Evidence of Ca^{2+}-Dependent Carbohydrate Association through Ion Spray Mass Spectrometry

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Abstract: The interactions of the cell-surface carbohydrate, Le^x (Gal-β-1→4-(Fuc-α-1→3)-GlcNAc) and the Le^x-containing glycosphingolipid, Le^x-LacCer, and their analogues were examined by ion spray mass spectrometry. Both Le^x and Le^x-LacCer complexed the divalent cations, Ca^{2+}, Mn^{2+}, and Mg^{2+}, to form $[nLe^x + \text{cation}^{2+}]^{2+}$ and $[nLe^x\text{–LacCer} + \text{cation}^{2+}]^{2+}$ (n = 1, 2, or 3) with greater stability observed for the dimer (n = 2) relative to the trimer (n = 3). No evidence of association was obtained when these compounds were analyzed under the same conditions in the presence of monovalent cations, Li^+, Na^+, and K^+. Collision-induced decomposition (CID) experiments were performed on the noncovalent dimers, $[2Le^x\text{–LacCer} + Ca^{2+}]^{2+}$ and $[2Le^x + Ca^{2+}]^{2+}$, with results indicating that the Ca^{2+} complexation site was within the Le^x moiety. Furthermore, CID results implied, through the loss of one or both fucose residues from the intact dimers, that both fucose residues may be exposed on the Le^x and Le^x–LacCer dimers. Le^x–LacCer was also found to associate with LacCer, GalCer, or Cer in the presence of the divalent cations. CID studies further implied that the binding affinity of Le^x–LacCer with Le^x–LacCer was greater than that with LacCer, and decreased in order with GalCer and Cer. Space-filling models and energy minimization calculations of Le^x in its solution conformation with Ca^{2+} affirmed that the molecules may bind through Ca^{2+} in a homotypic interaction. This predilection for homotypic Le^x interactions may correlate to the biological utility of this surface carbohydrate in the cellular adhesion process.

Introduction

The interaction of glycosphingolipids during cellular adhesion has been suggested[1-4] to occur prior to protein–carbohydrate and protein–protein interactions. Specifically, Lewis x (Le^x) (Gal-β-1→4-(Fuc-α-1→3)-GlcNAc) on the embryo cell surface at the 8-32 cell (morula) stage[5] is correlated with the onset of compaction. Since the compaction event is inhibited by Le^x haptens,[6] the Le^x determinant expressed on the morula stage embryos has been suggested to be recognized by other cell-surface Le^x sugars in homotypic cell interactions. Hakomori et al.[7] have demonstrated the association of liposomes conjugated with the Le^x-containing glycolipid and that this interaction is Ca^{2+} dependent.

The function of Ca^{2+} in these interactions is not clearly understood; however, its incorporation into this model for cellular interaction uniquely qualifies mass spectrometric analysis to investigate its possible role. Ion spray mass spectrometry has recently been shown to be useful in detecting noncovalent protein complexes[8-10] through its capacity to observe multiply charged ions and intact native noncovalently bound oligomers. In addition, recent results[11] have indicated that the gas-phase ions formed under ion spray conditions may reflect the solution-phase properties.

Le^x glycosphingolipids exist on cell surfaces often with extreme heterogeneity in their carbohydrate structures. Recent advances in synthetic organic chemistry[12] have been utilized to produce homogeneously pure glycosphingolipids as well as the carbohydrate Le^x, for use to simulate biological conditions without obscuring the mass spectral data with this heterogeneity. Thus, the availability of these substances and the capabilities of ion spray mass spectrometry applied with collision-induced decomposition (CID) have allowed us to investigate and further explore the nature of these cation-dependent carbohydrate–carbohydrate interactions.

