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Fine carbohydrate recognition of *Euphorbia milii* lectin ☆,☆☆

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Abstract

Glycans are key structures involved in biological processes such as cell attachment, migration, and invasion. Information coded on cell-surface glycans is frequently deciphered by proteins, as lectins, that recognize specific carbohydrate topology. Here, we describe the fine carbohydrate specificity of *Euphorbia milii* lectin (EML). Competitive assays using various sugars showed that GalNAc was the strongest inhibitor, and that the hydroxyl axial position of C4 and acetamido on C2 of GalNAc are critical points of EML recognition. A hydrophobic locus adjacent to GalNAc is also an important region for EML binding. Direct binding assays of EML revealed a stereochemical requirement for a structure adjacent to terminal GalNAc, showing that GalNAc residue is a necessary but not sufficient condition for EML interaction. The capacity of EML to bind epithelial tumor cells makes it a potentially useful tool for study of some over-expressed GalNAc glycoconjugates.

Keywords: Carbohydrate-binding protein; *Euphorbia milii* lectin; GalNAc glycoconjugate; Ligand loci

Glycans play a central and crucial role in a range of biological phenomena, since carbohydrate–protein recognition is involved in many functions of multicellular organisms [1]. Sugars exhibit a great diversity of oligosaccharide configurations from a small number of monomers, based on (i) the fact that glycosidic linkages can be α- or β-anomeric (ii) to different hydroxyl C-positions of the adjacent monosaccharide, and (iii) the branching capacity of oligosaccharide chains [2]. This diversity of possible configurations, not found in amino acids or nucleotides, and the flexibility of glycosidic linkages, give rise to a high number of different glycan topologies that construct the sugar code [3]. The fine topological recognition of sugar by protein is the key property that defines the well-known "lectins," for which a carbohydrate-recognition domain (CRD) of

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☆☆ *Abbreviations:* BSA, bovine serum albumin; Bzl, benzyl residue; CELLA, competitive enzyme-linked lectin assay; CellELLA, cell enzyme-linked lectin assay; CRD, carbohydrate-recognition domain; ELLA, enzyme-linked lectin assay; EML, *Euphorbia milii* lectin; HMC, human mononuclear cells; HPA, *Helix pomatia* agglutinin; HRP, horseradish peroxidase; IC_{50}, concentration required for 50% inhibition; Me, methyl residue; MPL, *Maclura pomifera* lectin; MUC, mucin; PBS, phosphate-buffered saline; PBS-T, PBS with 0.05% Tween 20; PDB, protein data bank; Phl, phenyl residue; pNP, *p*-nitrophenyl residue; RIP, relative inhibitory potency; RT, room temperature; SBA, soy bean agglutinin; TFD, Thomsen–Friedenreich disaccharide; UDP, uridine diphosphate.

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the protein is involved in the interaction, rather than a catalytic (enzymatic) or antibody domain [4,5]. Lectins play critical roles in several biological processes where oligosaccharide chains represent sorting tags involved in quality control of glycoprotein folding [6], intra- and extra-cellular trafficking of glycoconjugates [7], or cell-to-cell recognition and communication [8]. Aberrant mucin-type O-glycosylation on epithelial tumor cells is associated with a specific glycosyltransferase deficiency, and with expression of related Tn (GalNAcα-Ser/Thr) antigens [9]. Over-expression of these cryptic structures is related to the tumor "strategy" of immunosuppression of the host organism, and tumor cell dissemination (metastasis) mediated by endogenous lectins [10,11].

Complex glycans are recognized by specific molecules (e.g., selectins) that play crucial roles in the inflammatory process [12]. Plant lectins can trigger neutrophil migration, mimicking the effect of endogenous mammalian lectins. *Euphorbia milii* lectin (EML) induces neutrophil migration through its sugar recognition capacity [13]. Here, we elucidated the specific carbohydrate recognition of EML. Both the presence of terminal GalNAc in the ligand and its oligosaccharide stereochemistry are essential for this particular carbohydrate–protein interaction. The finding has implications for inflammation research and aberrant glycosylation of epithelial tumor cells.

