Biophysical Journal
Biophysical Letter

Biophysical Society

Glycolipid Crosslinking Is Required for Cholera Toxin to Partition Into and Stabilize Ordered Domains

Krishnan Raghunathan,[1] Tiffany H. Wong,[1] Daniel J. Chinnapen,[2] Wayne I. Lencer,[2] Michael G. Jobling,[3] and Anne K. Kenworthy[1,4,*]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee; [2]Harvard Medical School and the Harvard Digestive Diseases Center, Boston, Massachusetts; [3]Department of Immunology and Microbiology, University of Colorado, Aurora, Colorado; and [4]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee

ABSTRACT Current models of lipid rafts propose that lipid domains exist as nanoscale compositional fluctuations and these fluctuations can potentially be stabilized into larger domains, consequently better compartmentalizing cellular functions. However, the mechanisms governing stabilized raft assembly and function remain unclear. Here, we test the role of glycolipid crosslinking as a raft targeting and ordering mechanism using the well-studied raft marker cholera toxin B pentamer (CTxB) that binds up to five GM1 glycosphingolipids to enter host cells. We show that when applied to cell-derived giant plasma membrane vesicles, a variant of CTxB containing only a single functional GM1 binding site exhibits significantly reduced partitioning to the ordered phase compared to wild-type CTxB with five binding sites. Moreover, monovalent CTxB does not stabilize membrane domains, unlike wild-type CTxB. These results support the long-held hypothesis that CTxB stabilizes raft domains via a lipid crosslinking mechanism and establish a role for crosslinking in the partitioning of CTxB to ordered domains.

The plasma membrane is hypothesized to contain dynamic subdiffraction-sized coexisting liquid-ordered (l_o, raft) and liquid-disordered (l_d) phases (1). Although not readily apparent in intact cells, micron-sized l_o and l_d phases have been observed in isolated giant plasma membrane vesicles (GPMVs). It has been proposed that these micron-sized phase separations are related to the nanoscale organization of membranes (2). Thus, GPMVs can be used as a model system for understanding the origins of lipid-mediated membrane organization (2,3). Several proteins are known to associate with the l_o phase in GPMVs, suggesting they are selectively targeted to raftlike domains (4). However, mechanisms that control the partitioning of proteins to rafts remain incompletely understood.

Cholera toxin (CTx), an AB5 toxin, is a well-known lipid raft marker that has been used extensively to study the origins and functionality of lipid rafts (5). Its homopentameric B subunit (CTxB) recognizes and binds up to five molecules of its glycolipid receptor ganglioside GM1 (5). Previous studies have established that CTxB partitions into the l_o phase, defining it as a raft marker (6–8). (Working definitions of terminologies used are in the Supporting Material.) This preference of CTxB for the l_o phase is thought to arise in part from the intrinsic phase preference of GM1 itself (9). Moreover, binding of CTxB can drive the formation of both nanoscale and large-scale domains in cell-derived vesicles and model membranes that are close to a demixing point (6–8,10). Thus, CTxB not only functions as a raft marker but also as a domain inducer and raft stabilizer. It is widely assumed that these properties of CTxB depend on its ability to bind and cluster multiple GM1 molecules (6). However, this model remains to be formally tested. In this Letter, we test this hypothesis by comparing the behavior of wild-type forms of CTx and CTxB that can bind up to five GM1 molecules with corresponding monovalent variants (mCTx and mCTxB) that can bind only a single GM1 (11).

We first examined whether the ability of CTxB to bind multiple copies of its glycolipid receptor stabilizes micron-sized domains in GPMVs. To test this, we compared the effect of wild-type (WT) versus mCTxB binding on the miscibility transition temperature (T_{misc}) of GPMVs. T_{misc} is defined as the temperature at which 50% of the GPMVs contain coexisting l_o and l_d phases and provides a measure of the energetics of mixing behavior of the two phases (12,13). Treatment of GPMVs with WT CTxB has been

Submitted July 6, 2016, and accepted for publication November 11, 2016.

