Cell Adhesion

The Natural Lewis^X-Bearing Lipids Promote Membrane Adhesion: Influence of Ceramide on Carbohydrate–Carbohydrate Recognition

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Carbohydate–carbohydrate recognition has recently emerged as a potentially important interaction in cell adhesion processes.[1] One carbohydrate, the Lewis^X determinant (Le^X), is involved in murine embryogenesis,[2] although the precise mechanism underlying this role is as yet unclear. Ca^{2+}-mediated homotypic interaction between two Le^X determinants has been proposed to initiate cell adhesion during the compaction stage of the embryo.[3,4] Several recent studies support the existence of such calcium-mediated homotypic recognition[5,6] and have also provided a body of information on the geometry, structural requirements,[7–9] and energetics[10–12] of a Le^X–Le^X interaction. However, in these studies the local environment of the Le^X was always very different from that existing at a typical cell surface. In cells, the Le^X-bearing molecules are usually composed of a ceramide connected to the Le^X trisaccharide through a lactose group. This geometry considerably restricts the possible orientations of the Le^X [13] compared to those of soluble forms,[5,7,8] or to the large freedom in orientation provided by long flexible spacers.[5,7–9,14] The ceramide in the natural Le^X-bearing molecules may therefore have a very strong influence on the recognition of Le^X borne by opposite cells, by inhibiting or enhancing the recognition. To test more directly the hypothesis that Le^X could serve as a promoter for cell adhesion, the challenge is to determine if the natural Le^X-bearing molecules allow the Le^X–Le^X recognition between two membranes. Two natural glycosphingolipids have been synthesized for this purpose.

The first one, called CerLLe^X, is composed of a Le^X trisaccharide (Galβ1→4[Fucα1→3]GlcNAc) attached to a ceramide (Cer) unit (two hydrophobic tails: one sphingosine and one stearic acid) through a lactose (L) group (Figure 1a). The second one, CerLLe^a, is used as a control and is composed of the same ceramide and lactose moieties, but has a Lewis *a* (Le^a) trisaccharide as headgroup instead of a Le^X determinant. Le^a is an isomer of Le^X, and the only difference between the two determinants is the position of the

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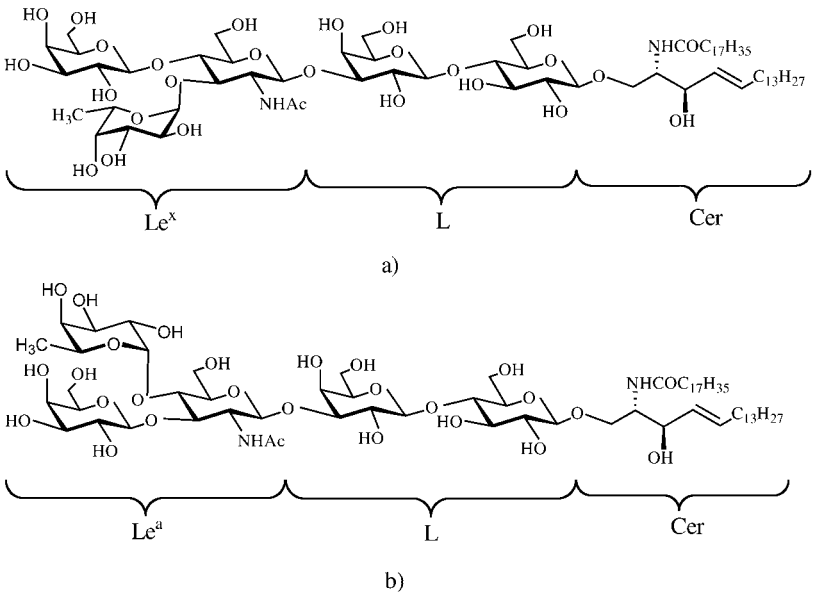


Figure 1. a) CerLLe^X: the Le^X determinant is a trisaccharide (Galβ1→4[Fucα1→3]GlcNAc). In a classical natural sphingolipid, it is attached to the ceramide (Cer) through a lactose (L) group. b) CerLLe^a: the Le^a determinant differs from the Le^X trisaccharide in the position of the fucose and galactose groups which are inverted. In these molecules the ceramide moieties impose an orientation on the headgroup that is perpendicular to the axis of the sphingosin.

fucose and galactose residues (Galβ1→3[Fucα1→4]GlcNAc) which are permuted (see Figure 1b). Both molecules are neutral.

