CyaA, as the activities of the CyaA-K983R protein (not shown). These results are in line with the earlier reported essential role of Lys^{983} acylation in toxin activity of CyaA on erythrocytes (*32*). The CyaA-K983R protein, however, exhibited a residual capacity to associate with erythrocyte membranes, which was comparable to that of the monoacylated CyaA-K860R and was clearly higher than that of nonacylated proCyaA (Table 1).

Lysine 983 Plays a Structural Role in Determining the Cation Selectivity of CyaA Channels, and Its Acylation Controls the Channel-Forming Propensity of CyaA. The reduced (15−20%) capacity of the CyaA-K860R and CyaA-K983R proteins to bind erythrocytes (Table 1) suggested that, despite being severely impaired in the capacity to translocate

Table 1: Toxin Activities of the Monoacylated CyaA Proteins on Sheep Erythrocytes

toxin	cell binding[a] (%)	cAMP intoxication[b] (%)	hemolytic activity[c] (%)
CyaA (Lys^{860} and Lys^{983} acylated)	100	100 ± 18	100
CyaA-K860R (only Lys^{983} acylated)	20 ± 4	10 ± 1	4 ± 2
CyaA-K983R (only Lys^{860} acylated)	15 ± 3	1 ± 0.4	3 ± 1
proCyaA (nonacylated)	<5	<1	<1

[a] Amount of AC enzyme activity associated with 5×10^8 RBCs upon incubation with 5 μg/mL of CyaA proteins for 30 min at 37 °C in the presence of 2 mM Ca^{2+} ions, determined as described previously (*36*) and expressed as the relative binding capacity taking intact CyaA binding as 100%. [b] pmols of cAMP accumulated per 5×10^8 RBCs following 30 min of incubation with 5 μg/mL of the various CyaA proteins were determined by a competitive ELISA assay as previously described (*37*). The cAMP intoxication is expressed in percent of the cAMP level produced by the intact CyaA (100% activity). [c] Hemolysis induced upon incubation of 5×10^8 RBCs with 5 μg/mL of CyaA in 5 h at 37 °C in the presence of 2 mM Ca^{2+} was measured as the amount of released hemoglobin (*10*, *42*). The results represent the average of values obtained in three independent experiments performed in duplicates ($n = 6$).

Table 2: Channel-Forming Capacities of the Monoacylated CyaA Proteins

toxin	membrane activity[a]	single channel conductance (pS)[b]	cation selectivity[c] P_{cation}/P_{anion}
CyaA	++++/+++++	50 ± 6	10.8 ± 1.0
CyaA-K860R	+++	43 ± 5	10.7 ± 1.2
CyaA-K983R	++	45 ± 5	6.5 ± 0.8
CyaA-K983V	++	43 ± 7	6.0 ± 0.5
proCyaA	+	47 ± 6	10.7 ± 1.1

[a] Channel-forming activity was compared after 30 min of incubation of the toxins with asolectin planar lipid bilayer membranes (~0.4 mm^2) in 1 M KCl and 10 mM HEPES-KOH at pH 7, at 50 mV membrane potential and a protein concentration of 12.5 ng/mL. The number of plus signs refers to the approximate power of the number of CyaA channels formed per asolectin membrane under the indicated conditions. [b] Single-channel conductance of the proteins was determined at 12.5 ng/mL CyaA in 1 M KCl and 10 mM HEPES-KOH at pH 7 at 50 mV membrane potential, as above. The single-channel conductance (±SD) was calculated as the mean of at least 100 events. [c] The ion selectivity (cation/anion permeability) ratio P_{cation}/P_{anion} (±SD) of CyaA channels formed in planar lipid bilayer membranes was calculated using the Goldman−Hodgkin−Katz equation (*39*) and the mean value of at least four individual experiments for each set of experimental conditions.

the AC domain across the erythrocyte membrane and/or to induce cell lysis, both monoacylated CyaA proteins still inserted into the membrane to some extent. In contrast to nonacylated proCyaA, the CyaA-K860R and CyaA-K983R proteins could not be stripped-off from the erythrocyte membranes by a 0.1 M sodium carbonate (pH 10.5) wash, used to remove peripheral membrane proteins (*40*). Therefore, we examined the capacity of the monoacylated CyaAs to form the cation-selective channels in artificial planar lipid bilayer membranes.

