Characterization of *Streptococcus agalactiae* CAMP factor as a pore-forming toxin

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SUMMARY

A recombinant form of CAMP factor of *Streptococcus agalactiae* has been expressed as glutathione S-transferase / CAMP fusion protein in *Escherichia coli*. After thrombin cleavage of the fusion protein, the recombinant CAMP factor exhibited hemolytic activity comparable to that of the native form. Osmotic protection experiments with polyethylene glycols show that CAMP factor forms discrete transmembrane pores with a diameter upward of 1.6 nm on susceptible membranes; electron microscopy reveals circular membrane lesions of heterogeneous size, up to 12-15 nm in diameter. Liposome permeabilization studies show that pore-formation is a highly cooperative process, which suggests that it involves the oligomerization of CAMP factor. Chemical cross-linking experiments also support an oligomeric mode of action.
INTRODUCTION

The CAMP reaction consists in a distinct zone of hemolysis on blood agar plates produced by Streptococcus agalactiae when grown near the colonies of Staphylococcus aureus (1). It has been used in diagnostic microbiology to identify the Streptococcus agalactiae strains ever since its discovery in 1944 by Christie, Atkins and Munch-Petersen. The proteins responsible for the CAMP reaction are sphingomyelinase from Staphylococcus aureus and CAMP factor, a protein secreted by Streptococcus agalactiae that has a molecular weight of 25 kDa and a pI of 8.9 (2).

Sphingomyelinase initially hydrolyzes sphingomyelin to ceramide, which renders the erythrocytes susceptible to the lytic activity of CAMP factor. Erythrocytes from different mammalian species support the CAMP reaction to different extents, depending on the sphingomyelin content in their cell membranes (3). Sheep red blood cells are the most susceptible, in keeping with their sphingomyelin content as high as 51% (by moles) (4). Human red blood cells and rabbit red blood cells, with 26% and 19% mole of sphingomyelin, respectively (4;5), were reported not to be sensitive to CAMP factor after sphingomyelinase treatment (3). The latter were, however, rendered susceptible to the CAMP factor after phospholipase C treatment, which converted the glycerophospholipids to diacylglycerol (6), indicating that ceramide is not specifically required for CAMP activity. Previous work with liposomes also suggested that the fraction of cholesterol in the membrane influenced the activity of CAMP factor (6;7).

CAMP factor has long been known as a pathogenic determinant, which exerted lethal effects when administered to rabbits and mice (8). Apart from its membrane-damaging activity, CAMP factor was found to bind to the Fc fragments of immunoglobulins, and it was therefore designated as protein B in analogy to protein A of Staphylococcus aureus (9).
The CAMP factor genes of *Streptococcus agalactiae* (10), *Streptococcus uberis* (11) and *Streptococcus pyogenes* (12) have been cloned in *Escherichia coli*, and their sequences were found to be highly homologous with each other (12). Co-hemolytic phenomena have also been reported with various other bacterial genera, but the proteins responsible for those, although sometimes referred to by the name ‘CAMP factor’ as well, are not closely related to streptococcal CAMP factor.

In this study, we expressed the CAMP factor as a glutathione S-transferase fusion protein in *E. coli*. The fusion protein was purified to homogeneity and cleaved by thrombin to yield the recombinant CAMP factor, which exhibited similar hemolytic activity to the native protein. We show that CAMP factor forms pores of finite, yet heterogeneous size in membranes of susceptible cells. Additional studies with liposomes show that the process of CAMP factor pore formation is highly cooperative, supporting an oligomeric nature of the membrane lesion.
MATERIALS AND METHODS

Cloning, plasmids and bacterial strains. The pGEX-KG (13) was a generous gift from Jingya Li, National Center for Drug Screening, Shanghai, China. The coding sequence of CAMP factor mature peptide was amplified from Streptococcus agalactiae genomic DNA. The following primers was used: forward, 5’-TTT GCC GAT CAA GTG ACA ACT CCA C; reverse, CAA TCA TGG TAG TAC AAA ATC ACG A. The amplified DNA was cloned into the pET-30a+ plasmid. The insert was then digested with NcoI and XhoI, and ligated into NcoI-XhoI-digested pGEX-KG vector. The insert from pET-30a+ was sequenced and matched the published mature peptide sequence for CAMP factor (14).

