Internal Lysine Palmitoylation in Adenylate Cyclase Toxin from *Bordetella pertussis*

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A number of bacterial protein toxins, including adenylate cyclase (AC) toxin from *Bordetella pertussis*, require the product of an accessory gene in order to express their biological activities. In this study, mass spectrometry was used to demonstrate that activated, wild-type AC toxin was modified by amide-linked palmitoylation on the ε-amino group of lysine 983. This modification was absent from a mutant in which the accessory gene had been disrupted. A synthetic palmitoylated peptide corresponding to the tryptic fragment (glutamine 972 to arginine 984) that contained the acylation blocked AC toxin–induced accumulation of adenosine 3′,5′-monophosphate, whereas the nonacylated peptide had no effect.

Adenylate cyclase toxin is a virulence factor from *Bordetella pertussis*, the bacterium that causes pertussis or "whooping cough." This disease is responsible for an estimated 340,000 deaths per year, largely among children in developing countries. It is also a cause of morbidity for children and adults in Europe and the United States (*1*). AC toxin is so named because it is an adenylate cyclase enzyme of bacterial origin that enters eukaryotic cells and catalyzes the production of supraphysiologic amounts of adenosine 3′,5′-monophosphate (cAMP) from adenosine triphosphate in the host cell (*2*). As a consequence of its insertion into target cell membranes, the toxin is also hemolytic for sheep red blood cells (RBCs) (*3*). AC toxin is expressed from its structural gene, *cyaA* (*4*), as a 177.5-kD protein consisting of a single peptide chain that contains no cysteines. Its toxin and hemolytic activities, however, are dependent on the product of an accessory gene, *cyaC* (*5*), which is believed to be involved in a posttranslational modification of AC toxin.

AC toxin is required as a virulence factor, and *B. pertussis* organisms that are defective in its production have been shown to be unable to establish a lethal infection in suckling mice (*6*). The increased intracellular concentrations of cAMP caused by the toxin inhibit the normal physiologic processes of phagocytic leukocytes, and it is hypothesized that impairment of immune effector cell function is the major contribution of AC toxin to the pathogenesis of pertussis (*2*). Activated AC toxin is also a protective antigen that is a candidate for inclusion in acellular pertussis vaccines (*7*).

AC toxin is a member of a rapidly growing family of Gram-negative bacterial cytolysins called "repeat in toxin" (RTX) toxins. This name refers to a series of glycine- and aspartic acid–rich nonameric repeats found in the COOH-terminal third of each toxin protein and thought to be involved in calcium binding (*8*). Many of these toxins share with AC toxin the dependence on an ancillary gene, such as *cyaC*, for their biological activities. Activation of *Escherichia coli* hemolysin, HlyA, can be achieved in vitro by transfer of a fatty acyl group from acyl carrier protein to prohemolysin (*9*), which leads to the hypothesis that HlyC is an acyltransferase. However, neither the type nor the site of acylation that occurs in vivo has been reported.

To investigate the posttranslational modification of AC toxin, we used purified AC toxins from both wild-type *B. pertussis* (BP338) and a *cyaC*-deficient mutant strain (BPDE386) (*10*) and analyzed the toxin structure by mass spectrometry. The toxins were digested with trypsin, and the resulting peptides were separated by high-performance liquid chromatography (HPLC). The profiles of the peptides generated from BP338 (Fig. 1A) and BPDE386 were similar. Each HPLC fraction was analyzed by matrix-assisted laser desorption–ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (*11*). The MALDI-TOF MS data (Fig. 1B) from the HPLC fractions revealed that approximately 180 fragments were derived from each AC toxin, whereas 137 fragments were expected on the basis of the

