Glycoconjugate Journal (1994) **11**:238–248

Further studies on cell adhesion based on Le^x-Le^x interaction, with new approaches: embryoglycan aggregation of F9 teratocarcinoma cells, and adhesion of various tumour cells based on Le^x expression

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Received 25 February, revised 31 March 1994

We previously proposed specific interaction of Le^x (Galβ1 → 4[Fucα1 → 3]-GlcNAcβ1 → 3Gal) with Le^x as a basis of cell adhesion in pre-implantation embryos and in aggregation of F9 teratocarcinoma cells, based on several lines of evidence (Eggens *et al.*, *J Biol Chem* (1989) **264**:9476–9484). We now present additional evidence for this concept, based on autoaggregation studies of plastic beads coated with glycosphingolipids (GSLs) bearing Le^x or other epitopes, and affinity chromatography on Le^x-columns of multivalent lactofucopentaose III (Le^x oligosaccharide) conjugated with lysyllysine. Comparative adhesion studies of Le^x-expressing tumour cells *vs* their Le^x-non-expressing variants showed that only Le^x-expressing cells adhere to Le^x-coated plates and are involved in tumour cell aggregation, in analogy to F9 cell aggregation. The major carrier of Le^x determinant in F9 cells is not GSL but rather polylactosaminoglycan ('embryoglycan'), and we demonstrated autoaggregation of purified embryoglycan in the presence of Ca^{2+}, and reversible dissociation in the absence of Ca^{2+} (addition of EDTA). Defucosylated embryoglycan did not show autoaggregation under the same conditions. Thus, Le^x-Le^x interaction has been demonstrated on a lactosaminoglycan basis as well as a GSL basis. A molecular model of Le^x-Le^x interaction based on minimum energy conformation with involvement of Ca^{2+} is presented.

Keywords: multivalent LFP III, Le^x-dependent aggregation, Ca^{2+}-carbohydrate complex, energy-minimized PKC model

Abbreviations: BSA, bovine serum albumin; CHO, carbohydrate; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; GP, glycopeptide; GSL, glycosphingolipid; LAG, lactosaminoglycan; Le^x, Galβ1 → 4[Fuc-α1 → 3]GlcNAcβ1 → R; LFP, lacto-*N*-fucopentaose; LysLys-OH, lysyllysinol; *M*r, relative molecular weight; PBS, phosphate-buffered saline; PG, paragloboside (Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer); TBS, Tris-buffered saline (10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl); TC, tumour cell.

Introduction

The molecular mechanisms of cell adhesion are crucial to understanding developmental processes in multicellular organisms. Failure of cell adhesion has been implicated in many pathological processes. Dramatic changes of specific carbohydrates (CHOs[1]) have been observed at every step of ontogenic development, and in oncogenic transformation, and their functional role in cell adhesion has been proposed [1, 2]. In a mouse model of pre-implantation embryo, expression of 'stage-specific embryonic antigen 1' (SSEA-1) was first observed at the eight-cell stage, maximal at the 16- or 32-cell cleavage stage, and declined rapidly after compaction, being restricted to the inner cell mass of the blastocyst [3]. SSEA-1 was subsequently identified as Le^x [4, 5]. The compaction process was inhibited and the once-compacted embryo 'decompacted' by Le^x, or more efficiently by the multivalent LFP III (Le^x oligosaccharide).

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Thus compaction, the first cell adhesion event of ontogenic development, is clearly mediated in part by Le^x. Cadherin [6] and uvomorulin [7, 8] are also involved in cooperative mediation of the compaction process.

We previously studied the mechanism of Ca^{2+}-dependent autoaggregation of F9 teratocarcinoma cells, based on the assumption that the compaction process is analogous to aggregation of F9 cells. Since autoaggregation of F9 cells was inhibitable by Le^x, involvement of an Le^x-binding molecule present at the surface of F9 cells was suspected. We attempted to identify such a molecule, and succeeded in demonstrating the presence of two glycoproteins (separated on gel filtration) that inhibit binding of anti-Le^x antibody to Le^x [9]. Further studies along this line led us to the conclusion that the molecule that recognizes Le^x is Le^x itself, and that compaction of mouse embryo and autoaggregation of F9 teratocarcinoma cells are mediated by highly specific Le^x-Le^x interaction. The reaction requires the presence of bivalent cation (Ca^{2+} or Mg^{2+}) [10, 11]. Le^x-Le^x interaction has been documented by several lines of evidence in our earlier studies [11], and also by a recent study with ion spray mass spectrometry by Siuzdak *et al.* [12]. In the present study, we further document this phenomenon employing autoaggregation of Le^x-coated beads and affinity chromatography. We also demonstrate Le^x-dependent aggregation of LAG or embryoglycan purified from F9 cells, and Le^x-dependent adhesion of various tumour cells. Specificity and molecular basis of Le^x-Le^x interaction were studied using minimum-energy modelling techniques. Our results indicate that Le^x-Le^x interaction can be based not only on Le^x GSL but also on highly multivalent Le^x-bearing glycoprotein glycans such as embryoglycan.

Materials and methods

Preparation of GSL-coated beads

Each GSL was dissolved in ethanol at a concentration of 50–250 μg ml^{-1}. One mg of beads (3 μm diameter, Pandex, Mundelein, IL, USA) was suspended in 1 ml of ethanol containing GSL, evaporated, and resuspended in 0.5 ml ethanol. The suspension was sonicated for 10 min, added to 0.5 ml water, sonicated, stirred overnight, and evaporated. GSL-coated beads were resuspended in 1 ml water and stored at 4 °C. Amount of GSL coated on bead surface was estimated by indirect fluorescence-activated cytometry. In the case of Le^x-coated beads, fluorescence intensity increased with increasing GSL concentration.

Aggregation of GSL-coated beads

One hundred μl of bead suspension (1 mg ml^{-1}) was added to 900 μl TBS. This suspension was placed in a quartz cuvette (1 cm width), and turbidity measured at 340 nm for 5 min. Then 10 μl of 1 M $CaCl_2$ or 1 M EDTA (pH 7.4) was added, the suspension gently stirred by pipetting, and turbidity monitored at 340 nm for 120 min.

Alternatively, 10 μl of bead suspension was added to 90 μl of TBS containing 10 mM $CaCl_2$ or 10 mM EDTA. After 30 min, aggregation of beads was determined by light microscopy, or by measurement of forward scatter using an EPICS flow cytometer (Coulter Corp., Hialeah, FL, USA).

Preparation of trivalent oligosaccharide-[^3H]LysLys-OH conjugate and GSL-bound C18 column

LysLys-OH labelled at the C-terminus by reductive tritiation of carboxylester was prepared as previously described [13]. [^{3}H]LysLys-OH thus prepared (2 mg, 1×10^6 cpm mg^{-1}), lactose, or LFP III (10 mg each) were dissolved in 200 μl of 0.2 M KH_2PO_4 buffer, pH 8.0. $NaBH_3CN$ (5 mg) was added and the mixture was incubated for 72 h at 37 °C. The mixture was subjected to chromatography on a Sephadex G-25 column (1.5 × 120 cm). 2 ml of each fraction was collected, and 50 μl aliquots were counted. Materials eluted in the void volume were collected and dried. Total counts were: trivalent lactose-[^{3}H]LysLys-OH, 8.0×10^5 cpm; trivalent LFP III-[^{3}H]LysLys-OH, 7.2×10^5 cpm.