Expression and purification of CAMP factor. The protein was expressed in BL21 (DE3) cells. A starter culture (20 ml LB1 containing 100 µg/ml ampicillin) of transformed cells was grown at 37°C overnight. This overnight culture was then inoculated into 1 liter LB containing 100 µg/ml ampicillin. The culture was shaken vigorously at 30°C until the OD600 reached 0.8. IPTG was added to a final concentration of 1 mM, and the culture was grown for an additional 4 h. Cells were harvested by centrifugation and stored at -20°C overnight. The frozen cells were thawed on ice for 15 min and resuspended in 20 ml of PBS buffer (16 mM K2HPO4, 150 mM NaCl, pH 7.2) containing protease inhibitor cocktail (Sigma, Oakville, ON). The cells were then lysed by sonication. Triton X-100 was added to the bacterial lysate to a final concentration of 1% (w/v). The lysate was centrifuged at 10,000 g for 10 min at 4°C. The fusion protein was purified from the supernatant. The purification procedure was essentially as described by Guan and Dixon.

1 The abbreviations used are: LB, Luria Bertani broth; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate buffered saline; GST-CAMP, glutathione S-transferase / CAMP factor fusion protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; PEG, polyethylene glycol; PC, phosphatidylcholine; C12-ceramide, N- lauroryl-D-erythro-sphingosine. C20-ceramide, N-arachidoyl-D-erythro-sphingosine.
The supernatant was mixed with 20 ml of 50% (v/v) glutathione-agarose beads and gently shaken at 4°C for 30 min. The beads were washed four times with 10 ml PBST buffer (PBS buffer with 1% Triton) at 4°C, once with PBS buffer and twice with thrombin cleavage buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0) at room temperature. After centrifugation, the agarose beads were mixed with 2 ml of thrombin cleavage buffer containing 12 μg of thrombin. The suspension was incubated with gentle shaking at room temperature for 1 h and centrifuged. The beads were washed with thrombin cleavage buffer twice to retrieve the cleaved protein, and the supernatants were pooled. This procedure typically yielded about 4 mg of purified CAMP factor per liter of liquid culture. Protein concentrations were determined by the Bradford method.

Purification of glutathione S-transferase/CAMP fusion protein (GST-CAMP) (15). The GST-CAMP protein bound to the glutathione agarose beads was eluted three times with 2 ml of 50 mM Tris-HCl, 10 mM glutathione (pH 7.5).

Circular dichroism. CD spectra were measured using a Jasco J-715 circular dichroism spectrometer (Jasco, Tokyo, Japan) with a 1 mm path length cuvette. The protein (0.08 mg/ml, 3 μM) was in 8 mM potassium phosphate buffer, pH 7.2. The samples were scanned 10 times from 180 to 250 nm with a 0.5 nm interval. CD spectra were corrected for background and analyzed for protein secondary structure using the CONTIN, CDSSTR and SELCON program with the 43-protein reference set (http://cryst.bbk.ac.uk/cdweb/) (16-18). The secondary structure was also analyzed with the prediction program GOR IV (http://npsa-pbil.ibcp.fr).

Lysis of sheep red blood cells. Sheep red blood cells (Cedarlane, Hornaby, ON) were washed 5 times in hemolysis buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) by centrifugation. The erythrocytes were then resuspended in hemolysis buffer to 0.5% (v/v). The cells were incubated...
at room temperature for 5 min in the presence of 10 mM MgCl₂ and with or without 50 mU/ml sphingomyelinase from *Staphylococcus aureus* (EC 3.1.4.12, Sigma). 180 µl of cell suspension was added to the wells of a microplate containing different amounts of CAMP factor in 20 µl of the former buffer. Hemolysis was measured by the decrease in turbidity (OD₆₅₀) using a 96-well plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA). For osmotic protection by PEG, the red cells were made to 5% (v/v) suspension, followed by incubation with 500 mU/ml sphingomyelinase at room temperature. 20 µl of the erythrocyte suspension was added to 180 µl of hemolysis buffer containing CAMP factor and polyethylene glycols (PEGs) of various molecular weights at 30 mM. Hemolysis was assayed by cell turbidity as above in the experiments depicted in Fig. 4A, or by measuring the OD of the released hemoglobin at 540 nm (after spinning down cells and membranes) in the experiments shown in Fig. 4B. Control samples without CAMP factor remained stable during the assay period.

