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

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Received August 31, 1992

Abstract: The interactions of the cell-surface carbohydrate, Le\textsuperscript{1} (Gal-\(\beta\)-1\(\rightarrow\)4(Fuc-\(\alpha\)-1\(\rightarrow\)3)-GlcNAc) and the Le\textsuperscript{1}-containing glycosphingolipid, Le\textsuperscript{1}-LacCer, and their analogues were examined by ion spray mass spectrometry. Both Le\textsuperscript{1} and Le\textsuperscript{1}-LacCer complexed the divalent cations, Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, and Mg\textsuperscript{2+}, to form [nLe\textsuperscript{1} + cation\textsuperscript{2+}]\textsuperscript{2+} and [nLe\textsuperscript{1}-LacCer + cation\textsuperscript{2+}]\textsuperscript{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\textsuperscript{+}, Na\textsuperscript{+}, and K\textsuperscript{+}. Collision-induced decomposition (CID) experiments were performed on the noncovalent dimers, [2Le\textsuperscript{1}-LacCer + Ca\textsuperscript{2+}]\textsuperscript{2+} and [2Le\textsuperscript{1} + Ca\textsuperscript{2+}]\textsuperscript{2+}, with results indicating that the Ca\textsuperscript{2+} complexation site was within the Le\textsuperscript{1} 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\textsuperscript{1} and Le\textsuperscript{1}-LacCer dimers. Le\textsuperscript{1}-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\textsuperscript{1}-LacCer with Le\textsuperscript{1}-LacCer was greater than that with LacCer, and decreased in order with GalCer and Cer. Space-filling models and energy minimization calculations of Le\textsuperscript{1} in its solution conformation with Ca\textsuperscript{2+} affirmed that the molecules may bind through Ca\textsuperscript{2+} in a homotypic interaction. This predilection for homotypic Le\textsuperscript{1} 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\textsuperscript{1-4} to occur prior to protein–carbohydrate and protein–protein interactions. Specifically, Lewis x (Le\textsuperscript{1}) (Gal-\(\beta\)-1\(\rightarrow\)4(Fuc-\(\alpha\)-1\(\rightarrow\)3)-GlcNAc) on the embryo cell surface at the 8-32 cell (morula) stage\textsuperscript{5} is correlated with the onset of compaction. Since the compaction event is inhibited by Le\textsuperscript{1} hapten,\textsuperscript{6} the Le\textsuperscript{1} determinant expressed on the morula stage embryos has been suggested to be recognized by other cell-surface Le\textsuperscript{1} sugars in homotypic cell interactions. Hakomori et al.\textsuperscript{7} have demonstrated the association of liposomes conjugated with the Le\textsuperscript{1}-containing glycolipid and that this interaction is Ca\textsuperscript{2+} dependent.

The function of Ca\textsuperscript{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\textsuperscript{8-10} through its capacity to observe multiply charged ions and intact native noncovalently bound oligomers. In addition, recent results\textsuperscript{11} have indicated that the gas-phase ions formed under ion spray conditions may reflect the solution-phase properties.