For preparation of the GSL-adsorbed C18 column, GSL (15 mg) was dissolved in prewarmed water (5 ml) and applied to the C18 silica gel column (0.55 × 0.8 cm, Analytichem International, Harbor City CA, USA) until saturation. The column was washed with 2 ml of water and equilibrated with TBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Trivalent oligosaccharide-[^{3}H]LysLys-OH conjugate (1×10^4 cpm) was dissolved in 0.1 ml of the same buffer and applied to the GSL-adsorbed C18 column. The column was stepwise eluted with 7.5 ml each of TBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, TBS containing 10 mM EDTA, and TBS containing 10 mM EDTA and 1% propanol. Fractions (0.5 ml) were collected and 100 μl aliquots were counted.

Liposome binding assay

[^{14}C]Cholesterol-labelled liposomes containing Le^x-related GSLs were prepared (25 000 cpm per well, 1 nmol GSL) and incubated in PBS (pH 7.2, containing 0.9 mM Ca^{2+} and 0.1 mM Mg^{2+}) for 16 h in GSL-coated 96-well flat-bottom assay plates at room temperature [11, 13]. Wells were subsequently washed with TBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} with slow suction, remaining liposomes were extracted with 2-butanol:hexane:water (55:25:20), and radioactivity was counted.

Cell adhesion assay

The procedure was essentially as described previously [11, 13, 14]. Cells (F9, PYS-2, B16/BL6, B16/F1, B16/WA4, and HRT-18) were maintained in DMEM supplemented with 10% FCS. For preparation of Le^x GSL-coated solid phase, 100 μl of a 50% aqueous ethanol solution of GSL

(50 μg ml^{-1}) was appropriately diluted, placed in each well of 96-well flat-bottom microtitre plates (Probind, Falcon, Lincoln Park, NJ, USA), and dried at 37 °C. GSL-coated solid phase was treated with 1% BSA in PBS for 1 h at room temp, and washed twice with PBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} before use. Cells were labelled with 0.5 μCi ml^{-1} of [^{3}H]thymidine in an overnight culture, harvested with 0.02% EDTA/PBS solution, washed twice with PBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} and suspended in DMEM containing 1% BSA at a concentration of 5×10^5 cells per ml. Labelled cells (100 μl, 5×10^4 cells) were placed in the GSL-coated wells of 96-well plates and the plates were centrifuged at 1000 rpm for 1 min. After incubation for 15 min at 25 °C, wells were washed three times with DMEM containing 1% BSA under slow suction, remaining cells were detached with EDTA, and radioactivity was counted.

Preparation of LAG-GP from F9 teratocarcinoma cells

F9 or PYS-2 cells were cultured in 15 cm tissue culture dishes (Falcon) coated with 0.1% gelatin (for F9 cells) in 25 ml of DMEM containing 10% FCS. Cells were placed at a density of 2×10^4 cells per ml. After 36 h, the medium was changed to DMEM/DMEM without glucose (1:4), and 100 μCi of [1-^{3}H]GlcN (Dupont NEN, Boston, MA) was added. After 24 h labelling, cells were harvested by rubber scraper and washed with PBS.

[^{3}H]GlcN-labelled cells were extracted with 50 mM Tris-HCl, pH 7.4, 1% octyglucoside, 150 mM NaCl (buffer A) containing 5 mM EDTA for 30 min at 4°C, and centrifuged at 1000 × **g** for 10 min. Supernatant was centrifuged at 100 000 × **g** for 60 min. Resulting supernatants were dialysed against buffer A containing 5 mM $CaCl_2$ overnight at 4 °C, and centrifuged at 20 000 × **g** for 60 min. Cell extract from F9 showed aggregation during dialysis against a buffer containing 5 mM Ca^{2+}, whereas that from PYS-2 did not. After dialysis, about 40% of radiolabelled material was recovered in precipitate from F9 cells, whereas less than 1% was recovered for PYS-2. No radiolabelled material was observed in the precipitate when an extract from either cell line was dialysed against buffer A containing 5 mM EDTA. The precipitate from 20 000 × **g** centrifugation of F9 cell extract was dissolved in buffer A containing 5 mM EDTA, and subjected to chromatography on a Bio-gel A 1.5 m column (2 × 120 cm) equilibrated with the same buffer. Radioactive materials eluted near the void volume, which were recognized by anti-Le^x mAb SH1, were collected, dialysed against buffer A containing 5 mM $CaCl_2$, and centrifuged at 20 000 × **g** for 60 min. The resulting precipitate was dissolved in 4 ml of 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and digested with pronase (2 mg) for 36 h at 37 °C under a toluene layer. 1 mg pronase was added after 36 h and digestion was terminated after 72 h by the addition of 10% (final) trichloroacetic acid. The digest was centrifuged, and supernatant was neutralized and dialysed against 10 mM Tris-HCl, pH 8.0, and 100 mM NaCl (buffer B). The digest was then applied to DEAE-Sephadex A-25 column equilibrated with buffer B and eluted with the same buffer. The pass-through fraction was collected, concentrated, dialysed, and applied to a Sephadex G-50 column (1.5 × 120 cm) equilibrated with buffer B. Fractions (2 ml) were collected, radioactivity in 100 μl of each fraction was counted, and radioactive material eluted at the void volume was collected, dialysed, lyophilized, and used as LAG-GP.

Gel filtration and glycosidase treatment of LAG-GP

LAG-GP from F9 cell extract was dissolved in 2 ml water, and 1 ml of LAG-GP was dialysed against 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 10 mM $CaCl_2$, or containing 150 mM NaCl and 10 mM EDTA, for over 24 h. LAG-GP dialysed against Ca^{2+}-containing buffer was subjected to chromatography on a Sepharose CL-6B column (1.5 × 120 cm) equilibrated with the same buffer. Fractions (2 ml) were collected and radioactivity of each fraction was counted. The same column was re-equilibrated with 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 10 mM EDTA. LAG-GP dialysed against EDTA-containing buffer was applied to the column equilibrated with EDTA-containing buffer.

LAG-GP was treated with 50 mU of α-fucosidase from bovine kidney (Sigma Chemical Co., St. Louis, MO, USA) in 10 mM citrate buffer, pH 5.5, for 24 h at 37 °C. Resulting LAG-GP was subjected to chromatography on the same column equilibrated with 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 10 mM $CaCl_2$.

Molecular modelling of Le^x-Le^x interaction

The minimum energy conformation of LFP III (Le^x oligosaccharide) was established by use of HSEA (Hard-Sphere Exo-Anomeric) calculations with the GESA (Geometry of Saccharides) program (used with permission of Dr Bernd Meyer, Department of Biochemistry, University of Georgia). The conformation comprised the following glycosidic torsion angles (ϕ/ψ): Galβ1 → 4GlcNAc (54°/9°), Fucα1 → 3GlcNAc (49°/24°), GlcNAcβ1 → 3Gal (57°/–10°), and Galβ1 → 4Glc (55°/2°). Based on the energy-minimized conformation, a Covey-Pauling-Koltun (CPK) model was constructed and visualized using the SYBYL® molecular graphics program (Tripos Associates, St Louis, MO, USA). The initial configuration for the Le^x-Le^x complex was set to achieve maximal intermolecular hydrophobic surface contact. Interaction energy was minimized by varying the relative configurations. Potential Ca^{2+}-binding sites in this complex were investigated based on the Ca^{2+} ion coordination shell [15], giving intermolecular oxygen-oxygen distances of 2.6–3.3 Å. Calcium ions were then placed at the potential binding sites and the distances between Ca^{2+} and oxygen atoms were compared with the literature value (2.3–2.6 Å) [15].