Materials and methods

Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO). EML was obtained from latex of *E. milii* var. *milii*, and purified by ammonium sulfate precipitation and affinity chromatography using an Ultrogel AcA44 (LKB, France) as affinity support. The retained protein by Ultrogel AcA44 column was eluted using 0.2 M galactose and dialyzed against PBS. Homogeneity of lectin was checked by SDS–PAGE, and molecular weight was estimated by molecular filtration using Sephadex G-100. The molecular weight was estimated as 60 kDa and comprised of two apparently identical 30 kDa subunits (Fig. 1). Similarly sized peptide subunits were previously reported for *Euphorbia marginata* lectin [14] and *Euphorbia neriifolia* lectin [15]. Purified EML was labeled with biotin using *N*-hydroxy-succinimidobiotin [16]. Purified EML was conjugated to horseradish peroxidase using $NaIO_4$ [17]. Labeled lectin was re-purified by affinity chromatography using ε-aminocaproylgalactosamine immobilized on beaded agarose to remove free peroxidase, which was eluted of column with 0.2 M GalNAc. GalNAc was removed of labeled EML by extensive dialysis against PBS and kept at −20 °C until use. MUC1 180-mer peptide, corresponding to 9 tandem repeats of mucin-core amino acid sequence (HGVTSAPDTRPAPGSTAPPA), was GalNAc O-glycosylated using recombinant GalNAc-T2 and -T4 transferases [18]. GalNAc–MUC1 glycopeptide was purified by HPLC using a reversed-phase column. The fraction corresponding to glycopeptide was dried and kept at −20 °C until use. Glycolipids N3, N4, N5a, N5c, and N6 were obtained from *Calliphora vicina*; Gb4 from human red blood; Forssman from sheep erythrocyte; and A blood group from human meconium [19,20].

Hemagglutination assays. Assays of hemagglutination and its inhibition were performed according to Sueyoshi et al. [21] using human A, B and O blood group erythrocytes stabilized with glutaraldehyde [22].

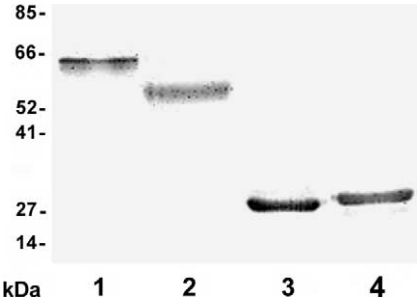

Fig. 1. SDS–PAGE of purified *E. milii* lectin obtained by affinity chromatography. Native and biotinylated EML (5 μg) in PBS were mixed with SDS sample buffer without 2-mercaptoethanol. Lanes 1 and 3, native EML. Lanes 2 and 4, biotinylated EML. Samples from lanes 1 and 2 were loaded into polyacrylamide gel without preheating, whereas samples from lanes 3 and 4 were heated at 95 °C for 5 min before loading. Gel was stained for proteins with Coomassie brilliant blue R-250. Purified native EML showed a homogenous band (1) whose electrophoretic mobility was changed by biotinylation (2). A ~30 kDa EML subunit was observed after heat treatment (3); its molecular weight was slightly increased by biotinylation (4).

Competitive enzyme-linked lectin assay. Competitive enzyme-linked lectin assay (CELLA) was performed as previously described [23]. Briefly, polystyrene microtitration plates (Corning, NY) were coated with 2 μg/ml GalNAc–MUC1 (100 μl/well) in 0.1 M carbonate buffer, pH 9.0, overnight at 4 °C, and saturated with PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl) with 0.05% Tween 20 (PBS-T). Carbohydrates were preincubated with 0.2 μg/ml biotin-EML or HRP-*Maclura pomifera* lectin (MPL) for 1 h at room temperature (RT), and then added at 100 μl/well. Plates were incubated for 2 h at RT, washed five times with PBS-T, incubated with 1 μg/ml HRP-streptavidin in PBS-T for 30 min at RT (EML plates only), and washed four times with PBS-T. Color reaction was developed using 2 mg/ml *o*-phenylenediamine and 0.02% H_2O_2 in sodium citrate, pH 5.0, at RT for 10 min. The reaction was stopped by addition of 0.5 M sulfuric acid (100 μl/well). Absorbance values were read at 492 nm with a microplate reader. MPL was assayed in parallel to EML, for validation of methodology and comparative analysis of the two lectins.

