COMMENTARY

Membrane deformation by the cholera toxin beta subunit requires more than one binding site

Raluca Groza^a and Helge Ewers^{a,1}

Cholera is an acute diarrheal disease caused by the bacterium *Vibrio cholerae* that still results in 20,000 to 140,000 deaths per year worldwide according to the World Health Organization. Once the bacterium reaches the small intestine after ingestion, it hangs on to the intestinal mucus and starts producing a proteinaeous multimeric toxin consisting of one catalytic A subunit and a pentamer of glycolipid-binding B subunits. This AB₅ organization is shared by a family of bacterial toxins that includes the toxins of *Bordetella pertussis*, pathogenic strains of *Escherichia coli*, and *Shigella dysenteriae*. The mechanism of toxicity relies on the delivery of the catalytic A subunit to the cell via endocytosis mediated by the B subunit. This homopentameric B subunit itself is assembled into a pentagon shape that binds to the plasma membrane and carries the A subunit into cells, and the biophysical basis of cellular internalization of the cholera toxin is the topic of Kabbani et al. (1) in PNAS. Interestingly and abundantly exploited in the investigation of endocytic mechanisms, the B subunits of several AB₅ toxins alone are capable of being endocytosed by themselves. They do so by binding to glycolipids in the plasma membrane in a pentameric arrangement of binding sites, which are spaced 3 nm apart. Such a pentavalent 3-nm spacing of binding sites to glycolipids is, interestingly, shared by polyomaviridae such as SV40 and the murine polyomavirus (2). Glycolipid-binding AB₅ toxins and glycolipid-binding polyomaviridae can deform membranes in vitro and in energy-depleted cells (3, 4) and are internalized by clathrin-independent endocytosis (3, 5, 6). The unifying principle of this so far incompletely understood uptake pathway seems to be the reliance of extracellular ligands on binding to several tightly clustered carbohydrate moieties on small (lipidic) receptors and the induction of membrane curvature in membranes that can lead to internalization (3). The finding that not only viruses and bacterial toxins but also endogenous sugar-binding ligands (lectins) employ glycolipid-dependent membrane deformation for endocytosis lead to the glycolipid–lectin hypothesis of clathrin-independent

endocytosis (7). Of the molecules that are endocytosed in this way, the cholera toxin beta subunit (CTxB) together with its receptor, the ganglioside GM1, are by far the best-studied system. CTxB is a marker for glycolipid clustering-mediated phase separation in vitro and in cell membranes (8, 9), and its interaction with GM1 in model membranes has elucidated our understanding of transbilayer coupling (10) and multivalent binding (11).

The tight multivalent clustering of GM1 into a 3-nm-side-length pentagon seems mechanistically important, since not all forms of GM1 cross-linking lead to membrane separation or deformation. The cross-linking of GM1 via antibodies results in a far less tight cross-linking on the nanoscopic scale and does not lead to phase separation or membrane deformation (4, 12). Unlike CTxB, endocytosed anti-GM1 antibodies do not become transported to the Golgi apparatus but are rather recycled back to the plasma membrane (13, 14), suggesting an important role of multivalent clustering of GM1 by CTxB in the endocytosis and intracellular transport of this agent. To investigate the role of multivalent binding of CTxB in endocytosis and toxicity, an important tool has been developed: recombinant CTxB with a controlled number of active binding sites in the pentamer, from zero to five. This can be achieved by assembling the pentamer in vitro from recombinantly expressed mutant B subunits that cannot bind to GM1 together with wild-type (wt) subunits at controlled molar ratio. In this way, it could be shown that monovalent but otherwise still pentameric CTxB exhibits greatly reduced toxicity when coupled to its toxic A subunit (15, 16).

So how many GM1 glycolipids must be cross-linked for effective membrane deformation? Could one be sufficient? Clearly, multivalent ligands such as CTxB or SV40 gather several GM1 receptors (10, 16, 17), but the glycolipid binding pulls both CTxB and SV40 very close to the membrane, possibly leading to deformation of the membrane by intrusion into the membrane–water interface, and this may play a role in Shiga toxin membrane deformation (18).

^aInstitute of Chemistry and Biochemistry, Department of Biology, Chemistry, and Pharmacy, Freie Universität Berlin, 14195 Berlin, Germany

Author contributions: R.G. and H.E. wrote the paper.

The authors declare no competing interest.

Published under the [PNAS license](#).

