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Studies of the carbohydrate-carbohydrate interaction between lactose and GM_3 using Langmuir monolayers and glycolipid micelles

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This paper describes studies of the carbohydrate-carbohydrate interaction (CCI) between micelles of a lactosyl lipid and monolayers of the glycosphingolipid GM_3. The lactose Lac·GM_3 interaction is involved in B16 melanoma cell adhesion and signaling processes, and a thorough understanding of the molecular details of this CCI is important for the design of new anti-adhesive and anti-metastatic agents. In this paper, we examine the influence of variations in divalent cations and subphase ionic strength on the Lac·GM_3 interaction. Our results indicate that, in the absence of divalent cations, the Lac·GM_3 CCI is strengthened at higher sodium chloride concentrations in the subphase. In contrast, when divalent cations are present in solution, the CCI is not as sensitive to ionic strength. These results suggest a role for both cation dependent as well as independent interactions in the Lac·GM_3 CCI.
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Keywords: **membranes, cell adhesion, melanoma, calcium, ionic strength**

Abbreviations: **CCI—carbohydrate carbohydrate interaction; LacCer—lactosyl ceramide; LacC14—*N*-tetradecyl-O-lactosylglycolamide; MaltC14—*N*-tetradecyl-O-maltosylglycolamide; CelC14—*N*-tetradecyl-O-cellobiosylglycolamide; GalCer—galactosyl ceramide; CBS—cerebroside sulfate.**

Introduction

Interactions between cell surface glycoconjugates have been implicated in various cell adhesion events which occur in the course of cell development, metastasis, fertilization, myelin compaction, and sponge cell aggregation [1]. The adhesion of a B16 melanoma cell to an endothelial cell is mediated by a multivalent carbohydrate-carbohydrate interaction (CCI) between the melanoma cell surface ganglioside sialosyllactosylceramide (GM_3, **1**) and the endothelial cell glycolipid lactosylceramide (LacCer, **2**) [2–4]. The GM_3 mediated CCI of B16 cells occurs primarily from within glycolipid enriched microdomains within the lipid membrane [5,6]. These microdomains contain a variety of proteins and enzymes that have important signaling functions within the cell. Synthetic glycosphingolipids can perturb both the structure and function of the glycolipid signaling domain in B16 cells by reducing GM_3 clustering on the cell surface and diminishing signaling [7].

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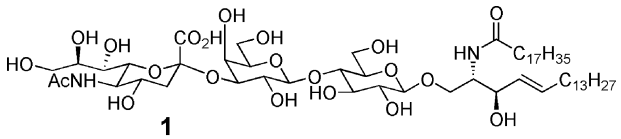

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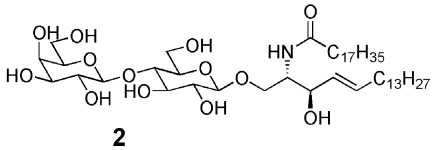

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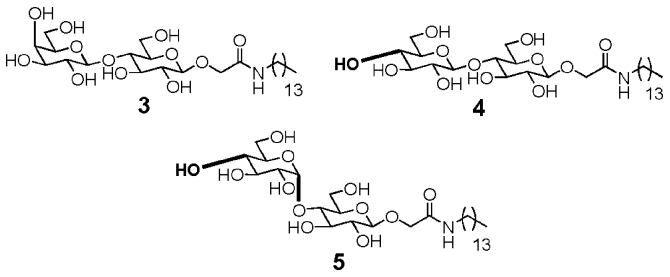
The LacCer·GM_3 interaction has been identified as a possible target for anti-metastatic cancer therapy [6]. A correlation between the metastatic potential of a cell and its ability to interact with lactose has been identified in four variants of B16 cell lines. The administration of LacCer containing liposomes or other multivalent lactosylated glycoconjugates to mice with aggressive melanoma reduces the development of metastatic lung tumors in the animals [6,8]. A recent report has demonstrated that lactosylated nanoparticles are also successful in inhibiting metastasis in a mouse model [9].

Various model systems for studying sugar-sugar interactions have been developed [10–12]. The CCI of GM_3 with Gg_3,

lactose, and other carbohydrates have been studied by examining the interactions between a glycopolymer and a glycolipid monolayer using Langmuir monolayers, a quartz crystal microbalance (QCM), and surface plasmon resonance (SPR) [13–15]. The latter methods permit quantitation of the association, and has also been used to study the $Le^x \cdot Le^x$ interaction [12] and CCI mediated sponge cell adhesion [16]. We have reported the development of a biophysical assay for studying CCI which uses Langmuir monolayers and glycolipid micelles as model systems [17]. Self-assembled structures such as monolayers and micelles permit the rapid preparation of multivalent carbohydrate presentations without extensive synthetic effort [18].

