**Clostridium difficile** Binary Toxin CDT Induces Clustering of the Lipolysis-Stimulated Lipoprotein Receptor into Lipid Rafts

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ABSTRACT: *Clostridium difficile* is the leading cause of antibiotics-associated diarrhea and pseudomembranous colitis. Hypervirulent *C. difficile* strains produce the binary toxin ADP-ribosylating toxin CDT (C. difficile transferase), in addition to the Rho-glucosylating toxins A and B. We recently identified the lipolysis-stimulated lipoprotein receptor (LSR) as the host receptor that mediates uptake of CDT into target cells. Here we investigated in H1-HeLa cells, which ectopically express LSR, the influence of CDT on the plasma membrane distribution of the receptor. We found by fluorescence microscopy that the binding component of CDT (CDTb) induces clustering of LSR into subcompartments of the plasma membrane. Detergent extraction of cells treated with CDTb, followed by sucrose gradient fractionation, uncovered accumulation of LSR in detergent-resistant membranes (DRMs) that contained typical marker proteins of lipid rafts. Membrane cholesterol depletion with methyl-β-cyclodextrin inhibited the association of LSR with DRMs upon addition of CDTb. The receptor-binding domain of CDTb also triggered LSR clustering into DRMs. CDTb-triggered clustering of LSR into lipid rafts could be confirmed in Caco-2 cells. Our data suggest that CDT forces its receptor to cluster into lipid rafts and that oligomerization of the B component might enhance but is not essential for this process.

**IMPORTANCE** C. difficile binary toxin CDT is a member of the iota-like, actin ADP-ribosylating toxin family. The mechanism that mediates endocytic uptake of these toxins still remains elusive. Previous studies highlighted the importance of lipid rafts for oligomerization of the binding component of these toxins and for cell entry. Recently, the host cell receptor for this toxin family, namely, the lipolysis-stimulated lipoprotein receptor (LSR), has been identified. Our study now demonstrates that the binding component of CDT (CDTb) induces clustering of LSR into lipid rafts. Importantly, LSR clustering is efficiently induced also by the receptor-binding domain of CDTb, suggesting that oligomerization of the B component of CDT is not the main trigger of this process. The current work extends our knowledge on the cooperative play between iota-like toxins and their receptor.
receptor with CDT for cell entry (12). LSR is a type I single-pass transmembrane protein expressed mainly in the liver but also in the intestine and various other tissues (13, 14). Early reports identified LSR as a hepatic receptor for triglyceride-rich lipoproteins (13, 15). Recently, an additional role of LSR in the organization of tricellular junctions that are involved in epithelial barrier function has been described (16).

Less is known about the mechanism underlying the LSR-mediated uptake of CDT into host cells. In the current study, we aimed to characterize the molecular processes at the plasma membrane in more detail, upon binding of CDTb to LSR. Our principal finding is that CDTb triggers clustering of LSR into detergent-resistant membrane (DRM) subcompartments that contain typical marker proteins of lipid rafts. Moreover, we observed that the receptor-binding domain (RBD) of CDTb also induces clustering of LSR into DRMs, assuming that the oligomerization of CDTb is not essentially involved in this process. Previous reports that highlighted the importance of lipid rafts for cell entry of iota-like toxins are now supported and extended by studies focusing on the toxin’s receptor, which was not known until recently.

RESULTS

CDTb colocalizes with LSR at distinct foci at the cell periphery.

It has been shown in previous reports that oligomers of the B component of iota toxin (Ib) localize to lipid rafts prior to binding of the A component (17, 18). At that time, however, the host receptor of iota-like toxins was unknown and it remained unclear whether the LSR is involved in the association of the iota-like toxins with lipid rafts. We therefore attempted to visualize by fluorescence microscopy the cell surface distribution of the B component of CDT (CDTb) on H1-HeLa cells that ectopically express LSR protein [H1-HeLa (+LSR) cells]. A dot-like distribution of DyLight488-labeled CDTb (CDTbDL488) on the cell surface was observed, suggesting an accumulation of the protein in membrane subcompartments. Immunolabeling of LSR uncovered overlapping fluorescent signals of LSR and CDTb in membrane clusters at the cell periphery (Fig. 1). Notably, a fraction of LSR was immunolabeled in intracellular vesicles and, as expected, these fluorescent signals of LSR do not colocalize with cell surface-associated CDTb.

