Identification of the Cellular Receptor of *Clostridium spiroforme* Toxin

Panagiotis Papatheodorou, Claudia Wilczek, Thilo Nölke, Gregor Guttenberg, Daniel Hornuss, Carsten Schwan, and Klaus Aktories

Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albert-Ludwigs-Universität Freiburg, Freiburg, Germany

*Clostridium spiroforme* produces the binary actin-ADP-ribosylating toxin CST (*C. spiroforme* toxin), which has been proposed to be responsible for diarrhea, enterocolitis, and eventually death, especially in rabbits. Here we report on the recombinant production of the enzyme component (CSTA) and the binding component (CSTB) of *C. spiroforme* toxin in *Bacillus megaterium*. By using the recombinant toxin components, we show that CST enters target cells via the lipolysis-stimulated lipoprotein receptor (LSR), which has been recently identified as the host cell receptor of the binary toxins *Clostridium difficile* transferase (CDT) and *Clostridium perfringens* iota toxin. Microscopic studies revealed that CST, but not the related *Clostridium botulinum* C2 toxin, colocalized with LSR during toxin uptake and traffic to endosomal compartments. Our findings indicate that CST shares LSR with *C. difficile* CDT and *C. perfringens* iota toxin as a host cell surface receptor.

**MATERIALS AND METHODS**

**Cell cultivation.** H1-HeLa and CaCo-2 cells were grown in Dulbecco modified Eagle medium (DMEM) (12 mM t-glutamine) supplemented with 10% fetal calf serum (FCS), penicillin (4 mM), streptomycin (4 mM), 1% nonessential amino acids (NEA), and for CaCo-2 cells only, 1% sodium pyruvate (1 mM). Cells were incubated at 37°C with 5% CO2 under humidified conditions. Generation of H1-HeLa cells stably transduced with a retroviral element expressing an N-terminally FLAG-tagged version of LSR (FLAG-LSR) has been described elsewhere (16).

**Cloning procedures.** For cloning of CSTa and CSTb into the *B. megaterium* expression system for recombinant production of CSTa and CSTb. The availability of both toxin components enabled us to elucidate the target cell receptor of CST. We recently identified the lipolysis-stimulated lipoprotein receptor (LSR) as the target molecule for binding and internalization of the clostridial binary toxins *C. difficile* transferase and *C. perfringens* iota toxin (16). Here, we demonstrate that *C. spiroforme* toxin also uses the LSR for target cell entry.

**Expression and purification of CSTa and CSTb.** Briefly, *B. megaterium* protoplasts, transformed with either pPH1522/CSTa-6His or pPH1522/CSTb-6His (according to the manufacturer’s instructions [MoBiTec, Göttingen, Germany]), were grown at 37°C in LB medium to an optical density at 600 nm (OD600) of 0.8. Protein expression was induced by addition of 5% (wt/vol) xylose followed by incubation overnight.

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Address correspondence to Klaus Aktories, klaus.aktories@pharmakol.uni-freiburg.de.

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"Clostridium spiroforme" is frequently found in the digestive tracts of rabbits, where it is proposed to be responsible for severe diarrhea, enterocolitis, and eventually death of the host (7). The virulence of *C. spiroforme* strongly correlates with the production of an enterotoxin termed CST (*C. spiroforme* toxin) (6) that belongs to the family of clostridial iota-like binary toxins, including *C. perfringens* iota toxin and *C. difficile* transferase (CDT). CST is an A-B-type toxin, with an enzyme component (CSTa) harboring mono(ADP-ribosyl)transferase activity and a separate binding component (CSTb) that forms heptamers, binds to target cells, and is responsible for the cell entry of the enzyme component. After endocytosis and traffic to acidic endosomes, the heptameric binding component forms pores in endosomal membranes, which allow the translocation of the enzyme component into the cytosol of target cells (1, 2). In the cytosol, CSTa ADP-ribosylates G-actin, thereby leading to actin depolymerization and disruption of microfilaments (18, 19, 21).