In our initial experiments using this model system, we prepared Langmuir monolayers composed of a 1:9 molar ratio of GM_3 and dipalmitoylphosphatidylcholine (DPPC). Micelles of the lactosyl lipid LacC14 (**3**) were injected into the subphase underneath the monolayer. The resulting CCI between LacC14 micelles and the monolayer causes a change in surface pressure ($\Delta\pi$) at the air-water interface, which is readily measured using a microbalance. The magnitude and pattern of these pressure changes is used to identify CCI.



Our initial studies showed that only LacC14 interacted with the GM_3 monolayer. This interaction is very specific, as very weak or no interactions are observed with the closely related compounds CelC14 (**4**) or MaltC14 (**5**), derived from cellobiose and maltose respectively. The association of LacC14 with the GM_3 monolayer was only observed above the critical micelle concentration (cmc) of LacC14, which is consistent with a multivalent interaction between the two glycolipids. Our results [17], as well as those from the Kobayashi group, indicate that these carbohydrates interact with each other in a very specific manner when presented in a multivalent format [13,15]. In the current paper, we examine the dual role of calcium ions and ionic strength in the CCI between LacC14 micelles and a GM_3 containing monolayer.

Experimental methods

The preparation of the glycolipid LacC14 has been previously described [17]. GM_3 and Tween-80 were obtained from Calbiochem. Phospholipids were obtained from Avanti. All salt solutions were prepared from their chloride salts. Monolayer binding experiments were performed on a Kibron μ trough S. A Kibron Teflon 4-well plate (well volume—3 mL) with a side-injection port was utilized. Monolayers were spread from a 9:1 chloroform:methanol solution until an equilibrium pressure of 30 mN/m was achieved. Once this pressure was reached, the subphase was stirred gently with a magnetic stir bar, and the monolayer was equilibrated for 5–10 min prior to micelle injection. The binding experiment was initiated by removing an aliquot of the subphase, equal in volume to that of the micelle aliquot to be injected, through the injection port. The micelle solution was then injected into the subphase under the monolayer. Injection volumes were either 15 μL or 25 μL. The final concentration of LacC14 or Tween-80 in the subphase was 25 μM. The change in pressure (π) from that at the time of injection ($\pi_{\Delta t=0}$) was monitored as function of the time elasped since injection (Δt). The $\Delta\pi$ value for the experiment was defined as the value of π at $\Delta t = 50$ min minus $\pi_{\Delta t=0}$ ($\Delta\pi = \pi_{\Delta t=50} - \pi_{\Delta t=0}$). All reported $\Delta\pi$ values are the average of at least two experiments.

Results

The change in surface pressure observed when a micelle interacts with a monolayer arises from insertion of glycolipids from the subphase into the extant monolayer. The magnitude of the insertion process (i.e. the $\Delta\pi$ value) is a function of both a specific CCI-derived event as well as a non-specific surfactant component. In order to separate these two different phenomena, we used Tween-80 micelles as a control surfactant for comparison purposes. Tween-80 is a non-ionic poly-hydroxy detergent with a cmc value similar to that of LacC14 [17]. Any changes in pressure observed with Tween 80 are solely derived from a non-specific surfactant interaction with the monolayer, not from CCI.

We determined the non-specific surfactant properties of LacC14 and Tween-80 prior to examining CCI between LacC14 and GM_3. This was accomplished by measuring both the intrinsic surface activity of these compounds as well as the $\Delta\pi$ values obtained upon their interaction with a pure DPPC monolayer. These results are shown in Table 1. The surface activity is a measure of the propensity of a compound to form a monolayer at the air-water interface. These were measured by monitoring the increase in surface pressure when a solution of micelles was injected into a water solution with no monolayer present. LacC14 and Tween-80 have comparable surface activities at low and high salt concentrations. Likewise, changes in surface pressure resulting from injection of LacC14 or Tween-80 under a monolayer of pure DPPC are also very similar.

Initial experiments examined the role calcium plays in the $LacC14 \cdot GM_3$ interaction. The interaction of LacC14 micelles or Tween-80 micelles with a GM_3/DPPC monolayer at various calcium concentrations was examined. The results of these experiments are shown in Figures 1 and 2. When the binding experiment is conducted using a subphase of water in the absence of calcium ions (Figure 1), a large $\Delta\pi$ is observed with Tween-80. However, the magnitude of this interaction steadily decreases as the calcium concentration is increased to 50 mM.

