Acylation of Lysine 983 Is Sufficient for Toxin Activity of Bordetella pertussis Adenylate Cyclase

SUBSTITUTIONS OF ALANINE 140 MODULATE ACYLATION SITE SELECTIVITY OF THE TOXIN ACRYLTRANSFERASE CyaC*

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The capacity of adenylate cyclase toxin (ACT) to penetrate into target cells depends on post-translational fatty-acylation by the acyltransferase CyaC, which can palmitoylate the conserved lysines 983 and 860 of ACT. Here, the in vivo acylation capacity of a set of mutated CyaC acyltransferases was characterized by two-dimensional gel electrophoresis and mass spectrometric analyses of the ACT product. Substitutions of the potentially catalytically serine 20 and histidine 33 residues ablated acylating activity of CyaC. Conservative replacements of alanine 140 by glycine (A140G) and valine (A140V) residues, however, affected selectivity of CyaC for the two acylation sites on ACT. Activation by the A140G variant of CyaC generated a mixture of bi- and monoacylated ACT molecules, modified either at both Lys-860 and Lys-983, or only at Lys-860, respectively. In contrast, the A140V CyaC produced a nearly 1:1 mixture of monoacylated pro-ACT with ACT monoacylated almost exclusively at Lys-983. The respective proportion of toxin molecules acylated at Lys-983 correlated well with the cell-invasive activity of both ACT mixtures, which was about half of that of ACT fully acylated on Lys-983 by intact CyaC. These results show that acylation of Lys-860 alone does not confer cell-invasive activity on ACT, whereas acylation of Lys-983 is necessary and sufficient.

The whooping cough agent, Bordetella pertussis, secretes a 1706-residue-long RTX1 adenylate cyclase toxin-hemolysin (ACT, AC-Hly, or CyaA), which can invade a variety of eukaryotic cells (1, 2). ACT delivers into cells a catalytic adenylate cyclase domain (AC) that is activated by intracellular calmodulin and catalyzes unregulated conversion of ATP to cAMP (3–6). This impairs microbial functions of immune effector cells and induces apoptosis of lung macrophages (7, 8). In addition, ACT has the capacity to form small cation-selective membrane channels that account for its weak hemolytic activity (7–13).

The capacity of ACT to form hemolytic channels and to penetrate target cell membranes and deliver the AC domain (cell-invasive activity) depends on a covalent post-translational fatty-acyl modification (14–16). This is catalyzed by a dedicated protein acyltransferase, CyaC, which can acylate the ε-amino groups of two internal lysine residues of ACT, Lys-983 and Lys-860, located within conserved RTX acylation sites (14–17). The mechanism of this novel type of protein acylation was recently analyzed in substantial detail for the prototype RTX toxin-activating and acyl-ACP-dependent protein acyltransferase HlyC, which acylates the homologous lysines 564 and 690 of the Escherichia coli a-hemolysin HlyA (18–21). Several residues, including Ser-20 and His-23, were identified as being potentially involved in acyl transfer catalysis by HlyC (22–24). A model of the reaction mechanism was proposed, and formation of an intermediary acyl-ACP:HlyC complex was demonstrated (22–25). Various acyl-ACP-carrying fatty acids, including the most common in E. coli, the palmitoyl (C16:0) and palmitoleyl (C16:1) residues, were found to be efficiently used in vitro as acyl donors for modification of HlyA (18, 26). In vivo, however, HlyC exhibits a high selectivity for C14:0 myristic acid, which represents only about 2% of total E. coli fatty acids but was found to constitute about 68% of the acyl chains covalently linked to lysines 564 and 690 of native HlyA (27). Moreover, the extremely rare odd carbon-saturated C15:0 and C17:0 fatty-acyl residues constituted the rest of the in vivo acylation of HlyA from two different E. coli strains (27). The functional consequences of this particular in vivo acylation of HlyA remain unknown.