See companion article, “Structured clustering of the glycosphingolipid GM1 is required for membrane curvature induced by cholera toxin,” [10.1073/pnas.2001119117](#).

¹To whom correspondence may be addressed. Email: helge.ewers@fu-berlin.de.

First published July 8, 2020.

Fig. 1. The structured cross-linking of several glycosphingolipids is required for CTxB-induced membrane deformation. Shown is a schematic drawing of a membrane bilayer containing the ganglioside GM1 (orange). Areas that exhibit positive or negative curvature as seen from the perspective of the toxin are marked by “+” and “-” signs, respectively. CTxB subunits capable of GM1 binding are shown in green. (A) Monovalent mutant of CTxB binds to one GM1 glycolipid receptor and does not cluster or generate membrane curvature. (B) The wt CTxB can bind up to five copies of the GM1 receptor and induces the formation of negative curvature into the membrane, subsequently resulting in the formation of a dome whose size is directly influenced by the stoichiometry of CTxB binding to GM1.

In PNAS, Kabbani et al. (1) use a powerful assay for nanoscale membrane deformation based on a combination of single-molecule localization microscopy of lipids (19) with fluorescence polarization microscopy they developed earlier (20). This assay allowed them, at the time, to ask whether they could detect membrane deformation by CTxB binding to GM1 in supported membrane bilayers via polarized emission from lipid dyes in the membrane bilayer, that would exhibit a different polarization angle in negatively curved membrane. They found that, indeed, the addition of wt CTxB to GM1-containing supported membrane bilayer patches induces the formation of 50- to 100-nm-diameter membrane protrusions within minutes (21). Similarly, when forming supported membrane bilayers above small glass beads, they could demonstrate that CTxB is enriched at the region of negative curvature at the perimeter of such membrane buds, consistent with earlier observations of membrane deformation by CTxB (4) and simulations on the induction of negative membrane curvature and membrane invaginations by CTxB binding (22). These *in silico* studies suggested that the protruding alpha helices in the center of the CTxB pentamers might push the membrane down while the binding sites for GM1, that are more to the sides of the helices, might pull the membrane upward, leading to curvature (22). Lateral interaction of toxins via the Kasimir effect would then lead to large-scale invaginations (18). What was missing from this picture was the connection of the process of membrane deformation to effective multivalent binding by CTxB. In the present work, now, the authors combine their convincingly established imaging system with the use of monovalent CTxB to ask whether multivalent nanoscale clustering of GM1 is required for membrane deformation through CTxB. They could show that the number of available receptors controls the size of membrane deformations, with membrane deformations vanishing, when the theoretical number per CTxB approached single receptors. Furthermore, the observed membrane deformation was

independent of acyl-side chain composition of the glycolipid GM1, ruling out a phase separation-mediated effect. When the authors then used a monovalent, mutant CTxB (mCTxB), they found that it was not capable of membrane deformation, even when further cross-linked by antibodies (Fig. 1). Importantly, they found that their assay also allowed them to image CTxB-induced membrane deformation in live cells, and they could demonstrate that mCTxB did not induce the formation of negative curvature patches in live cell membranes either.

The work presented here (1) delivers important further insight into the mechanism of membrane deformation in artificial membrane systems and cellular membranes as induced by clustering of glycolipids. It effectively rules out that mere attachment of the CTxB to membranes via a single binding site is sufficient for membrane deformation, at least on supported membrane bilayers and on the plasma membrane of live cells. It will be important to investigate, in future work, whether the assay developed by the authors is effective for other structures and is compatible with the temporal resolution required to investigate the dynamic formation of membrane invaginations after multivalent glycolipid binding and in endocytosis in general. If this were the case, this assay will likely yield important insight into nanoscopic membrane deformation processes beyond clathrin-independent endocytosis.

However, the work on the CTxB–GM1 interaction in cellular plasma membrane is not finished with this work (1). Important questions remain. Monovalent CTxB is still endocytosed (16) and was even found to localize to plasma membrane-associated tubular membrane invaginations in cells (23). Monovalent CTxB may, in these cases, merely exploit preexisting endocytic and tubular structures, or it may not. Further studies are required to quantify and mechanistically separate the exact contribution of the membrane deformation capabilities of CTxB in its endocytosis. The assay presented by the authors will provide an important capability toward this aim.