Results and Discussion

Association of Le^x and Le^x–LacCer in the Presence of Divalent Cations. The ion spray mass spectra of Le^x {(Gal-β-1→4-(Fuc-α-1→3)-GlcNAc-O-allyl, $C_{23}H_{39}O_{15}N_1$, monoisotopic mass = 569.2} and Le^x–LacCer ($C_{68}H_{124}O_{27}N_2$, monoisotopic mass = 1400.8) were acquired under a variety of conditions. Analyses were performed in aqueous, aqueous/methanol, and methanol solutions containing either the monovalent or divalent cations of Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, and Mn^{2+}. The effect of varying declustering potential was also explored.

Le^x was analyzed in both aqueous and neat methanol solutions with no discernible differences in the results. As expected,[13] the methanol solvent promoted greater signal intensities and therefore higher sensitivity. The glycosphingolipids were analyzed in aqueous, aqueous/methanol, and neat methanol solutions. It was found that insufficient solubility of the glycosphingolipids in

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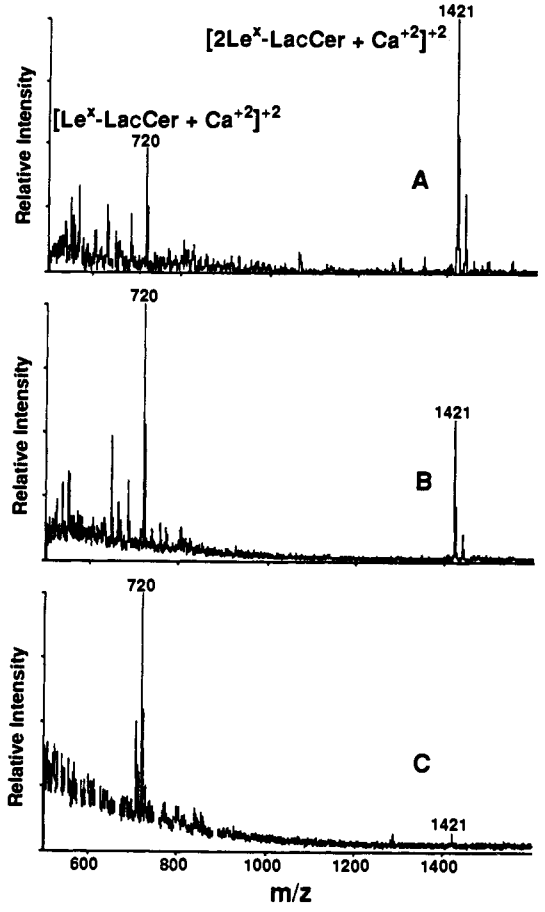

Figure 1. Ion spray mass spectra of Le^x–LacCer with Ca^{2+} at declustering potentials of (A) 250 V, (B) 150 V, and (C) 75 V; monomer = [Le^x–LacCer + Ca^{2+}]$^{2+}$ and dimer = [2Le^x–LacCer + Ca^{2+}]$^{2+}$.

aqueous solutions made mass analysis impossible. However, analyses were successfully performed in up to 30% aqueous/methanol solutions with results identical (albeit lower intensities) with those obtained with the methanol solution. At greater than 30% aqueous/methanol concentrations, no Le^x–LacCer ions were detected.

The ion spray mass analysis of Le^x (in water) and Le^x–LacCer (in methanol) with the monovalent cations resulted in spectra of the monomer ions, [Le^x + cation$^+$]$^+$ or [Le^x–LacCer + cation$^+$]$^+$, with no evidence of association (e.g., [2Le^x + cation$^+$]$^+$ or [2Le^x–LacCer + 3cation$^+$]$^{3+}$ were not observed). In contrast, when analyses were performed with the divalent cations, monomers, dimers, and trimers, [nLe^x + cation^{2+}]$^{2+}$ and [nLe^x–LacCer + cation^{2+}]$^{2+}$ (n = 1, 2, or 3), were observed; the monomer ions were also observed as [Le^x + cation^{2+}–H$^+$]$^+$ and [Le^x–LacCer + cation^{2+}–H$^+$]$^+$. The intensities of all of these ions were highly dependent on the declustering potential.