Le\textsuperscript{1} glycosphingolipids exist on cell surfaces often with extreme heterogeneity in their carbohydrate structures. Recent advances in synthetic organic chemistry\textsuperscript{12} have been utilized to produce homogeneously pure glycosphingolipids as well as the carbohydrate Le\textsuperscript{1}, 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\textsuperscript{1} and Le\textsuperscript{1}-LacCer in the Presence of Divalent Cations. The ion spray mass spectra of Le\textsuperscript{1} [(Gal-\(\beta\)-1\(\rightarrow\)4(Fuc-\(\alpha\)-1\(\rightarrow\)3)-GlcNAc-O-allyl, C\textsubscript{23}H\textsubscript{39}O\textsubscript{15}N\textsubscript{1}, monoisotopic mass = 394.2) with and without Ca\textsuperscript{2+} and [nLe\textsuperscript{1}-LacCer + cation\textsuperscript{2+}]\textsuperscript{2+} (n = 1, 2, or 3) 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\textsuperscript{+}, Na\textsuperscript{+}, and K\textsuperscript{+}. Collision-induced decomposition (CID) experiments were performed on the noncovalent dimers, [2Le\textsuperscript{1}-LacCer + Ca\textsuperscript{2+}]\textsuperscript{2+} and [2Le\textsuperscript{1} + Ca\textsuperscript{2+}]\textsuperscript{2+}, with results indicating that the Ca\textsuperscript{2+} complexation site was within the Le\textsuperscript{1} 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\textsuperscript{1} and Le\textsuperscript{1}-LacCer dimers. Le\textsuperscript{1}-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\textsuperscript{1}-LacCer with Le\textsuperscript{1}-LacCer was greater than that with LacCer, and decreased in order with GalCer and Cer. Space-filling models and energy minimization calculations of Le\textsuperscript{1} in its solution conformation with Ca\textsuperscript{2+} affirmed that the molecules may bind through Ca\textsuperscript{2+} in a homotypic interaction. This predilection for homotypic Le\textsuperscript{1} interactions may correlate to the biological utility of this surface carbohydrate in the cellular adhesion process.

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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 vra LacCer ions were detected.

The ion spray mass analysis of Le i (in water) and Le v LacCer (in methanol) with the monovalent cations resulted in spectra of the monomer ions, [Le + cation*]+ or [Le v LacCer + cation*]+, with no evidence of association (e.g., [2Le + cation]+ or [2Le v LacCer + 3cation]+) were not observed. In contrast, when analyses were performed with the divalent cations, monomers, dimers, and trimers, [nLe + cation2+]2+ and [nLe v LacCer + cation2+]2+ (n = 1, 2, or 3), were observed; the monomer ions were also observed as [Le + cation3+-H]+ and [Le v LacCer + cation2+-H]+. The intensities of all of these ions were highly dependent on the declustering potential.

The association of Le i 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 Kentga with those obtained with the methanol solution. At greater than 30% aqueous/methanol concentrations, no Le v LacCer ions were detected.

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van der Waals interactions. Additionally, these results indicate 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 Lea-LacCer.

Fucose loss from the monomer, mB1f, and the loss of two fucose residues from the intact dimer, mB1g. 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 fucosyl moieties, indicating that Ca2+ may be bound to the Glc-Lea-LacCer sugar. Further investigation of which ions covalently incorporate Ca2+ into the dimer reported that association was not observed for Lex-LacCer with Ca2+, or Cer, or both, mB1g, of the fucose residues. Weak formation of the monomer, [Lex + Ca2+]2+, was also observed. The loss of both fucose residues from the Lea and Lea-LacCer dimers indicates that both fucose saccharides may be exposed on the dimer complex.

**Ca2+-Dependent Association of Lea-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 Lea-LacCer in the presence of LacCer (C48H91013Nl, monoisotopic mass = 889.6 Da), GalCer (C47H89012Nl, monoisotopic mass = 727.6 Da), Cer (C37H7103Nl, monoisotopic mass = 565.5 Da), and the monovalent and divalent cations. Association was not observed for Lea-LacCer with LacCer, GalCer, or Cer at any declustering potential with the alkali salts. Lea-LacCer analysis with Ca2+ and LacCer, GalCer, or Cer resulted in the heterodimers [Lex-LacCer + LacCer + Ca2+]2+ and [Lex-LacCer + GalCer + Ca2+]2+ at intensities nearly equivalent to those observed for [2Lex-LacCer + Ca2+]2+. Complexes also dimerized with Lea-LacCer to form [Lex-LacCer + Cer + Ca2+]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 Ca2+-dependent dimerization was not limited to the self-association of Lea-LacCer. Previous results have indicated that in addition to the self-association of Lea-LacCer, it also had a low affinity for another glycolipid, paragloboside. To determine the relative affinity of Lea-LacCer with Lea-LacCer, LacCer, GalCer, and Cer, CID measurements were employed.