It has previously been shown (*19*) that, irrespectively of the different acylation status, the CyaA and proCyaA proteins form channels of identical size (conductance). The acylation status appears, indeed, to affect only the propensity of CyaA to form channels in planar lipid bilayers (membrane activity), with the nonacylated proCyaA being 2−3 orders of magnitude less active than acylated intact CyaA (*19*). In line with this, the loss of acylation at Lys^{860} or Lys^{983} residues, because of K860R and K983R or K983V substitutions, had no effect on the conductance (size) of individual channels formed by the CyaA-K860R, CyaA-K983R, and CyaA-K983V proteins, respectively, which remained similar to that of the CyaA and proCyaA channels. It can, hence, be concluded that the K860R, K983R, and K983V substitutions did not affect the overall structure of the CyaA channel. However, the membrane activities of the mutant CyaA proteins differed importantly from that of intact CyaA. The channel-forming activity of the CyaA-K860R mutant was about 1 order of magnitude lower and that of the CyaA-K983R and CyaA-K983V proteins was almost 2 orders of magnitudes lower, as compared to intact CyaA. The CyaA-K983R and CyaA-K983V were, however, still about an order of magnitude more active than intact but nonacylated proCyaA (Table 2). Hence, acylation of the Lys^{860} residue could only partially substitute for acylation of the Lys^{983} residue in supporting the channel-forming activity of CyaA.

The fatty-acyl modification of Lys^{983} could be modulating the channel-forming capacity of CyaA by enhancing its oligomerization propensity, as well as by facilitating lipid bilayer insertion and translocation of toxin segments involved in formation of the transmembrane channel. Both possibilities would be compatible with the data summarized in Table 2, and the role of acylation of Lys^{983} in channel formation would resemble its requirement for the capacity of CyaA to translocate the AC domain across the erythrocyte membrane (*32*). However, acylation-independent structural consequences of the substitution of Lys^{983} might also have contributed to the decrease of the channel-forming activity of the CyaA-K983R and CyaA-K983V proteins. As also shown in Table 2, both CyaA-K983R and CyaA-K983V constructs formed channels that were about 2 times less selective for cations and reproducibly exhibited a cation−anion permeability ratio (P_{cation}/P_{anion}) of ~6, as compared to the value ~11 determined for channels formed by intact CyaA, the CyaA-K860R mutant, or the nonacylated proCyaA, respectively. Furthermore, the result obtained with the nonacylated proCyaA, bearing a positive charge at Lys^{983}, shows that the mere absence of acylation and presence of a positive charge at Arg^{983} of CyaA-K983R did not account as such for the reduced cation selectivity of CyaA-K983R channels. The results rather suggest a specific structural requirement for the lysine side chain at position 983. Hence, independent of its acylation status, the interactions of the side chain of the Lys^{983} residue itself appear to play an important role in toxin structures determining the distribution of charged residues at the entry or within the CyaA channel that would control the channel selectivity for cations. These structures appear to be perturbed upon replacement of the ϵ-amino group of Lys^{983} with either the bulkier guanyl group of an arginine residue or by a shorter aliphatic chain of a valine residue.

