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Hetero-multivalent Binding of Cholera Toxin Subunit B with Glycolipid Mixtures

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Abstract

GM_1 has generally been considered as the major receptor that binds to cholera toxin subunit B (CTB) due to its low dissociation constant. However, using a unique nanocube sensor technology, we have shown that CTB can also bind to other glycolipid receptors, fucosyl-GM_1 and GD_1b. Additionally, we have demonstrated that GM_2 can contribute to CTB binding if present in a glycolipid mixture with a strongly binding receptor (GM_1/fucosyl-GM_1/GD_1b). This hetero-multivalent binding result was unintuitive because the interaction between CTB and pure GM_2 is negligible. We hypothesized that the reduced dimensionality of CTB-GM_2 binding events is a major cause of the observed CTB binding enhancement. Once CTB has attached to a strong receptor, subsequent binding events are confined to a 2D membrane surface. Therefore, even a weak GM_2 receptor could now participate in second or higher binding events because its surface reaction rate can be up to 10^4 times higher than the bulk reaction rate. To test this hypothesis, we altered the surface reaction rate by modulating the fluidity and heterogeneity of the model membrane. Decreasing membrane fluidity reduced the binding cooperativity between GM_2 and a strong receptor. Our findings indicated a new protein-receptor binding assay, that can mimic complex cell membrane environment more accurately, is required to explore the inherent hetero-multivalency of the cell membrane. We have thus developed a new membrane perturbation protocol to efficiently screen receptor candidates involved in hetero-multivalent protein binding.

Graphical abstract

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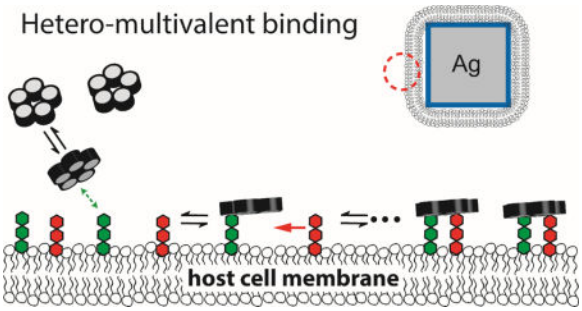

Keywords

Cholera Toxin; multivalent binding; supported lipid bilayer; glycolipid; nanocubes

Introduction

Many proteins recognize glycolipid receptors in cell membranes via multivalent binding mechanisms.[1] Such dynamic binding, driven by a series of binding domains, brings a protein to a membrane surface and initiates biological processes. Interactions between a single glycolipid receptor and a protein binding subunit are often weak, and therefore multivalency enhances the protein binding avidity and specificity to cell surfaces. Cholera toxin (CTx), the virulence factor of *Vibrio cholerae*, is a type of multivalent glycolipid binding protein. This AB_5 toxin consists of a single A subunit associated with five identical B subunits. The B pentamer binds to cell membranes and delivers the catalytic A subunit into the cytoplasm. A potential stepwise reaction of pentavalent cholera toxin subunit B (CTB) binding to the cell membrane [2, 3] is shown in Fig. 1. (1) CTB moves from the solution phase to the membrane surface, followed by one of its binding sites attaching to a glycolipid receptor; (2) Free glycolipids diffuse two dimensionally, encounter the bound CTB, and then enable subsequent binding. The synergistic effort amongst various binding pockets, membrane receptors, and membrane dynamics dramatically influences the overall association.[4]

We recently developed a unique nanocube sensor by integrating supported lipid bilayer and plasmonic sensing technologies.[5] This new tool has enabled label-free detection of protein binding to model membrane surfaces by using a standard laboratory spectrophotometer to observe the extinction spectrum shift of the quadrupolar localized surface plasmon resonance (LSPR) peak.[6] The nanocube sensor was used to investigate the multivalent binding principle of CTB interacting with various glycolipids.[6] We observed that the amount of CTB binding onto the surface containing fucosyl-GM_1 was higher than GM_1 although the dissociation constant of GM_1 was an order of magnitude lower than that of fucosyl-GM_1. This unintuitive result might be attributed to a reduced binding cooperativity between fucosyl-GM_1 receptors leading to an increased binding capacity.[6] Our previous findings indicated that dissociation constants cannot exclusively represent multivalent CTB bindings and that binding cooperativity also plays an essential role in determining CTB-cell membrane recognition.

Multivalent binding can be either homo-multivalent (i.e. a protein binds to multiple copies of the same type of receptor) or hetero-multivalent (i.e. a protein simultaneously binds to two or more different types of receptors).[7] Due to the complexity of hetero-multivalency, most studies have focused on homo-multivalency. However, homo-multivalent models neglect the inherent heterogeneity of cell membranes. We recently reported that adding a weak glycolipid receptor (GM_2) to a model membrane containing fucosyl-GM_1 significantly increased the total amount of bound CTB.[6] This was unexpected, as GM_2 receptors have negligible binding avidity in bilayers with GM_2 as the only glycolipid receptor. A few other studies have also reported that lectin binding to glycan mixtures is stronger than the binding to a single glycan.[8–11] However, the mechanism of such hetero-multivalency is not clear.