Table 1. Non-specific interactions of LacC14 and Tween-80

	Surface activity π [mN/m]		*Binding to 100% DPPC monolayer $\Delta\pi$ [mN/m]*	
Subphase	*1 mM $CaCl_2$*	*1 mM $CaCl_2$ 100 mM NaCl*	*1 mM $CaCl_2$*	*1mM $CaCl_2$ 100 mM NaCl*
LacC14	26.5	33.9	12.5	13.9
Tween 80	27.7	33.1	14.5	14.1

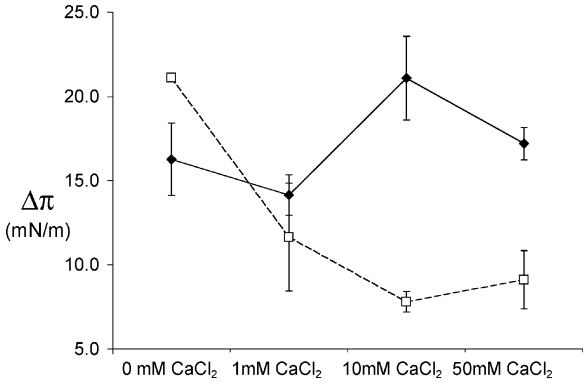


Figure 1. $\Delta\pi$ values as a function of calcium concentration. Subphase—Water (♦—LacC14; □—Tween-80).

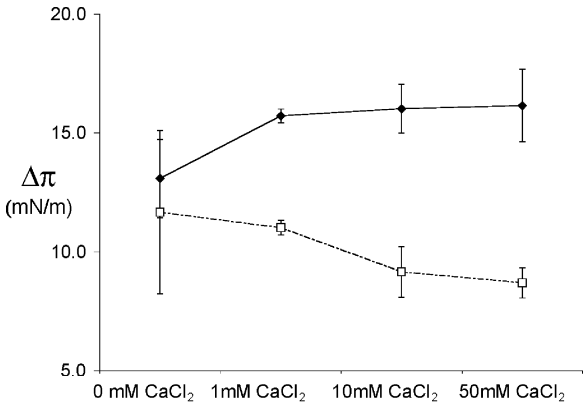


Figure 2. $\Delta\pi$ values as a function of calcium concentration. Subphase—100 mM NaCl (♦—LacC14; □—Tween-80).

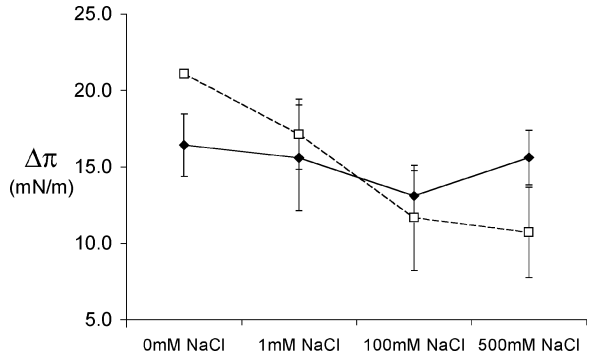


Figure 3. $\Delta\pi$ values as a function of NaCl concentration. Subphase—Water (♦—LacC14; □—Tween-80).

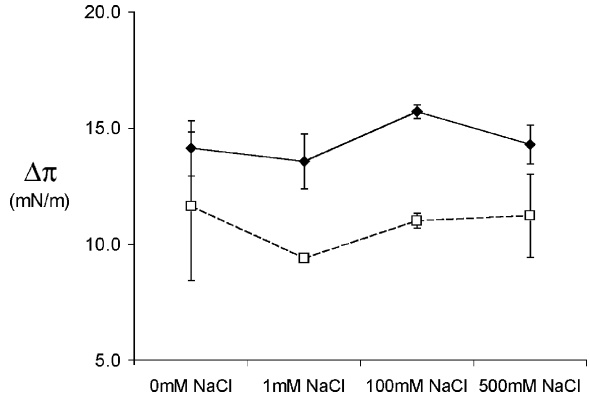


Figure 4. $\Delta\pi$ values as a function of NaCl concentration. Subphase—1 mM $CaCl_2$ (♦—LacC14; □—Tween-80).

In contrast, the $\Delta\pi$ for LacC14 consistently remains above 14 mN/m, and increases upon going from 1 mM to 10 mM in calcium.