Results

Turbidometric determination of aggregation of GSL-coated plastic beads

We previously demonstrated that Le^x GSL-containing liposomes specifically bound to Le^x GSL-coated solid phase in the presence of bivalent cations. To clarify the specific interaction of Le^x with Le^x, we quantitatively determined intensity of interaction by turbidometry using plastic beads coated with defined GSLs as described under Materials and methods. GSLs adhere in quantitative fashion to fine plastic beads, and expression of GSL on each bead can be quantitated by flow cytometric analysis using a specific antibody. Expression of Le^x coated on beads clearly correlated with the Le^x GSL concentration applied (Fig. 1). Aggregation of GSL-coated beads was monitored by turbidometry at 340 nm light transmittance. PG-coated beads showed no change of turbidity in the presence of Ca^{2+} during 120 min. Turbidity of Le^x-coated beads was decreased within 20 min in the presence of Ca^{2+}, whereas turbidity of Le^x-coated beads in the presence of EDTA decreased only slightly (Fig. 2A). Bead aggregation obviously correlated with the quantity of GSL applied (Fig. 2B).

Le^x-coated beads showed aggregation in the presence of Ca^{2+} under light microscopy (Fig. 3A, B), while PG-coated beads showed no such aggregation (Fig. 3E). The aggregation of Le^x-coated beads was abolished in the presence of EDTA (Fig. 3C, D). No aggregation was observed for beads coated with GM3 or with Gg3 (Fig. 3F, G). However, clear heterotypic aggregation was observed in a mixture of GM3-coated beads and Gg3-coated beads (Fig. 3H), as expected based on our previous observations of GM3-Gg3 interaction [14]. The aggregation was abolished by EDTA (data not shown). Bead aggregation was estimated by measuring forward scatter (which reflects the size of particles) using a flow cytometer. Results corresponded to observations under microscopy or turbidometric analysis (Fig. 4). Autoaggregation of Le^x-coated beads was dependent on surface Le^x expression and the presence of bivalent cations. These results, in conjunction with previous

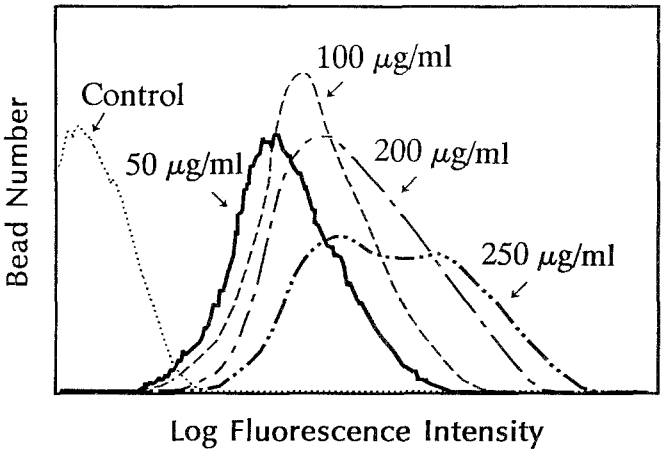

Figure 1. Indirect flow cytometry of Le^x GSL-coated beads. Plastic beads (1 mg, 3 μm diameter) were suspended in 50, 100, 200, or 250 μg ml^{-1} solutions of Le^x GSL in ethanol (represented by four different lines in Figure) and treated as described in Materials and methods. Quantity of Le^x GSL coated on the bead surface was estimated by flow cytometry, using anti-Le^x mAb SH1 as primary antibody and FITC-labelled goat anti-mouse IgG as secondary antibody.

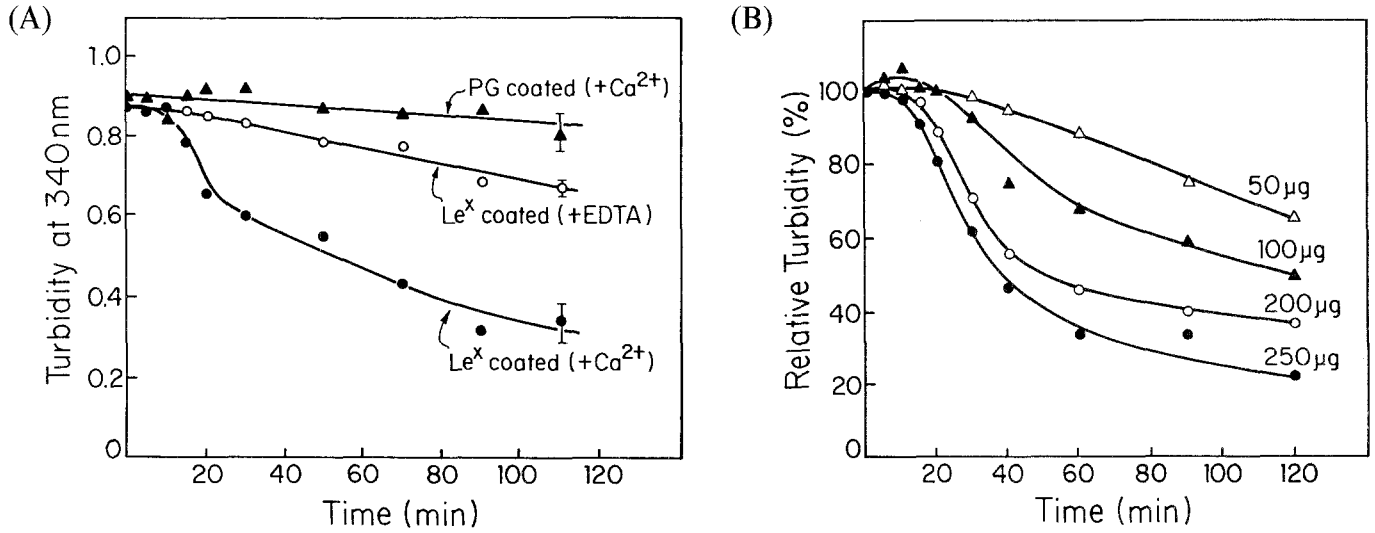

Figure 2. Autoaggregation of Le^x-coated beads in the presence of Ca^{2+}. (A) one hundred μl of GSL-coated beads (150 μg GSL per mg beads) was mixed with 900 μl TBS. After sonication, one tube was mixed with 10 mM (final concentration) $CaCl_2$, and a second tube with 10 mM EDTA. Turbidity of the suspension was measured at 340 nm during 120 min. See Materials and methods for details. (B) Turbidity of a suspension of beads coated with various amounts (50–250 μg per mg beads) of Le^x GSL in the presence of $CaCl_2$ was measured as in Panel A, and expressed as % turbidity before addition of $CaCl_2$.