The goal of this study was to gain insight into the mechanism of hetero-multivalent CTB binding. We first investigated the binding cooperativity of CTB to various glycolipid mixtures. Positive cooperativity was observed when GM_2 was mixed with any of the other three strongly binding receptors (GM_1, fucosyl-GM_1, and GD_1b). We hypothesized that the increase of CTB binding is caused by a reaction rate enhancement mechanism, “reduction of dimensionality” (Fig. 1). Once CTB has attached to a strong receptor, subsequent binding events are confined on the 2D membrane surface. Therefore, even a weak GM_2 receptor could now participate in second or higher binding events because its surface reaction rate is around 10^4 times higher than the rate in bulk solution. To test this hypothesis, we modulated the fluidity and heterogeneity of the model membrane by adding cholesterol or altering fatty acid composition of phospholipids and observed significant changes in the heterogeneous binding cooperativity. This complies with the surface reaction’s strong dependence on the membrane environment. Our results indicated that the traditional protein binding assay, which detects protein interactions with a specific receptor one by one (e.g. microarray technology), is not appropriate to explore multivalent binding interactions. To discover all possible receptors which could participate in a binding process, we designed a new membrane perturbation protocol that can efficiently screen possible glycolipid receptors involved in multivalent protein binding.

Materials and methods

Materials

Monosialoganglioside GM_1 (NH_4^+salt) (Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc-Ceramide, GM_1), monosialoganglioside GM_2 (NH_4^+salt) (GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc-Ceramide, GM_2), monosialoganglioside GM_3 (NH_4^+4salt) (Neu5Acα2-3Galβ1-4Glc-Ceramide,GM_3), fucosylated monosialoganglioside GM_1 (NH_4^+4salt) (Fucα1-2Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc-Ceramide, fucosyl-GM_1) and disialoganglioside GD_1b(NH_4^+salt) (Galβ1-3GalNAcβ1-4(Neu5Acα2-8)(Neu5Acα2-3)Galβ1-4Glc-Ceramide,GD_1b) were purchased from Matreya LLC (State College, PA). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine - sodium salt (DOPS), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine – sodium salt (DMPS) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholera Toxin B subunit (CTB, lyophilized powder) from *Vibrio cholerae*, cholesterol and casein from bovine milk were

purchased from Sigma-Aldrich. GM_1 oligosaccharide (GM_1os) (Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc) sugar was purchased from Elicityl (Crolles, France). All the CTB binding experiments were performed in Tris-buffered saline-TBS (Sigma Aldrich).

Methods

Synthesis & calibration of the nanocube sensor—Silica coated silver nanocubes were prepared as reported in our previous publication.[6] The silver nanocube synthesis was based on the polyol method. The silica shell synthesis over nanocubes was performed in a scaled-up synthesis batch using 2-propanol as solvent. The quality of the nanocube sensor, including silica shell thickness, nanocube size and uniformity, was confirmed by transmission electron microscopy (FEI Technai G2 F20 FE-TEM). (Fig. S1) The refractive index sensitivity of silica coated silver nanocubes was reported as peak shift (reported in nm) per refractive index unit (RIU). (Fig. S2) Since the change in refractive index is directly proportional to the amount of bound proteins, LSPR peak shift allows an estimation of the amount of protein bound.[5]

Supported lipid bilayer preparation—Lipids stored in organic solvents (chloroform for DOPC, DOPS, DMPC, and DMPS or chloroform/methanol/water mixture for glycolipids) were mixed to obtain the desired final composition. They were then dried using a rotary evaporator (Heidolph Hei-VAP Value®), followed by rehydration with Milli-Q® water. Small unilamellar vesicles (SUVs) were prepared by the standard extrusion protocol described in our prior publication.[6] A previously established modified vesicle fusion technique[6] was used to form supported lipid bilayers. The lipid bilayer coated nanocubes were incubated with 0.5 mg/ml casein in 1X TBS solution for 1 hour to prevent nonspecific binding of CTB.

CTB binding measurement—The lipid bilayer coated nanocubes were incubated with the required CTB concentration for 1.5 hours. Blank solutions were also prepared for each CTB concentration by mixing buffer and CTB corresponding to that composition. The extinction spectra of the solutions were measured in a 384 well plate with a UV/Vis microplate spectrophotometer equipped with a CCD (FLUOstar Omega®, BMG-Labtech). All measurements were carried out at room temperature, except the membrane fluidity experiment involving DMPC. The location of the quadrupolar LSPR peak was calculated by fitting the measured absorption spectra to a seventh order polynomial. Each protein binding measurement was repeated in eleven wells. Each data point is represented as the mean ± standard deviation (S.D.) where n = 11. The experimental conditions for each binding measurement are described below.

Combinatorial glycolipid array: To acquire binding curves for pure glycolipid systems (1 mol% glycolipid along with 89 mol% DOPC and 10 mol% DOPS), the CTB concentration was varied from 0 to 1726 nM. For the binary mixture of glycolipids (1 mol% of each glycolipid along with 88 mol% DOPC and 10 mol% DOPS), the CTB concentrations used were 706 nM and 1726 nM.

GM_1os pre-bound CTB binding experiment: 345 nM CTB was incubated at various sugar (GM_1os) concentrations (0 ~ 38.1 μM) prior to the binding measurement. The resulting GM1os-CTB complex was incubated with the bilayer containing 2 mol% glycolipid along with 88 mol% DOPC and 10 mol% DOPS.