*Correspondence: anne.kenworthy@vanderbilt.edu

Editor: Claudia Steinem.

http://dx.doi.org/10.1016/j.bpj.2016.11.008

© 2016 Biophysical Society.

previously reported to increase T_{misc}, indicating that rafts are stabilized in the presence of CTxB (7). Consistent with this, we found that GPMVs isolated from COS-7 cells and subsequently treated with WT CTxB exhibited an increased T_{misc}. This change in T_{misc} was dependent on the concentration of CTxB (Fig. 1). Similar results were obtained in a second cell line and when COS-7 cells were labeled with CTxB before GPMV isolation (Fig. S3 in the Supporting Material). In contrast, binding of mCTxB to the GPMVs did not change T_{misc} over the range of concentrations studied (Fig. 1). Thus, while WT CTxB binding stabilizes microscopic domains in the membrane, mCTxB is incapable of doing so.

We next compared the phase preference of WT and monovalent CTx and CTxB (refer to Supporting Materials and Methods and Fig. S4 for details). As expected (2), WT CTxB and CTx exhibited l_o phase preference (Fig. 2). In contrast, mCTx and mCTxB, which cannot cluster GM1, had reduced raft preference (Fig. 2). This difference in partitioning was not a consequence of the decreased avidity of binding of monovalent compared to the WT toxins (Fig. S4). Thus, preferential partitioning of CTx and CTxB into the l_o phase appears to be an emergent behavior dependent on its binding to multiple molecules of GM1.

To confirm if clustering is sufficient to drive raft partitioning and raft stabilization, we examined the effects of antibody crosslinking. Crosslinking of mCTxB increased the T_{misc} of the GPMVs (Fig. S5) and induced mCTxB to strongly partition into the l_o phase (Figs. S6 and S7), consistent with the role of clustering in enabling raft partitioning.

Our results have two major implications for our understanding of raft stabilization and partitioning mechanisms. First, they strongly suggest that the previously observed stabilization of microscopic phases by CTxB (6–8) indeed has its origins in the ability of CTxB to bind multiple molecules of GM1. While the impact of toxin binding is observed at the micron scale in our experiments, this likely translates into differences in plasma membrane organization at the nanoscale under physiological conditions (refer to the Supporting Material for further discussion).

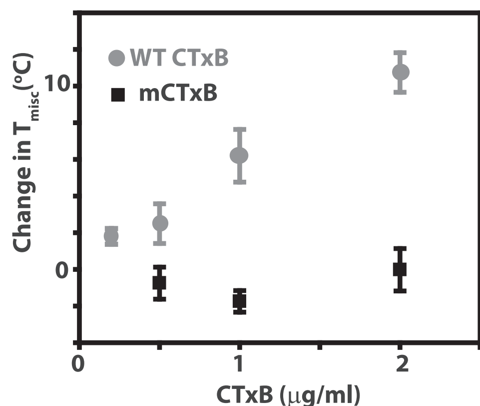

FIGURE 1 WT CTxB but not mCTxB increases T_{misc} of GPMVs isolated from COS-7 cells. See text for details. Data represent mean ± SE ($N \geq 3$).

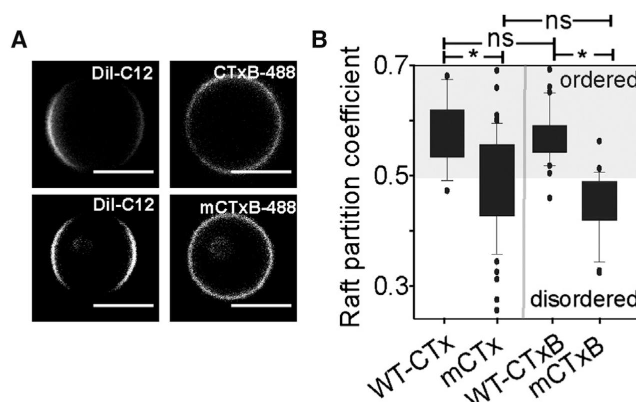

FIGURE 2 Monovalent and WT variants of CTx and CTxB exhibit different phase partitioning preferences in GPMVs derived from COS-7 cells. (*A*) Representative images of GPMVs labeled with WT or monovalent CTxB and the l_d phase marker DiI-C12. Scale bars, 5 μm. (*B*) Quantification of raft partition coefficients for WT and monovalent CTx and CTxB.