The association of Le^x significantly improved at the lower declustering potentials, while at the higher potentials the monomer dominated the spectrum. This effect has been noted by Baca and Kent[9a] with HIV-1 protease ternary complexes where dissociation of the noncovalent complex occurred at high potentials. Contrary to this dependence, the intensity of the Le^x–LacCer oligomers (dimer and trimer), relative to the monomer, increased as the declustering potential was increased (Figures 1 and 2). This increase in oligomer formation may initially appear to be counterintuitive; however, it may be related to the formation of micelles considering the nonpolar lipid and the highly polar carbohydrate functionalities of Le^x–LacCer. Thus, the higher collisional energies resulting from increasing the declustering potential would promote micelle dissociation apparently to a stable component of the micelle, the dimer.

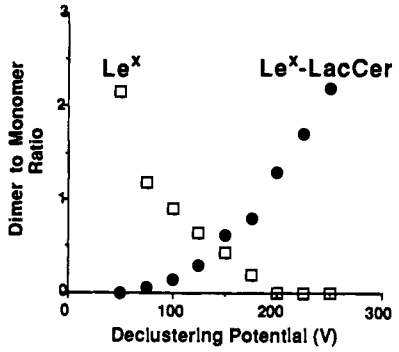

Figure 2. Ratio of Ca^{2+} dimers to Ca^{2+} monomers of Le^x–LacCer (●) and Le^x (□) as a function of declustering potential.

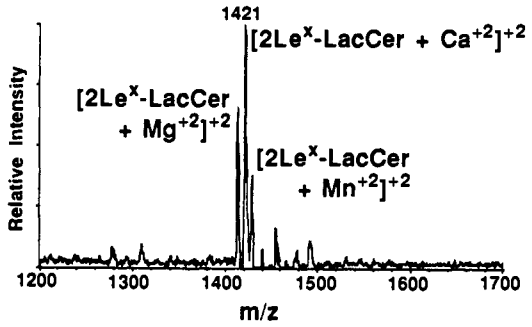

Figure 3. Dimers of Le^x–LacCer formed with equivalent concentrations of Mg^{2+}, Ca^{2+}, and Mn^{2+}: [Le^x–LacCer + Mg^{2+}]$^{2+}$, [Le^x–LacCer + Ca^{2+}]$^{2+}$, and [Le^x–LacCer + Mn^{2+}]$^{2+}$. The ions from 1435 to 1500 most likely correspond to additional divalent cation adducts: [2Le^x–LacCer + 2H$^+$ + 2Ca^{2+}]$^{2+}$, [2Le^x–LacCer – 2H$^+$ + 2Mn^{2+}]$^{2+}$, and [2Le^x–LacCer – 6H$^+$ + 2Mn^{2+} + 2Ca^{2+}]$^{2+}$.

This supposition was further supported by an additional experiment.[9b] The first two quadrupoles were held in rf-only mode and the third was scanned from m/z 300 to 2400; the change in the dimer to monomer and trimer to monomer ratios was then observed when argon was introduced into the second quadrupole collision cell. Both ratios increased upon addition of argon by as much as 150%. These results further support that oligomer formation may be caused by the breakup of much larger ions. Interestingly, the predominant ions produced in both of these experiments were the divalent cation complex of the dimer.

To gain further insight into the moieties responsible for Ca^{2+} complexation in the associated Le^x and Le^x–LacCer oligomers, CID studies were performed. Prior to CID analysis, samples containing equivalent concentrations of the Mg^{2+}, Ca^{2+}, and Mn^{2+} salts were analyzed with Le^x and Le^x–LacCer; the Ca^{2+} adduct was found to produce the greatest ion signals (Figure 3). In order to preserve the limited supply of these samples, CID studies were performed with Ca^{2+}.