Membrane Perturbation protocol: The reference bilayer comprised of 0.25 mol% of each glycolipid (GM_1, GM_2, GM_3, fucosyl-GM_1 and GD_1b), 10 mol% DOPS and 88.75 mol% of DOPC. For the perturbed membranes, one of the glycolipids was increased to 2 mol% while other glycolipids were maintained at 0.25 mol% along with 10 mol% DOPS and 87 mol% DOPC. Each experiment was treated with 0.5 mg/ml Casein in 1× TBS buffer to block non-specific binding and then incubated with 1726 nM CTB for 2 hours.

Results

CTB binding to glycolipid pairs

Our previous study demonstrated that mixing GM_2, a weak binding receptor, with fucosyl-GM_1 could enhance the overall CTB binding.[6] In order to understand the mechanism of the hetero-multivalency, we constructed a combinatorial array of glycolipids to evaluate cooperativity of CTB binding. The array was composed of glycolipids like GM_1, GM_2, GM_3, fucosyl-GM_1, and GD_1b (Fig. 2a). We first examined CTB binding to model membranes containing 1 mol% of a glycolipid (Fig. 2b). The shift in the location of the LSPR peak with respect to the control is directly proportional to the amount of CTB bound. CTB exhibited significant binding to the bilayers containing GM_1, fucosyl-GM_1, or GD_1b. (Fig. 2b) GM_2 and GM_3 showed negligible binding with CTB even at the highest CTB concentrations (1726 nM); this result is consistent with prior studies.[2, 12, 13] Thus, we categorized GM_1/fucosyl-GM_1/GD_1b as strongly binding receptors and GM_2/GM_3 as weakly binding receptors.

The combinatorial array was prepared by mixing two glycolipids in a 1:1 ratio (1 mol% of each glycolipid). The amount of CTB bound to the glycolipid mixtures was measured at two different CTB concentrations (706 nM and 1726 nM). From the CTB-glycolipid binding curves (Fig. 2b), we can see that CTB binding to the model membrane is approximately saturated at 1726 nM. Thus, we used this value to estimate the maximum binding capacity of the model membrane. We also measured the CTB binding at a lower CTB concentration (706 nM) to observe the influence of CTB concentration on binding cooperativity.

To quantify the binding cooperativity of hetero-multivalency, we have defined heterogeneous binding cooperativity (θ) as:

$$\theta = \frac{\text{LSPR shift when CTB binds to a bilayer containing paired glycolipids}}{\text{Sum of LSPR shift when CTB binds to a bilayer containing each individual glycolipid}}$$

Equation 1

If there is no cooperativity between two glycolipids, θ should equal 1. When θ is larger or smaller than 1, it represents positive or negative cooperativity, respectively. The calculated heterogeneous cooperativity was reported in Table 1. We observed positive cooperativity when GM_2 was mixed with any of the strongly binding receptors (GM_1, fucosyl-GM_1, and GD_1b) at both CTB concentrations. Since negligible CTB binding was observed with the model membrane surface containing GM_2 as the only glycolipid receptor, the strongly binding receptors seemed to have activated GM_2 receptors which led to a higher CTB binding. However, no significant cooperativity was observed when GM_3 was mixed with strongly binding receptors. In addition, cooperative action between strong receptors was negligible.

Possible causes of heterogeneous cooperativity

To the best of our knowledge, positive cooperativity between GM_2 and other glycolipid receptors has not yet been reported. Several possible reasons may cause this heterogeneous cooperativity, including induced glycolipid cluster formation, allosteric regulation, and reduction of dimensionality. Each hypothesis has been considered and discussed in the following.

Cremer and his coworkers have demonstrated that increasing GM_1 density in a model membrane induces the formation of GM_1 clusters, leading to weaker CTB binding.[14] If mixing GM_2 had induced the disturbance of glycolipid clusters leading to increased CTB binding, the addition of other glycolipids should have altered the clustering of glycolipid receptors and caused some change in binding cooperativity. However, we observed cooperative interactions only between GM_2 and other strongly binding glycolipids. Furthermore, the glycolipid concentration was kept relatively low (less than 2 mol%) to minimize any heterogeneous distribution of glycolipids on the membrane surface. Therefore, we believe that it is less likely for induced heterogeneity to be the major cause of positive cooperativity.

Allosteric regulation is another possible cause of positive cooperativity. The bound glycolipids (GM_1/fucosyl-GM_1/GD_1b) could have enhanced the binding energy between GM_2 and its adjacent binding sites, enabling GM_2 to participate in the CTB binding process and leading to a higher binding capacity (Fig. 3a). To test this hypothesis, we modified the saturation binding assay developed by Leach et al. for detection of allosteric interactions.[15] Klassen and his coworkers have reported that at the equilibrium state CTB forms a binding complex with GM_1 oligosaccharide (GM_1os), an allosteric modulator that contains the same glycan structure as the GM_1 glycolipid without its ceramide tail.[16] We first incubated CTB with various concentrations of GM_1os oligosaccharide Then, we measured the binding of GM_1os-CTB complex to a model membrane containing 2 mol% glycolipid (GM_2 or fucosyl-GM_1) at a fixed CTB concentration (345 nM) (Fig. 3b). If the bound GM_1os had altered the energetics of the adjacent CTB binding subunit, the allosteric effect should have initiated the attachment of GM_1os-CTB complex to the membrane containing GM_2. Instead, negligible CTB binding to the lipid bilayer having GM_2 was still observed. For the lipid bilayer containing 2 mol% of fucosyl-GM_1, the amount of bound GM_1os-CTB complex decreased with increased GM_1os concentration (Fig. 3b). This is due to competitive