**Binding of CAMP factor to erythrocyte membranes.** Sheep erythrocytes were lysed osmotically in 5 mM phosphate buffer and washed several times until the membrane pellets became white. Membranes were suspended in hemolysis buffer with 10 mM MgCl₂ and pretreated with or without 0.18 U/ml sphingomyelinase for 10 min at 37°C. CAMP factor protein or GST-CAMP fusion protein was added. After incubation at 37°C for half an hour, the mixtures were centrifuged at 14,000 rpm for 10 min and washed twice with the hemolysis buffer. The membrane pellet was dissolved in 10 mM Tris, 150 mM NaCl, 10% sodium deoxycholate (w/v), pH 8.0. The dissolved membrane pellets were analyzed by SDS-PAGE or by gel filtration. For SDS-PAGE, 300 µg/ml lipids and 17 µg/ml of protein were used. For gel filtration, the concentration of lipids and CAMP factor was 150 µg/ml and 174 µg/ml, respectively. The
protein concentration of the membrane was determined by the Bradford method and the lipid concentration was estimated by assuming the lipid to protein ratio is 1:1 by weight.

**Preparation of liposomes.** All lipids were purchased from Avanti Lipids (Alabaster, AL), with the exception of bovine brain ceramide (Sigma). The lipids, dissolved in chloroform, were mixed in various molar ratios (see results) and dried down under nitrogen in a round-bottom flask and then dried under vacuum for an additional 3 h to remove the residual solvent. The dried lipids were hydrated at room temperature for 1 h in 1 ml of 20 mM HEPES, 150 mM NaCl, 50 mM calcein, pH 7.4 by vigorous vortexing. For ceramide-containing liposomes, the suspension was warmed to 45°C during hydration. The suspension was frozen at -20°C and thawed at room temperature. The lipid suspension was sized down to unilamellar liposomes using a liposome extruder (Northern Lipids, Vancouver, BC) by extruding 10 times through a polycarbonate membrane with a 100 nm pore size. The extruder was thermostated to 45°C to facilitate processing of the ceramide-containing liposomes. The non-entrapped calcein was removed by gel filtration on Sephadex G-50 pre-equilibrated with 20 mM HEPES, 150 mM NaCl, pH 7.4. The lipid concentration was determined by an enzymatic cholesterol assay (Molecular Probes, Eugene, OR). For liposomes without cholesterol, the lipid concentration was estimated from the encapsulated liposome volume (19).

**Calcein release assay.** All fluorescence measurements were performed on a Fuorolog 3 spectrofluorimeter (Instrument S. A., JOBIN YVON/SPEX Division, Edison, NJ). The liposomes with entrapped calcein (final lipid concentration 60 μg/ml) were mixed with various amounts of CAMP factor in 10 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl₂, pH 7.4 buffer. After incubation at 37°C for 10 min, the samples were diluted with the same buffer, and calcein fluorescence intensity was measured immediately (excitation: 480 nm; emission: 520 nm). The
fraction of calcein released was calculated by deducting the fluorescence intensity of a control sample incubated without CAMP factor. 100% release was obtained from a liposome sample lysed by sodium deoxycholate.

**Gel filtration.** Gel filtration chromatography of solubilized membranes and CAMP factor was performed using a Sephacryl HR 300 (Amersham Pharmacia biosciences, Baie d’urfé, Quebec) column (diameter: 16 mm; length: 25 cm). The column was equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 6 mM sodium deoxycholate, pH 8.0. The samples containing erythrocyte membranes were pelleted and dissolved in 500 μl buffer (50 mM Tris-HCl, 150 mM NaCl, 10% sodium deoxycholate, pH 8.0), applied to the column and eluted with equilibration buffer. Fractions of 1 ml were collected and analyzed by SDS-PAGE.

**Chemical cross-linking of CAMP factor.** CAMP factor (final concentration 0.17 mg/ml) was first incubated with the liposomes (total lipids: 0.2 mg/ml) in 10 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, pH 7.4 at 37°C for 30 min. The cross-linking reagent glutaraldehyde (Sigma) was then added to 2.5 mM and the samples were incubated again at 37°C for 30 min. The reaction was stopped by addition of Tris to 0.1 M. The samples were then analyzed by SDS-PAGE.