CID of [2Le^x–LacCer + Ca^{2+}]$^{2+}$ and [2Le^x + Ca^{2+}]$^{2+}$. The CID experiments of the [2Le^x–LacCer + Ca^{2+}]$^{2+}$ dimers resulted in a neutral loss of covalently and noncovalently bound species (Figure 4). The CID mass spectra of the dimer produced six predominant fragment ions,[14] two corresponding to the loss of the ceramide moiety through either direct loss of Cer, $^dB_{4\alpha}$, or GalCer loss, $^dC_{3\alpha}$. The most predominant fragment was that with a loss of fucose, $^dB_{1\beta}$, from the dimer, in addition to the

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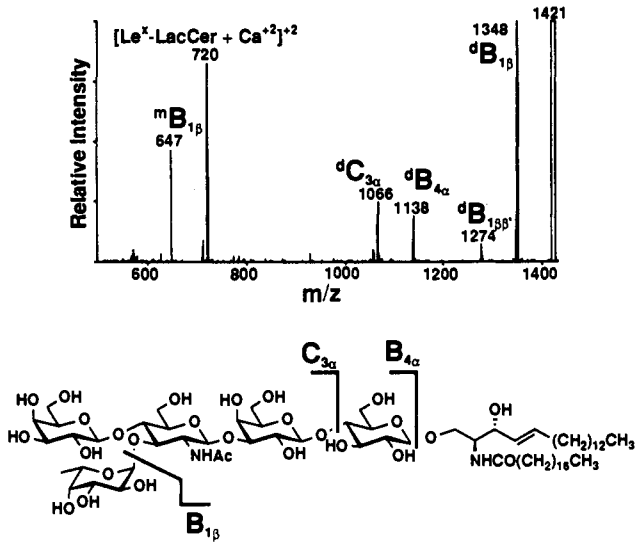

Figure 4. CID mass spectrum of the dimer $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ (m/z = 1421) including fragmentation nomenclature.[14] d designates that the fragment ion includes the noncovalent dimer; m designates that the fragment ion includes only the monomer and is no longer noncovalently bound to the other Le^x–LacCer.

fucose loss from the monomer, ${}^{m}B_{1\beta}$, and the loss of two fucose residues from the intact dimer, ${}^{d}B_{1\beta\beta'}$. These results indicate that the calcium cation complexes the sugar and further suggests that the Cer and GalCer functionalities may not perform an essential role in stabilizing the dimer. The ions observed in the CID mass analysis of the monomer corresponded to the loss of the GalCer and fucose moieties, indicating that Ca^{2+} may be bound to the Glc–Le^x sugar. To further determine how the sugar incorporated Ca^{2+} into the dimer complex, CID experiments were performed on the $[2Le^x + Ca^{2+}]^{2+}$ dimer.

The CID analyses of $[2Le^x + Ca^{2+}]^{2+}$ were similar to those of $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$. Neutral losses arose only from either one, ${}^{d}B_{1\beta}$, or both, ${}^{d}B_{1\beta\beta'}$, of the fucose residues. Weak formation of the monomer, $[Le^x + Ca^{2+}]^{2+}$, was also observed. The loss of both fucose residues from the Le^x and Le^x–LacCer dimers indicate that both fucose saccharides may be exposed on the dimer complexes.

Ca^{2+}-Dependent Association of Le^x–LacCer with Itself, LacCer, GalCer, and Cer. In order to determine whether the Cer moiety plays a role in these association processes, ion spray experiments were performed on Le^x–LacCer in the presence of LacCer ($C_{48}H_{91}O_{13}N_1$, monoisotopic mass = 889.6 Da), GalCer ($C_{42}H_{81}O_8N_1$, monoisotopic mass = 727.6 Da), Cer ($C_{37}H_{71}O_3N_1$, monoisotopic mass = 565.5 Da), and the monovalent and divalent cations. Association was not observed for Le^x–LacCer with LacCer, GalCer, or Cer at any declustering potential with the alkali salts. Le^x–LacCer analysis with Ca^{2+} and LacCer, GalCer, or Cer resulted in the heterodimers $[Le^x\text{–}LacCer + LacCer + Ca^{2+}]^{2+}$ and $[Le^x\text{–}LacCer + GalCer + Ca^{2+}]^{2+}$ at intensities nearly equivalent to those obtained for $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$. Cer also dimerized with Le^x–LacCer to form $[Le^x\text{–}LacCer + Cer + Ca^{2+}]^{2+}$, however, at less than 10% of the intensity observed with the LacCer and GalCer glycolipids. The lack of association with the alkali cations indicates that association was not significantly affected by the presence of the Cer moiety through van der Waals interactions. Additionally these results indicate that Ca^{2+}-dependent dimerization was not limited to the self-association of Le^x–LacCer. Previous results[4] have indicated that in addition to the self-association of Le^x–LacCer, it also had a low affinity for another glycolipid, paragloboside. To determine the relative affinity of Le^x–LacCer with Le^x–LacCer, LacCer, GalCer, and Cer, CID measurements were employed.