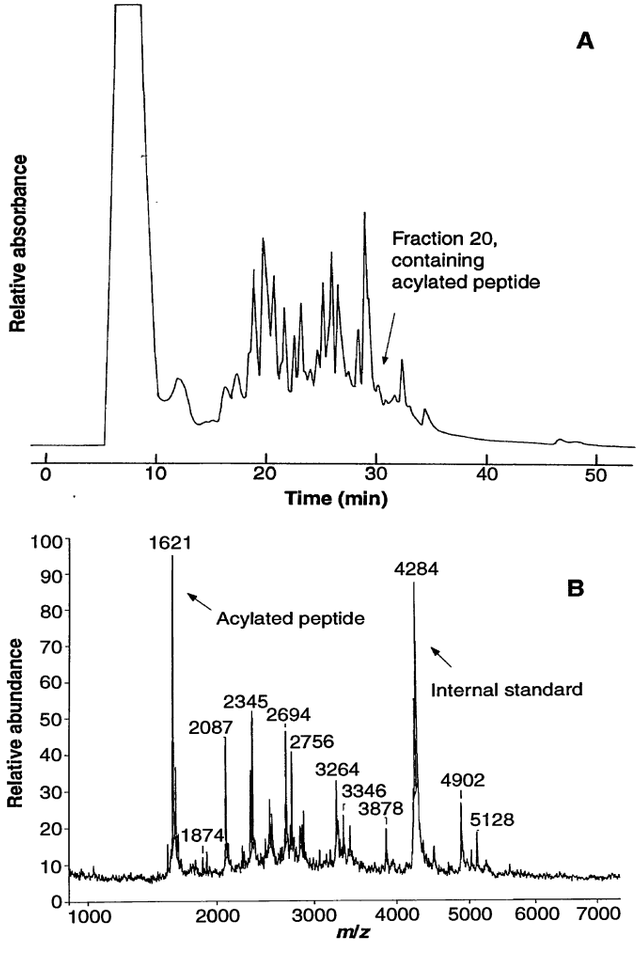

Fig. 1. Preliminary screening of tryptic fragments from wild-type AC toxin. (**A**) HPLC trace, monitored at 214 nm (1.0 absorbance unit full scale), of the tryptic digest from BP338. The palmitoylated peptide was identified in fraction 20 of the 24 collected and analyzed by MALDI-TOF MS. A 2.1-mm by 3-cm C4 Bu 300 HPLC column (ABI) was used with a 60-min binary gradient at 200 μl/min with 10-min delay, 500-μl injection, and eluted with a gradient of 10% to 100% solvent B. Solvent A, H_2O and 0.1% trifluoracetic acid (TFA); solvent B, 60% acetonitrile, 30% *N*-propanol, 10% H_2O, and 0.085% TFA. (**B**) MALDI mass spectrum of HPLC fraction 20 containing palmitoylated peptide at *m/z* 1621. Several nonacylated peptides were also present. α-Cyano-4-hydroxycinnamic acid (*20*) matrix was used with 100 fmol of bovine ubiquitin ($[M + 2H]^{++}$ = 4284) as an internal standard for mass calibration. The instrument used an N_2 laser with excitation at 337 nm and was constructed in-house (*21*).

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predicted amino acid sequence (*4*). Most of the differences could be attributed to incomplete digestion and to a small number of anomalous cleavages.

To avoid the task of sequencing every tryptic fragment, we analyzed further only those peptides that were unique to either the wild-type or mutant strain, as defined by their apparent molecular masses, or were contained in HPLC fractions in which a peptide with a lipid modification might be expected to elute, on the basis of retention times of synthetic acyl peptide standards. Tryptic fragments selected for collisionally activated dissociation (CAD) were sequenced with electrospray ionization microcapillary liquid-chromatography tandem mass spectrometry (ESI LC-MS-MS). Of 22 peptides characterized by CAD, only a fragment from the wild-type toxin corresponding to amino acid residues Glu^{972} to Arg^{984} appeared to have a modification. This peptide (Figs. 1A and 2A) gave an $[M + H]^+$ (the mass of the molecule plus a proton) at a mass-to-charge ratio (m/z) of 1621 (Figs. 1B and 2B). The CAD spectrum of the $[M + 2H]^{++}$ ion (the mass of the molecule plus two protons) at an m/z of 811 yielded a nearly complete series of Y ions (Fig. 2C). The mass difference between Y_1 and Y_2 indicated that Lys^{983} was 238 mass units greater than the expected lysine residue mass of 128. This value suggested palmitoylation at the ϵ-amino group. Further evaluation of this peptide with methyl esterification and acetylation yielded $[M + H]^+$ values of m/z 1649 and 1663, which is also consistent with the structure shown in Fig. 2C. The HPLC elution time, MALDI spectrum, and free acid CAD spectrum of the synthetic palmitoylated peptide based on this sequence served to confirm the proposed structure.