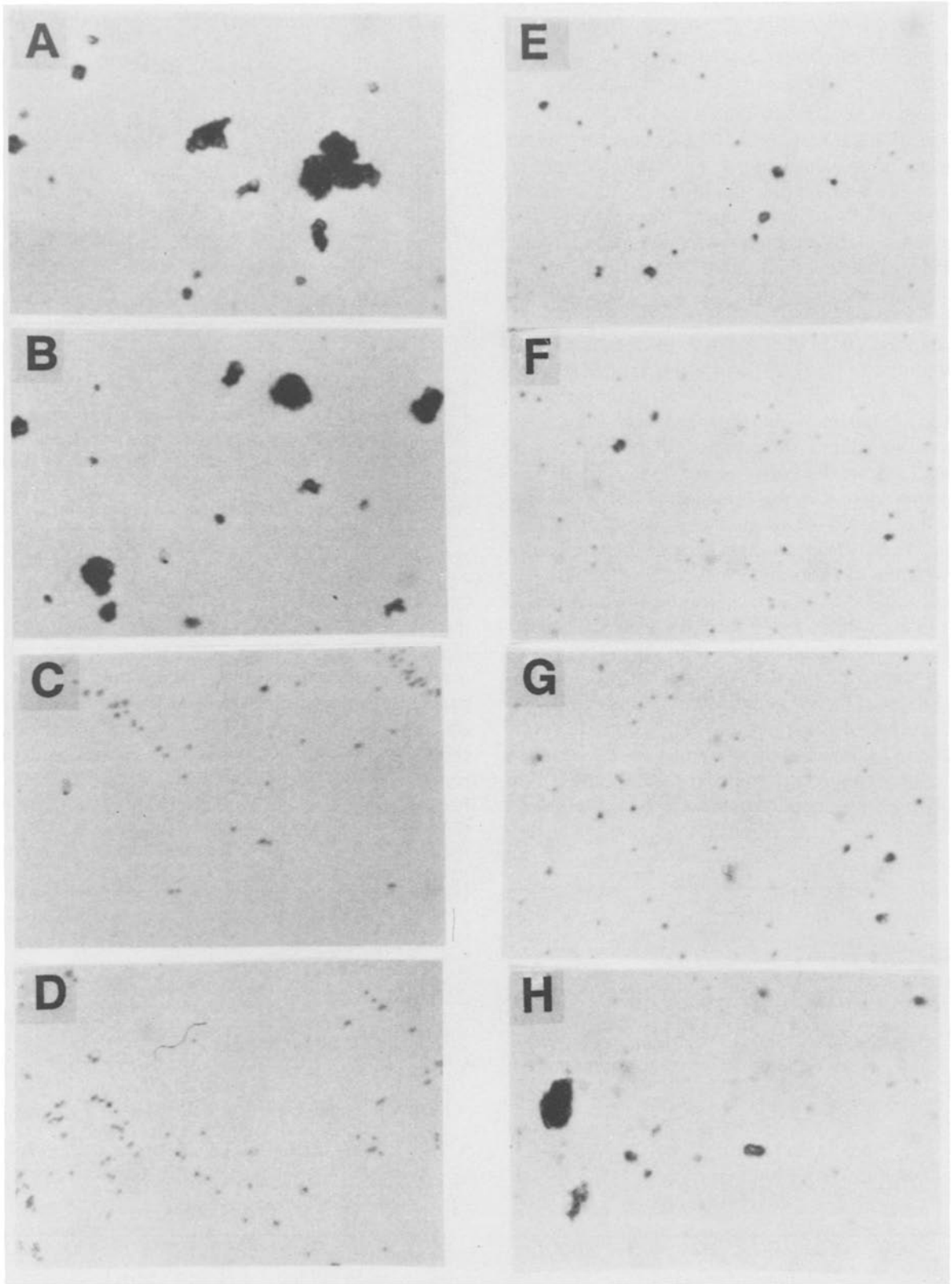

Figure 3. Autoaggregration of beads coated with various GSLs. (A) Le^x GSL-coated beads (250 μg GSL per mg beads) in the presence of Ca^{2+}. (B) same as A but 150 μg GSL per mg beads. (C) same as A but with EDTA instead of Ca^{2+}. (D) same as B but with EDTA instead of Ca^{2+}. (E) PG-coated beads in the presence of Ca^{2+}. (F) GM3/Ca^{2+}. (G) Gg3/Ca^{2+}. In E, F, and G, 150 μg GSL was coated per mg beads. (H) mixture of GM3-coated beads and Gg3-coated beads in the presence of Ca^{2+}. Ten μl of this bead suspension was added to 90 ml TBS containing 10 mM $CaCl_2$ or 10 mM EDTA, sonicated, and left at room temperature for 30 min.

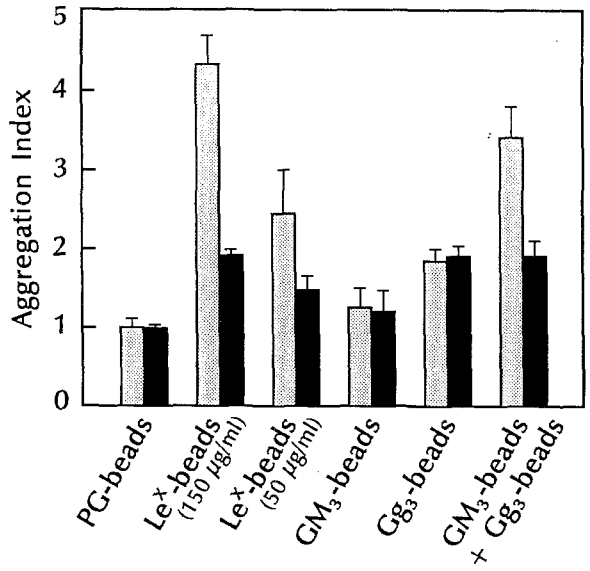

Figure 4. Comparative aggregation of beads coated with various GSLs as determined by flow cytometry. Ten μg of bead suspension was added to 90 μl TBS containing 10 mM $CaCl_2$ (shaded columns) or 10 mM EDTA (solid columns), as in Fig. 3. Forward scatter of bead aggregation was measured using an EPICS flow cytometer. Distribution of forward scatter of non-GSL-coated beads was measured first. Ninety per cent of beads showed forward scatter intensity < 1.00. Next, forward scatter of GSL-coated beads was measured. Beads showing intensity > 1.00 were regarded as being aggregated. Aggregation index as shown in the Figure was calculated as [(% GSL-coated bead aggregation)/(% control bead aggregation)]$^{-1}$.

observations, clearly document GSL-based Le^x-Le^x interaction.

Interaction of lysyllysine-conjugated LFP III with Le^x GSL affixed on a column

When LFP III conjugated with [^{3}H]LysLys-OH was applied to the Le^x GSL-adsorbed C18 column in the presence of Ca^{2+} and eluted with Ca^{2+}-containing buffer, about 60% of radioactive material was eluted in the pass-through fraction and some was eluted in delayed fractions. Interestingly, most of the remaining radioactivity was eluted with EDTA-containing buffer (Fig. 5, upper panel). On the other hand, lactose conjugated with labelled LysLys-OH was eluted only in pass-through fraction and no radioactivity was eluted with EDTA (Fig. 5, lower panel). The finding that LFP III can be affinity-adsorbed on a Le^x column indicates that Le^x-Le^x interaction can take place on an oligosaccharide-GSL basis as well as GSL-GSL basis.

