Acyltransferase-mediated selection of the length of the fatty acyl chain and of the acylation site governs activation of bacterial RTX toxins

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In a wide range of organisms, from bacteria to humans, numerous proteins have to be posttranslationally acylated to become biologically active. Bacterial repeats in toxins (RTX) cytolysins form a prominent group of proteins that are synthesized as inactive protoxins and undergo posttranslational acylation on e-amino groups of two internal conserved lysine residues by co-expressed toxin-activating acyltransferases. Here, we investigated how the chemical nature, position, and number of bound acyl chains govern the activities of Bordetella pertussis adenylate cyclase toxin (CyaA), Escherichia coli α-hemolysin (HlyA), and Kingella kingae cytotoxin (RtxA). We found that the three protoxins are acylated in the same E. coli cell background by each of the CyaC, HlyC, and RtxC acyltransferases. We also noted that the acyltransferase selects from the bacterial pool of acyl–acyl carrier proteins (ACPs) an acyl chain of a specific length for covalent linkage to the protoxin. The acyltransferase also selects whether both or only one of two conserved lysine residues of the protoxin will be posttranslationally acylated. Functional assays revealed that RtxA has to be modified by 14-carbon fatty acyl chains to be biologically active, that HlyA remains active also when modified by 16-carbon acyl chains, and that CyaA is activated exclusively by 16-carbon acyl chains. These results suggest that the RTX toxin molecules are structurally adapted to the length of the acyl chains used for modification of their acylated lysine residue in the second, more conserved acylation site.

RTX cytolysins (RTXA) are synthesized as inactive protoxins (proRTXA) and undergo a posttranslational acylation by toxin-activating acyltransferases (RTXC) co-expressed with the protoxins (2–6). As first demonstrated for the Escherichia coli α-hemolysin (HlyA), its cognate acyltransferase HlyC cannot use acyl-CoA as acyl chain donor and uses only fatty acyl residues carried by acyl carrier protein (ACP). Modification of proHlyA occurs through an amide-linked acylation of the e-amino groups of the Lys-564 and Lys-690 residues of HlyA (7, 8). The two lysine residues of HlyA were found to be predominantly acylated in uropathogenic E. coli by myristoyl chains (C14:0; ~68%), and the remaining acyl chains were initially identified as the extremely rare odd-carbon pentadecanoyl (C15:0; ~26%) and heptadecanoyl (C17:0; ~6%) fatty acyl groups (9). The myristoyl chain was also found to be the major modification of the Lys-558 (~18%) and Lys-689 (~71%) residues of the recombinant RtxA cytotoxin of Kingella kingae, whereas the remaining toxin molecules were modified by hydroxymyristoyl (Lys-558, ~5%; Lys-689, ~18%), lauroyl (Lys-689, ~2%), and palmitoleyl (Lys-689, ~8%) chains (5). In contrast, the Bordetella pertussis RTX adenylylate cyclase toxin (CyaA) was initially found to be acylated by single C16:0 palmitoylation on the Lys-983 residue (10), and when overproduced in B. pertussis, palmitoylation was detected also on its Lys-860 residue (11). This was recently confirmed for a number of clinical isolates producing the native CyaA (12). When CyaA was overproduced in E. coli together with its activating acyltransferase CyaC, a mixed acylation by predominantly palmitoyl (Lys-860, ~46%; Lys-983, ~22%) and palmitoleyl (Lys-860, ~44%; Lys-983, ~56%) chains with a low level of C14:0 myristoylation was observed (13–16).

Acylation of the RTX toxins appears to be crucial for all of their known cytotoxic activities (2, 5, 6, 8, 17, 18). However, the precise molecular mechanism by which the acyl chains contribute to membrane insertion and formation of pores by the toxins remains poorly understood. The presence of the acyl chains was shown to play a structural role in the folding of CyaA into a biologically active conformation (19, 20) and in a productive and irreversible interaction of CyaA with cells expressing the complement receptor 3 (CR3; also known as the integrin α₃β₂, CD11b/CD18, or Mac-1) (17, 21). Acylation was also shown to be required for the irreversible insertion of HlyA to target membrane (22) and for protein-protein interaction in HlyA oligomerization within the membrane microdomains (23).

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This article contains supporting information.