The binding components of binary actin-ADP-ribosylating toxins have significant similarity with the protective antigen (PA), which is the binding component of anthrax toxin (25). Structural studies of anthrax toxin were most instrumental for the understanding of the mechanisms underlying the cellular uptake of binary actin-ADP-ribosylating toxins. All these binding components are 80- to 100-kDa proteins and consist of 4 domains. Domain 1 is involved in proteolytic toxin activation. Cleavage of an ~20-kDa N-terminal peptide of domain 1 allows heptamerization, which is supported mainly by domain 3. Domain 2 is involved in membrane insertion, which occurs at the low pH of endosomes and depends on the formation of a beta-barrel structure based on the structural rearrangement of domain 2. Domain 4 appears to be involved in receptor binding. This domain of CST has similarity with the binding components of iota-like binary actin ADP-ribosylating toxins but is unrelated to the respective domains of C2 toxin or PA.

Recently, recombinant clostridial proteins (e.g., clostridial protein toxins) have been successfully expressed in *Bacillus megaterium*. This system was extremely instrumental for expression of members of the family of large glucosylating clostridial toxins, which possess a mass of 250 to 308 kDa and are notoriously poorly expressed in *Escherichia coli* (22). We previously employed this procedure for recombinant production of the clostridial toxins *C. sordellii* lethal toxin, *C. novyi* alpha-toxin, and *C. difficile* transferase (11, 17, 20). The current study describes the usefulness of the *B. megaterium* expression system for recombinant production of CSTa and CSTb. The availability of both toxin components enabled us to elucidate the target cell receptor of CST. We recently identified the lipolysis-stimulated lipoprotein receptor (LSR) as the target molecule for binding and internalization of the clostridial binary toxins *C. difficile* transferase and *C. perfringens* iota toxin (16). Here, we demonstrate that *C. spiroforme* toxin also uses the LSR for target cell entry.
at 16°C (CSTa) or at 29°C (CSTb). Cells were then harvested by centrifugation, and the pellet was suspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 20 mM imidazole, 10% glycerol, and 0.5 mM EDTA) supplemented with Complete protease inhibitor mix (Roche, Mannheim, Germany). After lysis of bacteria using a Microfluidizer (Microfluidics, Newton, MA) at 15,000 lb/in², cell debris was removed by centrifugation (21,000 × g, 15 min, 4°C). Cytosolic extracts (30 μg cell total protein) were incubated with 1 μg CSTa, 1 μg CSTb per 1 million cells in 0.5 ml PBS prior to addition of 10 μg activated, Alexa488-labeled CSTb per 100,000 cells in 1 ml PBS. After incubation for 15 min on ice, cells were washed twice with PBS and subjected to fluorescence-activated cell sorter (FACS) analysis using the BD FACSCalibur platform. Cell surface-bound fluorescence was detected with an argon-ion laser (488 nm) and the 530-nm-band-pass filter (FITC). Competition experiments were performed by preincubating cells for 5 min on ice with 50 μg activated, unlabelled CSTb or 50 μg bovine serum albumin (BSA) (including trypsin and trypsin inhibitor, as used for the activation of CSTb) per 1 million cells in 0.5 ml PBS prior to addition of 1 μg activated, Alexa488-labeled CSTb. Following two washings of cells with PBS, cell surface-bound fluorescence was detected by FACS analysis.

Other toxins used in this study. The components of C. botulinum C2 toxin (C2I and C2II) and C. difficile transferase (CDTa and CDTb) were purified from Escherichia coli and Bacillus megaterium, respectively, as described elsewhere, and binding components (C2II and CDTb) were activated by protease treatment (3, 5, 20).

RESULTS
Cloning, expression, and cytotoxic effect of recombinant C. s. Toxin produced in B. megaterium. The genes encoding either the enzyme component (CSTa) or the binding component (CSTb) of C. s. toxin were amplified by PCR using genomic DNA from strain CS246 as a template (Fig. 1a). PCR products were then cloned into the B. megaterium expression vector pHS1522 to generate C-terminally 6×His-tagged constructs, and plasmids were transformed separately into B. megaterium protoplasts. Tetracycline-resistant clones were grown in liquid LB medium, and protein expression was induced by the addition of xylose. The His-tagged proteins were then purified from crude Lasers, respectively. Images were processed with MetaMorph imaging software (Universal Imaging). For immunostaining of LSR, polyclonal rabbit anti-LSR antibody (sc-133765; Santa Cruz Biotechnology) and secondary Alexa 568- or Alexa 488-conjugated anti-rabbit antibodies (Invitrogen, Karlsruhe, Germany) were used.

