BRIEF COMMUNICATION
PUBLISHED ONLINE: 3 APRIL 2011 | DOI: 10.1038/NCHEMBIO.551
nature chemical biology

Cholesterol modulates glycolipid conformation and receptor activity

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We document a new dimension of surface recognition in which communication is controlled through the collective behavior of lipids. Membrane cholesterol induces a tilt in glycolipid receptor headgroup, resulting in loss of access for ligand binding. This property appears to organize erythrocyte blood group presentation and glycolipid receptor function during the activation of sperm fertility, suggesting that lipid 'allostery' is a means to regulate membrane recognition processes.

Surface glycosphingolipids (GSL) are important communication devices used by cells. They contain an oligosaccharide head group covalently linked to the membrane with a hydrophobic ceramide anchor and function as receptors in signaling, microbial and cellular adhesion processes and the display of immunological identity[1]. Here, glycolipids have been proposed to be capable of displaying more than one membrane-regulated receptor epitope from a single carbohydrate sequence[2]. We now identify membrane cholesterol as a key molecule that regulates glycolipid conformation and receptor function. This molecule changes receptor availability by inducing a membrane-parallel glycolipid head group configuration, a feature that seems to modulate the presentation of erythrocyte blood groups and also the exposure of sperm sugar residues during conversion to the fertile state.

We began by reconstituting surface recognition (**Supplementary Methods**) for two well-described GSL receptor systems[3]: the binding of cholera toxin and verotoxin to liposomes containing their respective GSL receptors monosialotetrahexosylganglioside (GM1) and globotriaosyl ceramide (Gb_3, also referred to as the P^K blood group). The resulting dissociation constants of toxin binding were similar to those reported by surface plasmon resonance[3]. Our results also demonstrated that in the presence of cholesterol, the B_{max} of binding was significantly lowered for both toxins, suggesting that sterol was inducing an 'unavailable' receptor fraction (**Table 1**, **Supplementary Results**, **Supplementary Fig. 1**). It has been demonstrated that for ganglioside at this concentration, GM1 forms nanoscale clusters that can decrease cholera toxin binding affinity[4]. However, we find that under these conditions, the B_{max} of toxin binding remains unaffected (**Supplementary Fig. 2**). Notably, changes in capacity rather than affinity have recently been shown to be a way in which proteins can selectively interact with membrane surfaces[5].

To understand the structural basis for these results, we ran atomic level molecular dynamics simulations of our membrane recognition surface (**Table 1**; **Supplementary Figs. 3** and **4**, **Supplementary Tables 1** and **2**). In the presence of cholesterol, the glycan moiety of GM1 was found to adopt a conformation that is significantly tilted toward the membrane plane. This is induced by the shielding of sterol from unfavorable contact with water and by hydrogen bonding of glycan to the membrane surface. To test this experimentally, we used dual polarization interferometry to measure the thickness (mass per unit area) of GM1 in cholesterol-containing membranes (**Supplementary Fig. 5**). We observed that this value decreased as a function of cholesterol content, indicative of GM1 having a less vertical orientation.

This suggested that cholesterol is able to constrain key features of the glycan toxin-binding site[6] and, in so doing, regulate receptor availability. If GSL receptor activity is decreased by cholesterol bringing the receptor glycan closer to the membrane surface, then the inhibition of binding should be related to the position of the sugar relative to the membrane plane. Upon varying this position (increasing the GSL fatty acid chain length in palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membranes), we found that the cholesterol-induced reduction in toxin binding was lower at higher GSL-membrane mismatch (**Supplementary Fig. 6**). In the more extreme mismatching condition, the inhibition by cholesterol was rescued by kinking (shortening) the GSL long chain fatty acid with an unsaturated bond, demonstrating that the effect of cholesterol was maximal in a membrane that minimizes mismatch stress. Receptor inhibition was therefore related to the relative plane of the membrane, supporting constrained GSL conformation toward the membrane surface as the molecular basis for glycolipid receptor masking by cholesterol.

To establish the biological relevance of cholesterol in glycolipid receptor activity, we examined our GSL receptor paradigms histologically. Verotoxin- and cholera toxin–GSL binding was assessed as function of methyl-beta cyclodextrin (MβCD)–mediated cholesterol extraction in human kidney tissue[7]. For both toxins, we observed a GSL-specific unmasking of receptor activity after cholesterol removal, with 'unseen' GM1 and Gb_3 residing in specific structures—the nephric tubules and renal glomeruli, respectively (**Fig. 1**, **Supplementary Figs. 7–9**)—underscoring a potential functionality to this phenomenon.