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The cytolytic (pore-forming) RTX toxins are important virulence factors of many Gram-negative bacterial pathogens (1). The RTX toxins permeabilize host cell membranes and share several characteristic features. These comprise (i) a C-terminal unprocessed secretion signal, recognized by the type I secretion system and mediating translocation of toxins directly from the bacterial cytosol into the extracellular milieu; (ii) characteristic C-terminal nonapeptide glycine- and aspartate-rich RTX repeats that upon binding of numerous calcium ions fold into a β-barrel structure; (iii) posttranslational modification of internal lysine residues within conserved acylated sites by covalent attachment of fatty acyl residues, and (iv) a hydrophobic pore-forming domain, respectively (1).
produced the nine pairwise combinations of the three protoxins activated by the acyltransferase CyaC, HlyC, or RtxC, respectively. To generate the constructs, the pT7CACT1 plasmid harboring the

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The toxin-activating acyltransferase genes are highly conserved between the rtx loci of various bacterial genera, and some of these acyltransferases were reported to activate also heterologous protoxins. For example, the HlyC-modified Actinobacillus pleuropneumoniae hemolysin ApxlA, as well as the ApxC-modified HlyA expressed in E. coli, exhibited a hemolytic activity on erythrocytes (24, 25). Similarly, the heterologously HlyC- or CyaA-activated Pasteurella hemolytica leukotoxin LktA exhibited the same activity and target cell specificity as the LktA activated by its cognate LktC acyltransferase. Nevertheless, the activation was not reciprocal, as the LktC-activated HlyA and CyaA produced in E. coli were neither hemolytic nor cytotoxic (26, 27). However, it has not been determined why some RTX protoxins are efficiently cross-activated by heterologous acyltransferases and some are not.

Here, we analyzed the activation of the CyaA, HlyA, and RtxA toxins, each acylated by one of the three CyaC, HlyC, or RtxC acyltransferases and produced in the same E. coli cell background, so as to eliminate the potential impact of differences in acyl-ACP pool composition of the original producer bacteria. The results reveal that it is the RTXC acyltransferase enzyme that selects the type of the acyl chain of adapted length that is covalently linked to the proRTXA protein, and the acyltransferase also selects whether a single lysine residue or both modification sites of proRTXA will be posttranslationally acylated, thereby conferring the biological activity on the RTX toxin.

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CyaA selects C16 fatty acyl chains, whereas HlyC and RtxC select C14 acyl chains and differ in recognition of acylation sites

To test the acyl residue and acylation site selectivity of the three homologous RTX toxin-activating acyltransferases, we produced the nine pairwise combinations of the three protoxins proCyaA, proHlyA, and proRtxA with the three acyltransferases CyaC, HlyC, and RtxC, respectively, in the same E. coli cell backbone. For this purpose, the expression signals of the pT7CACT1 plasmid for production of CyaC-activated CyaA toxin (28) were employed. The cyaC ORF in pT7CACT1 was replaced from its start to stop codon by hlyC or rtxC, and similarly, the cyaC ORF was replaced by hlyC or rtxC, respectively. B, a set of the nine pT7CACT1-derived constructs was used for the production of the RTXC-activated RTXA toxins in E. coli BL21/pMM100 cells. The proteins were purified close to homogeneity from urea-solubilized inclusion bodies by affinity chromatography on calmodulin-Sepharose (CyaA variants) or Ni-NTA agarose (HlyA and RtxA variants). The samples were analyzed on 7.5% polyacrylamide gels and stained with Coomassie Blue. St, molecular mass standards. C, ClustalW sequence alignment of acylated sites of CyaA (UniProt code: P0DKX7), HlyA (UniProt code: P08715), and RtxA (K. kingae isolate PYK081) with two conserved internal lysine residues (in boldface type and underlined) whose ε-amino groups are posttranslationally acylated. *, identity; **, strongly similar; †, weakly similar.

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Acylation patterns determine the levels of biological activity of the RTX toxins