Crystal violet staining. After CST treatment, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 4°C, followed by staining of cells with 0.5% crystal violet solution (in 70% ethanol) for 30 min at 4°C. Excess staining solution was removed by washing the cells three times with H2O. Stained cells were air dried at room temperature before imaging.

FACS analysis. Cells were detached from culture dishes by incubation with 10 mM EDTA in PBS and without trypsin to preserve cell surface proteins from degradation. The cell suspension was washed twice with PBS and kept on ice prior to addition of 10 μg activated, Alexa488-labeled CSTb per 100,000 cells in 1 ml PBS. After incubation for 15 min on ice, cells were washed twice with PBS and subjected to fluorescence-activated cell sorter (FACS) analysis using the BD FACS Calibur platform. Cell surface-bound fluorescence was detected with an argon-ion laser (488 nm) and the 530-nm-band-pass filter (FITC). Competition experiments were performed by preincubating cells for 5 min on ice with 50 μg activated, unlabelled CSTb or 50 μg bovine serum albumin (BSA) (including trypsin and trypsin inhibitor, as used for the activation of CSTb) per 1 million cells in 0.5 ml PBS prior to addition of 1 μg activated, Alexa488-labeled CSTb. Following two washings of cells with PBS, cell surface-bound fluorescence was detected by FACS analysis.

Microscopy. Cell morphology was analyzed in wells directly by using an inverted microscope (Axiovert 25; Carl Zeiss). Actin and nuclear stainings were performed with tetramethyl rhodamine isocyanate (TRITC)-conjugated phalloidin and ProLong Gold antifade reagent containing 4,6-diamidino-2-phenylindole (DAPI), respectively, and analysis was done by fluorescence microscopy (Axioptef; Carl Zeiss). Confocal fluorescence microscopy was performed with an inverted microscope (Axiovert 200 M; Carl Zeiss) equipped with a 40× Plan-Apochromat objective, a spinning-disk head (Yokogawa) with emission filters, and a CoolSNAP-HQ2 digital camera (Roper Scientific). Alexa488/fluorescein isothiocyanate (FITC) signals and Alexa568/TRITC signals were detected by illuminating the specimen with 488-nm- and 561-nm-wavelength solid-state
FIG 2 Cytotoxicity analysis of recombinant C. spiroforme toxin. (a) CST-induced cell rounding of CaCo-2 cells. Confluent CaCo-2 cell monolayers were incubated overnight with CSTa and/or CSTb (each at 10 nM) or were left untreated (w/o toxin) prior to microscopic analysis of cell morphology. (b) CST-induced collapse of the actin cytoskeleton. CaCo-2 cells were incubated with CSTa and CSTb (CSTa/b, each at 10 nM) for 8 h or were left untreated (w/o toxin) prior to fixation of cells and staining with TRITC-phalloidin (red, actin) and DAPI (blue, nuclei). Cells were then analyzed by fluorescence microscopy (using the 100× objective). (c) ADP-ribosylation of cellular actin with CST. CaCo-2 cells were treated for the indicated time intervals with CSTa and CSTb (each at 10 or 20 nM) or with CSTb only (20 nM). Subsequently, cells were lysed and cytosolic extracts subjected to in vitro ADP-ribosylation with C2I (modifies actin at the same amino acid residue as CST) and 32P-NAD as a cosubstrate. C2I-modified actin was visualized by SDS-PAGE and autoradiography.

bacterial lysates by nickel affinity chromatography. The purity of a representative preparation of recombinant C. spiroforme toxin is illustrated in Fig. 1b (CSTa) and Fig. 1c (CSTb, left lane, – trypsin). CSTb was further activated by incubation with trypsin, which is required to achieve full toxicity of CST toxin (Fig. 1c, right lane, + trypsin) (18). Typically, approximately 0.05 mg CSTa and 2 mg CSTb were purified from 1 liter of bacterial culture.