Heterogeneity in extent and nature of acyl residues linked to lysines 983 and 860 was observed previously for ACT from different sources. Native ACT produced by the Bordetella pertussis strain 338 (Bp-ACT) was initially found to be acylated exclusively by palmitoyl residues and only on the lysine 983 (16). In contrast, acylation by a mixture of palmitoleil (cis Δ9 C16:1), palmitoyl (C16:0), and myristoyl (C14:0) fatty-acyl residues was found for the recombinant r-Ec-ACT activated by CyaC in E. coli (17, 28). Moreover, in addition to acylation of polyacrylamide gel electrophoresis; LC/MS/MS, liquid chromatography-tandem mass spectrometry; GC, gas chromatography.
Lys-983, an incomplete (60%) acylation was found at Lys-860 of r-Ec-ACT (17, 28). r-Ec-ACT exhibited also a lower capacity to induce protective immune response against Bordetella infection in mice and a significantly lower channel-forming and hemolytic activity, as compared with Bp-ACT (13, 15, 17, 29, 30). Interestingly, however, the capacity of both forms of ACT to insert into target membranes and to deliver the AC domain into cells (cell invasiveness) was identical (15, 17). We have suggested earlier that acylation of Lys-860 might be an artifact of recombinant expression of ACT in E. coli and that it may selectively affect the propensity of r-Ec-ACT to form the oligomeric channels and to lyse erythrocytes (17). Recently, however, the r-Bp-ACT produced by a recombinant B. pertussis 18323 strain was found to be fully palmitoylated also on Lys-860, whereas its hemolytic activity was higher than that of r-Ec-ACT (47). This indicates that the extent of Lys-860 acylation may vary as a function of the strain and physiological state of the producing bacteria and the impact of Lys-860 acylation on biological activity of ACT needs to be established.

In a previous study, we have shown that Lys-860 is by itself important for ACT function and that a conservative substitution of Lys-860 by an arginine residue drastically reduced the capacity of ACT to insert into and translocate across target cell membrane (28). This substitution, however, also prevented the acylation of Lys-860 and it could not be rigorously excluded that this acylation itself, independent of the K860R substitution, was important for cell-invasive activity of ACT. In this study we have generated a set of mutant CyaC acyltransferases, aiming at a definition of residues that are critical to the process of ACT acylation. It is shown, by the use of monoclonal variants of CyaC, that in contrast to acylation of Lys-860, the acylation of Lys-860 alone does not confer cell-invasive activity on ACT.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Plasmids**—The E. coli K12 strain XL1-Blue (Stratagene) was used throughout this work for DNA manipulation and for expression of ACT. Bacteria were grown at 37 °C in LB medium supplemented with 150 μg/ml ampicillin. pT7CACT1 is a construct derived from pCACT3 (29), which was designed for enhanced production of recombinant CyaC-activated ACT in E. coli (r-Ec-ACT) under control of the IPTG-inducible lacZα promoter (28). To construct pT7NFLAG-CACT1, a synthetic oligonucleotide, 5'-TATGGATTATAAAGATGACGATGACAAATCACG, encoding the FLAG epitope (DYKDDDDK), was inserted in-frame into the unique Ndel site encompassing the ATG start codon of cyaC. The fusion was verified by DNA sequencing.

**Site-directed Mutagenesis of cyaC**—The individual substitutions were introduced into the cyaC gene by a two-step PCR mutagenesis procedure using Taq DNA polymerase and appropriate mutagenic and amplification primer pairs listed in Table I. In a first step, two complementary mutagenic primers were used in two separate amplification reactions on the cyaC template DNA to introduce the mutation into two partially overlapping PCR products. These were purified on agarose gel and used as templates in a second PCR amplification with an assembly pair of primers (5'-GGGATATACATATGATTATACGAAAG and 5'-CTAGAAGTCTGATGCCCCTGGGT) to assemble the whole mutagenized cyaC gene with the desired substitution. The final product was recloned as an Ndel-BamHI fragment into the appropriately digested pT7NFLAG-CACT1, to replace the original cyaC allele. The presence of desired substitutions and the absence of any additional secondary site mutations were systematically reverified by sequencing the entire mutagenized cyaC alleles within the pT7NFLAG-CACT construct.