The set of nine toxins with defined numbers and lengths of attached acyl chains enabled us to assess how the single or double acylation and the length of the attached acyl chains affect the biological activities of these proteins. For the three differently acylated CyaA variants, we first determined their capacity to bind, penetrate, and lyse sheep erythrocytes, respectively, using red blood cells as surrogate target cells lacking the CyaA receptor CR3. As documented in Fig. 2A, compared with double acylation of the CyaA<sub>HlyC</sub> protein by predominantly the C14:0 and/or C16:1 chains, monoacylation of the Lys-983 residue by predominantly the C14:0 or C14:0-OH chains reduced the relative capacity of CyaA<sub>HlyC</sub> (by ~62%) and CyaA<sub>RtxC</sub> (by ~70%) to bind erythrocytes. The modification by the C14 acyls decreased even more the relative cell-invasive capacity of both toxin variants (by ~94%) (Fig. 2A). Moreover, at the rather high concentration of 10 μg/ml that was used, the CyaA<sub>HlyC</sub> and CyaA<sub>RtxC</sub> proteins were unable to provoke any lysis of erythrocytes over time, whereas complete erythrocyte lysis was provoked by the same amount of CyaA<sub>CyaC</sub> within 5 h of incubation (Fig. 2B). The relative AC-translocating and lytic capacities of the CyaA<sub>HlyC</sub> and CyaA<sub>RtxC</sub> proteins remained low even when their input concentration was increased 2.5-fold over that of CyaA<sub>CyaC</sub> to achieve binding of equal amounts of the three CyaA variants to erythrocyte cells (Fig. 2, C and D). The C14:0 or C14:0-OH monoacylated CyaA<sub>HlyC</sub> and CyaA<sub>RtxC</sub> toxins bound with a reduced capacity (~53% and ~60% of CyaA<sub>CyaC</sub> binding) also to mouse J774A.1 macrophages that express the CyaA receptor J774A.1 (Fig. 2E). However, the binding of CyaA<sub>HlyC</sub>...
and CyaARtxC to J774A.1 cells was CR3 receptor–specific, as it could be blocked by the competing antibody M1/70 that binds the CD11b subunit of CR3 (29, 30) (Fig. 2E). Nevertheless, compared with the C16 biacylated CyaA_{HlyC} the C14 monoacylated CyaA_{HlyC} and CyaARtxC proteins were strongly impaired in their capacity to translocate the AC domain into the cytosol of J774A.1 cells to elevate the intracellular cAMP levels (Fig. 2F). This functional defect on J774A.1 cells was most likely not due to the lack of acylation of the Lys-860 residue of the CyaA_{HlyC} and CyaARtxC toxins, as a CyaA-K860R mutant acylated by CyaC only on the Lys-983 residue (17) exhibited similar binding (Fig. 2E) and translocate its AC domain (Fig. 2F) into J774A.1 cells as the intact doubly acylated CyaA_{CycC} toxin. Hence, the low cell-invasive activity of the monoacylated CyaA_{HlyC} and CyaARtxC proteins was rather due to the modification of the Lys-983 residue by the shorter C14 acyl chains, which conferred a much lower specific membrane penetration capacity on CyaA than the modification of the Lys-983 residue by the C16 acyl chains.
In agreement with the residual cytolytic capacity on erythrocytes, the CyaA_HlyC and CyaA_RtxC variants exhibited also a very low overall membrane activity on artificial lipid bilayers made of 3% asolectin (Fig. 3A). However, as calculated from single-pore recordings (Fig. 3B), the most frequent conductances of pores formed by CyaA_HlyC (12 pS) and CyaA_RtxC (12 pS) were quite comparable with that of CyaA_CyaC (11 pS) (Fig. 3C). Similarly, the most frequent lifetimes of pores formed by CyaA_HlyC (1140 ms) and CyaA_RtxC (1129 ms) were comparable with that of CyaA_CyaC (1083 ms) (Fig. 3D). It can thus be concluded that the difference in the number (one versus two acylated lysine residues) and the length and chemical nature (C16:0/C16:1 versus C14:0/C14:0-OH) of attached acyl chains affected strongly the propensity of formation but not the overall characteristics of the individual pores generated by the differently acylated CyaA toxin variants.

A different picture was observed for the HlyA toxin variants modified by the CyaC, HlyC, or RtxC acyltransferases. As documented in Fig. 4A, the HlyACyaC protein, acylated almost exclusively on the single Lys-690 residue by the C16:1 and C16:0 chains, was still exhibiting a similar capacity to lyse erythrocytes as the HlyC-activated HlyAHlyC toxin acylated on both Lys-564 and Lys-690 residues by the shorter C14:0 and C14:0-OH acyl chains. Moreover, the RtxC-modified and predominantly monoacylated HlyARtxC toxin was as hemolytic as HlyAHlyC. Furthermore, irrespective of the length (C16 versus
C14) or number (one or two) of attached acyl residues, all three differently acylated HlyA toxin variants were comparably cytotoxic to human macrophage THP-1 cells, as determined by their capacity to elicit loss of capacity of mitochondrial reductases to convert the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate (WST-1) to formazan (Fig. 4B). Activities represent average values ± S.D. from three independent determinations performed in duplicate with three different toxin preparations. B, mitochondrial functionality in HlyA-treated THP-1 cells (1.5 × 10⁴) was determined after 2 h as the capacity of mitochondrial dehydrogenases to reduce the tetrazolium salt WST-1 to its formazan product. Activities represent average values ± S.D. from three independent determinations performed in triplicate with two different toxin preparations.