Adhesion of Le^x-related GSL-liposomes to Le^x-related GSL-coated solid phase

We previously studied Le^x-liposome binding to Le^x-coated solid phase using III^{3}FucnLc$_4$Cer as Le^x GSL. In the present study, we compared specific interaction of Le^x-

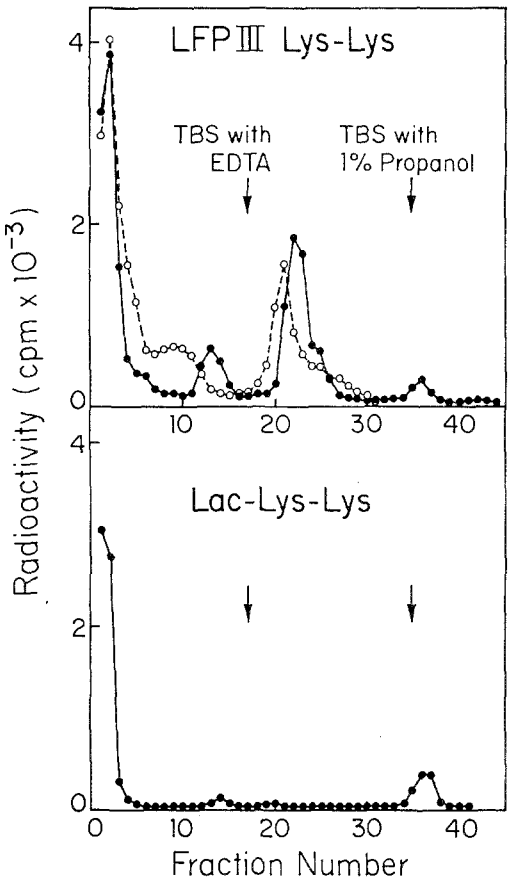

Figure 5. Binding of trivalent Le^x-[^{3}H]LysLys-OH conjugate to Le^x GSL affixed on C18 column. Trivalent LFP III conjugated with [^{3}H]LysLys-OH, and similar trivalent lactosyl conjugate, were prepared as described in Materials and methods. Le^x GSL was adsorbed on to C18 column, extensively washed with TBS containing 1 mM $CaCl_2$, and equilibrated in the same buffer. One hundred nmol oligosaccharide-[^{3}H]LysLys-OH conjugate (1×10^5 cpm) was applied to the Le^x-C18 column and eluted with 7.5 ml TBS containing 1 mM $CaCl_2$, and subsequently with 7.5 ml TBS containing 10 mM EDTA, and 7.5 ml TBS containing 10 mM EDTA and 1% propanol as indicated by arrow. 0.5 ml Fractions were collected, and a 0.1 ml aliquot of each fraction was counted by scintillation counter. Upper panel, elution curves from two batches of Le^x-[^{3}H]Lys-Lys-OH conjugate, shown respectively as solid line with solid circles and dotted line with open circles. Note that only Le^x-[^{3}H]LysLys-OH conjugate bound to the column and eluted as a radioactive peak with TBS/EDTA. Lower panel, elution curve of lactose-[^{3}H]LysLys-OH conjugate.

related GSLs. The results are summarized in Table 1. Le^x-GSL (III^{3}FucnLc$_4$)-liposome interacted with solid phase coated with extended Le^x-GSL (V^{3}FucnLc$_6$) and dimeric Le^x-GSL (V^{3}FucIII^{3}FucnLc$_6$) as well as Le^x-GSL, but not with solid phase coated with Le^y-GSL (IV^{2}FucIII^{3}FucnLc$_4$) or Le^y/Le^x-GSL (VI^{2}FucV^{3}FucIII^3-FucnLc$_6$). Liposomes containing extended Le^x or dimeric Le^x interacted with extended Le^x- or dimeric Le^x-coated solid phase as well as Le^x-coated solid phase. Neither

Table 1. Interaction of Le^x-related GSLs present in liposomes and coated on solid phase.

Liposome containing[b]	*Solid phase coated with*[a]					
	No GSL	nLc_4	$III^3FucnLc_4$	$V^3FucnLc_6$	V^3Fuc-$III^3FucnLc_6$	VI^2FucV^3Fuc-$III^3FucnLc_6$
$III^3FucnLc_4$	1830 ± 70	2020 ± 170	6210 ± 1080	6010 ± 1120	5240 ± 1030	2370 ± 580
$V^3FucnLc_6$	1880 ± 105	1930 ± 60	6340 ± 720	5230 ± 440	6550 ± 1870	2110 ± 320
$V^3FucIII^3FucnLc_6$	1970 ± 120	2180 ± 710	5930 ± 920	5035 ± 1080	4370 ± 530	2010 ± 460
VI^2FucV^3Fuc-$III^3FucnLc_6$	1945 ± 60	1870 ± 100	2530 ± 750	1870 ± 480	2010 ± 120	1050 ± 330

[a] Each well of 96-well plate was coated with 1 nmol GSL. Each experiment was performed in quadruplicate.
[b] Liposomes were prepared from [^{14}C]cholesterol, phosphatidylcholine, and GSL in a molar ratio of 1:1:0.2. 100 µl of liposome solution (0.5 nmol GSL; 25 000 cpm) was added to each well.

Le^y-liposome nor Le^y-Le^x($VI^2FucV^3FucIII^3FucnLc_6$)-liposome showed such interaction with Le^x-related GSL-coated solid phase. These results indicate that the essential structure for specific Le^x-Le^x interaction is the terminal Le^x structure, and that length of the saccharide chain is not crucial.

Le^x-dependent cell adhesion

We previously demonstrated Le^x-dependent autoaggregation of F9 teratocarcinoma cells, and adhesion of F9 cells to Le^x-coated plates. In the present study, we compared adhesion of Le^x-expressing F9 cells *vs* non-Le^x-expressing PYS-2 teratocarcinoma cells, and of Le^x-expressing mouse melanoma B16/WA4 variant cells *vs* non-Le^x-expressing B16/BL6 (or B16/F1) cells to plastic plates coated with Le^x or other GSLs. PYS-2 is a differentiated teratocarcinoma cell line derived from the same parent (129 murine teratocarcinoma OTT6050) as F9 cells. HRT-18 is a typical human colorectal carcinoma cell line expressing a high quantity of Le^x. The Le^x-expressing cells (F9, B16/WA4, and HRT-18) showed clear adhesion to Le^x-coated plates, but did not adhere to PG-coated or non-coated plates (Fig. 6, left side). The non-Le^x-expressing cells (PYS-2 and B16/BL6) showed no specific adhesion to Le^x-coated plates (Fig. 6, right side). Adhesion of cells as a function of quantity of Le^x coated on plates is shown in Fig. 7. For Le^x-expressing cells (F9 in Fig. 7A, B16/WA4 in Fig. 7B), there was a four- to five-fold increase in adhesion as quantity of coated Le^x increased from 0.01 to 1 µg. For non-Le^x-expressing cells (PYS-2 and B16/F1), no such dramatic increase was observed.