When the corresponding set of experiments is conducted using a subphase of 100 mM NaCl (Figure 2) instead of pure water, a significant difference is observed. High salt concentration attenuates the effect of calcium for both LacC14 and Tween-80. In this case, there is a slight increase in the LacC14 responses in going from 0 mM to 1 mM in calcium, and higher calcium concentrations do not result in further significant changes. Tween-80 continues to exhibit diminishing responses as calcium concentration is increased, but the decline is not as steep as observed in the absence of NaCl.

We then further examined the effect of sodium chloride on the LacC14·GM_3 interaction, and the results of these studies are shown in Figures 3 and 4. The role of salt concentration in the absence of calcium was examined (Figure 3). As seen above, in the absence of calcium over a pure water subphase, Tween-80 interacts with the monolayer more strongly than LacC14. However, as the sodium chloride concentration of the solution is increased to 500 mM, the responses from Tween-80 consistently diminish, while those of LacC14 remains largely invariant. Several differences are observed when calcium chloride (1 mM) is present in the subphase (Figure 4). As before, the LacC14 consistently affords a stronger response than Tween-80 under

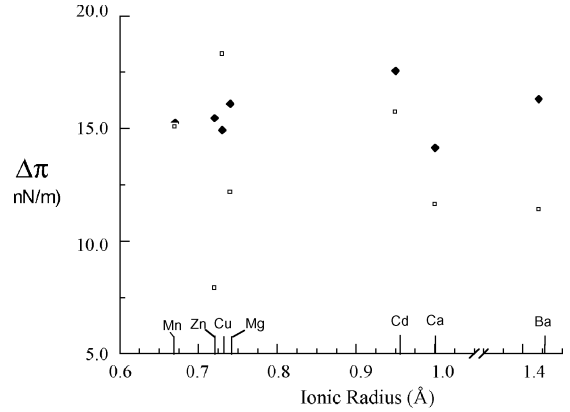


Figure 5. $\Delta\pi$ values as a function of divalent cation. Subphase—Water (♦: LacC14; □: Tween-80).

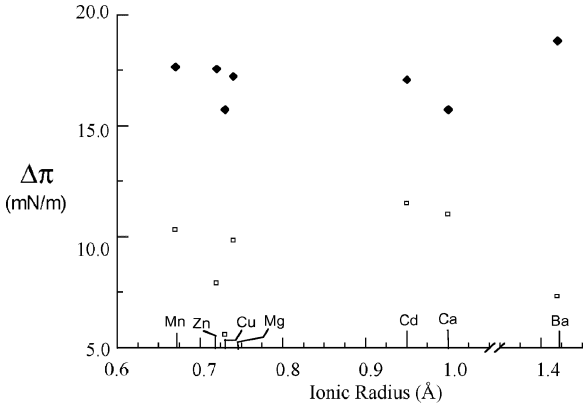


Figure 6. $\Delta\pi$ values as a function of divalent cation. Subphase—100 mM NaCl (♦: LacC14; □: Tween-80).

these conditions. Additionally, responses for both LacC14 and Tween-80 do not vary greatly with changes in sodium chloride concentration from 0 to 500 mM when calcium is present in solution.

In order to study whether the LacC14·GM_3 interaction was specific for calcium or whether an alternative divalent cation would suffice, the effect of varying the divalent cation present in solution was examined. Binding studies were performed with different cations in the subphase, both in the presence and absence of 100 mM NaCl. The interactions between LacC14 or Tween-80 and the GM_3 containing monolayer in the presence of various divalent cations (1 mM) are shown in Figure 5. In all but one instance, the $\Delta\pi$ for LacC14 is equal to or greater than the $\Delta\pi$ observed for Tween-80. While the responses from LacC14 are centered closely around a value of 15 mN/m, the responses from Tween-80 display a much wider range. When the effect of divalent cations is examined in the presence of 100 mM NaCl, in almost every case the responses from LacC14 are greater than those observed with pure water (Figure 6). In contrast, the responses from Tween-80 are universally lower. Additionally, the range of $\Delta\pi$ values is narrower for both LacC14 and Tween-80 with a 100 mM NaCl subphase.