binding between GM_1os and fucosyl-GM_1 receptors. In addition, three different research groups independently evaluated the allosteric effect of GM_1os-CTB binding and found that the affinity constants increased by only twofold when the neighboring binding sites were occupied.[13, 16, 17] Turnbull et al. have estimated the dissociation constant for CTB binding with GM_2 to be 2 mM.[13] Thus, even twofold enhancement of affinity constant (leading to ~1mM dissociation constant) is not sufficient to promote CTB binding to GM_2 at the physiological concentrations. Although we cannot completely exclude the allosteric regulation between GM_2 and other strong receptors, it is probably not the major cause for the observed positive cooperativity.

Another possible cause for positive heterogeneous cooperativity is the influence of reduced dimensionality. Searching for reaction partners is much more efficient on a two-dimensional membrane surface than in 3D space. In 1968, Adam and Delbrück first proposed that organisms can shorten the diffusion time of dilute reactants by adsorption to cell membrane surfaces in order to enhance the reaction rates of the biological processes.[18] Many researchers have validated this concept and provided a comprehensive theory to describe this mechanism.[19–24] Recently, Sengers et al. also reported that reduced dimensionality can improve the binding efficiency of a bivalent monoclonal antibody interaction with membrane bound targets by about 10^4-fold.[25] Thus, it is possible that reduction of dimensionality enhanced the CTB binding to GM_2.

The influence of reduced dimensionality

We hypothesized that CTB first moves from the solution phase to the membrane surface and attaches to one of the strongly binding receptors (GM_1, fucosyl-GM_1, and GD_1b). Jobling et al. have shown that a single active binding site on CTB pentamer is sufficient for cell binding and intoxication;[26] therefore, we expected CTB could form a relatively stable membrane-bound state with a single strongly binding receptor (Fig. 1). Once CTB is anchored to the surface, the effective concentration of GM_2 on 2-D membrane surface dramatically increases for subsequent bindings. Although the weak binding between GM_2 and CTB implies a short lifetime of the CTB-GM_2 complex, the enhanced effective concentration allows GM_2 to continuously participate in the process to bind to CTB leading to an increase in binding capacity. This hypothesis requires the presentation of a strongly binding receptor in order to anchor CTB to the membrane surface.

In order to verify this hypothesis, we first evaluated the 2D and 3D reaction rates using the established theoretical models.[22–24] The reaction rate, ϕ, can be written as[23]:

$$\phi = k_{obs} C_A C_B \quad \text{Equation 2}$$

Where C_A and C_B are the number densities of the two reactants, and k_{obs} is the empirical rate constant. In diffusion controlled reactions, k_{obs} is a function of diffusion coefficients ($D_{3D\,or\,2D}$), the radius of diffusion spaces (b), and the encounter radius of the target receptor (a). Based on our experimental conditions, the bulk concentration of CTB (species A) and glycolipid (species B) were estimated as: $C_A = 3 \times 10^{-7}$ *mol/L*, $C_B = 3 \times 10^{-7}$ *mol/L*. 3D diffusivities of CTB and glycolipid containing liposome were estimated using the Stokes-

Einstein equation as $D_{A,3D} = 9.77 \times 10^{-11}\ m^2/s$ and $D_{B,3D} = 4.88 \times 10^{-12}\ m^2/s$. The measured diffusivity of bound CTB was acquired from literature ($D_{A,2D} = 2.5 \times 10^{-13}\ m^2/s$). [27, 28] The DOPC lipid diffusivity was $D_{B,2D} = 8.25 \times 10^{-12}\ m^2/s$.[29] Using different fluorescent labeling approaches, previous researchers have also reported the diffusivity of GM_1 in DOPC bilayer to be around $3.6\sim8 \times 10^{-12}\ m^2/s$. [30, 31]

We estimated the 3D reaction rate using Smoluchowski's relation which gives a steady-state rate constant for fast reactions,[23]

$$k_{obs,3D} = 4\pi a(D_{A,3D} + D_{B,3D}) \quad \text{Equation 3}$$

Prior studies derived the approximate solution of k_{obs} for 2D membrane reactions using Smoluchowski theory, mean-passage time theory, and statistical thermodynamic theory (the models are summarized in Supplementary Note).[22–24] Based on our experimental conditions, we found that the 2D reaction rate can be up to 10^4 higher than 3D reactions. The increased reaction rate implies that effective concentration of reactants on the membrane surface is enhanced by about 10^4-fold. This calculated enhancement factor has the same order of magnitude of the value in antibody system reported by Sengers et al.[25] In such a case, the reduction of dimensionality could raise the effective GM_2 concentration close to or higher than the dissociation constant of CTB-GM_2 (2mM). Thus, it is possible that this significant enhancement of reaction rate between bound CTB and GM_2 led to higher CTB binding.