**CID of Lea-LacCer Associated with LacCer, GalCer, and Cer.** CID experiments were performed on [Lea-LacCer + LacCer + Ca2+]2+ and [Lea-LacCer + GalCer + Ca2+]2+. The CID analyses of [2Lex-LacCer + Ca2+]2+ were similar to those of [2Lex-LacCer + Ca2+]2+. Neutral losses arose only from either one, mB1f, or both, mB1g, of the fucose residues. Weak formation of the monomer, [Lex + Ca2+]2+, was also observed. The loss of both fucose residues from the Lea and Lea-LacCer dimers indicates that both fucose saccharides may be exposed on the dimer complex.

**Figure 4.** CID mass spectrum of the dimer [2Lea-LacCer + Ca2+]2+ (m/z = 1421) including fragmentation nomenclature. 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 Lea-LacCer.

**Table I. Covalent Dissociative Pathways Observed for Lea-LacCer from the Homodimer and Heterodimers**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>CID Mass Spectrum</th>
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<tbody>
<tr>
<td>[Lea-LacCer + Ca2+]2+</td>
<td>[Lex-LacCer + LacCer + Ca2+]2+</td>
</tr>
<tr>
<td>[Lex-LacCer + GalCer + Ca2+]2+</td>
<td>[Lex-LacCer + Cer + Ca2+]2+</td>
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**Figure 5.** CID mass spectra from the ions: (A) [2Lex-LacCer + Ca2+]2+, (B) [Lex-LacCer + LacCer + Ca2+]2+, (C) [Lex-LacCer + GalCer + Ca2+]2+, and (D) [Lex-LacCer + Cer + Ca2+]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 previously employed for determining relative proton and alkali cation binding affinities. The consequence of Ca2+ remaining preferentially bound to Lea-LacCer, with a complete lack of formation of the [Lex-LacCer + Ca2+]2+, [GalCer + Ca2+]2+, and [Cer + Ca2+]2+ monomers, makes quantitative assignment of the relative binding strengths impossible; however, qualitative analysis was possible.

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 [2Lex-LacCer + Ca2+]2+ to the heterodimer [Lex-LacCer + Cer + Ca2+]2+ (Table I). The second observation was that, while the number of detected fragmentation pathways decreased, the intensity of the monomer, [Lea-LacCer + Ca2+]2+, increased. 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.
of the heterodimers. Ultimately (spectrum D), only noncovalent dissociation was observed to form the \([\text{LeLLacCer} \text{Ca}^2+]\) previously resulting in covalent dissociation consistent with previous experiments demonstrating the \(\text{Ca}^2+\)-dependent formation of the \([\text{LacCer} \text{Ca}^2+]\) also greater with its biological counterpart, GM~. In addition, 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 \(\text{Le}^-\text{LacCer}\) to the heterodimers of \(\text{Le}^-\text{LacCer} \text{Ca}^2+\text{Gg}_3\). Similar to this \(\text{Le}^-\text{LacCer}\) study, \(\text{Gg}_3\) adhesion was also greater with its biological counterpart, \(\text{GM}_3\). In addition, the complete lack of formation of the \([\text{LacCer} + \text{Ca}^2+]j\), \([\text{GalCer} + \text{Ca}^2+]j\), and \([\text{Cer} + \text{Ca}^2+]j\) monomers further demonstrated the preferential homotypic \(\text{Le}^+\) complexation of \(\text{Ca}^2+\).