Autoaggregation of LAG (embryoglycan) of F9 cells

LAG of embryonal carcinoma cells, termed 'embryoglycan', is a high-*Mr* heterogeneous glycan characteristically found in various types of embryonal carcinoma, teratocarcinoma, or early embryo [16,17]. While the chemical structure of the embryo-glycan is not clearly known, it was identified as the major carrier of Le^x epitope in these cells [18]. We therefore investigated whether Le^x-bearing LAG chains showed aggregation by themselves, based on gel filtration of LAG-GP in the presence of Ca^{2+} or EDTA. Preliminary studies showed that [^{3}H]GlcN-labelled material extracted from F9 cells by octylglucoside and EDTA was recovered in precipitate during dialysis against Ca^{2+}-containing buffer, whereas no [^{3}H]GlcN-labelled material was observed in precipitate during dialysis of PYS-2 cell extract (see Material and methods). In the presence of EDTA, there was no aggregation of extract from F9 or PYS-2 cells. F9 cell extract was extensively digested with pronase, and LAG-GP

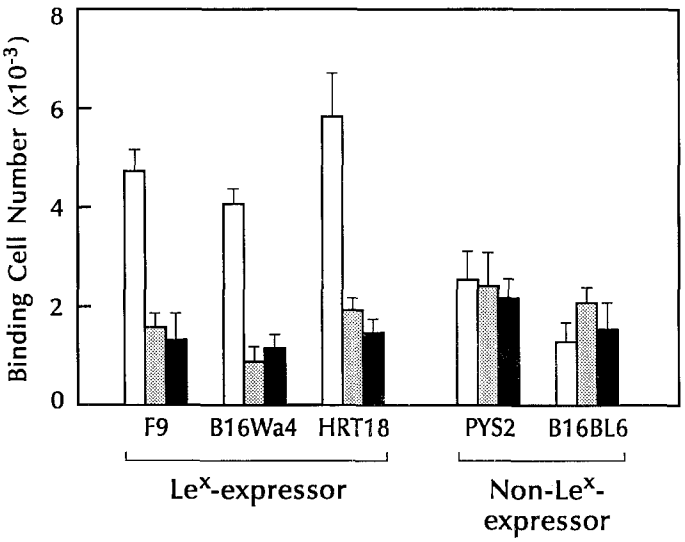

Figure 6. Adhesion of Le^x-expressing and -non-expressing TC lines to Le^x- and PG-coated plates. Multi-well plastic plates were coated with Le^x GSL (open columns), PG (shaded columns), or no coating (solid columns; control). Each well was then blocked by incubation with 1% BSA in PBS for 1 h, and washed with PBS. Various [^{3}H]thymidine-labeled TCs (see abscissa) were added to each plate (5×10^4 cells per plate). Binding cell number (ordinate) was calculated from radioactivity adhering to each plate.

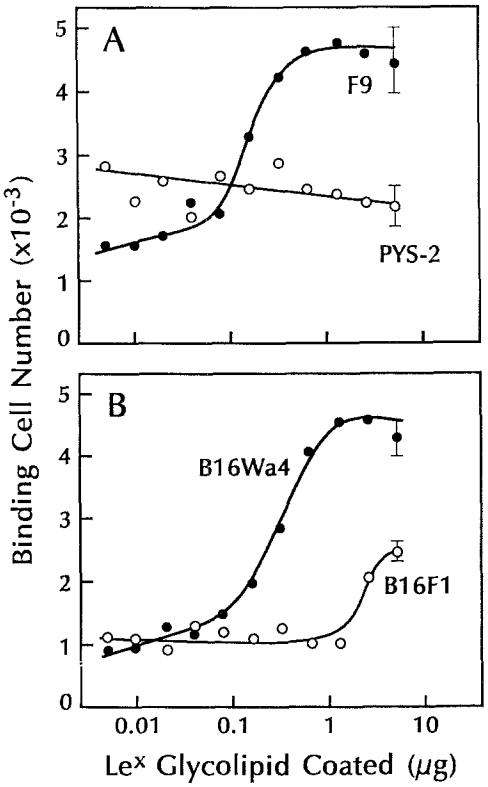

Figure 7. Adhesion of Le^x-expressing and -non-expressing TC lines as a function of quantity of Le^x coated on plates. Ninety-six-well plastic plates were coated with various quantities (0.01–10 μg) of Le^x GSL. [^{3}H]Thymidine-labelled cells were added to plates and adhesion was determined as in Fig. 6. (A) F9 cells (Le^x-expressor) and PYS-2 cells (Le^x-non-expressor). (B) B16/WA4 cells (Le^x-expressor) and B16/F1 cells (Le^x-non-expressor). Strong adhesion occurred only for Le^x-expressing cells on plates coated with 0.1–1.0 μg of Le^x.

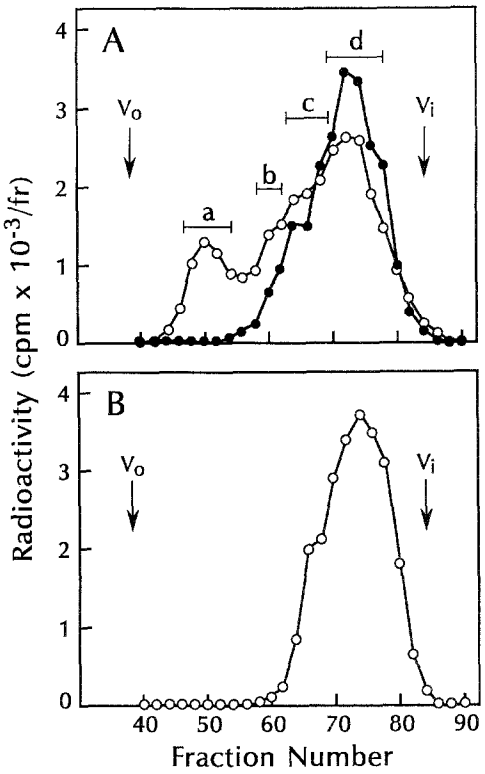

Figure 8. Aggregation of LAG-GP as demonstrated by gel filtration. (A) LAG-GP metabolically labelled with [^{3}H]GlcN was prepared from F9 cells as described in Materials and methods, dialysed against TBS containing 10 mM $CaCl_2$ or 10 mM EDTA, subjected to gel filtration on Sephacryl CL-6B column (1.5 × 120 cm), and equilibrated with TBS containing 2 mM $CaCl_2$ and/or 2 mM EDTA. Two ml fractions were collected and radioactivity of a 0.1 ml aliquot of each fraction was determined by scintillation counter. ○, gel filtration pattern of LAG-GP in the presence of $CaCl_2$. ●, in the presence of EDTA, v_o, void volume. v_i, column volume. Peaks a, b, c, and d are described in Results. (B) LAG-GP was treated with 0.1 U bovine liver α-fucosidase and incubated for 24 h at 37°C. The resulting solution was dialysed in TBS containing $CaCl_2$, and gel filtration performed.

containing both [^{3}H]GlcN and Le^x was purified by chromatography on Sephadex A-25 and G-50 columns.