Second, we find that the mere binding of CTxB to GM1 is not sufficient to ensure its preference for partitioning into the l_o phase. One potential explanation for these findings is that clustering of GM1 induced by CTxB binding increases its l_o phase preference. This model stems from earlier experimental observations that CTxB binds initially to the disordered phase in GUVs and with increased time partitions into the l_o phase (14). Such a rationale is further supported by our current findings that an increase in clustering induced through antibody crosslinking of the toxin increases raft partitioning. However, additional factors could also potentially contribute to differences in the phase preference of WT and monovalent CTxB. For example, binding of CTxB to GM1 is cooperative (15,16), depends on the orientation of the GM1 headgroup (17–19), and is sensitive to the local density and clustering of GM1 (17,20). Another major factor that can impact the partitioning of CTxB is the structure of GM1's ceramide moiety (21). Thus, even though clustering of GM1 by CTxB stabilizes large-scale domains and is linked to CTxB's phase preference, properties of GM1 itself could also influence these behaviors.

For CTx, it is known that loss of even a single GM1 binding site results in significant loss of toxicity (11,22). This loss of toxicity has been hypothesized to reflect a requirement for multivalent binding to scaffold GM1 into nanodomains to support subsequent membrane trafficking steps (11). Our results demonstrating a role of multivalent binding in CTxB's raft partitioning and domain stabilization strongly support this hypothesis. Finally, we note that many toxins and viruses utilize a multivalent glycolipid

binding strategy to gain access to host cells (23,24). Further, clustering of glycolipids has been hypothesized to play a role in many cellular functions (25–28). Our results validate the underlying assumption in these biological scenarios that clustering of glycolipids enables raft partitioning and stabilization.

SUPPORTING MATERIAL

Supporting Materials and Methods, seven figures, and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)31035-9.

AUTHOR CONTRIBUTIONS

A.K.K., K.R., W.I.L., and M.G.J. designed research; K.R., T.H.W., D.J.C., and M.G.J. performed research; K.R. contributed analytic tools; K.R., T.H.W., and M.G.J. analyzed data; K.R., A.K.K., M.G.J., W.I.L, and D.J.C. wrote the article; and all authors reviewed the final manuscript.

ACKNOWLEDGMENTS

Supported by NIH grant No. R01 GM106720 (to A.K.K.), the Stanley Cohen Innovation Fund (to A.K.K.), and grant Nos. DK048106, DK084424, and DK034854 (to W.I.L.) from the Harvard Digestive Disease Center.

SUPPORTING CITATIONS

References (29–47) appear in the Supporting Material.