**Electron microscopy.** Sheep red blood cells were treated with sphingomyelinase as above, incubated and lysed with 70 μg/ml of CAMP factor, and the membranes collected by centrifugation. The membranes were washed three times with 5 mM HEPES buffer (pH 7.4) and mounted onto carbon-coated grids (formvar / copper, 200 mesh, Marivac Ltd), followed by negative staining with sodium phosphotungstate (2% w/v, pH 7.4) for 5 seconds. They were then examined in a LEO 912 AB Omega electron microscope, operated at 100 kV.
RESULTS

Cloning, recombinant expression and purification of CAMP factor. The coding sequence for CAMP factor was amplified by PCR, cloned and sequenced from a laboratory strain of *Streptococcus agalactiae*. A BLAST (http://www.ncbi.nlm.nih.gov) search was performed. The coding sequence was found to deviate at two positions from the published sequence of CAMP factor of *Streptococcus agalactiae* (20) at both nucleotide and amino acid levels. However, the coding sequence of the CAMP factor mature peptide matched exactly the amino acid sequence of protein B, the alias of CAMP factor of *Streptococcus agalactiae* (14). These differences may arise from the different sources of the bacterial strains.

We initially tried to clone the CAMP factor gene into a high-copy number vector pT7-α vector in *Escherichia coli*, but failed to get any positive clones. This result may be related to previous reports on the cloning CAMP factor genes from different bacterial strains (10-12), which either used low-copy number plasmids, resulting in very low expression level in E. coli, or recovered clones carrying mutations in the promoter region. This suggests that CAMP factor may be toxic to the bacterial cells. We next tried to express the CAMP factor, including its signal peptide, in *Bacillus subtilis*, reasoning that secretion from a gram-positive cell should be more similar to the physiological situation in streptococci and, therefore, be less hampered by toxicity. However, when expression was induced by xylose (21), the cultures became lytic, and very low CAMP activity was obtained. Therefore, toxicity appears to pertain to gram-positive cells as well.

We finally used the pGEX-KG expression vector, which yields a protein expressed as glutathione S- transferase / CAMP factor fusion protein. The 53 kDa fusion protein was purified following a well-established protocol (13;15). The fusion protein absorbed to the glutathione agarose beads was directly cleaved by thrombin and the 27 kDa cleavage product was released
Streptococcus agalactiae CAMP Factor Is a Pore-Forming Protein

immediately from the agarose beads (Fig. 1A). The cleaved protein has 20 amino acids derived from the vector attached to the N-terminus of the wild-type CAMP factor. This N-terminal extension had no apparent effect on the activity of CAMP factor.

**Secondary structure prediction and circular dichroism.** The GOR IV secondary structure prediction program suggests the protein mainly consists of α-helix (42%) with 16% β strand. The percentage of α helix is somewhat lower in the estimate derived from the observed CD spectrum of the CAMP factor (Fig. 1B). The three programs, CONTIN, SELCON and CDSSTR yielded similar results, which indicate that there is about 30% α helix, 19% β strands, 22% turns and 29% random coil.

**Hemolytic activity of CAMP factor on sheep erythrocytes.** We found that 50% hemolysis could be achieved in 25 min at 25°C with 1.25 ng/ml of CAMP factor (Fig. 2A). The hemolytic activity of the recombinant CAMP factor thus is comparable to the previous data obtained with wild type CAMP factor, for which values of 1.75 ng/ml (22) or 1 ng/ml (7) have been reported.

The CAMP factor could lyse the sheep red blood cells alone even without sphingomyelinase treatment. However, a concentration of 12 μg/ml CAMP factor was needed to achieve an equivalent extent of hemolysis (also note that the kinetics is somewhat different; Fig. 2B). The untreated cells thus are about 10,000 times less susceptible to CAMP factor than the sphingomyelinase-treated ones. The uncleaved GST-CAMP fusion protein possessed very low hemolytic activity, being about 5,000 times less active than the thrombin-cleaved protein (data not shown). This finding may be related to the observed lack of toxicity during expression.