No evidence of a peptide with an $[M + H]^+$ at m/z 1621 was found in any HPLC fraction from BPDE386. The tryptic fragments containing nonacylated Lys^{983} in BPDE386 were not found in a digest separated under HPLC conditions optimized for the less polar acyl peptides. However, fragments giving an $[M + H]^+$ at m/z 1226 and 1383, corresponding to peptides at amino acid positions 972 to 983 and 972 to 984, were recovered from a digest of the mutant toxin that had been run under HPLC conditions favoring recovery of hydrophilic peptides (*12*). CAD analysis of this fragment from BPDE386 at m/z 1383 confirmed its identity as the unmodified sequence from Glu^{972} to Arg^{984}. A very small amount of peptide at m/z 1226 was observed in the BP338 digest, as determined by HPLC and MALDI-TOF MS. This suggests that the acylation may not be complete in the wild-type toxin, although the acylated peptide is present in much greater quantity than the nonacylated form.

Two well-known types of protein acylation are palmitoylation through an oxy- or thioester linkage to serine, threonine, or cysteine residues and myristoylation through an amide linkage to the NH_2-terminal glycine (*13*). There is, however, a group of myristoylated proteins in which the acylation is resistant to hydroxylamine treatment, which suggests an amide linkage, and in which there is no glycine present at the NH_2-termini (*14*). AC toxin activity is also resistant to hydroxylamine treatment (*15*), which suggests that no ester-linked modification is required for its biological effects, and the structure reported here (Fig. 2C) demonstrates the amide linkage nature of the palmitoylation.

Two domains are common to all known RTX toxins. A hydrophobic domain is located in the NH_2-terminal half of the toxin protein, and the putative calcium-binding domain, containing the nonameric repeats, occupies a site toward the COOH-terminal third of the toxin. Palmitoyl-Lys^{983} in AC toxin lies between these two domains. The lysines conserved among the RTX toxins (besides Lys^{983}) include Lys^{758} and Lys^{860} in the AC toxin. Tryptic peptides with molecular masses at m/z 1368 and 2521, derived from toxins produced by both the BP338 and BPDE386 strains, were observed by MALDI-TOF MS and have the values expected for residues 747 to 758 and 759 to 782, if Lys^{758} is not modified. CAD analysis of the peptide with $[M + H]^+$ at m/z 2521 in AC toxin from both the mutant and wild-type strains confirmed its origin in the sequence spanning Met^{759} to Lys^{782} and matched the spectrum of a synthetic peptide consisting of the same sequence. Similarly, a fragment observed at m/z 1358, derived from the sequence Ser^{861} to Lys^{872}, indicated the presence of a tryptic cleavage site at Lys^{860}, which would not have been subject to proteolysis if the site was acylated. This peptide was sequenced and its CAD spectrum matched that of a synthetic peptide of the proposed sequence. The