CID of Le^x–LacCer Associated with LacCer, GalCer, and Cer. CID experiments were performed on $[Le^x\text{–}LacCer + LacCer + Ca^{2+}]^{2+}$, $[Le^x\text{–}LacCer + GalCer + Ca^{2+}]^{2+}$, and $[Le^x\text{–}LacCer + Cer + Ca^{2+}]^{2+}$ and compared with the results obtained for $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$. These analyses were run under similar conditions to allow for a semiquantitative assessment of dimer binding strengths through the use of a kinetic method[15,16] previously employed for determining relative proton and alkali cation binding affinities.[15-19] The consequence of Ca^{2+} remaining preferentially bound to Le^x–LacCer, with a complete lack of formation of the $[LacCer + Ca^{2+}]^{2+}$, $[GalCer + Ca^{2+}]^{2+}$, and $[Cer + Ca^{2+}]^{2+}$ monomers, made quantitative assignment of the relative binding strengths impossible; however, qualitative analysis was possible.[20]

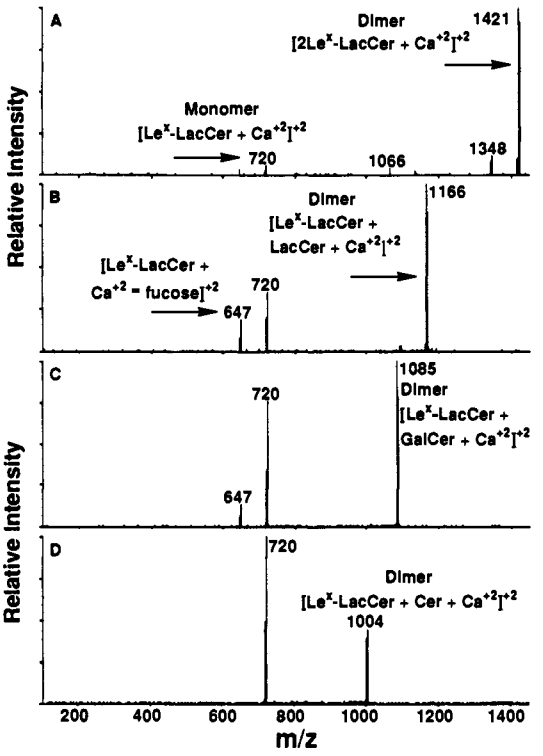

Figure 5. CID mass spectra from the ions: (A) $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$, (B) $[Le^x\text{–}LacCer + LacCer + Ca^{2+}]^{2+}$, (C) $[Le^x\text{–}LacCer + GalCer + Ca^{2+}]^{2+}$, and (D) $[Le^x\text{–}LacCer + Cer + Ca^{2+}]^{2+}$.