Acylation of Lysine 860 Is Sufficient for Tight CD11b/CD18 Receptor Binding and Partial Toxin Activity of CyaA on Murine Macrophage-Like Cells. It was possible that evidence for a functional role of acylation of Lys^{860} could not be obtained using erythrocytes as target cells, because these cells do not express the toxin receptor CD11b. Therefore, the capacity of the monoacylated CyaA-K860R

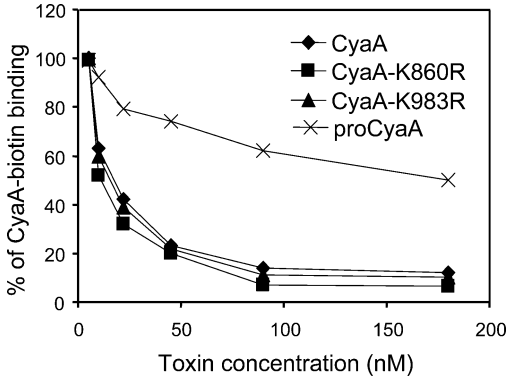

FIGURE 3: Competitive binding of CyaA mutants to the CD11b/CD18-expressing CHO cells. CHO-CD11b$^+$ cells were preincubated with different concentrations of CyaA variants for 30 min on ice. Then, CyaA-biotin (30 nM) was added and the amount of surface-bound CyaA-biotin was determined by flow cytometry, as previously described (*27*). Results are expressed as percent of CyaA-biotin bound in the presence of the various CyaA competitors, determined as (bound CyaA-biotin in the sample)/(maximal CyaA-biotin binding in the absence of competitor CyaA) × 100%. The results are representative of two independent experiments performed in triplicate.

and CyaA-K983R proteins to compete with acylated CyaA-biotin for binding to cells expressing CD11b/CD18 was examined (*27*). This assay is based on our previous observations that acylation of CyaA is essential for a functionally productive interaction of CyaA with CD11b$^+$ cells (*9*, *27*).

In contrast to proCyaA, the CyaA-K860R and CyaA-K983R toxin variants were indistinguishable from intact CyaA in their capacity to outcompete CyaA-biotin from binding to CD11b/CD18 expressed by transfected CHO cells, as shown in Figure 3. Hence, a single acylation of either the Lys^{860} or Lys^{983} residue, respectively, allowed a tight interaction of CyaA with the integrin receptor and an apparently "irreversible" association of CyaA with CD11b$^+$ cells.

Furthermore, as shown in Figure 4 and summarized in Table 3, the high capacity to bind CD11b/CD18 allowed the monoacylated CyaA-K860R and CyaA-K983R proteins to exhibit a capacity to lyze the CD11b-expressing mouse J774A.1 monocyte/macrophage cells in 3 h to an extent similar to that of intact and doubly acylated CyaA. The activities of CyaA-K860R were barely different from that of intact CyaA also when compared for the specific capacity to penetrate cells and raise intracellular cAMP levels and to cause the loss of mitochondrial dehydrogenase activity, because of ATP consumption and cAMP accumulation (Figure 4 and Table 3). These results, hence, show that the single acylation on the Lys^{983} residue was sufficient for CyaA activity on J774A.1 cells by all three used criteria. The results further show that interaction with the CD11b/CD18 receptor imposed on CyaA-K860R a conformation and/or mode of membrane interaction, which allowed it to overcome the structural defect introduced by the K860R substitution that accounted for the impairment of the capacity of CyaA-K860R to translocate across the erythrocyte membrane-lacking CD11b (cf. Tables 1 and 3). More importantly, while the protein monoacylated on Lys^{860} was essentially inactive on erythrocyte targets (cf. Table 1), it exhibited a substantial cytotoxic activity on the CD11b$^+$ J774A.1 cells, as also shown in Figure 4 and summarized in Table 3 for CyaA-K983R and also found for CyaA-K983V carrying a valine substitution at residue 983 (data not shown).