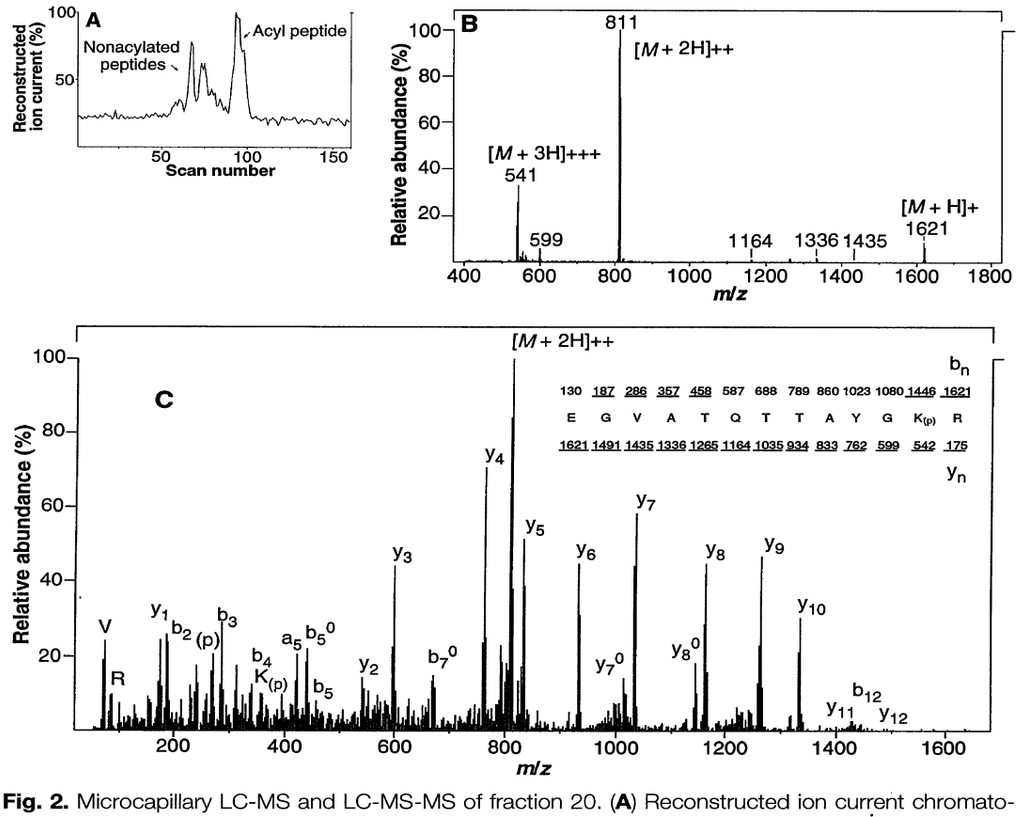

Fig. 2. Microcapillary LC-MS and LC-MS-MS of fraction 20. (**A**) Reconstructed ion current chromatogram (RIC) from the microcapillary ESI LC-MS analysis of the off-line HPLC fraction shown in Fig. 1A (*22*). Note the elution of the palmitoylated peptide, which is baseline-separated from the nonacylated peptides. (**B**) ESI mass spectrum of about 1 pmol of the acyl peptide. Shown are the singly, doubly, and triply charged ions associated with the intact molecule. Several Y-type fragment ions were also observed at low abundance, probably because of CAD-like processes occurring in the ESI ion source. (**C**) CAD spectrum of the $[M + 2H]^{++}$ ion at m/z 811. A, B, and Y series ions are so noted (in lowercase letters), with those observed in the spectrum underlined in the sequence at the appropriate one-letter code for the residue (*23*). The "0" superscript indicates the loss of water. Diagnostic low-mass ions for the presence of specific amino acids are indicated by one-letter codes. The general approach to sequencing peptides by low-energy CAD has been described (*24*).

NH_2-terminal side of Lys^{860} contains five tryptic sites between residues 853 and 860. These short hydrophilic peptides were not recovered under our HPLC conditions.

A monoclonal antibody, designated D12, binds to the wild-type form of *E. coli* hemolysin (HlyA) but not to the *hly*C-deficient hemolysin (*16*). The D12 epitope was mapped to a specific region of HlyA. In the corresponding region of AC toxin, Lys^{983} is the most likely acylation site.

AC toxin from BPDE386 has wild-type amounts of AC enzymatic activity but no toxin or hemolytic activity (*5*). Although it was speculated that the absence of these activities in BPDE386 was due to a lesion in membrane insertion and catalytic domain delivery, the molecular basis of the defect remains to be determined. To explore the role of the palmitoyl group in the biological activities of AC toxin, peptides with the sequence shown in Fig. 2C were synthesized with and without the palmitoyl group. These synthetic peptides were tested for their effects on toxin and hemolytic activities. The nonpalmitoylated peptide had no inhibitory effect on AC toxin activity. In contrast, the palmitoylated peptide reduced cAMP accumulation in a concentration-dependent manner, with 80% inhibition at 2.5 μM (Fig. 3). Similarly, the acylated peptide inhibited AC toxin–induced hemolysis (*17*).

Palmitoylation has been reported to mediate both protein-protein and protein-lipid interaction (*18*). Recent data indicate that the ability of AC toxin to lyse RBCs is related to its ability to create a transmembrane pore in an artificial lipid bilayer system and that the functional unit of the toxin is a trimer or larger (*19*). If monomers of AC toxin must combine within the membrane in order to generate pore formation, it is possible that the palmitoyl groups are involved in protein-protein interactions to facilitate oligomerization. This concept is supported by results from experiments in which the addition of palmitoylated peptide to AC toxin before addition of the mixture to cells was much more inhibitory of toxin effects than if the peptide was added to target cells before addition of the toxin (*15*).