REFERENCES

1. Lingwood, D., and K. Simons. 2010. Lipid rafts as a membrane-organizing principle. *Science.* 327:46–50.
2. Baumgart, T., A. T. Hammond, …, W. W. Webb. 2007. Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl. Acad. Sci. USA.* 104:3165–3170.
3. Veatch, S. L., P. Cicuta, …, B. Baird. 2008. Critical fluctuations in plasma membrane vesicles. *ACS Chem. Biol.* 3:287–293.
4. Sengupta, P., A. Hammond, …, B. Baird. 2008. Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles. *Biochim. Biophys. Acta.* 1778:20–32.
5. Day, C. A., and A. K. Kenworthy. 2015. Functions of cholera toxin B-subunit as a raft cross-linker. *Essays Biochem.* 57:135–145.
6. Lingwood, D., J. Ries, …, K. Simons. 2008. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci. USA.* 105:10005–10010.
7. Johnson, S. A., B. M. Stinson, …, T. Baumgart. 2010. Temperature-dependent phase behavior and protein partitioning in giant plasma membrane vesicles. *Biochim. Biophys. Acta.* 1798:1427–1435.
8. Hammond, A. T., F. A. Heberle, …, G. W. Feigenson. 2005. Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc. Natl. Acad. Sci. USA.* 102:6320–6325.
9. Sezgin, E., I. Levental, …, P. Schwille. 2012. Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. *Biochim. Biophys. Acta.* 1818:1777–1784.
10. Štefl, M., R. Šachl, …, M. Hof. 2012. Dynamics and size of cross-linking-induced lipid nanodomains in model membranes. *Biophys. J.* 102:2104–2113.
11. Jobling, M. G., Z. Yang, …, R. K. Holmes. 2012. A single native ganglioside GM1-binding site is sufficient for cholera toxin to bind to cells and complete the intoxication pathway. *MBio.* 3:1–9.
12. Gray, E., J. Karslake, …, S. L. Veatch. 2013. Liquid general anesthetics lower critical temperatures in plasma membrane vesicles. *Biophys. J.* 105:2751–2759.
13. Raghunathan, K., A. Ahsan, …, S. L. Veatch. 2015. Membrane transition temperature determines cisplatin response. *PLoS One.* 10:e0140925.
14. Bacia, K., P. Schwille, and T. Kurzchalia. 2005. Sterol structure determines the separation of phases and the curvature of the liquid-ordered phase in model membranes. *Proc. Natl. Acad. Sci. USA.* 102:3272–3277.
15. Lin, H., E. N. Kitova, and J. S. Klassen. 2014. Measuring positive cooperativity using the direct ESI-MS assay. Cholera toxin B subunit homopentamer binding to GM1 pentasaccharide. *J. Am. Soc. Mass Spectrom.* 25:104–110.
16. Lencer, W. I., S. H. Chu, and W. A. Walker. 1987. Differential binding kinetics of cholera toxin to intestinal microvillus membrane during development. *Infect. Immun.* 55:3126–3130.
17. Shi, J., T. Yang, …, P. S. Cremer. 2007. GM1 clustering inhibits cholera toxin binding in supported phospholipid membranes. *J. Am. Chem. Soc.* 129:5954–5961.
18. Mahfoud, R., A. Manis, …, C. A. Lingwood. 2010. A major fraction of glycosphingolipids in model and cellular cholesterol-containing membranes is undetectable by their binding proteins. *J. Biol. Chem.* 285:36049–36059.
19. Lingwood, D., B. Binnington, …, K. Simons. 2011. Cholesterol modulates glycolipid conformation and receptor activity. *Nat. Chem. Biol.* 7:260–262.
20. Šachl, R., M. Amaro, …, M. Hof. 2014. On multivalent receptor activity of GM1 in cholesterol containing membranes. *Biochim. Biophys. Acta.* 1853:850–857.
21. Chinnapen, D. J. F., W. T. Hsieh, …, W. I. Lencer. 2012. Lipid sorting by ceramide structure from plasma membrane to ER for the cholera toxin receptor ganglioside GM1. *Dev. Cell.* 23:573–586.
22. Wolf, A. A., M. G. Jobling, …, W. I. Lencer. 2008. Attenuated endocytosis and toxicity of a mutant cholera toxin with decreased ability to cluster ganglioside GM1 molecules. *Infect. Immun.* 76:1476–1484.
23. Ewers, H., R. W. Smith, …, L. Johannes. 2010. GM1 structure determines SV40-induced membrane invagination and infection. *Nat. Cell Biol.* 12:11–18.
24. Römer, W., L. Berland, …, L. Johannes. 2007. Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature.* 450:670–675.
25. Ledeen, R. W., and G. Wu. 2015. The multi-tasked life of GM1 ganglioside, a true factotum of nature. *Trends Biochem. Sci.* 40:407–418.
26. Ekyalongo, R. C., H. Nakayama, …, K. Iwabuchi. 2015. Organization and functions of glycolipid-enriched microdomains in phagocytes. *Biochim. Biophys Acta.* 1851:90–97.
27. Ikeda, K., T. Yamaguchi, …, K. Matsuzaki. 2011. Mechanism of amyloid β-protein aggregation mediated by GM1 ganglioside clusters. *Biochemistry.* 50:6433–6440.
28. Ichikawa, N., K. Iwabuchi, …, E. Arikawa-Hirasawa. 2009. Binding of laminin-1 to monosialoganglioside GM1 in lipid rafts is crucial for neurite outgrowth. *J. Cell Sci.* 122:289–299.
29. London, E. 2005. How principles of domain formation in model membranes may explain ambiguities concerning lipid raft formation in cells. *Biochim. Biophys. Acta.* 1746:203–220.
30. Wenz, J. J., and F. J. Barrantes. 2003. Steroid structural requirements for stabilizing or disrupting lipid domains. *Biochemistry.* 42:14267–14276.