**Binding of CAMP factor to sheep erythrocyte membranes.** The CAMP protein was incubated with red blood cell membranes with or without prior sphingomyelinase treatment. After incubation, the mixture was centrifuged and washed twice to remove the unbound protein. Fig. 3
shows that CAMP factor bound efficiently to the sphingomyelinase-treated membranes. It also shows that CAMP factor could bind to the untreated cell membranes, although the binding efficiency was much lower. GST-CAMP fusion protein also bound efficiently to the sphingomyelinase-treated red blood cell membranes. The intact binding but low hemolytic activity of GST-CAMP suggests that membrane binding and cell lysis are separate, successive events, and that the N-terminus of the CAMP molecule may be involved in the latter step.

CAMP factor is a pore-forming protein. Membrane-damaging toxins may act through two mechanisms. One mechanism involves enzymatic hydrolysis of phospholipids, which will directly disrupt the membrane structure and eventually lead to the cell lysis. In contrast, pore-forming proteins damage erythrocytes through a colloid-osmotic mechanism. They form channels on the target cell membranes, causing the leakage of small molecules such as ions and metabolites, while large molecules such as hemoglobin can’t pass through the channels. This will lead to an increased intracellular osmotic pressure and the influx of water into the cell, resulting in the burst of cells. Adding polymeric solutes to the cell suspension can inhibit this colloid-osmotic process. With growing molecular weight, these solutes will finally become too large to move freely through the pores, thus counterbalancing the osmotic pressure caused by the intracellular macromolecules. Therefore, the osmotic protection can be used to detect pores of defined size.

Previous studies on CAMP factor showed, after lysis of liposomes with CAMP factor, no change in lipid composition had occurred, so that enzymatic activity was not required for membrane damage (22). We therefore reasoned that CAMP factor might possess pore-forming activity. Fig. 4A shows that PEG 6000 and PEG 8000 cause a pronounced delay of hemolysis; lysis is even slower with PEG 10,000. In contrast, PEG 1000 and PEG 4000 have no protective effect on
CAMP factor mediated hemolysis. When cells were incubated with CAMP factor in the presence of PEG 10,000, washed twice in the same buffer and resuspended in buffer without PEG, hemolysis occurred immediately. This confirmed that pore-formation had indeed proceeded in the presence of PEG, indicating that PEG had worked by osmotic protection but not by directly inhibiting the binding or lytic action of CAMP factor. These data strongly suggest that CAMP factor forms pores with a defined diameter on susceptible membranes. Since the hydrodynamic radii of PEG 4000 and PEG 6000 are 1.6 nm and 2.7 nm, respectively (23), these experiments suggested that the diameter of CAMP factor pore is between these two values.

Prompted by the finding of larger pores by electron microscopy (see below, Fig. 5), we repeated the osmotic protection experiments with different dosages of CAMP factor. Fig. 4B shows that the osmo-protective effect of PEG 6000 is diminished and finally abrogated at higher toxin concentrations. This finding is in line with the assumption of heterogeneous pore size, with smaller pores being more prevalent than large ones at least at low toxin concentrations.

**Electron microscopy of CAMP factor pores.** On sheep red cell membranes, pre-treated with sphingomyelinase and then exposed to high concentrations of CAMP factor, we observed discrete, circumscribed membrane lesions of various sizes (Fig. 5). While the smallest pores were just discernible against the background, larger ones reached diameters of 12 nm and beyond. While some pores were round and regular in shape (Fig. 5D), others appeared quite irregular (Fig. 5B, 5C). Similar findings have been reported previously with streptolysin O (24;25). In comparison to the latter toxin, the pores created by CAMP factor appear to be lined by a more delicate seam of protein, which may reflect the smaller molecular mass of CAMP factor (27 kDa as compared to 60 kDa for streptolysin O).
**Streptococcus agalactiae CAMP Factor Is a Pore-Forming Protein**

*Oligomeric state of CAMP factor.* Gel filtration performed on CAMP factor showed that it was monomeric in solution, as it was eluted from a Sephacryl HR 300 column between β-lactoglobulin (35 kDa) and cytochrome C (12.4 kDa) markers (data not shown). With many pore-forming toxins, the pore oligomers are stable upon membrane solubilization by SDS or other detergents. However, we could not detect any oligomer formation on membranes by SDS-PAGE (cf. Fig. 6B, lane 4). To test whether the pore oligomer is stable in non-denaturing detergents, we incubated the CAMP protein with susceptible membranes, which were then solubilized with deoxycholate and analyzed by gel filtration. Again, only one peak was observed (Fig. 6A), which eluted at the same position as the toxin sample that had not been incubated with membranes. Therefore, no stable oligomers were observed after membrane solubilization with either denaturing or non-denaturing detergent, suggesting either that CAMP factor does not oligomerize, or that the oligomer is stable only on the lipid membrane.