This study involves two vesicles in tight contact, to simulate the geometry of two cells at the compaction stage. They are composed of a 1:9 mixture of glycosphingolipid and stearoyl-oleoylphosphatidylcholine (SOPC) and are referred to by the name of the glycolipid that they bear (CerLLe^X or CerLLe^a). CerLLe^X and CerLLe^a have two saturated chains, so one can expect the formation of domains in the vesicle membrane. We performed monolayer compression isotherm measurements of pure CerLLe^X, pure CerLLe^a, pure SOPC, and pure Le^X neoglycolipid,[11] which has three ramified chains (Figure 2). The ceramide moiety causes pure CerLLe^X and CerLLe^a monolayers at an air/water interface to undergo a phase transition upon compression, which indicates that clustering of glycolipids can occur at the membrane surface that influences Le^X–Le^X recognition.[15] By contrast, we observed that monolayers composed of a 1:9 ratio of CerLLe^X or CerLLe^a and SOPC show the same liquid shape as SOPC or Le^X neoglycolipid. This result is consistent with an NMR study on the effect of ceramide on phosphatidylcholine membranes, in which no phase separation was observed at either ambient or physiological temperatures for ceramide concentrations smaller than 15 mol%.[16] In the 1:9 glycosphingolipid/SOPC vesicles, the two components are therefore homogeneously mixed.

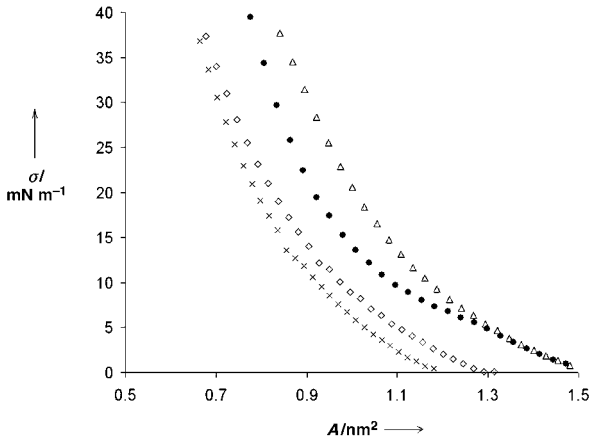


Figure 2. Isotherm compression measurements of lipid monolayers at an air/water interface. σ is the surface tension and A is the molecular area; pure SOPC (x), 1:9 SOPC/CerLLe^X (or CerLLe^a) mixture (◇), pure Le^X neoglycolipid (△),[11] and pure CerLe^X or CerLe^a (●).

The adhesion energies of CerLLe^X–CerLLe^X and CerLLe^X–CerLLe^a vesicle pairs were measured in NaCl and $CaCl_2$ aqueous solutions using a micropipette manipulation technique. This technique and the experimental conditions have been extensively described elsewhere.[11] Briefly, two vesicles (either both CerLLe^X, or one CerLLe^X and one CerLLe^a) in separate micropipettes are aspirated and brought into contact by displacement of the pipettes (Figure 3). The aspiration pressure in the pipettes

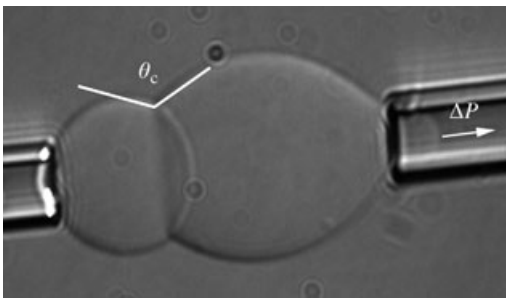


Figure 3. The two osmotically controlled vesicles held in micropipettes by aspiration are observed by interference contrast microscopy. The suction pressure applied to the micropipettes allows control of the tension of the vesicle bilayers. One of them (left) is pressurized into a tight, rigid sphere with large bilayer tension, whereas the adherent vesicle (right) is held with low pressure and remains deformable. The adhesion energy W_{adh} is obtained by determining the contact angle θ_c of the two vesicles and the tension τ_m of their membrane.[11]