To further verify this hypothesis, we altered the diffusivity of glycolipids by replacing DOPC with DMPC that has a gel phase transition temperature near room temperature (24 °C). We conducted the measurements of CTB binding to DMPC model membranes with 1 mol% GM_1 and GM_1:GM_2 mixture (1 mol%:1 mol%) at 15 °C and 45 °C. In the DOPC bilayer, which has transition temperature at −20 °C,[32] the cooperativity between GM_1 and GM_2 at 15 °C was quite similar to what we obtained at room temperature, which implies that such a temperature change does not alter CTB binding much (Fig 4). However, the diffusion of glycolipids in DMPC gel phase is two orders of magnitude lower when compared to the fluidic DMPC membrane.[33, 34] Goins et al. reported GM_1 diffusivity to be approximately $1–2 \times 10^{-13}$ m^2/s in DMPC below 20 °C.[35] Under this condition, the 2D reaction rate is only 400–500 times higher than the 3D reaction rate in DMPC gel phase. Thus, we expected that the rate enhancement via reduced dimensionality would be minimized in the DMPC system at 15 °C. Fig. 4 shows that mixing GM_2 with GM_1 in a DMPC bilayer did not enhance the overall CTB binding at 15 °C; in contrast, binding enhancement was observed in fluidic DMPC bilayer at 45 °C. This result further corroborates our hypotheses that reduction in dimensionality is influencing the binding of CTB with heterogeneous mixtures of glycolipids.

In addition, 10 mol% of cholesterol was added to DOPC bilayer in order to alter the fluidity and the heterogeneity of model membranes. Similar to the DMPC system, changing the membrane environment altered the heterogeneous binding cooperativity (Fig. 4). This result is not surprising because many studies have shown the compositions of fatty acids and

cholesterol in host cells can influence the toxin potency.[36, 37] Previous studies have also reported that surface diffusion and heterogeneity can influence the homo-multivalent CTB-GM_1 binding.[38] Our result indicated that the membrane environment is also essential in hetero-multivalent binding process.

The other question is why mixing GM_3 with the other receptor did not enhance CTB binding. The only difference in the structure of GM_2 and GM_3 is that GM_2 contains an additional N-acetyl galactosamine (GalNAc) in its glycan portion. The crystal structure of CTB-GM_1 complex indicates that the sugar groups of galactose (Gal), GalNAc, and sialic acid (Neu5Ac) in GM_1 were buried in the CTB binding subunit and contribute to 39%, 17%, and 43% of the contact surface area respectively.[39] CTB binding to GM_3 that has only one Neu5Ac epitope should be weaker than GM_2 receptor. In fact, Turnbull et al. estimated the dissociation constant for α-methyl sialoside, which contains only Neu5Ac epitope, to be 210 mM.[13] Even though the mechanism of reduced dimensionality could increase the reaction rate around 10^4-fold, the effective concentration of GM_3 on membrane surfaces is still far below the dissociation constant between CTB and sialic acid residual. Therefore, it wasn't surprising that no cooperativity was found between GM_3 and the other binding receptors.

A new perturbation protocol for screening glycolipid receptors in multivalent interactions

One of the difficulties in observing hetero-multivalency is that some receptors, such as GM_2, only exhibit significant binding when they form a partnership with other receptors. Traditional ligand-receptor binding assays (e.g. microarray technology) cannot reflect such hetero-multivalency because they screen only one specific receptor at a time. Thus, the contribution of GM_2 was often ignored since CTB binding to pure GM_2 was only detected at the CTB concentration far beyond physiologically relevant conditions. To address this issue, previous studies have developed combinatorial arrays that mix two different receptors in 1:1 ratio.[9] However, this labor-intensive method cannot observe hetero-multivalent binding involving more than two receptors.

In order to efficiently discover receptor candidates for multivalent binding proteins, we designed a new membrane perturbation protocol. This protocol first involves constructing a membrane that contains all receptor candidates with known compositions as a reference. The reference membrane is then perturbed by increasing the density of a desired glycolipid receptor. If a specific receptor can either directly bind to the target protein or indirectly form a binding complex with the assistance of other glycolipids; the perturbation will alter the overall protein binding irrespective of the mechanism.

As a proof-of-concept, we constructed a reference membrane consisting of GM_1, GM_2, GM_3, fucosyl-GM_1, and GD_1b (0.25 mol% of each glycolipid). We then perturbed the reference membrane by increasing one of the glycolipid receptor to 2 mol%. The CTB binding to the reference membrane and each perturbed membrane is shown in Fig. 5. As expected, CTB binding was significantly enhanced when the densities of GM_1, fucosyl-GM_1, and GD_1b were increased. The positive binding cooperativity between GM_2 and the other glycolipids present in the reference membrane also enhanced the overall CTB binding. In addition, increasing GM_3 density did not enhance CTB binding. Thus, we could exclude GM_3 as a CTB receptor candidate without conducting the entire combinatorial array

measurement. In order to identify receptors of multivalent protein from a large library of molecules, this perturbation method can be more efficient than combinatorial glycolipid arrays.