CDTb forces clustering of LSR into membrane microdomains of the cell surface. To address the issue of whether CDTb accumulates into already preformed LSR membrane clusters, we expressed an LSR-enhanced green fluorescent protein (LSR-EGFP) fusion protein in H1-HeLa cells that allows a direct investigation by time-lapse microscopy of the dynamics of the membrane distribution of LSR upon binding of CDTb. Interestingly, in the absence of CDTb, LSR-EGFP was evenly distributed at the plasma membrane, and within 30 min after CDTb addition, fluorescent signals of LSR accumulated at distinct foci at the cell periphery (Fig. 2, lower panel). Dot-like redistribution of LSR-EGFP did not occur when cells were incubated at 10°C, suggesting that a certain degree of membrane fluidity and/or intracellular signaling events are required for the movement of LSR into subcompartments of the plasma membrane (Fig. 2, upper panel). These microscopic observations suggested that LSR clustering into membrane microdomains is induced by CDTb.

CDTb induces clustering of LSR into detergent-resistant membranes. Membrane microdomains, such as lipid rafts, are commonly defined as detergent-resistant membranes (DRMs), due to the fact that they contain a high percentage of cholesterol and cannot be solubilized with nonionic detergents (e.g., Triton X-100) (19). We utilized this biochemical feature of DRMs to determine the submembranous localization of LSR before and after incubation of H1-HeLa (+LSR) cells with CDTb. Strikingly, with increasing concentrations of CDTb, LSR accumulated predominantly in the insoluble fraction of Triton X-100-solubilized cell membranes. Concomitantly, LSR signals decreased in the Triton X-100-soluble membrane fractions upon addition of CDTb (Fig. 3A). In line with our microscopic observations shown in Fig. 2, CDTb-dependent accumulation of LSR in the DRM fraction was time dependent (Fig. 3B) and temperature dependent (Fig. 3C), again indicating that LSR clustering is induced by CDTb.

Sucrose gradient fractionation uncovers colocalization of LSR with lipid raft marker proteins. We next aimed to biochemically characterize the DRM-associated LSR and performed discontinuous sucrose gradient centrifugation with Triton X-100-solubilized H1-HeLa (+LSR) cells. The buoyant density of the insoluble membrane fraction leads to its flotation at the top of the gradient, whereas the soluble membrane fraction is typically found at the bottom part. Importantly, preincubation of H1-HeLa (+LSR) cells with CDTb led to an increased accumulation of LSR into the top fractions of the sucrose gradient that also contained marker proteins of lipid rafts, such as flotillin-1 and caveolin-2 (Fig. 4A). ATP1A1 (Na+/K+ ATPase), a marker protein for the soluble membrane fraction, was found exclusively in the bottom fraction of the sucrose gradient (Fig. 4A). To substantiate the find-
ing that the presence of CDTb leads to partitioning of LSR into lipid rafts, we employed the lipid raft-destabilizing agent methyl-β-cyclodextrin (mβCD). To this end, H1-HeLa (+LSR) cells were incubated with increasing concentrations of mβCD prior to addition of CDTb to induce LSR clustering. As expected, CDTb-induced LSR clustering into DRMs was inhibited with increased destabilization of lipid rafts by mβCD (Fig. 4B). Stable LSR levels were detected in whole-cell lysates of H1-HeLa (+LSR) cells after treatment with increasing concentrations of mβCD, thereby excluding the possibility that mβCD treatment leads to extraction of LSR from membranes (Fig. 4C).

The RBD of CDTb independently binds to LSR-expressing cells. We next investigated whether the interaction of the receptor-binding domain (RBD) of CDTb is sufficient for triggering LSR clustering into lipid rafts. Marvaud and colleagues have shown previously that the receptor-binding domain of the iota toxin is represented by the C-terminal end of the B component (20). Accordingly, we recombinantly produced the putative receptor-binding domain (RBD) of CDTb (amino acids 677 to 876 of the precursor form) as a glutathione S-transferase (GST) fusion protein (designated RBD in this study). We first aimed to directly prove specific binding of RBD to LSR-expressing cells by fluorescence-activated cell sorter (FACS) analysis. To this end, DyLight488-labeled RBD (RBD_DL488) or CDTb (CDTb_DL488), respectively, was incubated with cell suspensions of either naive H1-HeLa cells (Fig. 5A) or H1-HeLa (+LSR) cells (Fig. 5B) prior to FACS analysis. As expected, RBD_DL488 as well as the control CDTb_DL488 did not bind to naive HeLa cells (Fig. 5A). However, both DL488-labeled proteins bound and increased the fluorescence of H1-HeLa cells that express the LSR protein (Fig. 5B). Moreover, when they were added together, the excess volume of nonlabeled RBD competitively inhibited the binding of CDTb_DL488 to H1-HeLa (+LSR) cells (Fig. 5B). These results, indicating that the recombinantly purified RBD is functional and capable of binding.
LSR independently, were confirmed with a human epithelial colorectal adenocarcinoma cell line (Caco-2) (Fig. 5C).