controls the mechanical tension of the vesicle membrane. Conditions are set such that one of the vesicles is pressurized into a tight, rigid sphere with large bilayer tension, whereas the other is held with low pressure and remains deformable. The adhesion energy W_{adh}[17] is obtained by determining the contact angle θ_c of the two vesicles (Figure 3) when equilibrium is reached. The appropriate relationship can be written as: $\Delta P = C W_{adh}$, where ΔP is the pressure applied in the pipette controlling the flaccid vesicle and parameter C depends only on the radius of the micropipette (r_p), the radius of the vesicle (r_v), which can both be measured, and θ_c. The value of θ_c was numerically deduced from geometrical

parameters[18] and was measured for several tension values of the flaccid vesicle membrane by decreasing and then increasing the aspiration to check the reversibility of the adhesion. The adhesion results for CerLLe^X–CerLLe^X and CerLLe^X–CerLLe^a pairs are displayed in Figure 4 as plots of ΔP versus C; W_{adh} is the slope of the linear regression. The adhesion energy values are reported in Table 1.

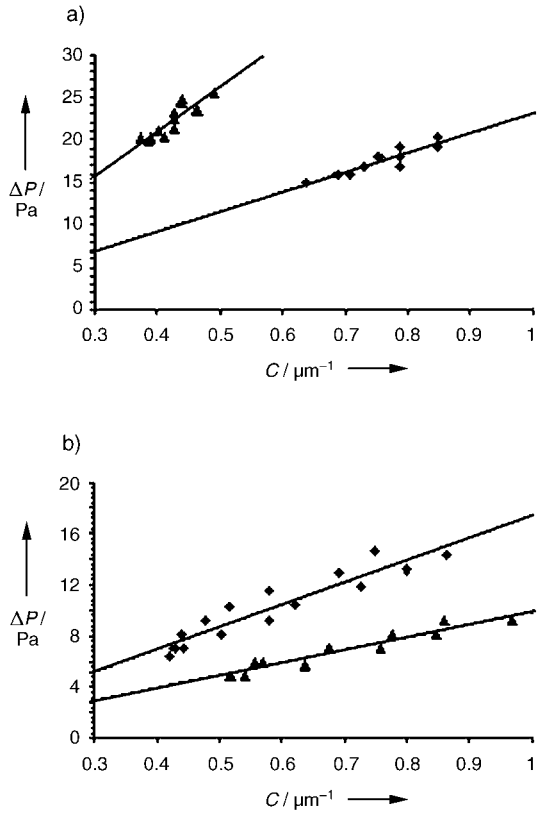


Figure 4. Aspiration pressure (ΔP) as a function of parameter C: a) CerLLe^X–CerLLe^X experiment (two vesicles with SOPC/CerLLe^X, 9:1); b) CerLLe^X–CerLLe^a experiment (one vesicle is SOPC/CerLLe^X, 9:1, and the other is SOPC/CerLLe^a, 9:1); $CaCl_2$ solution (▲) and NaCl (◆). The straight lines are least-squares fits.

Table 1: Adhesion energy of vesicle pairs in $CaCl_2$ or NaCl aqueous medium.

	Adhesion energy [μJ m^{-2}]	
Left vesicle–right vesicle	in $CaCl_2$ (0.11 M)	in NaCl (0.2 M)
CerLLe^X–CerLLe^X	67.8 ± 24.0	26.6 ± 2.1
CerLLe^X–CerLLe^a	15.1 ± 6.6	21.4 ± 5.8

CerLLe^a differs from CerLLe^X by only a structural isomeric change of the sugar headgroup, so interactions between two CerLLe^X vesicles or one CerLLe^X and one CerLLe^a vesicle should be equal unless there are specific effects. Nonspecific interactions (van der Waals attraction, Hefrich undulations, steric repulsions etc.) for the two systems are similar, as confirmed by the results obtained in a NaCl environment (Table 1) where, as expected, the substitution of the Le^X by Le^a has no significant effect on adhesion. By contrast, the adhesion energy of the CerLLe^X–CerLLe^X pair increases significantly in the presence of calcium ions, whereas that of the CerLLe^X–CerLLe^a pair actually decreases slightly. The strong specific enhancement obtained for the CerLLe^X–CerLLe^X pair proves that two Le^X determinants borne by natural molecules inserted in lipid bilayers can indeed recognize each other and produce additional adhesion.