To detect oligomers on membranes, we performed chemical cross-linking on CAMP factor incubated with liposomes consisting of ceramide (40%), cholesterol (45%) and phosphatidylcholine (15%). Fig. 6B shows that oligomeric forms were indeed produced upon treatment with glutaraldehyde. Considering the low susceptibility of the liposomal membranes to CAMP factor (see below), the relatively high efficiency of cross-linking also suggests the existence of non-lytic oligomeric stages on liposome membranes.

*Liposome permeabilization studies.* In previous studies, the activity of CAMP factor was found to be related with the amount of diacylglycerol or ceramide, and cholesterol in both natural and artificial membranes (6;7). Therefore, liposome membranes containing different concentrations of ceramide or diacylglycerol might offer a good model for studying the action of CAMP factor on bilayers, and for better understanding the effects of lipid composition on the activity of
CAMP factor. Fig. 7 shows the CAMP-induced calcein release from liposomes of various lipid compositions. The following observations were made:

1. The extent of liposome permeabilization is not linearly related to the CAMP factor concentration. Instead, there is very little permeabilization until a threshold concentration is reached; the percentage of calcein release then increases from 20% to 100% by merely doubling the protein concentration. This cooperative dose-effect correlation is in line with an oligomeric mode of action of CAMP factor during membrane permeabilization.

2. Contrary to what we had expected, ceramide did not augment the activity of CAMP factor on the liposomes, even at molar fractions as high as 40%; this applied to C12-ceramide (Fig. 7A), and to C20-ceramide as well as ceramide isolated from bovine brain (not shown). We found, however, that cholesterol was important. By increasing the cholesterol concentration from 25% to 45%, the liposomes became three times more sensitive, whereas cholesterol-free liposomes were insensitive (Fig. 7B). The latter finding concurs with previous reports that the cholesterol content in the membrane affected the CAMP activity on liposome membranes (6).

3. Even with the most susceptible model membranes used, the concentration of CAMP factor needed to elicit a substantial liposome permeabilization was more than a thousand times higher than that required with sphingomyelinase-treated red blood cells but very similar to the one required with untreated erythrocytes (cf. Fig. 2).

The low activity of CAMP factor on the liposomes might potentially arise from inefficient membrane binding, or from low activity of membrane-bound CAMP factor. To distinguish between these possibilities, we performed the following experiments: Firstly, the ratio of membrane lipids and CAMP factor was kept constant, while the absolute amounts of both were
varied. If binding were efficient and quantitative at low concentrations, then the extent of membrane permeabilization should be the same; this, however, was not observed (Fig. 8A). On the other hand, if binding were very inefficient, then increasing amounts of membranes should not significantly deplete CAMP factor from solution; a fixed amount of CAMP factor should therefore permeabilize any amount of liposomes to approximately the same degree, which was not observed either (Fig. 8B). This suggests that CAMP factor is partially depleted with increasing membrane concentration; the weak effect of CAMP factor on the liposome membrane therefore probably is not due to weak binding only but in part to low pore-forming activity of the bound toxin.

DISCUSSION

The present study shows that CAMP factor forms large, oligomeric pores in susceptible membranes. The size of these pores is between those of streptolysin O (24) and the terminal complement complex (and perforin) (24;26). With both streptolysin O and complement, pores may vary considerably in functional diameter (24-27), a finding that also applies to CAMP factor.