CID of \([3\text{Le}^-\text{LacCer} + \text{Ca}^2+]\) and \([3\text{Le}^-\text{Ca}^2+]\). Similar to the experiments performed with the heterodimers, the CID analyses of the \(\text{Le}^-\text{LacCer}\) and \(\text{Le}^+\) trimers, \([3\text{Le}^-\text{LacCer} + \text{Ca}^2+]\) and \([3\text{Le}^- + \text{Ca}^2+]\), resulted in significant noncovalent fragmentation (Figure 6). (1) Predominant fragmentation was observed via loss of either \(\text{Le}^-\text{LacCer}\) or \(\text{Le}^-\) to form the respective dimers, \([2\text{Le}^-\text{LacCer} + \text{Ca}^2+]\) and \([2\text{Le}^+ + \text{Ca}^2+]\). 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 \(\text{Ca}^2+\) to coordinate the \(\text{Le}^+\) sugar stimulated both energy minimization and CPK modeling studies of \(\text{Le}^+\) based on its NMR solution conformation.22

Space-Filling Model and Energy Minimization Calculations of \(\text{Le}^+\) and \(\text{Ca}^2+\). Although a previous NMR study23 indicated \(\text{Le}^+\) did not bind \(\text{Ca}^2+\), a CPK model was constructed based on NMR data,22 as well as an energy-minimized \(\text{Le}^+\) structure incorporating \(\text{Ca}^2+\). These models allowed for \(\text{Ca}^2+\) to be complexed through a crown-like cavity within the monomer (Figure 7).

Figure 6. CID spectra of \([3\text{Le}^- + \text{Ca}^2+]\) and \([3\text{Le}^-\text{LacCer} + \text{Ca}^2+]\). increased. These results indicate that the collisional energy previously resulting in covalent dissociation of \([2\text{Le}^-\text{LacCer} + \text{Ca}^2+]\) progressively resulted in more noncovalent dissociation of the heterodimers. Ultimately (spectrum D), only noncovalent dissociation was observed to form the \([\text{Le}^-\text{LacCer} + \text{Ca}^2+]\) 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 \(\text{Le}^-\text{LacCer}\) to the heterodimers of \(\text{Le}^-\text{LacCer} \text{Ca}^2+\text{Gg}_3\). These results are further suggestive of the dimers' greater stability.

Conclusion

This study presents data describing the homodimerization of the \(\text{Le}^-\) 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 \(\text{Ca}^2+\) ion binds the \(\text{Le}^+\) moiety to promote association to form a homodimer. Preliminary analyses of \(\text{Le}^+\) and \(\text{Le}^-\text{LacCer}\) in the presence of \(\text{Mn}^2+\) and \(\text{Mg}^2+\) also yielded results consistent with that in the presence of \(\text{Ca}^2+\). This association behavior was, however, not observed in the presence of monovalent cations.

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

A space-filling model as well as energy minimization calculations on the monomer further demonstrated the possibility that the molecules were bound through \(\text{Ca}^2+\) in a homotypic interaction. The homotypic \(\text{Le}^+\) 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 \(\mu\)L/min. The positive ions generated by the ion evaporation process entered the analyzer through an interface plate and a 100-\(\mu\)m orifice. The declustering potential was maintained between 50
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and 250 V to control the collisional energy 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 $2 \times 10^{-4}$ 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 Lex, Le^{2+}-LacCer, LacCer, GalCer, and Cer are described in detail elsewhere.\(^{(12)}\) The analysis of the Le^{2+}-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^{2+}-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^{2+}-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^{2+}-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^{2+}-LacCer was maintained at 200 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 $6 \times 10^{14}$ atoms/cm\(^2\)) in the second quadrupole produced dissociation. The third quadrupole mass-analyzed the resultant ions. Collision energies 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^{θ} (as noted in the text) and were compared to additional MM2 calculations performed for the solution conformation of Le^{θ}.

**Acknowledgments.** The author (G.S.) would like to thank Stephen B. H. Kent and Manuel Baca for many stimulating discussions and insightful observations; the authors are especially indebted to Professor Kent for allowing us to use his PE Sciex API III instrumentation.