Plasmids for coexpression of the truncated ACTα protein together with the mutant NFLAG-CyaC proteins were obtained as described previously, by introducing a TAA stop codon at position 1069 of the cyaA gene. This resulted in production of a truncated ACT protein (ACTα) with a C-terminal Asp-1068 residue (28). The nonacylated variants of ACT and ACTα were expressed from plasmids from which the cyaC gene was deleted as an Ndel-BamHI fragment.

**Production and Purification of the CyaA-derived Proteins**—The ACT and ACTα proteins were produced in the presence or absence of the various N-FLAG-CyaC acyltransferase derivatives, using the E. coli strain XL1-Blue (Stratagene) transformed with the appropriate plasmids.
TABLE II

Biological activities of the ACT activated in vivo by the CyaC variants

| Substitution in CyaC | Cell binding of ACT | Invasive AC activity | Hemolytic activity |
|----------------------|---------------------|----------------------|--------------------|
| None (wt CyaC)       | 100 ± 12            | 100 ± 9              | 100 ± 13           |
| None (N-FLAG-CyaC)   | 97 ± 13             | 101 ± 10             | 97 ± 8             |
| C67S                 | 101 ± 14            | 99 ± 9               | 102 ± 11           |
| S68T                 | 98 ± 9              | 96 ± 9               | 95 ± 14            |
| S30R                 | <10                 | <1                   | <1                 |
| H33S                 | <10                 | <1                   | <1                 |
| H33D                 | <10                 | <1                   | <1                 |
| A140G                | 61 ± 5              | 57 ± 7               | 43 ± 4             |
| A140V                | 52 ± 12             | 47 ± 11              | 32 ± 11            |
| R141L                | 98 ± 11             | 101 ± 8              | 97 ± 13            |
| R141K                | 102 ± 12            | 98 ± 9               | 100 ± 6            |

* Point substitutions in CyaC were introduced by site-directed PCR mutagenesis.

* Expressed as percentages of activity of ACT activated by intact CyaC. Target cell binding, cell-invasive AC, and hemolytic activities were determined as previously described (37) at 2 units of ACT per ml of sheep red blood cells (5 × 10⁷ cells/ml). The activity values are the average from three determinations performed in duplicates with three independent preparations of ACT (n = 6). Invasive activity was determined as the AC activity translocated into sheep erythrocytes and protected against digestion by extracellularly added trypsin (9).

mid derivative of pT7NFLAG-CACT. For ACT production, exponential 500-ml cultures were induced with IPTG (1 μM), and the extracts of insoluble cell debris after sonication were prepared in 8 M urea, 50 mM Tris-HCl, pH 8.0, 0.2 mM CaCl₂, as described previously (15). The differently acylated full-length ACT proteins were further purified by ion-exchange chromatography on DEAE-Sepharose and phenyl-Sepharose (Amersham Pharmacia Biotech) as described previously (31). In the final step, the proteins were eluted with 8 M urea, 50 mM Tris-HCl, pH 8.0, and stored frozen.

High Resolution Two-dimensional Gel Electrophoresis—The two-dimensional gel electrophoresis assay for acylation of the ACTΔ has previously been established and was performed as described in detail elsewhere (28). It allows separation of the non-, mono-, and biacylated forms of ACTΔ in whole cell extracts, due to the loss of one and/or two positive charges upon acylation of the Lys-860 and/or the Lys-983 residues, respectively, which causes alteration of the isoelectric point of the protein. Briefly, whole cell extracts of the different CyaC acyltransferase variants, were prepared from exponentially growing cultures induced by IPTG (1 μM) for 3 h. Total protein samples (20 μg) were analyzed by large format two-dimensional (IEF/SDS-PAGE) gel electrophoresis (32) using the Investigator system (Oxford Glycosystems). The gels were stained with Coomassie Blue. We have previously demonstrated that the two-dimensional gel electrophoresis of whole cell samples provides a convenient and unambiguous semiquantitative assay for assessment of ACTΔ acylation in vivo. It has further been shown by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) and tandem mass spectrometry of purified preparations that acylation of ACTΔ reflects well the acylation of full-length ACT produced under similar experimental conditions (28).