In contrast to many other pore-forming proteins (including streptolysin O and complement), the oligomers of CAMP factor are not stable following membrane solubilization. This finding is not unprecedented with pore-forming peptides and proteins. E.g., while evidence of oligomerization on membranes has been obtained with several pore-forming peptides such as melittin (28) or magainin (29) and with Bacillus thuringiensis δ-endotoxin (30), stable oligomers of these molecules have not been recovered from the membranes (with δ-endotoxin, aggregates do form
pores on brush border membrane vesicles; from these, only trimers were extracted whereas the stoichiometry of the oligomer was expected to be larger than 3 (31)).

CAMP factor displays little sequence homology to other pore-forming proteins, the closest match being colicin Ia, which scores 20% identity when allowing for 10 gaps in the alignment. Since this alignment does not span the putative pore-forming domains of colicin Ia (32), this homology does not permit the prediction of the tertiary structure of CAMP factor in the membrane-inserted, oligomeric form. Structure and structure-function relationships therefore await experimental elucidation.

As with other pore-forming proteins, two phases can be distinguished in the action of CAMP factor: membrane binding, and pore formation, which presumably involves oligomerization. While sphingomyelinase treatment very clearly increased membrane binding, this difference does not fully account for the observed difference in membrane susceptibility; it therefore seems that the oligomerization / pore-formation step is affected by the change in lipid composition as well.

While model liposomes were susceptible to CAMP factor to limited extent, CAMP factor was not any more active on liposomes containing ceramide than on those without. This is in striking contrast to the red cells, which were sensitized 10,000 fold by sphingomyelinase treatment, and suggests that the role of ceramide does not consist in a direct interaction with the protein. It remains to be determined what property or component of the red cell membrane accounts for the sensitizing effect of ceramide.

Another open question is what the physiological role of CAMP factor might be. The apparent toxicity of CAMP factor for bacterial cells, which was observed in the initial attempts to achieve expression, led us to speculate that bacteria might be relevant as a target of CAMP factor in vivo.
However, addition of purified CAMP factor to *Escherichia coli* or *Bacillus subtilis* cultures did not detectably affect cell viability; CAMP factor may therefore be toxic to bacterial cells only from the inside. Further studies on its mode of action need to be performed with different types of cells and model membranes to better understand the physiological significance of CAMP factor. While cholesterol has a strong effect on membrane susceptibility, the specificity of the sterol requirement has not yet been examined in detail, and the apparent toxicity of CAMP factor for bacterial cells suggests that it might be functionally replaced by non-sterol lipids in the target membrane.

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Fig. 1. (A) Expression and purification of CAMP factor in Escherichia coli (SDS-PAGE, 12%). M, molecular weight marker; Lane 1 and 2, E. coli protein extracts before and after IPTG induction, respectively; Lane 3, glutathione S-transferase / CAMP fusion protein (0.4 µg); Lane 4, CAMP factor released from GST-CAMP after thrombin cleavage (2 µg). (B) Circular dichroism spectrum of CAMP factor.

Fig. 2. Lysis of sheep erythrocytes by CAMP factor. (A) Lysis of cells pretreated with sphingomyelinase (50 mU/ml) and then exposed to various concentrations of CAMP factor (from 1.25 to 20 ng/ml). Hemolysis was assayed by the decrease in turbidity (A650). (B) Hemolysis of sheep erythrocytes by CAMP factor, without sphingomyelinase pretreatment. The amounts of CAMP factor ranged from 5 to 30 µg/ml. Note the widely differing amounts of CAMP factor used in A and B, respectively.

Fig. 3. Binding of CAMP factor to sheep erythrocyte membranes. CAMP factor or GST-CAMP fusion protein at 17 µg/ml were incubated with sphingomyelinase-treated and untreated sheep erythrocyte membranes. The membranes were washed twice by centrifugation and analyzed by SDS-PAGE (12%). M, molecular weight markers. Lane 1, CAMP factor. Lanes 2 and 3, CAMP factor bound to sphingomyelinase-treated and untreated sheep erythrocyte membranes, respectively. Lane 4, sheep erythrocyte membrane treated with sphingomyelinase (without CAMP factor). Lane 5, binding of the non-lytic GST-CAMP fusion protein to sphingomyelinase-treated sheep erythrocyte membranes. Lane 6, GST-CAMP fusion protein.