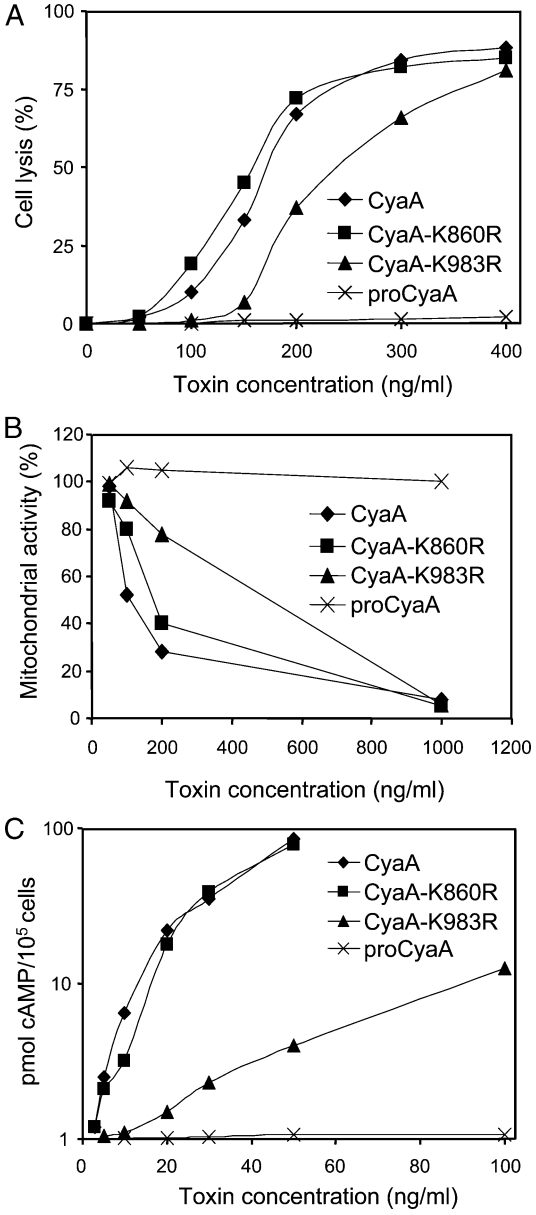

FIGURE 4: Toxin activities of the mutant CyaAs on mouse J774A.1 monocyte/macrophage cells. (A) CyaA cytotoxicity determination. Lysis of J774 cells (10^5 per well) was determined as the extent of LDH release following CyaA-induced cell death. (B) Mitochondrial functionality in toxin-treated cells was determined as the capacity of mitochondrial dehydrogenases to reduce the tetrazolium salt WST-1 (Roche) to its formazan product. (C) Mono-acylated toxins are able to induce an increase of the cAMP level in mouse macrophages J774. The concentration of intracellular cAMP was determined by ELISA (*37*) following 30 min of incubation of J774 macrophage cells (10^5 cells/well) with the indicated toxin concentrations. Cytotoxic activities of the toxins were compared on the basis of CyaA concentrations required to yield accumulation of 10 pmol of intracellular cAMP in 10^5 cells in 30 min of incubation. The results represent the average of values obtained in at least three independent experiments performed in duplicates ($n \geq 6$).

DISCUSSION

We report here that individual ablations of the two fatty-acylation sites on lysine 860 and 983 residues, respectively, affected differently the activities of CyaA toward different types of target cells. Pellett and Welch have, indeed, observed

Table 3: Toxin Activities of the Monoacylated CyaA Proteins on CD11b$^+$ J774A.1 Cells

toxin	cell lysis[a] CL_{50} (ng/mL)	mitochondrial activity[b] CV_{50} (ng/mL)	cAMP intoxication[c] $C_{10\_pmol\_cAMP}$ (ng/mL)
CyaA	179 ± 38	87 ± 25	13 ± 1
CyaA-K860R	145 ± 36	160 ± 28	16 ± 2
CyaA-K983R	235 ± 25	506 ± 45	76 ± 12
proCyaA	6273 ± 766	5374 ± 449	493 ± 107

[a] CL_{50} was determined as the concentration of added CyaA (ng/mL) that caused a half-maximal (50%) LDH release from 10^5 J774.1 cells in 3 h. [b] CV_{50} was determined as the concentration of added CyaA (ng/mL) that caused a half-maximal (50%) decrease of mitochondrial dehydrogenases capacity to reduce the WST-1 substrate, using 10^5 J774.1 cells incubated for 3 h with the toxin. [c] $C_{10\_pmol\_cAMP}$ was determined as the concentration of added CyaA (ng/mL) that caused accumulation of 10 pmols of cAMP in 10^5 J774.1 cells in 30 min of incubation with the toxin. The results represent the average of values obtained in at least three independent experiments performed in duplicates ($n \geq 6$).