Table I. Covalent Dissociative Pathways Observed for Le^x-LacCer from the Homodimer and Heterodimers

$[2Le^x\text{–}LacCer + Ca^{2+}]$	${}^{m}B_{1\beta}$	${}^{d}B_{1\beta}$	${}^{d}B_{1\beta\beta'}$	${}^{d}B_{4\alpha}$	${}^{d}C_{3\alpha}$
$[Le^x\text{–}LacCer + LacCer + Ca^{2+}]$	${}^{m}B_{1\beta}$	${}^{d}B_{1\beta}$			
$[Le^x\text{–}LacCer + GalCer + Ca^{2+}]$	${}^{m}B_{1\beta}$				
$[Le^x\text{–}LacCer + Cer + Ca^{2+}]$					

Considering the CID of the homodimer and heterodimers (Figures 4 and 5), it was observed that the detected fragmentation pathways were the same; however, two specific trends were observed progressing from spectrum A to D in Figure 5. The first observation was that the number of detected covalent fragmentation pathways decreased upon going from the homodimer $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ to the heterodimer $[Le^x\text{–}LacCer + Cer + Ca^{2+}]^{2+}$ (Table I). The second observation was that, while the number of detected fragmentation pathways decreased, the intensity of the monomer, $[Le^x\text{–}LacCer + Ca^{2+}]^{2+}$,

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(20) The collision cross-section and center-of-mass collision energy are unique for each dimer ion experiment; however, as a first approximation the differences would tend to cancel in multiple collision conditions such as this.

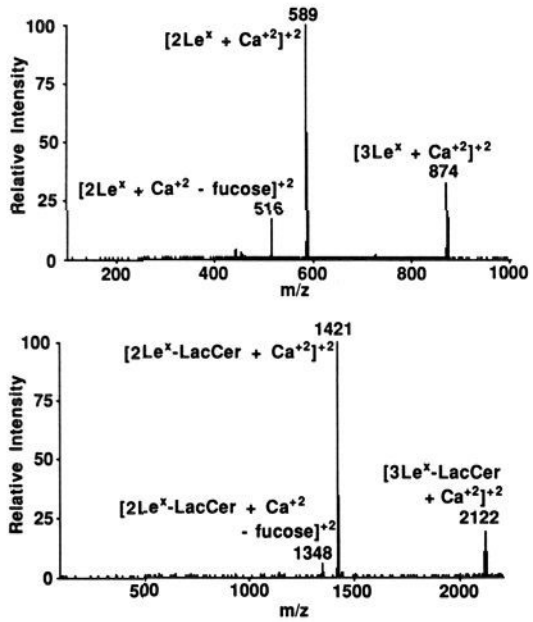

Figure 6. CID spectra of $[3Le^x + Ca^{2+}]^{2+}$ and $[3Le^x\text{–}LacCer + Ca^{2+}]^{2+}$.

increased. These results indicate that the collisional energy previously resulting in covalent dissociation of $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ progressively resulted in more noncovalent dissociation of the heterodimers. Ultimately (spectrum D), only noncovalent dissociation was observed to form the $[Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ monomer. Therefore, in analyzing spectra A through D, the progressive lack of covalent fragmentation and the increasing monomer intensity, relative to the dimer, suggested that the relative stability of the dimers decreased upon going from the homodimer of Le^x–LacCer to the heterodimers of Le^x–LacCer and LacCer, GalCer, and Cer, respectively. These results are consistent with previous experiments demonstrating the Ca^{2+}-dependent binding of LacCer to Gg_3,[21] also an adhesion carbohydrate. Similar to this Le^x–LacCer study, Gg_3 adhesion was also greater with its biological counterpart, G_{M3}. In addition, the complete lack of formation of the $[LacCer + Ca^{2+}]^{2+}$, $[GalCer + Ca^{2+}]^{2+}$, and $[Cer + Ca^{2+}]^{2+}$ monomers further demonstrated the preferential homotypic Le^x complexation of Ca^{2+}.