The specific adhesion energy (W_{spe}) caused exclusively by Le^X–Le^X recognition can be extracted from the measured adhesion energies for the vesicular interactions. As shown in Equation (1), W_{spe} is given by the difference between the

$$W_{spe}^{(Le^X-Le^X)} = W_{adh}^{(Le^X-Le^X)_{CaCl_2}} - \left[W_{adh}^{(Le^X-Le^X)_{NaCl}} + \left(W_{adh}^{(Le^X-Le^a)_{CaCl_2}} - W_{adh}^{(Le^X-Le^a)_{NaCl}}\right)\right] \quad (1)$$

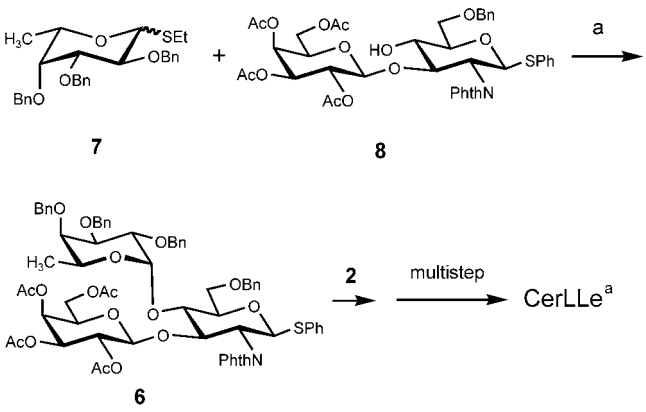
adhesion energy measured with calcium ions and that contributed by all other (nonspecific) interactions. The specific adhesion energy is about 47.5 μJ m^{-2}. In similar experiments performed on vesicles made of SOPC and a Le^X neoglycolipid mixed in the same 9:1 proportion,[11] the specific adhesion energy was only one-fifth ($\approx$9.5 μJ m^{-2}) of the value reported here with the natural molecule. What could be the explanation for such a large discrepancy?

The surface density (ρ) of molecules involved in Le^X–Le^X recognition can be determined directly from W_{spe}:[19] $\rho = W_{spe}/k_BT$. In this expression, ρ depends not only on the surface density of the glycolipids on each vesicle, but also on the Le^X accessibility and therefore on the architecture of the Le^X-bearing molecule. The surface densities were equal for both natural (CerLLe^X) and neoglycolipid systems. However, these glycolipids present some differences in their aliphatic tails. The natural molecule is based on a ceramide, whereas the neoglycolipid used in the previous study was composed of three alkyl chains linked to a long flexible spacer.[20] In the latter case the spacer provides the Le^X group with a high orientational freedom, whereas in CerLLe^X the rigid connection between the sugar headgroup and the ceramide restricts the possible conformations of the Le^X group.[13] The affinity of two Le^X groups for calcium ions depends strongly on their relative positions.[8] Therefore, the relative orientation of two Le^X groups is a predominant factor in Le^X–Le^X recognition. The specific adhesion energies experimentally obtained show that although the ceramide restricts the number of spatial orientations accessible to the Le^X group, the proportion of those suitable for Le^X–Le^X recognition is higher. This is possible only if the orientations provided by the ceramide chains in the natural molecule enhance Le^X–Le^X recognition.

The choice of the Le^a determinant as a control highlighted both the specificity of Le^X–Le^X interaction and the very high sensitivity of the recognition to structural changes. The weak adhesion energy obtained for the CerLLe^X–CerLLe^a pair with $CaCl_2$ salt shows clearly that the permutation of the fucose and galactose residues in the trisaccharide headgroup effectively prevents specific adhesion (Table 1) and therefore demonstrates that the molecular recognition involved is

highly specific. In another carbohydrate couple, lactose–GM3, some hints on the high sensitivity of recognition to molecular structure were obtained through surface tension measurements.[21] Taken together, these results illustrate the wealth of specific interactions that carbohydrates can provide through their wide variety of structures and spatial orientations.