Discussion

In this study, significant enhancement of CTB binding was observed when a strongly binding receptor was mixed with a weakly binding receptor (GM_2). When investigated further, the reduction of dimensionality looks like the most likely cause. If this mechanism is valid, a fraction of bound CTB should simultaneously bind to GM_2 and other strong binding receptors. Most recently, Klassen and his coworkers demonstrated the same heterogeneous binding cooperativity using catch-and-release electrospray ionization-mass spectrometry (CaR-ESI-MS) assay.[40, 41] Mass spectrometry allows identifying the types of receptors binding to CTB. Using CaR-ESI-MS assay, Klassen and his coworkers observed that CTB could bind to very weak binding receptors GM_2 and GM_3 when 7 different glycolipids (GM_1, GM_2, GM_3, GD_1a, GD_1b, GD_2, and GT_1b) were mixed in either picodiscs or micelles systems, but no binding was observed when GM_2 or GM_3 was the only receptor. Their results provide evidence that CTB can directly bind to weakly binding receptors when they are mixed with strongly binding receptors. It is worth noting that we did not observe binding cooperativity between GM_1 and GM_3, but Klassen and his coworkers observed CTB binding to GM_3. This is probably due to the difference of lipid bilayer conditions. In our experiment, surface density of glycolipid receptor was maintained at 1mol%. CaR-ESI-MS assay mixed 7 glycolipid receptors equally resulting in 14mol% of each glycolipid. The reaction enhancement via reduced dimensionality was higher in CaR-ESI-MS assay; thus, it is not surprising that Klassen and his coworkers observed CTB binding to GM_3.

Reduction of dimensionality provided a potential mechanism to answer a long-standing question, why CTB binding does not correlate with GM_1 level on cell surfaces.[42] Yanagisawa et al. observed strong reactivity between CTB and embryonic neuroepithelial cells in the absence of GM_1.[43] Kirkeby stained GM_1 with CTB and anti-GM_1 antibody, and found that both labeling reagents were not co-localized.[44] In addition, GM_1 is of very low abundance (0.0015–0.003 mol% of glycosphingolipids) in human small intestinal epithelial cells[45]; thus, a recent publication raised a question, whether GM_1 is sufficient to induce cholera toxin attachment.[46] In the reduction of dimensionality model, high-affinity receptors can serve as initiators, and then activate weak receptors, leading to higher retention of CTB on the cell surface. Thus, the overall CTB binding is not simply controlled by a single GM_1 receptor; the weakly binding receptors can contribute to CTB binding via reduction of dimensionality. Surface diffusion and local density of membrane receptors can influence the 2D reaction rate, membrane fluidity and heterogeneity (i.e. lipid raft) which can also play essential roles in CTB binding process.

The mechanism of reduced dimensionality has also been used to explain unexpected phenomena in various multivalent binding studies.[3, 8, 25] For example, Mazor et al. observed that the binding avidity of a bispecific antibody to receptors confined in cell membrane surfaces were significantly higher than the binding avidity to free receptors in solution.[47] Sengers et al. established a mathematical model based on the reduced

dimensionality hypothesis to describe the mechanism of bivalent antibody binding to heterogeneous membrane targets, and estimated that the effective affinity of bivalently bound antibody can be enhanced by approximately 4 orders of magnitude.[25] These studies, combined with our own CTB binding measurements suggest the importance of the role of reduced dimensionality in multivalent protein-cell membrane recognition. Further kinetic studies are necessary in order to verify the hypothesis and establish a comprehensive model of hetero-multivalent recognition.

Since the complex interplay between multiple membrane receptors is critical, we also developed a new membrane perturbation protocol to efficiently screen receptor candidates. This protocol measured CTB binding to perturbed membranes that preserve all receptor candidates; therefore, the interplay between different receptors can be monitored. This new protocol is more efficient in screening the potential receptors than the combinatorial array, which detects proteins binding to the binary mixture of glycolipids. For example, if we plan to screen 20 receptor candidates, the membrane perturbation protocol required only 21 measurements instead of 190 measurements in a combinatorial array.

Conclusion

In summary, we elucidated the essence of hetero-multivalency in CTB-cell membrane recognition using a high-throughput and easy-to-use nanocube sensors. We believe that the detection protocols presented here can provide a systematic and efficient strategy to investigate multivalent protein-cell membrane recognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A weak receptor can bind to CTB when present with a high-affinity receptor.
- Reduced dimensionality of receptor diffusion causes the hetero-multivalent binding.
- Fluidity of membrane plays an essential role in multivalent binding.
- A new ligand-receptor binding assay is required to explore the hetero-multivalency.
- A new protocol is developed to efficiently screen receptor candidates.

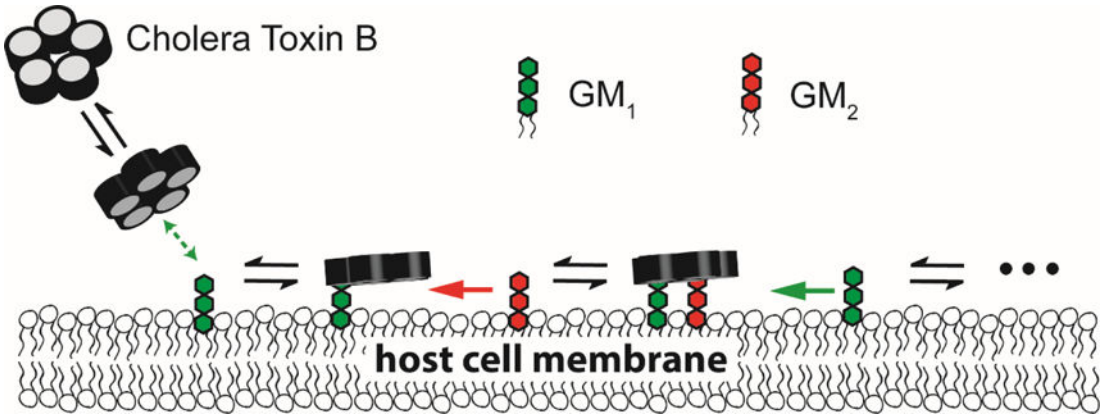

Fig. 1.