HPTLC-lectin staining. Glycolipids were separated on HPTLC silica gel 60 (Merck) in the running solvent chloroform/methanol/aqueous 0.2% $CaCl_2$ (45:45:10), using a tank, to obtain highly reproducible chromatograms [24]. Plates were air-dried for 15 min, coated by dipping in a 0.5% solution of polyisobutylmethacrylate (Plexigum P28, Rohm & Haas, Darmstadt, Germany) in hexane/chloroform (9:1) for 1 min, air-dried again for 10 min, incubated with 0.2 μg/ml biotin-EML or 5 μg/ml HRP-MPL in PBS-T overnight at 4 °C, washed with PBS-T, incubated (EML plates only) with 1 μg/ml HRP-streptavidin in PBS-T for 30 min at RT, and washed four times with PBS-T. Color reaction was developed using 0.1 mg/ml 4-chloro-1-naphthol and 0.02% H_2O_2 in methanol–PBS (1:29) for 20 min, and stopped by washing with distilled water [25].

Direct enzyme-linked lectin assay. Wells of microtiter plate were coated with purified glycolipids dissolved in methanol and evaporated at 60 °C, or with (glyco)peptides in 0.1 M carbonate buffer (pH 9.6) overnight at 4 °C, and saturated with PBS-T for 1 h at RT. Plates were incubated with various concentrations of HRP-lectin in PBS-T (100 μl/well) for 2 h at RT. Final washing and color reaction were performed as for CELLA. Enzyme-linked lectin assay (ELLA) data applied for measurement of affinity constants (K_a) between lectin and glycoconjugate were fitted by Scatchard plot and LIGAND software as described previously [26]. All assays were performed in triplicate.

Molecular modeling. Initial structures of the glycoconjugates (N4, N5a, Gb4, Forssman, blood group A and Tn) were generated with the

program SWEET [27], whereas for the rest of the glycosides a molecular editor was utilized. The glycoconjugate conformations were obtained by energy minimizing the structures from SWEET or molecular editor. Minimizations of all molecules were performed on the MM2 [28] energy function.

Fluorescence staining of epithelial tumor cells using EML as probe. Cell lines (human mammary carcinoma T47D, human colonic adenocarcinoma HT29 and mouse mammary carcinoma TA3 Ha) were grown on glass slides to ~60% confluence in media as recommended by American Type Culture Collection. Human mononuclear cells were obtained by using Histopaque-1077, and washed with PBS. Cells were fixed in ice-cold acetone for 7 min, washed with PBS, saturated with 3% BSA in PBS for 1 h at RT, and incubated with biotin-EML (0.5 μg/ml) in PBS for 2 h at RT. Sugar inhibition of cell staining was assayed by previous incubation of GalNAc (100 mM) with biotin-EML during 1 h at RT, and then added to cells. After incubation, the slides were washed five times with PBS, incubated with FITC-streptavidin (1 μg/ml) for 1 h at RT, and washed with PBS and water. Slides were mounted with glycerol containing *p*-phenylenediamine. Stained fluorescent cells were examined with a Zeis fluorescence microscope equipped with FITC-interference filters and a 75 W xenon lamp, using epi-illumination [29].

Cell enzyme-linked lectin assay (CellELLA) and competitive CellELLA. Tumor cell recognition was determined using T47D, HT29, and TA3 Ha cell lines grown to subconfluence in 96-well multi plates in media as recommended by American Type Culture Collection. After removal of medium, plates were saturated with PBS-T for 1 h at RT. CellELLA was performed by incubation of serially diluted labeled proteins (biotin-EML or biotin-BSA) against tumor cell lines for 2 h at RT in PBS, and washing four times with PBS. Wells were incubated with 1 μg/ml HRP-streptavidin in PBS for 30 min at RT, and washed four times with PBS. Color reaction was performed as for CELLA [30].

The procedure for competitive CellELLA was similar to that for CellELLA, except that various concentrations of carbohydrates were preincubated with 0.6 μg/ml biotin-EML for 1 h at RT before adding to wells. Plates were incubated for 2 h at RT and washed four times with PBS. HRP-streptavidin incubation, washing, and color reaction steps were as described for CellELLA.