In the presence of Ca^{2+}, LAG-GP prepared from F9 cells showed three peaks (termed a, b, and c) corresponding to high-*Mr* aggregates, in addition to the major peak d (Fig. 8A). In the presence of EDTA, peaks a and b completely disappeared, and c was greatly reduced (Fig. 8A). After extensive digestion of purified LSG-GP by bovine liver α-L- fucosidase (which cleaves off the fucosyl residue of Le^x), peaks a, b, and c were eliminated, even in the presence of Ca^{2+} (Fig. 8B).

Molecular modelling of Le^x-Le^x interaction

To understand the molecular basis of Le^x-Le^x interaction, a CPK model of LFP III was constructed. In this model, one side of the molecule was more hydrophobic than the other side. The hydrophobic surfaces of two Le^x molecules fit closely together when oriented properly (Fig. 9A). We hypothesized that interaction between two Le^x molecules is initiated by preferential approach when hydrophobic surfaces are facing each other, and that this interaction is subsequently strengthened by Ca^{2+} bridging. Accordingly, we investigated possible Ca^{2+}-binding sites after initial formation of a Le^x-Le^x complex through hydrophobic interactions. Crystallographic studies on Ca^{2+}-CHO complexes have shown that chelating oxygen atoms are located in the proper geometrical orientation to displace water molecules in the Ca^{2+} coordination shell [15, 19, 20]. The oxygen-oxygen distances between two Le^x molecules were compared with those in the Ca^{2+} shell [15].

Two possible Ca^{2+}-binding sites were discovered (Fig. 9B). One site was found where two Fuc residues were in close proximity. These Fuc residues could be coordinated to Ca^{2+} through their *O*-4 and *O*-5 (ring oxygen) groups (intermolecular *O*-4-*O*-4 distance: 3.59 Å; *O*-5-*O*-5 distance: 2.68 Å). Thus, the Ca^{2+} ion could reinforce the Le^x-Le^x interaction by cross-linking these Fuc residues. The second

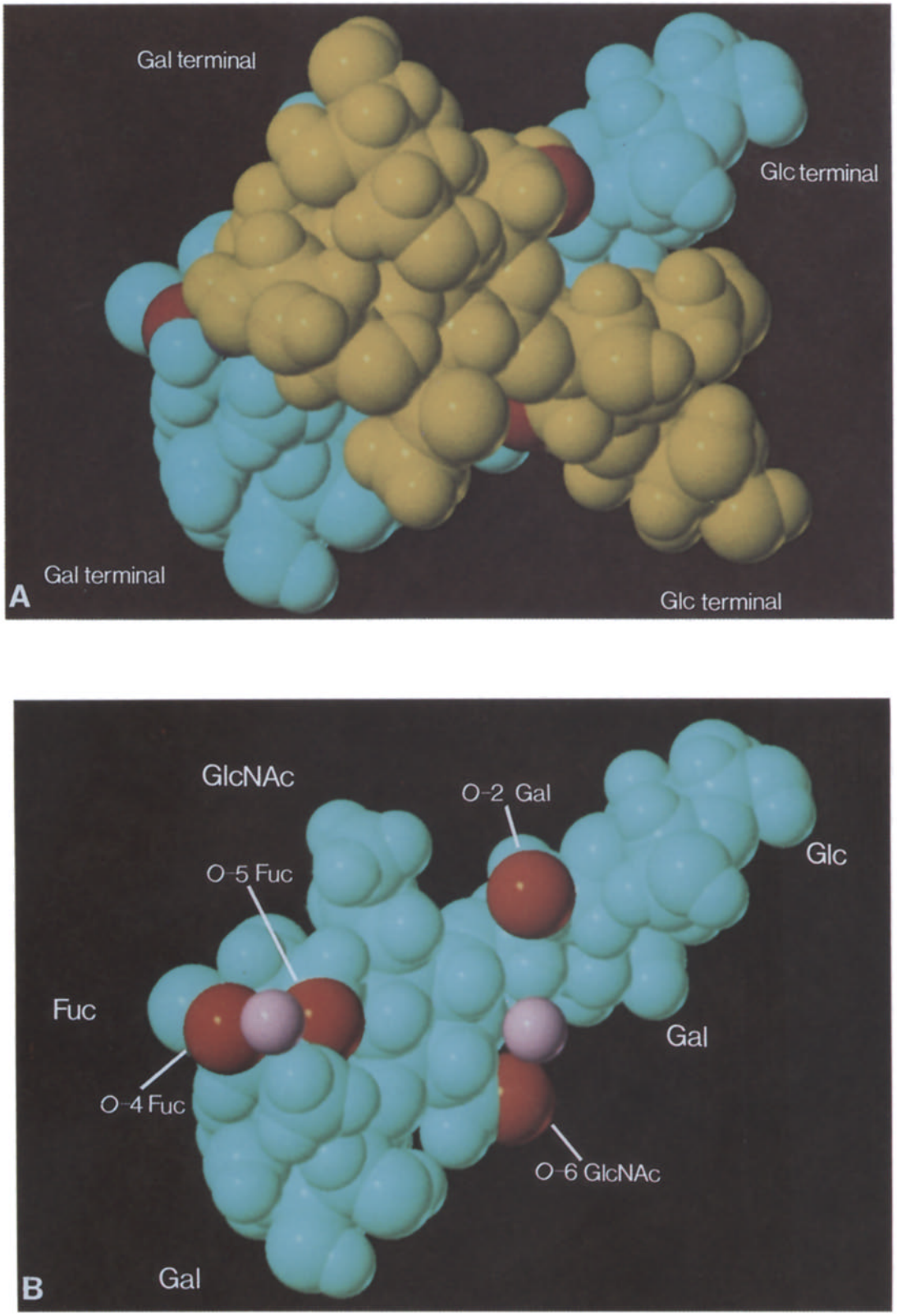

Figure 9. Minimum-energy molecular model of Le^x and its proposed interaction with Le^x ceramide pentasaccharide. Energy-minimized CPK model of the Le^x-Le^x complex with Ca^{2+}. Purple, Ca^{2+}. Red, chelating oxygen atoms. Blue and yellow, Le^x molecules. (A) Le^x-Le^x complex. (B) Removal of one Le^x molecule showing two possible Ca^{2+} binding sites. For clarity, the hydrogen atoms on the chelating oxygens are omitted.

site was where two GlcNAcβ1 → 3Gal moieties were close to each other. Two *O*-2 groups of the internal Gal residues and two *O*-6 groups of the GlcNAc residues were found to be in a geometrical arrangement suitable for Ca^{2+} coordination (intermolecular *O*-2-*O*-6 distances: 2.69 and 2.70 Å).