CID of $[3Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ and $[3Le^x + Ca^{+2+}]^{2+}$. Similar to the experiments performed with the heterodimers, the CID analyses of the Le^x–LacCer and Le^x trimers, $[3Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ and $[3Le^x + Ca^{2+}]^{2+}$, resulted in significant noncovalent fragmentation (Figure 6). Predominant fragmentation was observed via loss of either Le^x–LacCer or Le^x to form the respective dimers, $[2Le^x\text{–}LacCer + Ca^{2+}]^{2+}$ and $[2Le^x + Ca^{2+}]^{2+}$. The covalent losses of fucose were observed only from the dimer. Comparing the CID spectra of the dimer (Figure 4) with the trimer (Figure 6), it is obvious that covalent losses predominate the dimer spectra while noncovalent losses predominate the trimer spectra. Considering the similar conditions of these experiments, these results are further suggestive of the dimers' greater stability. Speculation on the ability of Ca^{2+} to coordinate the Le^x sugar stimulated both energy minimization and CPK modeling studies of Le^x based on its NMR solution conformation.[22]

Space-Filling Model and Energy Minimization Calculations of Le^x and Ca^{2+}. Although a previous NMR study[23] indicated Le^x did not bind Ca^{2+}, a CPK model was constructed based on NMR data,[22] as well as an energy-minimized Le^x structure incorporating Ca^{2+}. These models allowed for Ca^{2+} to be complexed through a crown-like cavity within the monomer (Figure 7).

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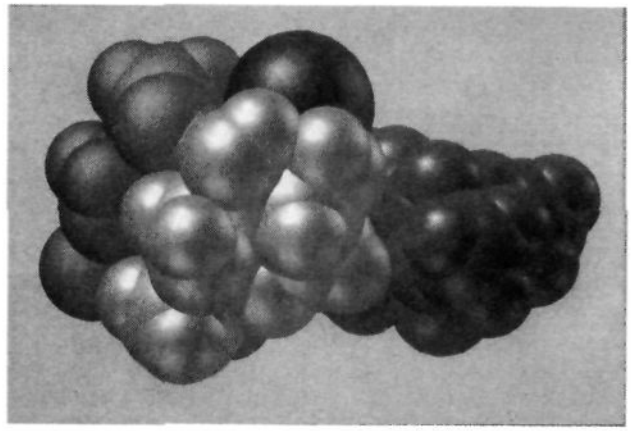

Figure 7. Energy minimized CPK of Le^x with Ca^{2+}; Ca^{2+} = black, Gal = medium gray, fucose = light gray, and GlcNAc = dark gray.

The addition of another Le^x molecule through the space-filling model allowed the second Le^x to complex Ca^{2+} in the same way as the first, with the fucose functionalities exposed and the LacCer functionalities positioned at opposite ends of the dimer complex. This dimerization model is consistent with experimental results and the expected interaction of these surface carbohydrates.[1,4] The exposure of the fucose residues in the dimer complex is consistent with ion spray CID evidence where both fucose residues were lost. Furthermore, the opposite orientations of LacCer on the dimer model is in accord with how cellular interaction might occur, where interaction of the opposing cells should procede through the surface carbohydrates in opposite configurations.

Conclusion

This study presents data describing the homodimerization of the Le^x glycosphingolipid and trisaccharide, a phenomenon dependent on the presence of divalent cations. Through qualitative observations of the ion spray spectra, it appears that a single Ca^{2+} cation binds the Le^x moiety to promote association to form a homodimer. Preliminary analyses of Le^x and Le^x–LacCer in the presence of Mn^{2+} and Mg^{2+} also yielded results consistent with that in the presence of Ca^{2+}. This association behavior was, however, not observed in the presence of monovalent cations.

In addition, Le^x–LacCer was found to undergo calcium-dependent homo- and heteroassociation with the glycosphingolipids Le^x–LacCer, LacCer, GalCer, and Cer, with decreasing binding affinities in this order. CID experiments on these dimers also indicated that the cation complexation site is within the Le^x moiety.

A space-filling model as well as energy minimization calculations on the monomer further demonstrated the possibility that the molecules were bound through Ca^{2+} in a homotypic interaction. The homotypic Le^x interactions may correlate to their biological utility as a cell-surface carbohydrate, and thus the Ca^{2+} dependent carbohydrate–carbohydrate interactions may provide a viable mechanism for cellular communication/interaction in the adhesion process.