Mass Spectrometric Analysis of Protein Acylation—The location and identity of the acyl modifications on ACT produced in the presence of certain NFLAG-CyaC variants were analyzed by LC/MS/MS and MALDI-TOF MS techniques as described previously (16, 17, 28). Tryptic and Asp-N fragments were generated according to standard protocols and separated on 50-μm inner diameter × 12-cm capillary column packed with Magic 5-μm, 200-Å C18 material (Michrom BioResources, Auburn, CA), at a flow rate of 150 nL-min⁻¹ with a 2–95% acetonitrile gradient (1% acetic acid) over 40 min. Peptide analysis by micropipet high pressure liquid chromatography coupled to a Finnigan TSQ 7000 electrospray tandem quadrupole mass spectrometer was performed as described in detail elsewhere (33, 34). Positive-ion MALDI-TOF spectra were measured on a Bruker BIFLEX-III reflector time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a SCOUT-384 inlet and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV, and the reflectron (ion mirror) voltage was set to 20 kV. For delayed extraction, a 4-kV potential difference between the probe and the extraction lens was applied with a time delay in the range of 200–400 ns after

![Figure 2](image-url)

**CyaC A cyltransferase Mutagenesis**

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FIG. 3. SDS-PAGE analysis of purified full-length ACT proteins acylated by different CyaC variants. The proteins were expressed in the presence of the activating protein CyaC in recombinant E. coli K12 strains and purified from urea extracts of cell debris by DEAE- and phenyl-Sepharose chromatography as described previously (31). Proteins (2 μg) were separated on 7.5% acrylamide gel and visualized by Coomassie Blue staining. Lane 1, ACT acylated by intact N-FLAG-CyaC; lane 2, ACT acylated by the A140V variant of CyaC; lane 3, ACT acylated by the A140G variant of CyaC.

![SDS-PAGE gel](image)

Each laser pulse, samples were irradiated at a frequency of 5 Hz by 337-nm photons from a pulsed Laser Science (Cambridge, MA) nitrogen laser. Typically, 20–50 shots were summed into a single mass spectrum. 4-Hydroxy-α-cyano-cinnamic acid was used as the MALDI matrix. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of a peptide standard (bombesin, Aldrich) and reprocessed by Bruker XMASS 5.0 software.

Identities of the C16:0 and C16:1 fatty acyl groups were confirmed by capillary GC/MS with electron impact ionization after a one-step extraction and derivatization procedure (35).

Assay of Adenylate Cyclase, Cell Binding, Cell-invasive, and Hemolytic Activities—Adenylate cyclase (AC) activities were measured as described previously in the presence of 1 μM calmodulin (36). One unit of AC activity corresponds to 1 μmol of cAMP formed per min at 30 °C, pH 8.0. Cell-invasive AC was determined as the amount of AC that became protected against externally added trypsin upon internalization into erythrocytes within 30 min of incubation (9). The hemolytic activity was measured as the hemoglobin released upon incubation of washed sheep erythrocytes (5 × 10⁶/ml) with the toxins for 270 min, respectively (9). Erythrocyte binding of the toxins was determined as described in detail previously (37).

RESULTS

Substitutions of Ser-20 and His-33 Residues Ablate Activity of CyA—The acyl-ACP-dependent acyltransferases activating RTX toxins exhibit a very high degree of sequence conservation. This makes identification of functionally essential residues of CyaC difficult. Substitution of several potentially catalytic serine, histidine, and cysteine residues of CyaC was, therefore, performed to probe their functional importance (Fig. 1).

The substitutions were introduced into a CyaC construct tagged by a FLAG epitope at its N-terminal end (N-FLAG-CyaC). This allowed tracking of mutant CyaC in vivo and discrimination between loss of function and enhanced degradation of CyaC. The N-FLAG-CyaC, carrying various substitutions, were coexpressed in E. coli with the full-length pro-ACT to assess the capacity of CyaC variants to confer cell-invasive activity on ACT. In parallel, the capacity of the CyaC variants to acylate in vivo a C-terminally truncated pro-ACΔ derivative of ACT was determined by a two-dimensional gel electro-
CyaC Acyltransferase Mutagenesis