Fig. 4: Osmotic protection of CAMP factor induced hemolysis by PEG. (A) Sheep erythrocytes (5% by volume) were pretreated with 50 mU/ml of sphingomyelinase for 10 min at 25°C and then diluted tenfold into 5 ng/ml CAMP factor solution with 30 mM PEGs of different molecular
weights. Hemolysis was assayed by the decrease in turbidity (A\textsubscript{650}). Erythrocyte suspension in PEG-containing solution will yield a smaller OD\	extsubscript{540} reading, therefore the initial OD reading of each sample was normalized to 1 and the subsequent readings were adjusted accordingly. (B) Sphingomyelinase-treated erythrocytes were incubated for 30 min with the amounts of CAMP factor indicated. The samples were centrifuged, and the extent of lysis determined from the OD\	extsubscript{540} in the supernatant. Mean values of duplicates are shown.

Fig. 5: Electron microscopy of CAMP factor on sheep red cell membranes (phosphotungstate negative staining). The erythrocytes were pre-treated with sphingomyelinase, incubated with CAMP factor at 70 \( \mu \text{g/ml} \), and the lysed membranes collected and repeatedly washed by centrifugation. Circumscribed lesions vary in size and shape. Magnification varies among panels A-D as indicated by the scale bars.

Fig. 6: Oligomeric state of CAMP factor. (A). Gel filtration. CAMP factor was incubated with the sphingomyelinase-treated sheep erythrocyte membranes at 37°C for 30 min. The membranes were washed by centrifugation and solubilized with deoxycholate. The dissolved sample was applied to a Sephacryl HR 300 column. The eluate fractions were analyzed by SDS-PAGE (12%). Fraction numbers are shown above the gel lanes (M: Molecular weight marker). The band labeled by a star identifies CAMP factor, as it was absent from a control sample containing sphingomyelinase-treated membranes only. (B) Chemical cross-linking with glutaraldehyde. CAMP factor was incubated with liposomes made from C12-ceramide/cholesterol/phosphatidylcholine (40/45/15% by mole) at 37°C for 30 min to allow for oligomerization. They were then exposed to glutaraldehyde at 37°C for another 30 min for cross-linking, and the reaction quenched with Tris. The cross-linked samples were heated to 95°C with SDS and analyzed by SDS-PAGE (10%). M, molecular weight marker. Lane 1, CAMP factor.
Lane 2, CAMP factor cross-linked by glutaraldehyde. Lane 3, CAMP factor incubated with liposomes and then cross-linked. Lane 4, CAMP factor incubated with liposomes but without glutaraldehyde.

**Fig. 7.** Permeabilization of liposomes by CAMP factor. Liposomes of different lipid composition were incubated with various amounts of CAMP factor at 37°C for 10 min. The samples were diluted and the fluorescence intensity was measured (excitation: 480 nm; emission: 520 nm). The fraction of released calcein was calculated from the increase of fluorescence over a control sample without CAMP factor. 100% release was determined using a sample solubilized by sodium deoxycholate. (A) Effect of ceramide content on the permeabilization of liposomes induced by CAMP factor. Liposomes with calcein trapped inside were made from cholesterol (25% by mole), C12-ceramide (molar fraction as indicated) and phosphatidylcholine (ad 100%).

(B) Role of cholesterol in the activity of CAMP factor. Liposomes with entrapped calcein were produced from 40% C12-ceramide, various amounts of cholesterol as indicated, and phosphatidylcholine (ad 100%).

**Fig. 8.** Effect of CAMP factor and of lipid concentrations on the efficacy of membrane permeabilization. Liposomes used were PC/cholesterol/C12-ceramide (55/25/20% by mole), and the assay procedure was the same as described in Fig. 5. (A) Effect of varying both concentrations at constant ratio (1μg of protein per 1.2 μg lipid). Data are averages of duplicates. (B) Effects of protein/lipid ratio on the CAMP factor induced permeabilization of liposomes. A fixed amount of CAMP factor (60 μg/ml) was incubated with different amount of lipids (7.5, 15, 30, 60, 120 μg/ml).
Figure 2

A

B

OD_{600}

Time (min)

OD_{600}

Time (min)
Figure 4
Figure 7
Figure 8

A

![Graph A]

B

![Graph B]
Characterization of streptococcus agalactiae CAMP factor as a pore-forming toxin
Shenhui Lang and Michael Palmer

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