In line with the comparable cytolytic capacities on erythrocytes, all three HlyA variants displayed comparable overall membrane activities on artificial lipid bilayers (Fig. 5A). As calculated from single-pore recordings (Fig. 5B), the HlyA<sub>HlyC</sub> variant formed pores with the most frequent conductance of 405 pS that was similar to that of pores formed by the HlyA<sub>CycA</sub> (322 pS) and HlyA<sub>RtxC</sub> (384 pS) proteins (Fig. 5C). The HlyA<sub>CycA</sub> and HlyA<sub>RtxC</sub> toxin variants also formed pores with most-frequent pore lifetimes similar to those of the pores formed by the HlyA<sub>HlyC</sub> (1788, 1654, and 1599 ns, respectively) (Fig. 5D).

All of these results demonstrate that the C16 acyl chains attached to the Lys-690 residue conferred on the HlyA<sub>CycA</sub> toxin a similar pore-forming and cytotoxic activity as did the naturally HlyC-mediated acylation by C14 acyl chains attached to both Lys-564 and Lys-690 residues in HlyA<sub>HlyC</sub> or the partial acylation of Lys-564 and full acylation of Lys-690 by C14 acyl chains in the HlyA<sub>RtxC</sub> toxin. This indicates that in contrast to CyaA, monoacylation of the single Lys-690 residue by either C14 or C16 acyl chains was sufficient for biological activity of HlyA.

Similarly, single acylation of the Lys-689 residue of RtxA by C14:0 or C14:0-OH acyl chains (Table 1) was sufficient for full cytolytic activity of the RtxA<sub>RtxC</sub> and RtxA<sub>HlyC</sub> toxins, acylated by RtxC or HlyC, respectively (Fig. 6). However, in contrast to HlyA, the RtxA toxin was not activated by CyaC despite almost complete modification of the Lys-689 residue by the C16:0 or C16:1 acyl chains. Hence, the biological activity of RtxA was supported only upon modification by C14 and not C16 acyl chains. In line with that, the RtxA<sub>RtxC</sub> and RtxA<sub>HlyC</sub> toxins displayed comparable overall membrane activities on planar lipid bilayers, whereas the RtxA<sub>CycA</sub> protein exhibited a low membrane activity (Fig. 7A). Nevertheless, the formed RtxA<sub>CycA</sub> pores (Fig. 7B) exhibited a comparable most-frequent conductance of 487 pS, like RtxA<sub>HlyC</sub> (454 pS) and RtxA<sub>RtxC</sub> (479 pS) pores, respectively (Fig. 7C). Similarly, the most frequent values of single-pore lifetimes of RtxA<sub>CycA</sub> (968 ms) were similar to those of RtxA<sub>HlyC</sub> (1002 ms) and of RtxA<sub>RtxC</sub> (1288 ms) (Fig. 7D). These results indicate that the RtxA<sub>CycA</sub> variant was impaired in its capacity to insert into the lipid bilayer and/or in its propensity to form oligomeric pores due to the modification by the longer C16 acyl chains.

**Discussion**

We report that of the three examined RTXA toxins, the CyaA toxin is only activated by predominant modification of its Lys-983 residue by C16 fatty acyl chains and the RtxA protein only by the modification of its Lys-689 residue by C14 acyl chains. Intriguingly, the HlyA toxin can be fully activated by modification with either C14 or C16 acyl chains linked to its Lys-690 residue. These results further show that it is the acyltransferase activating the RTX protoxin that selects the acyl chain of the functionally adapted length from the acyl-ACP pool of the producing bacterium. Furthermore, the acyltransferase also determines whether both or only one of the two conserved acylation sites in the respective RTX protoxin will be recognized and covalently modified by the linked acyl chains.