- 1 A. M. Kabbani, K. Raghunathan, W. I. Lencer, A. K. Kenworthy, C. V. Kelly, Structured clustering of the glycosphingolipid GM1 is required for membrane curvature induced by cholera toxin. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 14978–14986 (2020).
- 2 U. Neu, T. Stehle, W. J. Atwood, The Polyomaviridae: Contributions of virus structure to our understanding of virus receptors and infectious entry. *Virology* **384**, 389–399 (2009).
- 3 W. Römer et al., Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature* **450**, 670–675 (2007).
- 4 H. Ewers et al., GM1 structure determines SV40-induced membrane invagination and infection. *Nat. Cell Biol.* **12**, 11–18 (2010).
- 5 H. Ewers, A. Helenius, Lipid-mediated endocytosis. *Cold Spring Harb. Perspect. Biol.* **3**, a004721 (2011).

- 6 D. J.-F. Chinnapen, H. Chinnapen, D. Saslowsky, W. I. Lencer, Rafting with cholera toxin: Endocytosis and trafficking from plasma membrane to ER. *FEMS Microbiol. Lett.* **266**, 129–137 (2007).
- 7 L. Johannes, C. Wunder, M. Shafaq-Zadah, Glycolipids and lectins in endocytic uptake processes. *J. Mol. Biol.* **428**, 4792–4818 (2016).
- 8 A. T. Hammond et al., Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 6320–6325 (2005).
- 9 D. Lingwood, J. Ries, P. Schwille, K. Simons, Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 10005–10010 (2008).
- 10 K. M. Spillane et al., High-speed single-particle tracking of GM1 in model membranes reveals anomalous diffusion due to interleaflet coupling and molecular pinning. *Nano Lett.* **14**, 5390–5397 (2014).
- 11 N. Parveen, S. Block, V. P. Zhdanov, G. E. Rydell, F. Höök, Detachment of membrane bound virions by competitive ligand binding induced receptor depletion. *Langmuir* **33**, 4049–4056 (2017).
- 12 K. Raghunathan et al., Glycolipid crosslinking is required for cholera toxin to partition into and stabilize ordered domains. *Biophys. J.* **111**, 2547–2550 (2016).
- 13 W. Möbius, V. Herzog, K. Sandhoff, G. Schwarzmann, Gangliosides are transported from the plasma membrane to intralysosomal membranes as revealed by immuno-electron microscopy. *Biosci. Rep.* **19**, 307–316 (1999).
- 14 F. M. Ruggiero, A. A. Vilcaes, N. Yuki, J. L. Daniotti, Membrane binding, endocytic trafficking and intracellular fate of high-affinity antibodies to gangliosides GD1a and GM1. *Biochim. Biophys. Acta Biomembr.* **1859**, 80–93 (2017).
- 15 M. J. De Wolf, W. S. Dierick, Regeneration of active receptor recognition domains on the B subunit of cholera toxin by formation of hybrids from chemically inactivated derivatives. *Biochim. Biophys. Acta* **1223**, 285–295 (1994).
- 16 M. G. Jobling, Z. Yang, W. R. Kam, W. I. Lencer, R. K. Holmes, A single native ganglioside GM1-binding site is sufficient for cholera toxin to bind to cells and complete the intoxication pathway. *MBio* **3**, e00401–e00412 (2012).
- 17 O. M. Szklarczyk et al., Receptor concentration and diffusivity control multivalent binding of Sv40 to membrane bilayers. *PLOS Comput. Biol.* **9**, e1003310 (2013).
- 18 W. Pezeshkian et al., Mechanism of Shiga toxin clustering on membranes. *ACS Nano* **11**, 314–324 (2016).
- 19 A. Sharonov, R. M. Hochstrasser, Wide-field subdiffraction imaging by accumulated binding of diffusing probes. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18911–18916 (2006).
- 20 A. M. Kabbani, C. V. Kelly, The detection of nanoscale membrane bending with polarized localization microscopy. *Biophys. J.* **113**, 1782–1794 (2017).
- 21 A. M. Kabbani, C. V. Kelly, Nanoscale membrane budding induced by CTxB and detected via polarized localization microscopy. *Biophys. J.* **113**, 1795–1806 (2017).
- 22 W. Pezeshkian, L. J. Nãbo, J. H. Ipsen, Cholera toxin B subunit induces local curvature on lipid bilayers. *FEBS Open Bio* **7**, 1638–1645 (2017).
- 23 C. A. Day et al., Microtubule motors power plasma membrane tubulation in clathrin-independent endocytosis. *Traffic* **16**, 572–590 (2015).