**RBD efficiently induces clustering of LSR into DRMs.** To then test whether the receptor-binding domain of CDTb is sufficient for triggering LSR clustering into the DRM fraction, RBD was added to H1-HeLa (+LSR) cells and the amount of LSR in the DRM fraction was quantified after immunoblotting. In parallel, CDTb and the precursor form of CDTb (pCDTb), which is not able to oligomerize, were introduced in this experiment for comparison. Interestingly, both pCDTb and RBD were capable of inducing LSR clustering into DRMs; however, the resultant induction was slightly less efficient than that seen with CDTb (Fig. 6A).

**Verification of CDTb-induced DRM association of LSR in Caco-2 cells.** To verify our results with an additional cell line that expresses LSR endogenously, we repeated the experiment represented by Fig. 6A with Caco-2 cells. Strikingly, in this cell line also, CDTb-, pCDTb-, and RBD-induced clustering of LSR into DRMs could be confirmed (Fig. 6B). In addition, we were able to detect RBD in DRMs of Caco-2 cells, indicating that RBD binds to LSR and clusters with the receptor into lipid rafts (Fig. 6C).

Taken together, our data substantiate the hypothesis that CDT forces its receptor to localize into lipid rafts and that oligomerization of the B component of the binary toxin CDT is not essentially involved in this process.

**DISCUSSION**

We recently identified the lipolysis-stimulated lipoprotein receptor (LSR) as the host cell receptor for CDT and, moreover, showed
that all clostridial iota-like binary toxins share LSR for cell entry (11, 12). A series of previous studies, mainly performed with C. perfringens iota toxin, suggested the model that the B components of iota-like toxins bind to the cell surface receptor, are then activated by host proteases, and subsequently oligomerize to form a heptameric prepore. Alternatively, proteolytically activated B components can form prepores in solution that subsequently bind to the cell surface receptor. Upon binding of the A component to the receptor-bound prepore, endocytic uptake is initiated (8, 21–23).

Previously, it was shown by Nagahama et al. and Hale et al. that iota toxin prepores are essentially associated with cholesterol-rich, detergent-resistant membrane microdomains (lipid rafts) (17, 18). Since membrane cholesterol depletion was found to prevent oligomerization of iota b and to inhibit intoxication of cells with iota toxin, this finding suggests that iota-like toxins exploit lipid rafts for cell entry (17, 18). It is still unclear, which endocytic process mediates the uptake of iota-like toxins. However, Gilbert and colleagues found that dynamin, but not clathrin, is involved in the cellular uptake of the iota toxin. In addition, they observed colocalization of the iota toxin and the interleukin-2 (IL-2) receptor in endocytic vesicles (24), suggesting that LSR and the IL-2 receptor share similar endocytic routes. Interestingly, the IL-2 receptor has also been associated with partitioning into DRMs prior to endocytic uptake by a clathrin-independent mechanism (25).

To date, it remained unclear whether the association of iota-like toxins with lipid rafts engages also the host receptor of these toxins or whether this step occurs independently. By using the proteolytically activated B component of CDT (CDTb) and transduced H1-HeLa cells that overexpress a defined isoform of the LSR protein, we now show that LSR is involved in the clustering of the B components into lipid rafts. In the absence of CDTb, we find the majority of LSR in detergent-soluble membrane fractions. This finding is in line with an earlier report showing that iota b, which was incubated with cells at 4°C to prevent oligomerization, was predominantly found in the detergent-soluble membrane fractions (17). Our data indicate that accumulation of LSR into detergent-resistant microdomains occurs only after binding of CDTb. This relocalization process did not occur when cells were incubated with CDTb at low temperature. Since oligomerization of the B components of iota-like toxins at the cell membrane is also inhibited at low temperature (22, 23), this finding argues for a connection between LSR clustering and oligomer formation of CDTb. However, we found that the precursor of CDTb (pCDTb), which is not capable of oligomerizing, as well as the stand-alone receptor-binding domain of CDTb was able to trigger clustering of LSR into DRMs. These findings support the model that oligomerization of the B components of iota-like toxins occurs at the membrane and is enhanced by local accumulation of LSR-bound monomers into lipid rafts.