TABLE III
Semiquantitative mass spectrometric assessment of Lys-983 and Lys-860 acylation in ACT activated by the CyaC variants in vivo

| CyaC  | Lys-860 acylation | Lys-983 acylation |
|-------|------------------|------------------|
|       | Nonacylated | Acylated | Relative acylation | Nonacylated | Acylated | Relative acylation |
| wt    | 20 | 238 | 92% | ND | 310 | 100% |
| A140V | 1300 | 194 | 13% | 220 | 321 | 59% |
| A140G | 25 | 451 | 94% | 82 | 93 | 53% |

a The ACT was expressed in the presence of intact CyaC or in the presence of the indicated CyaC variant.
b Proteolytic fragments encompassing the acylation sites were recovered from the microcapillary high pressure liquid chromatography and identified by MALDI-TOF MS spectra and/or electrospray MS, as described under “Experimental Procedures.” To estimate the relative abundances, the value of absolute selected ion intensity from the electrospray data was used. It should be noted, that this represents only a semiquantitative estimation of the abundance of a given peptide. The error of the given values is difficult to estimate in the absence of an internal standard, but from past experience with known synthetic standards of the tryptic peptides of interest, the relative errors are on the order of 10%–15% (16, 17).
c Asp-N generated proteolytic fragments encompassing residues 828–873 and 821–873, respectively, were analyzed for presence and identity of acyl modifications at Lys-860 and behave similarly with respect to analytical precision.
d A tryptic-generated peptides, proteolytic fragments encompassing residues 972–983 and 972–984, respectively, were analyzed for presence and identity of acyl modifications at Lys-983.

e Comprises the sum of fragments acylated by both C16:0 Lys-983 palmitic and cis Δ9 C16:1 palmitoleic residues.
f The values given are the absolute selected ion intensity divided by 10^4.
g Calculated as the ratio of the total acylated peptide signal divided by the sum of the acylated and nonacylated peptide signal on a percentage basis.
h ND, not detected.

As summarized in Table II and documented in Fig. 2A (fields 1–4), the N-terminal addition of the FLAG epitope did not alter the toxin activation and acylation capacity of CyaC. Replacement of the highly conserved cysteine 67 by a serine residue (Cys-67 → Ser) and of the serine 68 by a threonine residue (Ser-68 → Thr), respectively, had no observable effect on the toxin-activating capacity of N-FLAG-CyaC in vivo, as documented in Table II and illustrated for the S68T-CyaC in Fig. 2A (fields 5 and 6). In contrast, the ACTα proteins produced in the presence of the Ser-30 → Arg (S30R), Ser-30 → Trp (S30W), His-33 → Asp (H33D), and His-33 → Ser (H33S) variants of CyaC, respectively, migrated in two-dimensional gels at the position of nonacylated pro-ACTα and resolved quantitatively from the more acidic monoaoylated ACTΔK860R and biacylated ACTΔ3 standards (28), when these were added to the samples (Fig. 2A, fields 7–15). In agreement with a complete loss of fatty-acetyl activation, the toxin activity of full-length ACT produced in vivo in the presence of the same CyaC variants was nil, as shown in Table II. However, normal levels of CyaC proteins carrying the substitutions of Ser-30 and His-33 were detected in cellular extracts by Western blotting (data not shown). Collectively, these results suggest that the Ser-30 and His-33 residues are specifically required for the acylating activity of CyaC. Indeed, while this work was in progress, the same conclusion on the role of the corresponding Ser-20 and His-23 residues of HlyC was drawn from an extensive in vitro characterization of intact and mutant HlyC variants (22–24).