In the first report analyzing the acylation of CyaA, the toxin isolated from the *B. pertussis* strain BP338 was found to be modified by a single amide-linked palmitoylation on the ε-amino group of Lys-983 (10). However, CyaA overproduced together with CyaC in the *B. pertussis* 18-323 strain was later found to be palmitoylated on both the Lys-860 and Lys-983 residues (11), and the double acylation was recently confirmed for CyaA extracted from seven clinical isolates of *B. pertussis* (12). Most of the modifications occurring in *B. pertussis* consisted in palmitoylation of the Lys-983 (up to ~90%) and Lys-860 (~70–100%) residues. However, partial myristoylation (~10–40%) was also observed on the Lys-983 residue of the native CyaA (12). Initial analysis of CyaA produced with CyaC in the *E. coli* K12 strain XL1-Blue revealed that the recombinant toxin was acylated by palmitoyl chains (~67%) at the Lys-860 residue and palmitoyl (~87%) and myristoyl (~13%) chains at the Lys-983 residue (15). A higher-resolution MS analysis later demonstrated
that the Lys-983 residue of the recombinant CyaA isolated from E. coli K12 was not only palmitoylated (C16:0), but also palmitoleylated (cis D9 C16:1) at a ratio of 1:2 (14). The palmitoyl (Lys-860, 46%; Lys-983, 22%) and palmitoleyl (Lys-860, 44%; Lys-983, 56%) chains were also identified later as the major fatty acyl modifications of the recombinant CyaA purified from the E. coli K12 strain (16).

In line with these findings, we found here that palmitoylation (Lys-860, 32%; Lys-983, 45%) and palmitoleylation (Lys-860, 34%; Lys-983, 56%) are the two major posttranslational modifications of the recombinant CyaA isolated from E. coli K12 strain BL21. In contrast, the HlyC- or RtxC-modified CyaA variants produced on the same genetic background were predominantly myristoylated and hydroxymyristoylated almost exclusively on the Lys-983 residue (CyaAHlyC, 77%; CyaARtxC, 85%). Modification by C16:0 and C16:1 acyl chains was negligible in CyaAHlyC (3%) and CyaARtxC (8%). The CyaAHlyC and CyaARtxC variants then exhibited substantially lower biological activities than CyaACyaC, and this was most likely not due to the missing acylation at the Lys-860 residue of CyaAHlyC and CyaARtxC. Indeed, we have demonstrated that acylation of the Lys-983 residue of CyaACyaC was necessary and sufficient for biological activities of the toxin on both CR3-negative erythrocytes (13) as well as on CR3-positive J774A.1 cells (Fig. 2 (E and F)) (17). It is therefore plausible to conclude that the 14-
Carbon myristoyl and hydroxymyristoyl chains are unable to functionally replace the 16-carbon palmitoyl and palmitoleoyl chains at the Lys-983 residue of CyaA. Thus, not only the absence of acylation itself, but also the length of the covalently linked acyl chains appears to play a crucial role in activation of CyaA and in conferring of biological activities on this toxin.

The specific binding of the myristoylated and hydroxymyristoylated CyaA_HlyC and CyaA_RtxC variants to CR3-positive J774A.1 cells was affected only mildly. However, compared with C16-acylated CyaA_CyaC, the C14 acyl-modified CyaA_HlyC and CyaA_RtxC variants were largely impaired in the capacity to deliver the AC enzyme across target cell membrane into the cytosol and to intoxicate cells by cAMP production. Hence, the two-carbon unit shorter C14 acyl chains were unable to deliver the AC enzyme across target cell membrane into the cytosol and to intoxicate cells by cAMP production. This suggests that a 16-carbon-long acyl chain of the Lys-983 residue of CyaA to impose the necessary membrane insertion and translocation of the CyaA polypeptide.

The remaining linked acyls were then identified as the very rare C15:0 (≈26%) and C17:0 (≈68%) odd-carbon fatty acyl chains (9). Here, we demonstrated that the recombinant HlyC-acylated HlyA toxin produced in the E. coli strain BL21 was acylated mostly by the C14:0 and C14:0-OH chains both at the Lys-564 (≈84%) and Lys-690 (≈93%) residue and partially by the C12:0, C12:0-OH, C16:0, and C16:1 chains. In contrast to the results of Lim et al. (9), we were unable to identify any C15:0 and C17:0 acyl groups attached to the HlyC-modified HlyA molecule despite the extreme sensitivity and accuracy of the current state-of-the-art analytical LC FT-ICR MS technology used. This indicates that uropathogenic E. coli isolates may have an acyl-ACP pool composition different from that of the E. coli B strain used here.

Intriguingly, the activation of HlyA exhibits a flexibility as to the length and nature of the attached acyl chains. When the Lys-690 residue of HlyA was predominantly acylated with the C16:0 and C16:1 (≈90%) acyl chains by CyaC, the HlyA_CyaC protein exhibited a similar capacity to reduce viability of THP-1 cells or to lyse erythrocytes, and it exhibited similar membrane properties on planar lipid bilayers as upon C14 modification on both Lys-564 and Lys-690 residues. Thus, in contrast to CyaA, the HlyA toxin can be modified by acyl chains of varying length (C14 versus C16) and still gains biological activity.