In order to prove that recombinant CSTa and CSTb (trypsin activated) are correctly folded, we added both components in combination or separately (each at 10 nM) to cultured CaCo-2 cells. Only cells that were treated with both components of the recombinant toxin displayed severe morphological alterations (cell rounding) after overnight incubation (Fig. 2a), implying that a binary toxin with full biological activity was formed by both purified CST components. Our next aim was to prove that recombinant toxin-induced cell rounding of CaCo-2 cells is due to the collapse of the actin cytoskeleton. Therefore, we intoxicated CaCo-2 cells for 8 h with recombinant CST (10 nM), fixed the cells, and visualized the actin cytoskeleton by TRITC-phalloidin staining followed by fluorescence microscopy. Depolymerization of actin filaments was obvious only in cells that were treated with toxin (Fig. 2b). Finally, we aimed to substantiate that recombinant CST modifies cellular actin by ADP-ribosylation. This was confirmed by probing the ADP-ribosylation state of actin in lysates of CST-intoxicated H1-HeLa and H1-HeLa (+ LSR) cells. The amount of actin in toxin-treated cells that is available for in vitro ADP-ribosylation (carried out with the enzyme component of C. botulinum C2 toxin (C2I)) decreased over time only in FLAG-LSR-transduced H1-HeLa cells (H1-HeLa (+ LSR)). Nontransduced H1-HeLa cells were resistant to toxin addition.

LSR-dependent intoxication was further confirmed by probing the ADP-ribosylation state of actin in lysates of CDT-intoxicated H1-HeLa and H1-HeLa (+ LSR) cells. The amount of actin in toxin-treated cells that is available for in vitro actin-ADP-ribosylation was observed exclusively in FLAG-LSR-transduced H1-HeLa cells and not in nontransduced cells (Fig. 3c).

To further confirm that LSR acts as a cell surface receptor and mediates binding of CST to the cell membrane, Alexa-coupled CSTb was incubated with suspensions of nontransduced and LSR-transduced H1-HeLa cells on ice to prevent endocytosis, and cell surface-bound fluorescence was then detected by FACS analysis. H1-HeLa cells expressing LSR protein showed considerably increased binding of fluorescence-labeled CSTb compared with native H1-HeLa cells (Fig. 3d). To substantiate the specificity of CSTb binding to LSR-expressing H1-HeLa cells, we performed a competition assay, where cells were preincubated with a 50-fold excess of activated, unlabeled CSTb or with the same amount of BSA (including trypsin and trypsin inhibitor). Importantly, binding of
Alexa-labeled CSTb was specifically reduced on cells that were preincubated with unlabeled CSTb (Fig. 3e). To validate that LSR binding mediates cell entry of CST, we performed colocalization studies with CaCo-2 cells. For this purpose, Alexa-coupled CSTb was added together with CSTa to CaCo-2 cells that were kept at 4°C to allow binding of the toxin to its receptor at the cell surface. Following an incubation period of 20 min at 37°C to induce endocytic uptake of the receptor/toxin complexes, cells were fixed, permeabilized, and subjected to immunostaining of endogenous LSR. Comparison of fluorescent signals of CST with signals of endogenous, immunolabeled LSR by confocal fluorescence microscopy confirmed colocalization of both proteins in endocytic vesicles (Fig. 4, CST). A similar colocalization pattern was observed when Alexa-labeled CDT was used as a positive control (Fig. 4, CDT). In contrast, endocytic vesicles containing Alexa-labeled C. botulinum C2 toxin, a more distantly CST-related actin-ADP-ribosylating toxin that enters cells via an asparagine-linked complex and hybrid carbohydrate structures (9), did not colocalize with LSR-containing endocytic compartments (Fig. 4, C2). Thus, our collective data demonstrate that CST is an additional member of the iota-like family of binary actin-ADP-ribosylating toxins that targets LSR for host cell entry.