A notable advantage of CyaC as a target for mutagenesis studies is the ability to perform the selective acylation of CyaC variants and to monitor the extent of acylation by a typical example of spectra shown in Fig. 4. Table III shows that semiquantitative mass spectrometric assessment of the acylation status of CyaC is employed in the present study. As shown in Fig. 2B (fields 5–12), both conservative substitutions of the alanine 140 residue of CyaC by glycine (A140G) and valine residues (A140V) resulted in nearly complete loss of production of the biacylated form of ACTΔ3. The ACTΔ3 preparations acylated in vivo by the A140G-CyaC consisted predominantly of monoaoylated ACTΔ3 and of a small amount (10–15% of total) of biacylated ACTΔ3, whereas no nonacylated pro-ACTΔ3 was detected by two-dimensional gel electrophoresis (Fig. 2B, field 5). In contrast, activation by the A140V-CyaC yielded a mixture of monoaoylated ACTΔ3 with the nonacylated pro-ACTΔ3 (Fig. 2B, field 9). Therefore, the full-length ACT produced in cells expressing intact, A140G and the A140V variants of CyaC, respectively, were purified close to homogeneity (Fig. 3) and analyzed by mass spectrometry (28) to clarify the acylation status of Lys-860 and Lys-983. For each ACT preparation, the relative abundance of acylated versus nonacylated peptides covering the two acylation sites were estimated semiquantitatively, from the relative intensities of selected ions in reconstructed ion current chromatograms. The identity of the peptides and the character of the linked acyl chains was further determined by partial sequencing of the peptides upon collisionally induced dissociation (CID) and analysis of daughter ion spectra, as illustrated by a typical example of spectra shown in Fig. 4.

In both preparations of ACT activated by either the A140G or the A140V variant of CyaC, respectively, a similar degree of acylation at the lysine 983 residue was detected, with about 50% of the peptides containing lysine 983 acylated by palmitic (C16:0) and/or palmitoleic (C16:1) fatty acyl groups, and the rest being nonacylated, as summarized in Table III. However, in the same ACT preparations a striking difference in the extent of acylation of the lysine 860 residue was observed. Although about 90% of Lys-860 residues were found acylated in ACT activated by A140G-CyaC, only about 10% of Lys-860 residues were acylated in ACT activated by A140V-CyaC. Almost no nonacylated pro-ACTΔ3 was present in the preparation acylated by A140G-CyaC. In contrast, about half of the ACT preparation activated by the A140V variant of CyaC consisted of nonacylated pro-ACT molecules, whereas the ACT molecules making the second half of the preparation were monoacylated substitutions of the corresponding Ala-140 and Arg-141 residues of CyaC on its in vivo acylating capacity.

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almost exclusively on the Lys-983 residue. Hence, both the A140G and A140V substitutions caused a moderate reduction in the capacity of ACT to acylate the Lys-983 residue, whereas the A140V substitution selectively caused a strong reduction in acylation of Lys-860 of ACT. Because even such conservative substitutions differentially affected the capacity of CyaC to acylate ACT, this suggests that alanine 140 is an important structural residue involved in interaction of CyaC with the two acylation sites of the protoxin.

Acylation of Only the Lysine 983 of ACT Is Necessary and Sufficient for Cell-invasive Activity of the Toxin—When half of the ACT preparation consisted of ACT monoacylated on Lys-860 (activation by A140G-CyaC), or when half of it was non-acylated pro-ACT (activation by A140V-CyaC), the membrane insertion and cell-invasive AC activities of both the ACT preparations were very similar, as shown in Table II. These activities were close to 50% of the activity of ACT acylated by intact CyaC, and there was a good correlation between the proportion of ACT molecules acylated on the Lys-860 residue and the cell-invasive activity of the ACT preparation, as defined by the capacity of ACT molecules to insert into the membrane of erythrocytes and to deliver the catalytic AC domain into a compartment where it was protected against externally added trypsin (cf. Tables II and III). These results clearly show that ACT molecules acylated uniquely at the Lys-860 residue were as noninvasive as the nonacylated pro-ACT molecules. Therefore, only ACT molecules acylated on Lys-983 contributed to cell-invasive activity.

**DISCUSSION**

The results reported here suggest that the Ala-140 residue plays an important structural role in activity of CyaC. The acylation pattern of Lys-983 and Lys-860 residues of coexpressed ACT was strongly affected by subtle changes to Ala-140, which consisted of removal of one methyl group in the A140G variant or addition of two methyl groups by the A140V substitution. The most straightforward explanation is that Ala-140 of CyaC directly interacts with the acylation sites on ACT. Substitutions of Ala-140 could then differentially affect binding and/or the catalytic efficiency of CyaC at the two sites, and this might well account for the observed acylation differences at Lys-983 and Lys-860 of ACT. It is conceivable that both glycine and valine side chains introduced at position 140 may cause a similar loss of binding and/or acylation efficiency of CyaC at the Lys-983 acylation site, whereas only the shorter glycine side chain might still allow productive CyaC interaction at the Lys-860 site. The bulkier valine side chain could then selectively interfere with CyaC binding and/or acylation at the sterically different Lys-860 site.