In summary, Ca^{2+}-dependent specific adhesion was firmly established for natural Le^X-bearing molecules inserted in fluid bilayer membranes. The choice of the Le^a determinant as the control molecule underscored the high sensitivity of Le^X–Le^X recognition to molecular structure. Moreover, the vesicle adhesion energy experiments demonstrate that in a geometry akin to that of a cell membrane, the possible orientations provided by natural Le^X-bearing molecules not only allow but also strongly favor Le^X–Le^X recognition.



7 8 6

Scheme 2. Reagent: a) NIS/TfOH, toluene, 82%.

Experimental Section

The synthesis of $CerLLe^X$ is depicted in Scheme 1. The previously prepared trisaccharide **1**[22] was condensed with the known diol **2**[23] to give regio- and stereoselectively the pentasaccharide **3** in 90% yield. After a sequence of deprotection and protection reactions, the obtained imidate **4** was coupled with azidosphingosine **5**[24] to afford a glycoside in 57% yield. Selective reduction of the azide group, followed by condensation with octadecanoic acid, gave an acylated Le^X compound, which was *O*-deacylated to provide the $CerLLe^X$ in 95% yield.

Similarly, $CerLLe^a$ was synthesized using the donor **6** instead of compound **1**. This trisaccharide was prepared in 82% yield by condensation of **7** and **8** (Scheme 2).

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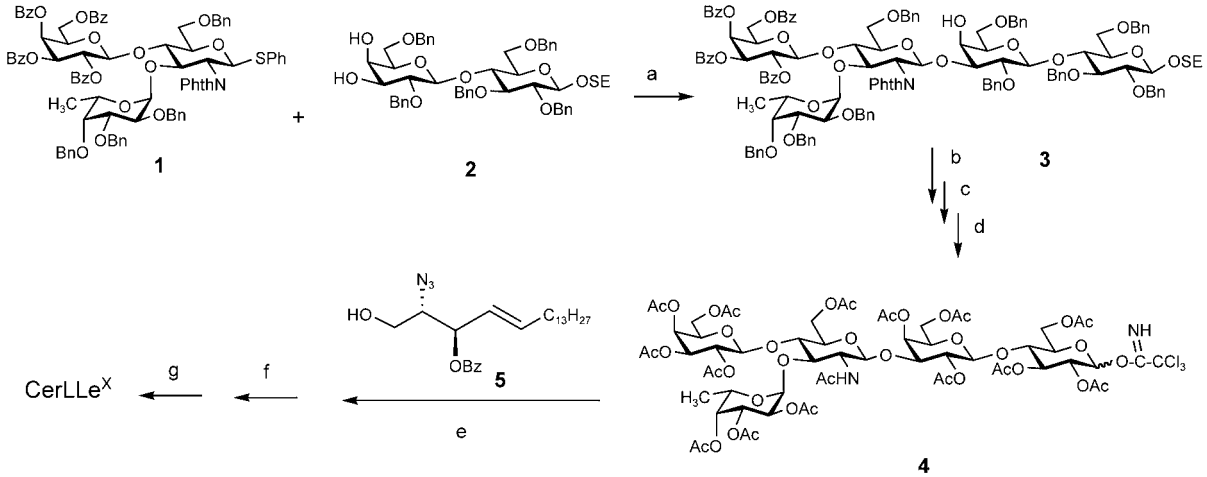

1 2 3 4 5

Scheme 1. Reagents: a) NIS/TfOH, CH_2Cl_2, 90%; b) 1. NH_2NH_2, EtOH, 2. Ac_2O, CH_2Cl_2, MeOH 78%; c) 1. H_2, Pd/C (10%), MeOH, EtOAc, 2. Ac_2O/Py, DMAP, 74%; d) 1. CF_3CO_2H, CH_2Cl_2, 2. CCl_3CN, DBU, CH_2Cl_2, 80%; e) TMSOTf, CH_2Cl_2, 57%; f) 1. PPh_3, benzene, H_2O, 2. octadecanoic acid, WSC, CH_2Cl_2, 72%; g) NaOMe, MeOH, 95%. Bz = benzoyl, Bn = benzyl, Phth = phthaloyl, SE = trimethylsilylethyl, NIS = *N*-iodosuccinimide, Tf = triflate, DMAP = 4-dimethylaminopyridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TMS = trimethylsilyl, WSC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

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