Results and discussion

EML recognizes mainly pNPβGalNAc structure

Preliminary experiments indicated that the minimum EML concentration required for A and B human blood group erythrocytes agglutination was similar (32 μg/ml), whereas against O blood group was four-time lower (8 μg/ml). Among a panel of simple sugars, GalNAc has the strongest inhibitory effect on EML hemagglutination (Table 1). Gal, disaccharides with terminal nonreducing Gal (lactose, melibiose, and TFD) and galactosamine showed a lesser inhibitory effect on EML hemagglutination, while Glc and GlcNAc had no such effect. This result showed the importance of GalNAc, and helped guide the search for a proper ligand for further study of fine carbohydrate recognition of EML. Based on the high affinity constant of GalNAc–MUC1 glycopeptide interaction with EML, this ligand was selected. Conditions for direct binding of GalNAc–MUC1 adsorbed on polystyrene microtitration plates against labeled lectins (EML and MPL) were previously optimized (data not shown). Table 2 summarizes the concentrations of various carbohydrates required for 50% inhibition (IC_{50}) of EML or MPL binding, and the relative inhibitory effect of the carbohydrates. The MPL results are in accordance with previously reported [31]. EML binding was inhibited strongly by GalNAc, to a lesser degree by galactose and disaccharides with terminal nonreducing Gal. C4 epimers of GalNAc (such as GlcNAc and GlcNAc derivatives) did not inhibit EML interaction, showing that the axial C4 hydroxyl position is a critical binding region. Acetyl residue on nitrogen atom of galactosamine improves its inhibitory ability 60-fold and is therefore considered an additional EML binding locus. This group is commonly part of hydrophobic interactions with proteins as previously described [32], and showed in PDB examples for GalNAc with *Dolichos*

Table 1
Inhibition by sugars of hemagglutinating activity from *E. milii* lectin[a]

Carbohydrate	Concentration required for 50% inhibition (mM)	Relative inhibitory potency
Gal	4.0	1
GalNAc	0.25	16
Glc	>100	<0.04
GlcNAc	>100	<0.04
Lactose	4.0	1
Melibiose	4.0	1
TFD	4.0	1

[a] Similar results were obtained using different human (A, B, and O) blood group erythrocytes.

Table 2
Inhibition of direct binding of lectins by using soluble carbohydrates

Carbohydrate	EML		MPL	
	IC_{50}	RIP	IC_{50}	RIP
Gal	1	1	25	1
MeαGal	0.5	2	1	25
MeβGal	0.3	3.3	>100	<0.25
GalNAc	0.05	20	5	5
BzlαGalNAc	0.04	25	0.3	83.3
PhlαGalNAc	0.03	33.3	1	25
pNPαGalNAc	0.02	50	0.2	125
pNPβGalNAc	0.005	200	5	5
UDPαGalNAc	0.5	2	>10	<2.5
Glc	>100	<0.01	>100	<0.25
GlcNAc	>100	<0.01	>100	<0.25
BzlαGlcNAc	>10	<0.1	>10	<2.5
BzlβGlcNAc	>10	<0.1	>10	<2.5
TFD	1	1	5	5
BzlαTFD	1	1	0.2	125
pNPαTFD	0.5	2	0.1	250
Lactose	0.4	2.5	>100	<0.25
N-Acetyllactosamine	0.4	2.5	>2	<12.5
Melibiose	1	1	6	4.2
Galactosamine	3	0.33	ND	ND

IC_{50}, concentration (mM) required for 50% inhibition; RIP, relative inhibitory potency; ND, no data.

biflorus lectin (1LU1) or GlcNAc with mouse glandular kallikrein-13 (1AO5). The addition of pNP β-anomeric residue on GalNAc enhanced its inhibitory effect on EML interaction 10-fold. Besides the hydrophobic nature of pNP, this residue also have the potential to form π–π staking with aromatic residues of the protein, increasing its EML recognition. These findings are well explained by the presence of hydrophobic loci adjacent to the GalNAc-binding site on EML. The existence of a hydrophobic subsite adjacent to the carbohydrate-binding site on EML is consistent with the lower binding when GalNAc in C1 position is linked to a polar residue, such as UDP. From comparative analysis of carbohydrate-binding specificity with other Euphorbiaceae lectins [14,15], EML shows the preferential interaction to GalNAc whereas Gal is the main ligand of rest. The recognition of acetyl residue of GalNAc is a differential ability of EML respect to other members of Euphorbiaceae lectin family.