We frequently observed a second band migrating a bit faster than that corresponding to LSR in anti-LSR immunoblots of DRM samples. This band was present only in samples where CDTb was added to induce LSR clustering. Future investigations will be necessary to characterize whether this band represents a cleavage product of LSR protein, we now show that LSR is involved in the clustering of the B components into lipid rafts. In the absence of CDTb, we find the majority of LSR in detergent-soluble membrane fractions. This finding is in line with an earlier report showing that iota b, which was incubated with cells at 4°C to prevent oligomerization, was predominantly found in the detergent-soluble membrane fractions (17). Our data indicate that accumulation of LSR into detergent-resistant microdomains occurs only after binding of CDTb. This relocalization process did not occur when cells were incubated with CDTb at low temperature. Since oligomerization of the B components of iota-like toxins at the cell membrane is also inhibited at low temperature (22, 23), this finding argues for a connection between LSR clustering and oligomer formation of CDTb. However, we found that the precursor of CDTb (pCDTb), which is not capable of oligomerizing, as well as the stand-alone receptor-binding domain of CDTb was able to trigger clustering of LSR into DRMs. These findings support the model that oligomerization of the B components of iota-like toxins occurs at the membrane and is enhanced by local accumulation of LSR-bound monomers into lipid rafts.

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VacA toxin and the protective antigen (PA), the binding component of anthrax toxin, both trigger the accumulation of the respective toxin receptors (RPTPbeta and ATR, respectively) into lipid rafts (31, 32).

Recently, Wigelsworth et al. suggested an important role for CD44 during intoxication of host cells by iota-like toxins (33). Interestingly, CD44 was primarily detected by quantitative profiling of DRMs obtained from Vero cells treated with the B component of the iota toxin (34). It is conceivable that CD44 facilitates clustering of LSR into lipid rafts, which might be an attractive issue for further investigations.

**MATERIALS AND METHODS**

Cultivation of mammalian and bacterial cells. H1-HeLa and H1-HeLa (+LSR) cells (described in reference 11) were incubated at 37°C with 5% CO₂ under humidified conditions and with Dulbecco’s modified Eagle’s medium (DMEM) (12 mM d-glutamine) medium supplemented with 10% fetal bovine serum (FCS), 4 mM penicillin/streptomycin, and 1% nonessential amino acids. *Escherichia coli* strains were grown in LB medium at 37°C.

**Transient expression of LSR-EGFP in H1-HeLa cells.** LSR was amplified by PCR with primers generating a 5’-NheI and a 3’-HindIII restriction site and by using the previously described plasmid pMXs-IRES-Blasticidin/FLAG-LSR as the template (11). The PCR product was then ligated into an NheI/HindIII-opened pEGFP-N1 vector to generate pEGFP-N1/LSR. Polyethyleneimine (PEI) was used for transfection of H1-HeLa cells with pEGFP-N1/LSR. Cells were analyzed 24 to 48 h after transfection.

**Cloning, expression, and purification of the receptor-binding domain of CDTb.** To generate a GST fusion of the receptor-binding domain (RBD) of CDTb (amino acids 677 to 876 of the precursor form), the corresponding DNA sequence (bp 2029 to 2631) was amplified by PCR with oligonucleotides generating a 5’-EcoRI and a 3’-XhoI restriction site and ligated into pGEX-4T3. The plasmid was then transformed in *E. coli* BL21 (DE3). Transformants were grown in LB medium at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.4 to 0.6 was reached prior to addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further incubation for 4 h at 37°C to induce protein expression. Then, cells were harvested by centrifugation (5,000 × g, 20 min, 4°C) and resuspended in lysis buffer (Tris-buffered saline [TBS] [pH 8] supplemented with 10% [wt/vol] glycerol, 10 mM beta-mercaptoethanol, and Complete protease inhibitor cocktail [Roche]). After lysis of the cells by the use of a microfluidizer at 15,000 lb/in², the cell debris was removed by centrifugation (14,000 × g, 30 min, 4°C) and the supernatant was applied to glutathione-Sepharose (Pharmacia), where the fusion protein was retained and eluted with phosphate-buffered saline (PBS) supplemented with 10% glycerol and 15 mM reduced glutathione. Finally, glutathione was removed by the use of PD10 desalting columns (GE Healthcare) pre-equilibrated with PBS-10% glycerol.

**Antibodies.** LSR was detected by using a polyclonal rabbit anti-LSR antibody (clone X-25) (sc-133765; Santa Cruz). Caveolin-2, flotillin-1, and flotillin-2 were detected with mouse monoclonal anti-caveolin-2 (C9992; Sigma-Aldrich), anti-flotillin-1 (F1180; Sigma-Aldrich), and anti-flotillin-2 (F1805; Sigma-Aldrich) antibodies, respectively. Sodium-potassium-transporting ATPase subunit alpha-1 (ATP1A1) was detected with mouse monoclonal anti-ATP1A1 antibody (ab7671; Abcam). GST was detected with goat polyclonal anti-GST antibody (27-4577-01; GE Healthcare).