To explore this possibility, we analyzed the role of cholesterol in regulating the surface recognition of the Gb_3 blood group in erythrocyte membranes. It is known that despite its presence as a blood group antigen, verotoxin only binds to erythrocytes held at 4 °C (ref. 8). We evaluated verotoxin binding to erythrocyte ghosts

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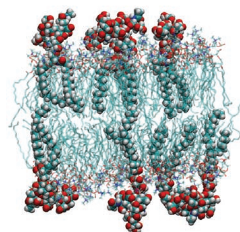
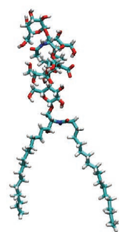
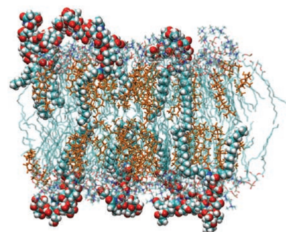
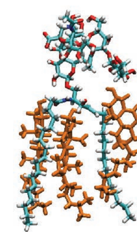
Table 1 | Cholesterol induces an 'unavailable' GSL receptor configuration

	B_{max} (rel. intensity)		K_d (nM)	
	POPC	POPC + cholesterol	POPC	POPC + cholesterol
GM1d18:1/C18:0	601,432 ± 18,521	451,416 ± 29,378	1.71 ± 0.10	2.37 ± 0.33
Gb_3d18:1/C16:0	79,047 ± 2,168	43,399 ± 798	36.29 ± 2.69	36.33 ± 1.86


POPC + GM1


Extended GSL


POPC + GM1 + Cholesterol


Tilted GSL

The B_{max} for cholera toxin and verotoxin GSL binding was significantly lowered ($P < 0.02$ for both toxins, two-tailed *t*-test, data represent mean values ± standard error, $n = 3$) when cholesterol was present in the membrane. Atomistic molecular dynamics simulations indicate that in the presence of cholesterol, the GM1 glycan moiety adopts a conformation that is tilted toward the membrane plane (see also **Supplementary Figs. 1** and **3–5**, **Supplementary Tables 1** and **2**).

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as a function of cholesterol extraction by MβCD. We find that in the absence of cholesterol, verotoxin recognizes the membrane surface (**Fig. 1**). The exposure of Band III, an erythrocyte membrane protein, was unaffected by cholesterol removal. As Gb_3 is the only receptor for verotoxin in this system[8], masking of the blood group by cholesterol is a likely explanation for its lack of reactivity, alluding to a role for sterol in recognition of self antigens. Given the unexpectedly high concentration of antibodies to the Gb_3 carbohydrate in healthy human serum[9], our results could suggest that cholesterol is a factor in preventing surface recognition and autoimmunity.

Having established biological relevance for membrane cholesterol as a modulator of glycolipid receptor activity, we reasoned that its functionality would be best illustrated in a physiologically relevant context in which membrane cholesterol levels are altered. One possibility is sperm capacitation, a collective term for the changes induced once inside the female reproductive tract that lead to activation to a fertile state[10]. This includes a reduction in membrane cholesterol levels[11], with as yet unclear implications. GSL are known to laterally interact with cholesterol in raft assemblies[12] that are proposed to undergo substantial reorganization during this process[13]. Notably, antibodies to sperm glycolipids inhibit egg binding, both for mice[14] and for humans[15]. It has been suggested that sperm surface sugar residues, important for subsequent binding to the egg membrane, become exposed during capacitation[10]. We hypothesized that cholesterol-based glycolipid conformational changes could be involved in this enigmatic activation process. To this end, we used MβCD and the more-standard 2-hydroxylpropyl-beta cyclodextrin (2-OHCD) to induce capacitation in mouse sperm[16] and then analyzed the molecular recognition activity of surface GM1, a GSL important in mediating this activation process[17]. By FACS analysis we observed a quantitative increase in sperm receptor activity for cholera toxin–Alexa 488 after cholesterol efflux with both compounds (**Fig. 2**). This increase was not because of membrane