A schematic of the proposed CTB binding mechanism. CTB first diffuses from the solution phase to a membrane surface. One of its binding subunit finds a strongly binding receptor and then forms a relatively stable membrane bound state. Free glycolipid receptors diffuse two dimensionally, encounter the bound CTB, and then enable subsequent binding. The reaction rate on the 2D membrane surface is significantly higher than the rate in 3D bulk solutions. Thus, a weakly binding receptor, such as GM_2, can participate in subsequent binding, leading to an enhanced binding capacity.

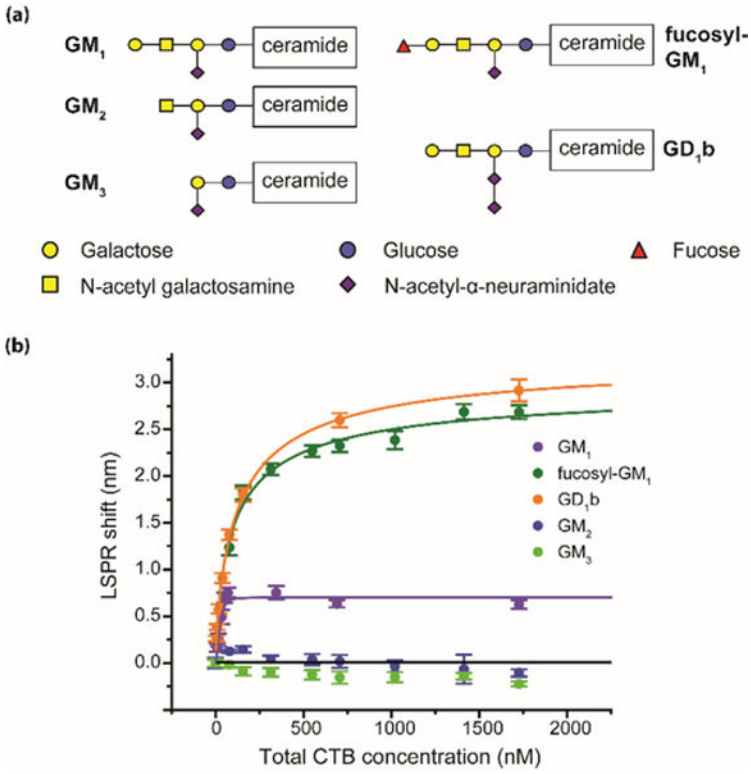

Fig. 2.

Homo-multivalent CTB binding. (a) Structures of glycolipids used in the study. (b) Equilibrium binding of CTB to pure glycolipids. The glycolipid composition in each case was 1 mol%. Data points are reported as mean ± S.D (n = 11).

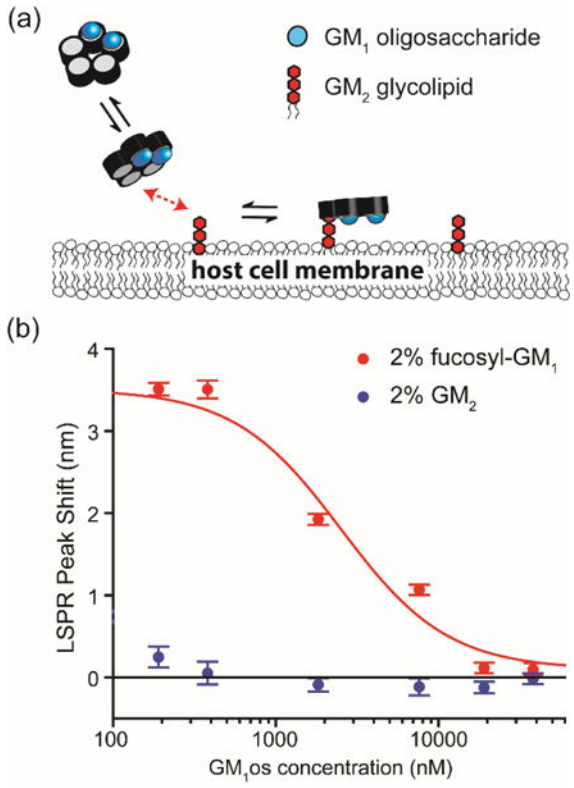

Fig. 3.

Evaluation of allosteric effect. (a) A schematic of the allosteric regulation hypothesis. CTB was incubated with GM_1os to form a GM_1os-CTB complex. Then, this GM_1os-CTB complex was bound to a model membrane containing GM_2. If GM_1os modulated the energetics of the adjacent CTB binding pocket, the attachment of GM_1os-CTB complex to the membrane containing GM_2 should be detectable. (b) Binding of CTB-GM_1os complex to membrane surfaces containing 2 mol% fucosyl-GM_1 and 2 mol% GM_2. Binding of CTB-GM_1os complex to the GM_2 surface was still negligible; thus, allosteric regulation may not be a major cause of the enhanced CTB binding.