earlier that residue substitutions within the individual acylation sites (Lys^{563} and Lys^{689}) of the somewhat homologous *E. coli* RTX α-hemolysin, HlyA, differentially affected activities of HlyA toward various cells (*41*). Although not rigorously the same type of substitutions and target cells were used in the two studies, some divergence in acylation requirements of the two toxins is apparent. For HlyA, the acylation of both conserved lysine residues was strictly required for any activity on human B cells (Raji), while acylation of either one of the lysines allowed a fairly high activity on BL-3 cells and some activity on erythrocytes. In contrast, acylation of the Lys^{983} was necessary and sufficient for a full capacity of *Bp*-CyaA or of the intact r-*Ec*-CyaA to penetrate and lyze erythrocytes (*30*). It also appears as sufficient for a full capacity of the CyaA-K860R protein to penetrate monocytes and raise the cellular cAMP levels and/or cause J774A.1 cell lysis, as shown here, although the CyaA-K860R construct has little activity in erythrocytes, because of the K860R substitution. Monoacylation of Lys^{860}, in turn, conferred on CyaA-K983R a reduced capacity to raise cAMP level in the CD11b$^+$ J774A.1 cells and did not support any AC domain penetration into erythrocytes.

It is further noteworthy that comparable concentrations of CyaA-K983R and intact CyaA induced 50% lysis of J774.1 cells in 3 h, while the mutant protein was about 6 times less effective in reaching the cell cytosol to raise intracellular cAMP levels and provoke mitochondrial dysfunction (Table 3). This suggests that the relation between cAMP accumulation and J774.1 death is indirect and that additional factors are involved in cell lysis. A plausible hypothesis would be that despite of its very low channel-forming and nil hemolytic activity in targets lacking CD11b, such as erythrocytes and lipid bilayers, the CyaA-K983R mutant may still possess a normal capacity to form membrane-permeabilizing toxin channels in CD11b$^+$ J774A.1 cells, because of its conserved capacity to tightly bind the CD11b receptor. Such pore-forming capacity can be expected to act in synergy with the cAMP-increasing capacity of the toxin in provoking J774A.1 cell lysis, and it might compensate in part for the reduced capacity of the CyaA-K983R construct to elevate cAMP levels in cells. Because of the extremely high catalytic power of the AC enzyme, indeed, a supraphysiological level of cAMP is reached in J774A.1 cells within minutes upon exposure to the used concentrations of both the intact CyaA and its CyaA-K983R derivative (data not shown), while lysis of cells occurs only in hours. Hence, a threshold cAMP concentration, sufficient to synergize with the pore-forming activity of the toxin in promoting cell lysis, may be reached early enough also upon CyaA-K983R interaction with cells. This would allow it to cause J774A.1 cell lysis at comparable concentrations and with similar kinetics as the intact CyaA.

In conclusion, it is intriguing that the CyaA molecule has conserved both RTX acylation sites over its diverging evolution from the ancestor RTX cytolysin, while a single acylation of Lys^{983} appears to be sufficient for full CyaA activity on both CD11b$^-$ cells and CD11b$^+$ cells (*23*, *30*, *33*). Previous studies indicated that some *Bordetella* strains may produce CyaA acylated at only the Lys^{983} residue, while other strains also accomplish acylation of the Lys^{860} residue (*23*, *31*). The present results provide the first evidence for a functional role of Lys^{860} acylation in CyaA toxin activity, albeit that this appears to be redundant with the role of Lys^{983} acylation in conferring on CyaA the capacity to penetrate CD11b$^+$ cells. It remains to be determined whether acylation of Lys^{860} is essential for toxin interaction with some as yet unidentified types of host cells or alternative toxin receptors during natural infections by *B. pertussis*.

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