Previously, using deleted HlyA protoxin variants and peptides as substrates in an in vitro acylation assay, Stanley et al. (31) demonstrated that HlyC possessed an about 4 times higher affinity for the segment encompassing the Lys-564 residue of HlyA than for that harboring the Lys-690 residue, resulting in acylation of 80% Lys-564 residues and only 20% of Lys-690 residues. Moreover, substitutions of the Lys-564 and Lys-690 residues revealed that both sites were required for hemolytic activity of HlyA (8). Interestingly, our results indicate that the affinity of the CyaC and RtxC acyltransferases in vivo was much higher to the segment harboring the Lys-690 residue, as this was quantitatively acylated by both enzymes in vivo, whereas only 7% to 27% of HlyA molecules were modified by CyaC or RtxC on the Lys-564 residue. Despite a substantially lower extent of acylation of the Lys-564 residue, the complete acylation of the Lys-690 residue conferred on the HlyA_CyaC and HlyA_RtxC proteins a full capacity to lyse erythrocytes. Moreover, the HlyA_CyaC and HlyA_RtxC proteins also exhibited an equally high membrane activity on planar lipid bilayers as the

Figure 6. The CyaC-acylated RtxA variant is inactive. The RtxA variants were expressed in E. coli BL-21 cells and purified from urea extract on Ni-NTA agarose. Sheep erythrocytes (5 x 10⁷/ml) were incubated at 37 °C in the presence of the RtxA variants (200 ng/ml) and in the presence of 2 mM Ca²⁺. Hemolytic activity was measured as the amount of released hemoglobin by photometric determination (A541) (n = 3). Activities represent average values ± S.D. (error bars) from three independent determinations performed in duplicate with two different toxin preparations.

EDITORS’ PICK: Specificity of toxin-activating acyltransferases

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native doubly acylated HlyA/HlyC protein. Hence, rather than acylation of Lys-564 being essential, the activity of HlyA was likely lost upon substitution of Lys-654 because of a structural role of the Lys-564 residue in toxin activity. Indeed, a similar conclusion was reached upon substitution of the homologous Lys-860 residue of CyaA, the acylation of which is by itself dispensable for CyaA activity on both erythrocytes, as well as on CR3-expressing cells (13, 17). However, the CyaA-K860R mutant is importantly affected in its specific membrane penetration activity on cells lacking the CR3 receptor (13, 17).

Recently, we reported that the recombinant RtxA produced together with RtxC in the E. coli B strain BL21 was primarily modified by C14:0 and C14:0-OH acyl chains (~89%) on the Lys-689 residue (5). Only a minor proportion of the RtxA molecules (~23%) was also found to be modified by C14:0 and C14:0-OH acyl chains on the Lys-558 residues (5). Here, we confirmed the type and extent of acylation of recombinant RtxA toxin on the Lys-689 residue, whereas a lower level (~2%) of modification of the Lys-558 residue was detected. This indicates that the extent of Lys-558 acylation may vary as a function of the physiological state of the producing bacteria, as discussed previously for the acylation of the Lys-860 residue of CyaA (13). Alternatively, differences in LC–MS configuration used for quantification of protein peptides between the reports may have accounted for the variation in the detected quantities of the acylated peptide (5). Indeed, reproducibility of MS-based peptide quantitation
was shown to vary by up to 20%, depending on the sample preparation, unique characteristics of reversed-phase columns used for separation of peptides, and LC–MS instrument configuration, respectively (32).

Similarly as RtxARtxC, the HlyC-modified RtxA was predominantly acylated by C14:0 and C14:0-OH acyl chains (~96%) on the Lys-689 residue, and only residual modification by C14:0 and C14:0-OH chains (~3%) was observed on the Lys-558 residue. The RtxARtxC and RtxA_HlyC variants with a similar acylation pattern then exhibited comparable capacities to lyse erythrocytes and similar membrane properties on planar lipid bilayers. However, unlike the fully biologically active C16-acylated HlyA_CyaC toxin, the RtxACyaC protein modified by CyaC at the Lys-689 residue by the C16:0 and C16:1 chains (~89%) was unable to lyse erythrocytes and exhibited only a residual overall membrane activity on planar lipid membranes. C16-acylated RtxACyaC was most likely impaired in binding/insertion to the lipid bilayer and/or in the propensity to form oligomeric pores, as once inserted into the membrane, it formed pores exhibiting single-pore conductances and lifetimes similar to those of RtxARtxC. In this respect, the RtxACyaC protein was similar to the unacylated proCyaA, proHlyA, and proRtxA pro-toxins, which despite highly decreased overall membrane activity, once inserted into the lipid bilayer, formed pores with properties similar to those of the acylated toxins (5, 17, 33, 34).