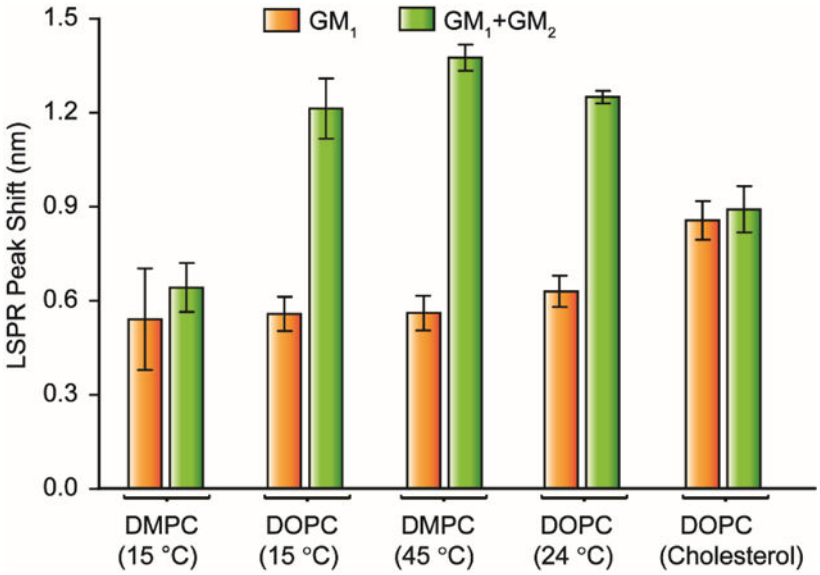

Fig. 4.

CTB binding to single glycolipid (orange) or paired glycolipids (green) in different membrane environments. (DMPC/DMPS (15 °C), DOPC/DOPS (15 °C), DMPC/DMPS (45 °C), DOPC/DOPS (room temperature) or DOPC/DOPS/cholesterol (room temperature)) The heterogeneous binding cooperativity between GM_1 and GM_2 depends on the fluidity and heterogeneity of membranes. Data points are reported as mean ± S.D (n = 11).

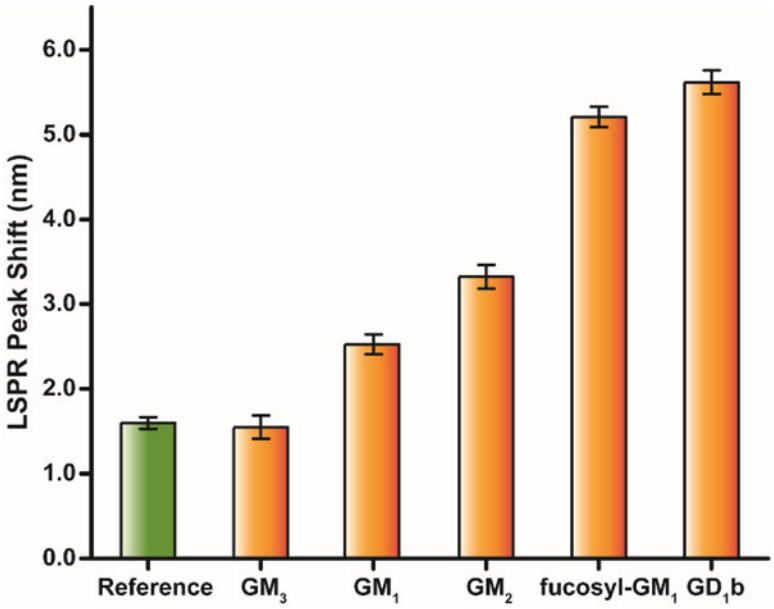

Fig. 5.

The demonstration of membrane perturbation protocol. 1726 nM CTB was bound to the reference and perturbed membranes that preserved all receptor candidates. The reference membrane contained 88.75 mol% DOPC, 10 mol% DOPS, 0.25 mol% of each GM1, GM2, GM3, GD1b and fucosyl-GM1. The reference membrane was perturbed by increasing the density of a specific glycolipid to 2 mol%. Data points are reported as mean ± S.D (n = 11).

Table 1

Calculated heterogeneous binding cooperativity between two glycolipids. Column and row headings represent the mixture of two glycolipids. Each cell contains two values that represent the calculated cooperativity at the two CTB concentrations, 706 nM (top)/1726 nM (bottom). Cooperativity values are reported as mean ± S.D (n = 11). The raw data of CTB binding was reported in Fig. S3–S4.

GM_1	**fucosyl-GM_1**	**GD_1b**	**GM_2**	**GM_3**	
	1.08 + 0.03 1.12 + 0.03	0.92 + 0.02 1.05 + 0.04	1.46 + 0.17 1.99 + 0.28	1.16 + 0.26 0.92 + 0.20	GM_1
		0.94 + 0.02 1.10 + 0.03	1.57 + 0.07 1.54 + 0.09	1.19 + 0.06 1.11 + 0.04	fucosyl-GM_1
			2.06 + 0.08 1.96 + 0.10	1.05 + 0.05 0.98 + 0.05	GD_1b
				1.00 + 0.77 1.00 + 0.12	GM_2
					GM_3