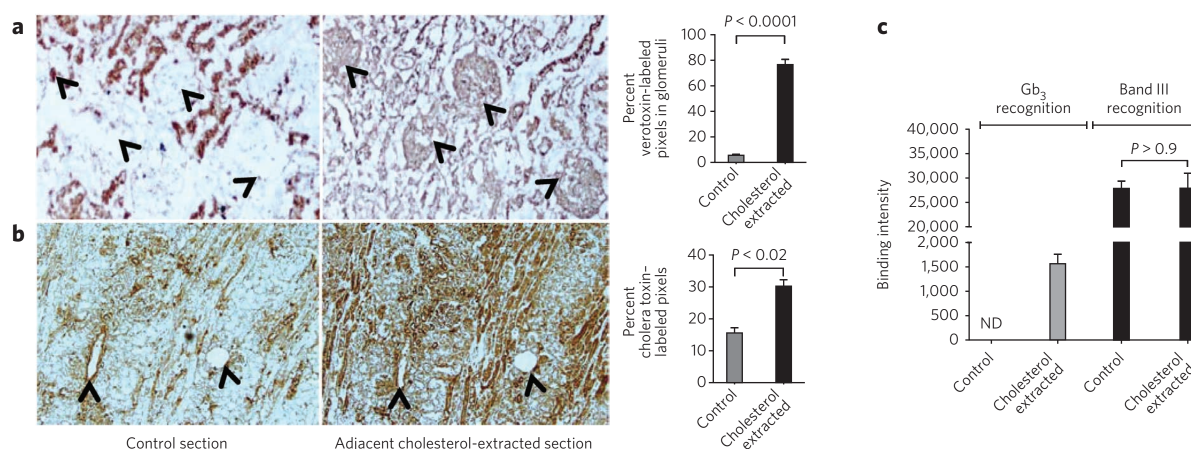

Figure 1 | Cholesterol alters GSL surface recognition in human tissue and blood group presentation in human erythrocyte membrane. (**a**,**b**) Serial frozen sections of human kidney (6 μm thick) were probed with verotoxin (**a**) or cholera toxin (**b**). After MβCD treatment of adjacent sections, nephric tubular GM1 labeling by cholera toxin dramatically increased, and verotoxin now stained Gb_3 in the renal glomeruli ($P < 0.02$ and $P < 0.0001$, respectively, Fisher's exact test (two-tailed), data represent mean values ± standard error, $n = 4$). Arrows denote glomeruli (**a**) and conserved morphology between adjacent sections (**b**). MβCD treatment removes tissue cholesterol, with the GSL recognition activity being specific to glycolipid and not protein antigens (**Supplementary Figs. 7–9**). (**c**) Verotoxin binding to the Gb_3 blood group of human red blood cell ghosts also increased from a nondetectable (ND) level after cholesterol extraction by MβCD. This cholesterol extraction did not affect the exposure of Band III, a protein antigen, to a commercial antibody ($P > 0.9$, paired *t* test, two-tailed, data represent mean values ± standard error, $n = 4$).

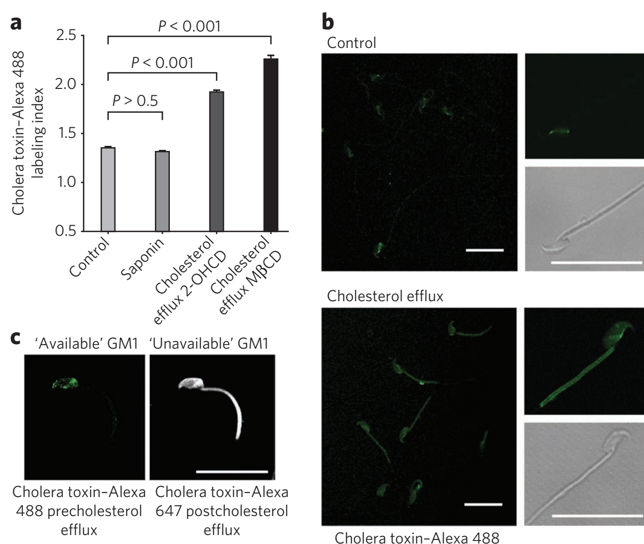

Figure 2 | Cholesterol depletion during capacitation enhances GSL recognition in the plasma membrane of mouse sperm. (**a**) After cholesterol efflux, by either MβCD or 2-OHCD, a quantitative increase in sperm surface receptor activity for cholera toxin–Alexa 488 was measured by FACS ($P < 0.01$, ANOVA, then Dunnett's comparison to a control, data represent mean values ± standard error, $n = 3$). (**b**) After cholesterol efflux, cholera toxin–Alexa 488 binding changed from the postacrosomal plasma membrane pattern defined for immobilized uncapacitated sperm to the head and midpiece binding pattern defined for immobilized sperm that underwent capacitation. (**c**) These uncapacitated and capacitated patterns represent two distinct GM1 populations, as they could be observed within the same sperm cell by two cholera toxin colors labeled pre- and postcholesterol efflux. Scale bars, 20 µm.