Palmitoylated Lys^{983} is not present in inactive AC toxin from a strain (BPDE386) in which the accessory gene, *cya*C, has been disrupted. These data strongly suggest that internal palmitoylation at this site represents the modification that is required for the toxin and hemolytic activities of AC toxin. The ability of the acylated peptide to inhibit toxin activities suggests new avenues for investigation of the mechanisms of action for the entire family of RTX toxins.

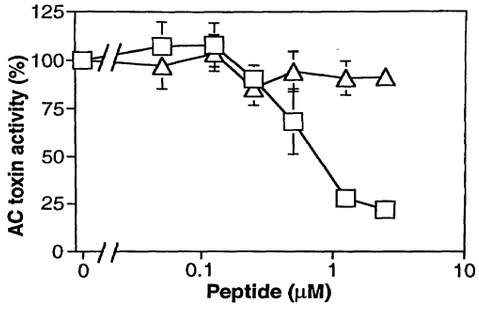

Fig. 3. Inhibition of AC toxin by the palmitoylated peptide (squares) but not the corresponding unmodified peptide (triangles). AC toxin activity was determined by quantitation of intracellular cAMP accumulation in Jurkat cells, as described in the purification procedure (*10*). The peptide and wild-type toxin were mixed together in 8 M urea (*25*), 10 mM tricine, and 0.5 mM EDTA-EGTA (pH = 8). Ten microliters of this mixture, containing 0.6 μg of toxin, was added to 1.0 ml of cells at a density of 1×10^6 cells per milliliter, yielding the final peptide concentration indicated on the *x* axis. After incubation at 37°C for 30 min, intracellular cAMP and cell protein were extracted and measured (*26*). The AC toxin activity is expressed as percent of the activity (3490 and 2840 pmol of cAMP per milligram of cell protein per microgram of AC toxin in two separate experiments) without any added peptide (mean ± SD; $n = 6$ from two separate experiments).

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10. BP338 is the wild-type strain derived from the Tohama I strain (*6*). BPDE386 is a mutant that contains a 4–base pair (bp) insert in *cyaC*, resulting in a molecule with no toxin or hemolytic activities (*5*). Organisms were grown, and AC toxins were purified by urea extraction, phenyl-Sepharose chromatography, sucrose density gradient centrifugation, and calmodulin-Sepharose affinity chromatography. Purified AC toxins were dialyzed against a suitable buffer before tryptic digestion [E. L. Hewlett, V. M. Gordon, J. D. McCaffery, W. M. Sutherland, M. C. Gray, *J. Biol. Chem.* **264**, 19379 (1989); E. L. Hewlett *et al.*, *ibid.* **266**, 17503 (1991)].
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12. To recover the hydrophilic peptides, an OD 300 C18 column (ABI, Foster City, CA) was used in place of the C4 column, with a 0 to 60% (acetonitrile) gradient over 60 min. For the ESI LC-MS-MS analysis, PRP-1 (Hamilton Syringe, Reno, NV) packing was used in the microcapillary column instead of Poros.
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16. The D12 epitope was mapped to amino acids 673 to 726 in HlyA, a sequence that contains five lysines [S. Pellett, D. F. Boehm, I. S. Snyder, G. Rowe, R. A. Welch, *Infect. Immun.* **58**, 822 (1990)]. In the corresponding region of CyaA (amino acids 967 to 1020), Lys^{983} is the only lysine present.
17. AC toxin–induced hemolytic activity was determined by measuring hemoglobin release from sheep RBCs (*3*). However, this assay could not tolerate the high concentrations of chaotrope or detergent necessary to fully solubilize the acylpeptide. When assayed without urea, acyl peptide at a concentration of 100 μM reduced AC toxin–induced hemolysis to 0%. It was suspected, given its amphipathic nature, of forming micelles. We believe that only the portion of the total amount that consisted of free peptide in solution was available to interact with the protein in a concentration-dependent manner.
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23. Single-letter abbreviations for the amino acid residues are: A, Ala; E, Glu; G, Gly; $K_{(p)}$, palmitoyl-Lys; Q, Gln; R, Arg; T, Thre; V, Val; and Y, Tyr.
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