Discussion

The $\Delta\pi$ values observed when LacC14 micelles are injected into the subphase under a GM_3/DPPC monolayer arise from both CCI-mediated and non-specific insertion of LacC14 molecules into the monolayer. Lipid insertion also occurs in the absence of GM_3, as LacC14 inserts into a monolayer of pure DPPC. However, Tween-80 inserts into a pure DPPC monolayer just as effectively as LacC14. Both Tween-80 and LacC14 have comparable surface activities and therefore have similar non-specific insertion behavior [19]. We therefore selected Tween-80 as a reference surfactant for our studies, since the magnitude of the Tween-80 response provides a measure of the surfactant, non-specific, component of the LacC14 response [20].

Initial binding experiments with varying calcium concentration indicated that over a subphase of pure water and in the absence of calcium, Tween-80 has a higher $\Delta\pi$ value than LacC14. This result suggests that the dominant interaction under these conditions is a non-specific surfactant response. However, the presence of calcium depresses the non-specific response while maintaining the strength of the LacC14 interaction. These results indicate that the LacC14·GM_3 CCI is enhanced in the presence of calcium, although the higher ionic strength may also be a factor at 50 mM $CaCl_2$. In the presence of 100 mM NaCl, CCI mediated insertion dominates over the non-specific process at all the calcium concentrations examined. The lack of a significant change in $\Delta\pi$ for either LacC14 or Tween-80 when calcium concentration is varied in 100 mM NaCl can arise from electrostatic screening of the divalent cation. These results demonstrate that calcium is involved in the LacC14·GM_3 interaction, when it is present. However, even in the absence of calcium, the observation that the LacC14 $\Delta\pi$ is larger than that for Tween-80 at high NaCl concentrations indicates that salt concentration also contributes to CCI mediated insertion. Taken together, these two sets of experiments illustrate that both bulk electrolye concentrations as well as calcium ions affect the CCI between LacC14 micelles and GM_3 containing monolayers. In subsequent experiments, we focused on each of these components individually.

A systematic comparison of the interactions of LacC14 and Tween-80 with the GM_3 monolayer as a function of increasing sodium chloride concentration in the absence of calcium indicated that higher concentrations enhance CCI, as seen in Figure 3. In contrast, the CCI appears to be less sensitive to sodium chloride concentration in the presence of 1 mM calcium (Figure 4). This is better illustrated by comparing the differences in $\Delta\pi$ ($\Delta\Delta\pi$), between LacC14 and Tween-80. The term $\Delta\Delta\pi$ is defined as: $\Delta\Delta\pi = \Delta\pi_{\text{LacC14}} - \Delta\pi_{\text{Tween-80}}$. The value of $\Delta\Delta\pi$ defines a measure of the contribution of the specific CCI to the observed response, as it subtracts out the non-specific surfactant contribution. The $\Delta\Delta\pi$ values are shown below in Figure 7.

The $\Delta\Delta\pi$ dependence on NaCl concentration in the absence of calcium clearly implicates ionic strength as playing a role in

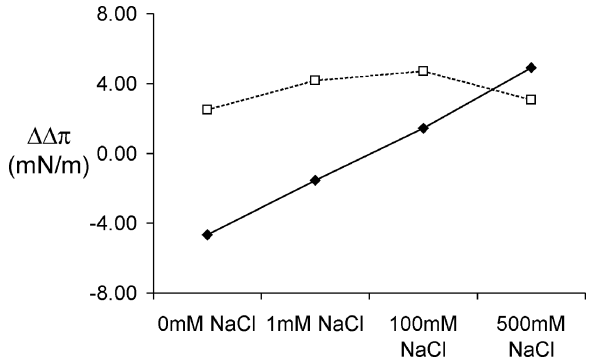


Figure 7. $\Delta\Delta\pi$ values as a function of NaCl concentration. (♦—Water Subphase; □—1 mM $CaCl_2$ Subphase).

this CCI. If calcium is not involved in the interaction, the $\Delta\pi$ values between Figures 1 and 2 or Figures 3 and 4 would not be expected to differ much. That there are significant differences indicates that calcium does play a role when it is present. These findings show that both calcium and ionic strength affect the LacC14·GM_3 interaction under our experimental conditions.

Boggs has found that the strength of the GalCer·CBS interaction is inversely correlated to the ionic radius of the divalent cation involved [10]. We find no such correlation in our case. The invariance of the LacC14 $\Delta\pi$ to variations in the divalent cation suggests that the identity of the cation does not matter, as long it is present. Hakomori has shown that calcium dependent adhesion of GM_3 coated liposomes to a Gg_3 coated surface is only partially inhibited by the addition of EDTA [21]. In contrast, the adhesion of the same GM_3 liposomes to immobilized Gg_3 containing liposomes does not occur in the absence of calcium, and Kobayashi has reported that calcium has no observable effect in their studies of the GM_3 interaction [13]. These results indicate that the manner in which carbohydrates are presented is important when studying these interactions.