permeabilization (**Fig. 2**, **Supplementary Fig. 10**), suggesting that capacitation includes an unveiling of cryptic GSL receptor at the sperm surface.

In free-swimming sperm, cholera toxin binds to the plasma membrane over the rostral head[18], and an intensification of signal over the apical acrosome is thought to accompany capacitation[13]. In mouse sperm, the pattern of cholera toxin labeling pre- and postcapacitation has also been described[19]. Although these patterns are different[18] and do not represent the actual GM1 distribution encountered by the egg, they are clearly defined and amenable to testing our prediction that the sperm surface contains differentially available populations of the same GSL receptor. After cholesterol efflux, we observed the 'diffuse pattern' indicating sperm have undergone capacitation (widespread localization of toxin to the head and midpiece, in contrast to uncapacitated immobilized sperm in which cholera toxin localizes to the postacrosomal region of the plasma membrane) (**Fig. 2**). To assess whether these staining profiles reflect one or two GM1 populations, we labeled with cholera toxin–Alexa 488 pre–cholesterol efflux and cholera toxin–Alexa 647 post–cholesterol efflux. After immobilization, we observed the uncapacitated pattern in the first color and the pattern for having undergone capacitation in the second color, all within the same sperm cell (**Fig. 2**). Although pleiotropic effects of cholesterol removal cannot be ruled out (for example, potential for disruption of cortical actin), the fact that the uncapacitated pattern does not turn into the capacitated pattern suggests there is an available GSL receptor population and an unavailable GSL receptor population in the same membrane surface.

The membrane recognition surface described here now provides a molecular explanation as to how glycolipid receptor activity could work during sperm activation. In the unactivated sperm, the membrane sterol induces a tilted, unavailable receptor configuration. Capacitation and cholesterol efflux elicits a change in GSL conformation, exposing sugars that could be recognized by lectins in the egg zona pellucida. The degree of this surface exposure could be internally tuned through the hydrolysis of cholesterol sulfate to cholesterol that also accompanies capacitation[20]. Lipid-dependent surface recognition is supported by membrane studies documenting cholesterol-modulated lipid receptor–ligand binding activity[21,22] and is in keeping with the clinically relevant fact that sperm fertilizing ability *in vitro* is improved by depletion of cholesterol[16].

In conclusion, the regulation of some surface recognition processes may be reduced to simple lipid-lipid interactions, ultimately adding membrane cholesterol to the cellular toolkit gating the flow of molecular information.

Received 19 July 2010; accepted 8 February 2011; published online 3 April 2011

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Acknowledgments

The authors thank I. Nuesslein, M. Gerl, I. Levental, H. Kaiser (Max Planck Institute for Molecular Cell Biology and Genetics), K. Hölig (Technische Univesität), P. Paroutis, M. Woodside (Hospital for Sick Children), U. Devi, W. Jones and M. Swann (Farfield Sensors) for their assistance in this project. This work was supported by a MPI-CBG stipend (D.L.) and funding to K.S. (EU FP6 Lipid PRISM Grant LSHB-CT2007-037740, DFG Schwerpunktprogramm 1175 Grant SI459/2-1, DFG Transregio 83 Grant: TRR83 TP02, BMBF ForMaT Grant: 03FO1212, ESF "LIPIDPROD" Grant: SI459/3-1); I.V. (Academy of Finland); and funding to C.A.L. (Canadian Institutes of Health Research Grant MT 13747, Ontario HIV Treatment Network and Canfar support).

Author contributions

Membrane and sperm recognition experiments were performed by D.L.; histology was by B.B.; molecular dynamics simulations were by T.R. and I.V.; DPI was performed by D.L., M.G. and U.C.; D.L., C.A.L. and K.S. formulated the project and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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