Experimental Section

Instrument. All experiments were performed on an API III PE Sciex triple-quadrupole mass spectrometer with an upper mass range of m/z 2400. The ion spray (pneumatically assisted electrospray) interface was used for sample introduction with the potential of the interface sprayer maintained at 5.0 kV. A curtain gas of ultrapure nitrogen (1.8 L/min) between the interface plate and the sampling orifice was applied to aid desolvation of the charged droplets and to prevent particulate matter from entering the analyzer region. Samples were introduced through the interface at a rate of 3.0 μL/min. The positive ions generated by the ion evaporation process entered the analyzer through an interface plate and a 100-μm orifice. The declustering potential was maintained between 50

and 250 V to control the collisional energy[9,24] and thus the ability to observe oligomers. A cryogenic pump was used to cool the surfaces within the spectrometer (14–18 K) maintaining a working pressure of 2×10^{-5} Torr in the analyzer region.

Solvents and Materials. The lithium, sodium, potassium, magnesium, calcium, and manganese chloride salts and methanol solvent were obtained from Aldrich at greater than or equal to 99.9% purity and were used without further purification. The preparation of Le^x, Le^x–LacCer, LacCer, GalCer, and Cer are described in detail elsewhere.[12] The analysis of the Le^x–LacCer and the other glycosphingolipids, including Cer, were performed in methanol, aqueous/methanol, and aqueous solutions. The analyses of the sugars without the Cer functionality were performed in both aqueous and methanol solutions.

Association Experiments. Samples were mass analyzed in the presence of either monovalent or divalent cations. The sample concentrations were typically 20 pmol/μL, while salt concentrations were maintained at 200 pmol/μL. Each analysis included the acquisition of data in multichannel analysis (MCA) mode with unit mass resolution over a mass range dependent on the experiment. All spectra were acquired with PE Sciex software.

The effect of varying Ca^{2+} concentration from 20 to 1200 pmol/μL at fixed Le^x–LacCer concentrations (20, 100, and 200 pmol/μL) was examined. Significant monomer and dimer formations were not reached until a Ca^{2+} concentration of 80 pmol/μL, with monomer and dimer increasing uniformly in intensity until a concentration of 200 pmol/μL of Ca^{2+} was obtained; from 200 to 1200 pmol/μL of Ca^{2+} the intensity of the ions remained relatively unchanged. The concentration of Ca^{2+} was maintained at 200 pmol/μL for most of these experiments since higher concentrations of the salt increased background chemical noise.

To further determine the effect of Le^x–LacCer concentration on the ion spray signal, a concentration range of 20 to 200 pmol/μL was examined at 20-pmol/μL increments; the calcium concentration was maintained at 200 pmol/μL. In these experiments it was found that the dimer increased relative to the monomer, as Le^x–LacCer concentration was increased, by 5–30%, depending on the declustering potential. At 200 pmol/μL the signal intensity was an order of magnitude greater; however, for most of these experiments the concentration of Le^x–LacCer was maintained at 20 pmol/μL to preserve the limited quantity of the sample.

CID Experiments. The CID experiments were performed with ultrapure argon as a collision gas. The positive ion MS/MS spectra were acquired by mass selecting the precursor ion with the first quadrupole; collisions with argon (target thickness of 6×10^{14} atoms/cm^2) in the second quadrupole produced dissociation. The third quadrupole mass-analyzed the resultant ions. Collision energies[24] of 80 eV were maintained in these experiments. CID spectra were the result of averaging from 200 to 3000 scans depending on the number of scans necessary to obtain a signal to noise greater than or equal to 50. Sample concentrations were maintained at 20 pmol/μL, while salt concentrations were maintained at 200 pmol/μL.

Molecular Modeling. Molecular modeling and reproductions described were performed and created using molecular modeling programs DISCOVER and INSIGHT II, from BIOSYM Technologies (Version 2.0.0) using the consistent valence force field [CVFF]. The integrity of the calculations was based on published solution-phase NMR studies of Le^x (as noted in the text) and were compared to additional MM2 calculations performed for the solution conformation of Le^x.

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