Discussion

F9 teratocarcinoma cells show clear Le^x-dependent autoaggregation, and mimic the compaction process of preimplantation embryos, which express high quantities of Le^x (SSEA-1). Our ongoing studies on Le^x-Le^x interaction were initiated by a general search for an Le^x-binding molecule expressed in F9 cells, which led to the detection of an Le^x-binding glycoprotein [9]. Subsequent studies indicated that Le^x-binding protein *per se* carries Le^x, suggesting the possibility of an Le^x-Le^x interaction. Further evidence for this interaction was provided by observations that: (i) Le^x GSL-liposomes adhered to plates coated with Le^x but not to those coated with other GSLs; (ii) liposomes containing Le^x, but not those containing other GSLs, adhered to Le^x-coated plates; (iii) Le^x-liposomes show autoaggregation in the presence of Ca^{2+}; (iv) LFP III shows preferential interaction with Le^x-liposome on equilibrium dialysis. In each of these systems, clear interaction required two factors: (a) either multivalent Le^x carried by ceramide which forms micellar or liposomal clusters or Le^x conjugated with LysLys; and (b) the presence of bivalent cations [11]. Wormald *et al.* [21] and our studies (Levery SB, Toyokuni T, Hakomori S, unpublished) failed to detect ^{1}H-NMR spectral changes characteristic of LFP III on addition of Ca^{2+}. This finding suggests that LFP III does not interact with itself in aqueous solution, since free oligosaccharides in solution move randomly [22] and may not get the opportunity to interact with each other in the absence of an appropriate carrier molecule. However, Siuzdak *et al.*, [12] using ion spray mass spectrometry, recently provided unequivocal physical evidence that chemically-synthesized Le^x oligosaccharide and Le^x GSL both dimerize or trimerize in the presence of bivalent cations, especially Ca^{2+}.

The present study provides strong additional evidence for Le^x-Le^x interaction, based on autoaggregation of plastic beads coated with Le^x GSL. Only Le^x GSL-coated beads showed strong aggregation. The degree of aggregation was correlated with the quantity of Le^x GSL coated on beads. Le^x-dependent cell adhesion, adhesion of Le^x GSL-liposomes to Le^x-coated plates, and autoaggregation of Le^x GSL-liposomes were observed in PBS containing Ca^{2+} (1 mM) and Mg^{2+} (0.5 mM) in our previous [10, 11] and present studies. However, Le^x-dependent aggregation of plastic beads coated with Le^x GSL, and that of embryoglycan glycopeptide, were best observed in the presence of 10 mM Ca^{2+}. Stewart and Boggs [23] recently described effects of bivalent cation concentration on sulfatide-cerebroside interaction, using GSL-coated beads.

Our systematic studies on interactions of various α1 → 3 fucosylated structures (Table 1) indicated that terminal Le^x structure is essential for Le^x-Le^x interaction. The experimental method used for homotypic aggregation is also applicable to heterotypic aggregation, as illustrated for a mixture of GM3-coated beads and Gg3-coated beads (Figs. 3H and 4). Thus, the method is useful for detection of GSL interactions in general. Stewart and Boggs used a similar approach with bead aggregation for detection of sulfatide-GalCer interaction [23]. The absorption of multivalent LFP III on a Le^x GSL-affixed column provided additional evidence of Le^x-Le^x interaction.

Le^x-Le^x interaction can only be demonstrated between Le^x epitopes multiply affixed on carrier molecules, e.g. GSLs bearing clusters of Le^x, or Le^x carried by embryoglycan. However, interaction between two Le^x molecules can be visualized through molecular modelling techniques. Our modelling results suggest that: (i) one surface of the Le^x molecule is more hydrophobic than the opposite surface; (ii) preferred interaction of two Le^x molecules involves contact between the two hydrophobic surfaces; (iii) there are two possible Ca^{2+} binding sites (see Results and Fig. 9B). Thus, initial Le^x-Le^x recognition could result from interaction between the two hydrophobic surfaces, and Ca^{2+} could subsequently cross-link the two Le^x molecules by being coordinated to four oxygens (two oxygens from each Le^x molecule). Additional coordination sites would be available through the oxygens on the surrounding water molecules. Although two Ca^{2+}-binding sites were found, it is is not certain if both sites are required for the stabilization of the Le^x-Le^x interaction. Recently, Siuzdak *et al.* [12] proposed a different molecular model for Le^x-Le^x interaction, where two Le^x molecules interact through Ca^{2+}. A variety of synthetic analogues of Le^x are required to determine which model is the most appropriate.

The results described in the present and previous studies are based on GSL-GSL interaction. However, Le^x-dependent adhesion of pre-implantation embryo, and autoaggregation of F9 teratocarcinoma cells, may not involve GSL since the major carrier of Le^x determinant in these cells is embryoglycan [18]. The glycan prepared from F9 cells after extensive proteolysis showed similar properties as previously described for embryoglycan [16, 17], and autoaggregation of embryoglycan in the presence of Ca^{2+} was clearly demonstrated based on gel filtration pattern on Sepharose CL 6B column chromatography. Autoaggregation of embryoglycan is abolished by defucosylation or the presence of EDTA. Thus, Le^x-Le^x interaction as clearly documented on a GSL basis may also occur with glycoproteins, particularly the high-*Mr* embryoglycan which contains a high density of Le^x epitope.

Mechanisms of cell adhesion, and molecules involved in adhesion, are characterized by a high degree of redundancy.

At least six classes of adhesion molecules are currently known: (i) integrins which recognize adhesive proteins or members of the Ig receptor superfamily [24, 25]; (ii) members of the Ig superfamily showing homotypic interaction with Ig receptors or recognized by integrins [26–28]; (iii) members of the CD44 family showing different splicing and recognizing different glycans [29]; (iv) members of the selectin family recognizing specific CHO structures such as sialosyl-Le^x, sialosyl-Le^a, and sulfated glycans [30–32]; (v) cell surface lectins [33] and glycosyltransferases recognizing specific CHOs [34]; and (vi) CHO-CHO interaction [35]. Our previous studies indicate that mechanism (vi) takes place more rapidly than some of the others and is highly specific, but is also weaker than the others [36]. Adhesion of morula-stage embryonic cells (i.e. compaction), is clearly initiated by rapid Le^x-Le^x interaction, followed and reinforced by Ca^{2+}-dependent adhesion of Ig receptors termed cadherin [6] or uvomorulin [8], and perhaps by other types of adhesion molecules.

Le^x-dependent cell adhesion is involved in not only embryonic compaction but also aggregation of Le^x-expressing TCs, in analogy to autoaggregation of F9 teratocarcinoma cells. The observed adhesion of Le^x-expressing cells (B16/WA4 melanoma, HRT-18 colonic carcinoma), but not Le^x-non-expressing cells (B16/F1, PYS-2) to Le^x-coated plates suggests that Le^x-Le^x interaction between TCs may lead to TC autoaggregation. However, TC autoaggregation seems to require expression of Le^x above a certain threshold value. This phenomenon is under active investigation.

In contrast to Le^x, Le^y and sialosyl-Le^x antigens do not show homotypic binding, but rather a repellant effect. Certain TCs expressing Le^x and also a high level of Le^y or sialosyl-Le^x may not undergo autoaggregation, but rather may be recognized by selectins. Studies on expression of various fucosylated and sialylated structures on TCs, and their correlation with adhesive and invasive properties, are in progress.

Acknowledgements

We thank Dr Stephen Anderson for preparation and scientific editing of the manuscript, and Ms Jennifer Stoeck for technical assistance in molecular modelling studies.

This study was supported by funds from The Biomembrane Institute, in part under a research contract with Otsuka Pharmaceutical Co., and by National Cancer Institute Outstanding Investigator Grant CA42505 (to S.H.).

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