In conclusion, we report here that RtxA has to be modified by 14-carbon fatty acyl chains to be biologically active, whereas HlyA remains active also when modified by 16-carbon acyl chains and CyaA is only activated by 16-carbon acyl chains. These results reveal the selection of acyl chains of appropriate lengths by the respective cognate RTX acyltransferase enzymes and suggest a structural adaptation of the RTXA toxin molecules to the length of the acyl chains used for modification of their crucial acylated residue in the second, more conserved acylation site.

Experimental procedures

Bacterial strains

The E. coli strain XL1-Blue (Stratagene, La Jolla, CA) was used throughout this work for DNA manipulations and was grown in Luria–Bertani medium at 37 °C. The E. coli strain BL21 (Novagen, Madison, WI) carrying the plasmid pMM100 used throughout this work for DNA manipulations and was

Cell lines
Murine monocytes/macrophages J774A.1 (ATCC, number TIB-67) were cultured at 37 °C in a humidified air/CO₂ (19:1) atmosphere in RPMI 1640 (Sigma–Aldrich) supplemented with 10% fetal calf serum (Gibco) and antibiotic antimycotic solution (0.1 mg/ml streptomycin, 1000 units/ml penicillin and 0.25 mg/ml amphotericin; Sigma–Aldrich).

Standard techniques

Determination of protein concentration and SDS-PAGE were performed according to standard protocols (36). PageRu-
EDITORS’ PICK: Specificity of toxin-activating acyltransferases

**LC–MS analysis**

The proteins were dissolved in 50 mM ammonium bicarbonate buffer (pH 8.2) to reach 4 M concentration of urea and digested with trypsin (Promega, Madison, WI, modified sequencing grade) at a trypsin/protein ratio of 1:50 for 6 h at 30°C. The second portion of trypsin was added to a final ratio of trypsin/protein of 1:25, and the reaction was carried out for another 6 h at 30°C. When the reaction was complete, the concentration of the resulting peptides was adjusted by 0.1% TFA to 0.1 mg/ml, and 5 μl of the sample were injected into the LC–MS system. The LC separation was performed using a desalting column (ZORBAX C18 SB-300, 0.1 × 2 mm) at a flow rate of 40 μl/min (Shimadzu, Kyoto, Japan) of 0.1% formic acid (FA) and a separation column (ZORBAX C18 SB300, 0.2 × 150 mm) at a flow rate of 10 μl/min (Agilent 1200, Santa Clara, CA) of water/acetoniitrile (MeCN) (Merck, Darmstadt, Germany) gradient: 0–1 min, 0.2% FA, 5% MeCN; 5 min, 0.2% FA, 10% MeCN; 35 min, 0.2% FA, 50% MeCN; 40 min, 0.2% FA, 95% MeCN; 40–45 min, 0.2% FA, 95% MeCN. A capillary column was directly connected to a mass analyzer. The MS analysis was performed on a commercial solarix XR FTMS instrument equipped with a 15 Tesla superconducting magnet and a Dual II ESI/MALDI ion source (Bruker Daltonics, Bremen, Germany). Mass spectra of the samples were obtained in the positive ion mode within an m/z range of 150–2000. The accumulation time was set at 0.2 s, LC acquisition was 45 min with a 5-min delay, and one spectrum consisted of accumulation of four experiments. The instrument was operating in survey LC–MS mode and calibrated online using Agilent tuning mix, which results in mass accuracy below 2 ppm.

**Data processing and interpretation**

MS data were processed by the SNAP version 2.0 algorithm of the DataAnalysis 4.4 software package (Bruker Daltonics, Billerica, MA, USA) generating a list of monoisotopic masses from deconvoluted spectra. The parameters were set as follows: export m/z range of 150–2000, maximum charge state of 8, signal/noise threshold of 0.75, and absolute intensity threshold 5 × 10^5. The extracted experimental data were searched against the FASTA of a single corresponding toxin molecule (CyaA: UniProtKB code P0DKX7; HlyA: UniProtKB code P08715; RtxA: UniProtKB code A0A1X7QNC7) using the home-built Linx software (RRID:SCR_018657). The Linx algorithm was set for fully tryptic restriction with a maximum of three missed cleavages and variable modification for methionine oxidation (m/z 15), acetylation (m/z 44), and monosaturated and hydroxylated variants. The mass error threshold was set to ±2 ppm, and all assigned peptides used for quantification were verified manually. The acylation status of lysine residues was determined by comparison of the relative intensity ratio between acylated peptide ions and their unmodified counterparts. Only lysine residues modified at specific positions (CyaA: 860,983; HlyA: 564,690; RtxA: 558,689) according to the sequence of the full-length proteins were investigated. All assigned peptide sequences, including posttranslational modifications, along with corresponding FASTA formats used within the search algorithm are listed in the supporting MS Data.