Potential roles for calcium in CCI include coordination to the sialic acid of GM_3 and the *cis* oriented 3- and 4-hydroxyl groups in lactose [22–26]. Identifying calcium binding sites on carbohydrates is challenging because of the weak association constants, although several models have been proposed for the position of calcium in the Le^x·Le^x interaction [27,28]. Sasaki has reported evidence for lactose·calcium complexation in a glycosylated peptide [29]. Boggs has suggested in the GalCer·CBS interaction that calcium induces a conformational change in the glycolipid [30]. It is interesting to note that both the GalCer·CBS and LacCer·GM_3 interactions involve one anionic and one neutral partner, but do not always display an absolute requirement for calcium. In contrast, the Le^x·Le^x interaction involves two neutral partners and has an absolute calcium requirement in model systems examined thus far [11,12,27,28, 31,32].

The effect of sodium chloride concentration is consistent with interactions between hydrophobic components of sugars as an important component of CCI [21,27,32]. Additionally, high ionic strengths also decrease electrostatic repulsion between neighboring GM_3 molecules and alter the presentation and clustering of this glycolipid within the monolayer [33,34]. Higher ionic strengths lead to increased aggregation of GalCer and CBS containing liposomes [10]. Studies of glycolipid bilayer adhesion using the surface force apparatus [35] and X-ray/osmotic stress [36] analysis have provided evidence of van der Waals and other attractive interactions between glycolipids on the apposing bilayers. Sasaki has recently shown that attractive lactose-lactose interactions driven by carbohydrate desolvation are strong enough to influence a peptide folding equilibrium [29]. Direct evidence for hydrophobic contacts also comes from NOE contacts identified in the Le^x·Le^x interaction [27]. In this context it is worth noting that many lectins utilize hydrophobic aromatic residues to make contacts with bound sugars [37].

In summary, we have shown that both calcium concentration and sodium concentration influence the CCI between lactose and GM_3. Under the appropriate conditions, both of these parameters maximize the specific CCI contribution to the interaction between LacC14 micelles and a GM_3 containing monolayer, while simultaneously minimizing non-specific insertion processes. We find that when divalent cations are absent, increases in bulk salt concentration significantly increase the CCI component of $\Delta\pi$ while simultaneously decreasing non-specific insertion. In contrast, when divalent cations are present, the CCI component dominates regardless of the salt concentration. Changes in cation or ionic strength can affect CCI in a *cis* (*i.e.* within glycolipids in the same membrane) or *trans* (*i.e.* within glycolipids on apposing membranes) manner. Distinguishing between *cis* interactions and *trans* interactions is a particular challenge in studies of CCI [38]. *Cis* interactions in Langmuir monolayers can be examined by analysis of monolayer compression isotherms or directly by microscopy of the monolayer at the air-water interface.

Although molecular details of the LacCer·GM_3 interaction remain elusive, several generalizations about CCI have begun to emerge. Calcium ions bound to carbohydrates located at the air-water interface can function as a locus around which the interacting carbohydrate residues are organized. Although the calcium carbohydrate interaction is weak, molecular recognition events such as metal ligand associations and hydrogen bonds are stronger at interfaces than in bulk aqueous solution [39,40]. Additional driving force for association can be derived from contacts between the hydrophobic components of the interacting oligosaccharides. In the case of the Lac·GM_3 interaction, the role of calcium is not critical, and presumably hydrogen bonding and non-polar interactions suffice to mediate association under some circumstances. Further study is needed to determine whether the cation is intrinsic to the native association or whether is it is an artefact of the particular model system being used. This highlights the need for multiple approaches and techniques for studying supramolecular interactions such as CCI.

Acknowledgment

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20 The glycolipid MaltC14, which has a similar surface activity (22 mN/m at 25 μM, subphase = 1 mM $CaCl_2$) to LacC14 was also considered as a reference glycolipid, as it too does not interact with GM_3 via a CCI. Unfortunately, MaltC14 has a weak non-specific insertion component ($\Delta\pi$ for insertion into a 100% DPPC monolayer: 6.4 mN/m, subphase = 1mM $CaCl_2$). The use of MaltC14 as a reference could potentially result in overestimation of the CCI component of the LacC14 response. We elected to err towards a conservative estimate of CCI and selected Tween-80 as the reference compound, although this may result in an underestimation of contribution of CCI in some instances.
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