**DISCUSSION**

Our study describes the recombinant production of the enzyme component (CSTa) and the binding component (CSTb) of the *C. spiroforme* toxin in the *Bacillus megaterium* expression system. The methodology was adapted from protocols that were previously established for the recombinant production of various other clostridial toxins (11, 17, 20, 22). The *B. megaterium* expression system often offers an advantage over conventional bacterial expression systems (e.g., *Escherichia coli*) in producing clostridial toxins, obviously due to a similar codon usage of the related genera *Bacillus* and *Clostridium*. However, we noticed that the efficiency of producing CSTa in *B. megaterium* was less than that for CSTb. A significant amount of CSTa protein was found in the insoluble fraction after lysis of bacteria and centrifugation of cell debris. Because we produced both CST components as C-terminally His-tagged proteins, we speculate that the His tag might negatively influence the stability of CSTa, resulting in increased aggregation and partially in the formation of inclusion bodies. As we show that the combined addition of both recombinantly produced CST components to cultured CaCo-2 cells led to the ADP-ribosylation of cellular actin, disruption of the actin cytoskeleton, and changes in cell morphology (cell rounding), our findings in-
dicate that recombinant CST produced in B. megaterium constitutes a fully functional actin-ADP-ribosylating toxin. Since the separate addition of the purified CST components did not lead to intoxication characteristics in CaCo-2 cells, possible toxic contaminants that might be copurified from the expression host can be excluded.

Clostridial actin-ADP-ribosylating toxins are subdivided into two families on the basis of their biological activities and immunological relatedness. One family corresponds to the C2 toxins from C. botulinum (C2 toxin family), and the other family (iota-like toxin family) includes the eponym C. perfringens iota toxin, as well as CST and C. difficile transferase (CDT) (8, 14, 15). Interestingly, binding and enzymatic components are mutually interchangeable among the members of the iota-like toxin family, to form fully active toxins, but not between the C2 toxin and the iota-like toxin group (8, 18). Further interfamily comparisons revealed that C2 toxin recognizes unique cell surface receptors for internalization (9, 10). These findings are in agreement with a recent study from our laboratory that identifies the cellular receptor of CDT and iota toxin, namely, the lipolysis-stimulated lipoprotein receptor (LSR), which does not mediate cell entry of C2 toxin (16). So far, it has not been investigated whether additional members of the iota toxin family, such as, for example, CST, also use the LSR for entry into host cells. The current study clearly provides evidence that the LSR also represents the target molecule for binding and internalization of CST. Our reasoning was based on H1-HeLa cells that do not produce LSR, thereby exhibiting increased resistance to the CST-related toxins CDT and iota toxin that can be circumvented by ectopic expression of LSR (16). In the current study, intoxication of H1-HeLa cells with CST and binding of the toxin to these cells was strictly dependent on the presence of LSR protein. The role of LSR in the cellular uptake of CST could be substantiated by using an additional cell line (CaCo-2) for colocalization studies, revealing overlapping fluorescence signals of Alexa-labeled toxin and immunolabeled LSR protein in endocytic vesicles.

LSR is a type I single-pass transmembrane protein of the cell surface, featuring an Ig-like V-type domain in the N-terminal, extracellular portion of the protein. The protein is expressed mainly in the liver but also in the intestine and in various other tissues (13, 24). Previous studies suggest that the LSR functions in the cellular uptake of triglyceride-rich, low-density lipoproteins for clearance of chylomicron remnants from blood circulation (4, 23). Recently, an additional role in the organization of tricellular junctions that are involved in epithelial barrier function has been attributed to the LSR protein (12). The finding that disruption of

FIG 4 CST colocalizes with LSR in endocytic vesicles of CaCo-2 cells. CaCo-2 cells were incubated with either CSTa/CSTbAlexa 488 (CST), CDTa/CDTbAlexa 568 (CDT), or C2Alexa 566/C2II (C2) for 30 min on ice to allow binding of the toxins to the cell surface. Subsequently, cells were shifted to 37°C to induce endocytic uptake of the receptor/toxin complexes. Following an incubation period of 20 min, cells were fixed, permeabilized, and subjected to immunostaining of endogenous LSR. Localization of CST, CDT, or C2 (green signals in merged images, Alexa) and endogenous LSR (red signals in merged images, anti-LSR) was analyzed by confocal fluorescence microscopy (scale bars, 10 μm). Yellow signals in the merged images (merge) indicate colocalization of Alexa signals with immunostained LSR. mag., magnification of boxed area in the merged image.
the LSR gene causes lethality in mice also reveals an important role of this protein in early steps during development (13). Our study now highlights that pathogenic clostridia producing iota-like toxins have evolved by hijacking the LSR protein as a membrane target for smuggling their toxins into host cells.

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