**Planar lipid bilayers**

Measurements on planar lipid bilayers (black lipid membranes) (39) were performed in Teflon cells separated by a diaphragm with a circular hole (diameter 0.5 mm) bearing the membrane. The RTX proteins were pre-diluted in TUC buffer (50 mM Tris-HCl (pH 8.0), 8 mM urea, and 2 mM CaCl2) and added into the grounded cis compartment with a positive potential. The membrane was formed by the painting method using soybean lecithin in n-decane–butanol (9:1, v/v). Both compartments contained 150 mM KCl, 10 mM Tris-HCl (pH 7.4), and 2 mM CaCl2, and the temperature was 25°C. The membrane current was registered by Ag/AgCl electrodes (Theta) with salt bridges (applied voltage, 50 mV), amplified by LCA-200-100G and LCA-200-10G amplifiers (Femto, Berlin, Germany), and digitized by use of a LabQuest Mini A/D converter (Vernier, Beaverton, OR). For lifetime determination, ~400 individual pore openings were recorded, and the dwell times were determined using QuB software (40) with a 100-Hz low-pass filter. The kernel density estimation was fitted with a double-exponential function using Gnuplot software. The relevant model was selected by the χ² value.

**Cell-binding and cell-invasive activities on sheep erythrocytes**

AC enzyme activities of the CyaA variants were measured in the presence of 1 μM calmodulin as described previously (41). One unit of AC activity corresponds to 1 μmol of cAMP formed/min at 30°C, pH 8.0. Cell-invasive AC activity was determined in TNC buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 2 mM CaCl2) as the amounts of the AC enzyme protected against inactivation by externally added trypsin upon internalization into sheep erythrocytes, as described previously (42). Erythrocyte binding of the CyaA variants was determined in TNC buffer as described previously (42). Activity of CyaC-activated CyaA was taken as 100%.

**Hemoglobin release assay**

Sheep erythrocytes stored in Alsever’s solution (Sigma–Aldrich) were repeatedly washed with TNC buffer. Washed erythrocytes (5 × 10⁸/ml) were then incubated with various acylated CyaA, HlyA, and RtxA variants in 1 ml of TNC buffer, and hemolytic activity was measured in time by photometric determination (A₅₄₁) of the hemoglobin release.

**Binding of CyaA to J774A.1 cells and determination of cAMP levels**

Prior to assays, RPMI was replaced with DMEM (which contains 1.9 mM Ca²⁺) without fetal calf serum, and the cells were allowed to rest in DMEM for 1 h at 37°C in a humidified 5% CO₂ atmosphere (43). J774A.1 cells (1 × 10⁶) were incubated in DMEM with 1 μg/ml of the CyaA variants for 30 min at 4°C, prior to removal of unbound toxin by three washes in DMEM. After the transfer to the fresh tube, the cells were lysed with 0.1% Triton X-100 for determination of cell-bound AC enzyme activities.
activity. For intracellular cAMP assays, 1.5 \times 10^6 cells were incubated at 37°C with the CyaA variants for 30 min in DMEM, the reaction was stopped by the addition of 0.2% Tween 20 in 100 mM HCl, samples were boiled for 15 min at 100°C and neutralized by the addition of 150 mM unbuffered imidazole, and cAMP was measured by a competitive immunoassay (42). Activity of CyaC-activated CyaA was taken as 100%.

**Cell viability**

Cell viability following exposure to the toxin was determined as the capacity of mitochondrial reductases to convert the tetrazolium salt WST-1 to formazan, using the WST-1 assay kit (Roche Applied Science) according to the protocol of the manufacturer.

**Data availability**

The MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD018859 (44).

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: RTX, repeats in toxin; ACP, acyl carrier protein; Ni-NTA, nickel-nitritolriacetic acid; FT-ICR, Fourier transform ion cyclotron resonance; pS, picoseconds; FA formic acid; DMEM, Dulbecco’s modified Eagle’s medium; WST-1, 3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate; KDE, kernel density estimation.

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