By analogy to the reaction mechanism of HlyC, CyaC is expected to form an intermediary acyl-ACP-CyaC complex prior to interaction with the pro-ACP substrate (23, 25). It cannot be excluded that the Ala-140 substitutions in CyaC may also affect the capacity of CyaC to form these complexes. However, a simultaneous decrease in acylation at both the Lys-863 and Lys-860 sites, proportional to the respective affinities of the complex for these two sites, would be expected if the substitutions in CyaC would affect formation and availability of the acyl-ACP-CyaC complexes. This was clearly not the case, because the A140V and A140G substitutions in CyaC caused simultaneously a similar decrease of acylation at Lys-983 and a very different effect on acylation at Lys-860. It is, therefore, more likely that the Ala-140 substitutions affect interaction and/or acyl transfer efficiency of CyaC at the Lys-983 and Lys-860 sites and not the formation of acyl-ACP-CyaC complex.

The results further show that the cell-invasive activity of ACT did not depend on acylation of Lys-860 but did correlate with the extent of acylation at the Lys-983 residue. Therefore, it can be concluded that acylation of the lysine 983 is necessary and sufficient at least for the capacity of ACT to insert into and translocate across the model target membrane of sheep erythrocytes. These results provide a rationale for the previous observations, that various preparations of native and/or recombinant ACT exhibited identical capacity to insert into the target membranes and to deliver the AC domain into cells, despite a variable extent of Lys-860 acylation (17). A stable and essentially complete acylation of Lys-983 by intact CyaC was, indeed, observed in those preparations. Moreover, recent mass spectrometric analyses show a batch to batch variation in the degree of Lys-860 acylation even for the native ACT produced by the same *Bordetella* strain, suggesting the influence of growth conditions on the extent of Lys-860 acylation of ACT by CyaC. The sum of the available data suggests that CyaC and/or its acyl-ACP-CyaC complex may have a higher affinity for the Lys-983 acylation site of ACT, as compared with the Lys-860 site, and this may account for the preferential acylation of Lys-983 under certain physiological conditions. Altogether, these results question the role of Lys-860 acylation in the biological activity of ACT and are in good agreement with our previous observation, that, regardless of its acylation status, Lys-860 is by itself an important structural residue, involved in toxin interaction with target membranes (28). In this respect, it is interesting that the sequence of the Lys-860 acylation site becomes better conserved among the known RTX toxins, relative to that of the Lys-983 acylation site. It will be important to determine in other target cell models, such as primary lung macrophages and neutrophils, whether the variable acylation of the conserved lysine 860 contributes to the biological activity of the AC toxin, or whether it is just an evolutionary relict.

We have previously suggested that the acylation of Lys-860 might account for the observed difference in the propensity of membrane-inserted recombinant (r-Ec-ACT) and native (Bp-ACT) to oligomerize to form channels (17). In contrast to other RTX toxins, such as HlyA, however, the primary biological activity of ACT appears to consist of delivery of an enzyme into cells and their intoxication by production of cAMP rather than in the formation of membrane channels (39). This may suggest why double acylation of HlyA at Lys-564 and Lys-690 is optimal for channel forming (cytotoxic) activity of HlyA, whereas a single acylation at the conserved Lys-983 residue appears to be sufficient for membrane insertion and cell-invasive activity of *B. pertussis* ACT.

ACT has recently gained a lot of attention as a candidate protective antigen for acellular pertussis vaccines and as a novel vector for delivery of viral and tumoral antigens into major histocompatibility complex class I antigen-presenting cells and induction of specific cellular immune responses (29, 40–45). It will be important to identify the factors determining the variability of ACT acylation and to define the relations between differences in acylation and the capacity of ACT to interact with various target cells.

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