31. Levental, K. R., J. H. Lorent, …, I. Levental. 2016. Polyunsaturated lipids regulate membrane domain stability by tuning membrane order. *Biophys. J.* 110:1800–1810.
32. Putzel, G. G., and M. Schick. 2009. Theory of raft formation by the cross-linking of saturated or unsaturated lipids in model lipid bilayers. *Biophys. J.* 96:4935–4940.
33. Machta, B. B., S. L. Veatch, and J. P. Sethna. 2012. Critical Casimir forces in cellular membranes. *Phys. Rev. Lett.* 109:138101.
34. Honigmann, A., S. Sadeghi, …, R. Vink. 2014. A lipid bound actin meshwork organizes liquid phase separation in model membranes. *eLife.* 3:e01671.
35. Edidin, M. 2003. The state of lipid rafts: from model membranes to cells. *Annu. Rev. Biophys. Biomol. Struct.* 32:257–283.
36. Simons, K., and W. L. C. Vaz. 2004. Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys.* 33:269–295.
37. de Almeida, R. F. M., L. M. Loura, …, M. Prieto. 2005. Lipid rafts have different sizes depending on membrane composition: a time-resolved fluorescence resonance energy transfer study. *J. Mol. Biol.* 346:1109–1120.
38. Sun, H., L. Chen, …, W. Fang. 2015. Nanodomain formation of ganglioside GM1 in lipid membrane: effects of Cholera toxin mediated cross-linking. *Langmuir.* 31:9105–9114.
39. Tian, A., and T. Baumgart. 2009. Sorting of lipids and proteins in membrane curvature gradients. *Biophys. J.* 96:2676–2688.
40. Safouane, M., L. Berland, …, P. Bassereau. 2010. Lipid cosorting mediated by Shiga toxin induced tubulation. *Traffic.* 11:1519–1529.
41. Day, C. A., N. W. Baetz, …, A. K. Kenworthy. 2015. Microtubule motors power plasma membrane tubulation in clathrin-independent endocytosis. *Traffic.* 16:572–590.
42. Jobling, M. G., and R. K. Holmes. 2000. Identification of motifs in cholera toxin A1 polypeptide that are required for its interaction with human ADP-ribosylation factor 6 in a bacterial two-hybrid system. *Proc. Natl. Acad. Sci. USA.* 97:14662–14667.
43. Jobling, M. G., and R. K. Holmes. 1991. Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis. *Mol. Microbiol.* 5:1755–1767.
44. Merritt, E. A., S. Sarfaty, …, W. G. Hol. 1997. Structural studies of receptor binding by cholera toxin mutants. *Protein Sci.* 6:1516–1528.
45. Fujinaga, Y., A. A. Wolf, …, W. I. Lencer. 2003. Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulum. *Mol. Biol. Cell.* 14:4783–4793.
46. Liljeqvist, S., S. Ståhl, …, M. Murby. 1997. Fusions to the cholera toxin B subunit: influence on pentamerization and GM1 binding. *J. Immunol. Methods.* 210:125–135.
47. Kang, D., Y. S. Gho, …, C. Kang. 2002. Highly sensitive and fast protein detection with Coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Bull. Korean Chem. Soc.* 23:1511–1512.