**Fluorescent dye labeling of proteins.** Purified proteins were coupled to DyLight 488 NHS ester (Thermo Scientific) in phosphate-buffered sa-
line (PBS) following the manufacturer’s instructions. Excess dye was removed with Micro Bio-Spin 6 columns (Bio-Rad Laboratories). The degree of labeling was 0.97 mol dye per mole protein for CDTb and 0.23 mol dye per mole protein for RBD.

**DRM preparation and sucrose gradient fractionation analysis.** H1-HeLa (+LSR) or Caco-2 cells grown in dishes were detached with PBS–10 mM EDTA, washed twice with PBS, and finally resuspended in DMEM–FCS (10%). Typically, 1 × 10^6 to 2 × 10^6 cells in 1 ml DMEM–FCS (10%) were incubated with CDTb for 30 min at 37°C (or as indicated). Cells were then washed again with PBS, resuspended in 500 μl ice-cold detergent buffer (1% [wt/vol] Triton X-100, 25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, Complete protease inhibitor cocktail), and incubated for 60 min at 4°C under conditions of rotation. Nuclei and debris were removed by centrifugation (1,500 × g, 5 min, 4°C) prior to centrifugation of the supernatant for 1 h at 21,100 × g and 4°C to precipitate the detergent-resistant membrane fraction. DRMs were resuspended in Laemmli buffer and boiled for 10 min at 95°C prior to analysis by SDS-PAGE and immunoblotting to detect LSR.

For sucrose gradient fractionation analysis of DRMs, CDTb– or mock-treated suspensions of H1-HeLa (+LSR) cells were solubilized with the detergent buffer mentioned above for 90 min at 4°C under conditions of rotation and then mixed with 60% (wt/vol) sucrose–10 mM Tris-HCl (pH 7.5) to obtain a 40% sucrose solution, placed at the bottom of a centrifuge tube, and overlaid with a 35% and a 15% sucrose–Tris–HCl (pH 7.5) solution. Following centrifugation of the sucrose gradient for 18 h at 174,000 × g and 4°C, fractions were collected from the top to the bottom and analyzed by SDS-PAGE and immunoblotting for the presence of LSR and marker proteins of lipid rafts or nonraft proteins.

To analyze the presence of LSR in Triton-soluble and -insoluble cell membranes, CDTb– or mock-treated suspensions of H1-HeLa (+LSR) cells were sedimented by centrifugation (400 × g, 1 h, 4°C), resuspended in ice-cold detergent buffer (mentioned above), and incubated for 90 min at 4°C under conditions of rotation. The Triton-soluble and -insoluble membrane fractions were separated by centrifugation at 21,100 × g for 20 min at 4°C, complemented with Laemmli buffer, and boiled for 10 min at 95°C prior to detection of LSR signals by SDS-PAGE and immunoblotting.

**Membrane cholesterol depletion with methyl-β-cyclodextrin.** H1-HeLa (+LSR) cell suspensions were obtained by detaching cells with PBS–10 mM EDTA. Following washing of the cells with PBS, cells were resuspended in depletion buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 4 mM KCl, 10 mM glucose). Methyl-β-cyclodextrin (mβCD) was then added at the indicated concentrations followed by incubation for 30 min at 37°C. Subsequently, cells were washed once with DMEM–10% FCS and resuspended again in medium.

**Microscopy.** Confocal fluorescence microscopy was performed with an inverted microscope (Axiovert 200 M; Carl Zeiss) equipped with Plan-Apochromat objectives, a spinning-disc head (Yokogawa) with emission filters, and solid-state laser lines (488 and 561 nm). For time-lapse series, cells were observed at the indicated temperature in a chamber providing a humidified atmosphere (6.5% CO₂ and 9% O₂). Images were collected with a digital camera (CoolSNAP-HQ2; Roper Scientific) and processed with Metamorphic imaging software (Universal Imaging).

**Fluorescence-based flow cytometry.** H1-HeLa, H1-HeLa (+LSR), or Caco-2 cells were detached from culture plates with PBS–10 mM EDTA, washed twice with PBS, and kept on ice prior to addition of the indicated amounts of DyLight488-labeled proteins to 3 × 10^6 cells in 1 ml PBS. For competition studies, nonlabeled proteins were added in excess together with labeled proteins at this stage. Following incubation for 10 min on ice, cells were washed twice with PBS and subjected to fluorescence flow cytometry using the BD FACSCalibur platform. Cell surface-bound fluorescence was detected with an argon-ion laser (488 nm) and a 530-nm-band filter (FITC).

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