Stereochemical requirement of ligand for GalNAc–EML interaction

A battery of glycoconjugates revealed differential capacity of lectins on direct carbohydrate-binding recognition (Fig. 2). Data from direct binding assays show that EML interacts with high affinity constants to N4 and N5a glycolipids and Tn antigen, which have GalNAc as terminal nonreducing monosaccharide (Table 3). Other oligosaccharides with terminal GalNAc (Gb4, Forssman, blood group A glycolipid) did not interact with EML, indicating that terminal nonreducing GalNAc structure is a necessary but not a sufficient condition for EML interaction. Comparative analysis of configuration from assayed glycolipids reveals that the first monosaccharide linked to ceramide is β-glucose. Oligosaccharide chains from N4 and Gb4 have β-anomeric GalNAc in terminal nonreducing position, while the other two sugars are different. Theoretical conformational studies allow us to predict the most probable conformer (Fig. 3). This analysis shows a breaking point in the oligosaccharide chain organization corresponding to glycosidic linkage between the second and third Gal through 1-4 α-anomeric conjugation for Gb4, which is not observed for N4 conformation. Topological study of carbohydrates adjacent to terminal nonreducing GalNAc revealed that GalNAcβ1-3Gal from Gb4 exposes hydroxyl groups in positions that could block EML binding. The C2 hydroxyl group of subterminal Gal from Gb4 is in a stereochemical position corresponding

Table 3
Affinity constants for the interaction of lectins with glycoconjugates[a]

Glycoconjugate	EML ($10^{-7} \times K_a$ M^{-1})	MPL ($10^{-7} \times K_a$ M^{-1})
Glycolipid		
N4	6.5 (±0.45)[b]	NB
N5a	1.9 (±0.35)	NB
Gb4	NB	0.041 (±0.012)
Forssman	NB	0.49 (±0.11)
(Glyco)peptide		
MUC1	NB	NB
GalNAc–MUC1	1.9 (±0.41)	2.1 (±0.31)

NB, no binding.
[a] Data of measurement (25 °C) were fitted as previously described [26].
[b] Parentheses indicate standard deviation ($n = 3$).

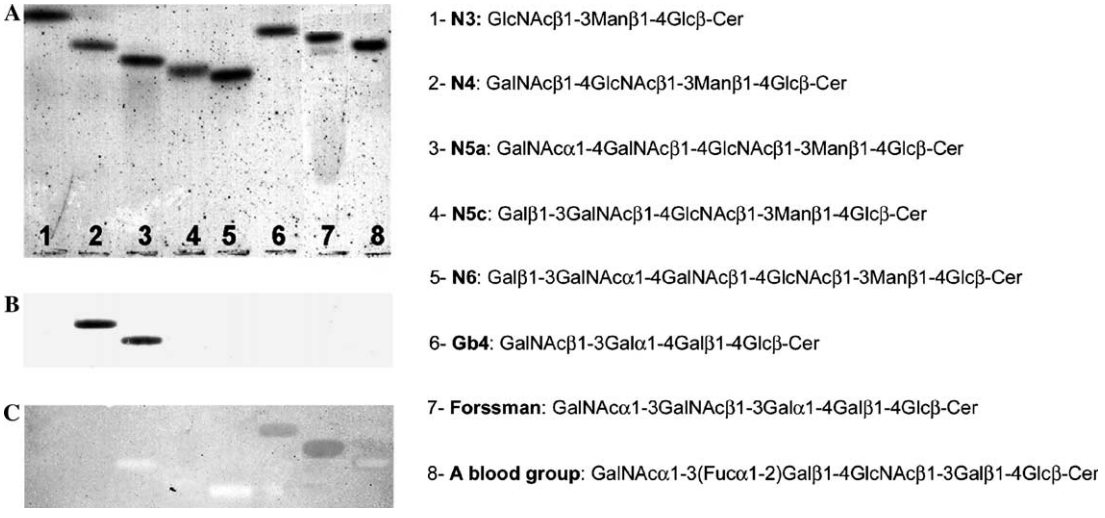

Fig. 2. HPTLC-lectin binding to glycolipids. N3 (1), N4 (2), N5a (3), N5c (4), N6 (5), Gb4 (6), Forssman (7), and blood group A (8) glycolipids were assayed on HPTLC plates using chloroform/methanol/aqueous 0.2% $CaCl_2$ (45:45:10) as running solvent. Glycolipids were visualized chemically using orcinol-sulfuric acid reagent for 5 min at 120 °C (A), or by lectin interaction through incubation with 0.2 μg/ml biotin-EML (B) or 5 μg/ml HRP-MPL (C), in PBS-T overnight at 4 °C. Samples were washed with PBS-T, incubated with 1 μg/ml streptavidin-HRP in PBS-T for 30 min at RT (EML plate only), and washed four times with PBS-T. Color reaction was developed using 0.1 mg/ml 4-chloro-1-naphthol and 0.02% H_2O_2 in methanol–PBS (1:29) for 20 min.

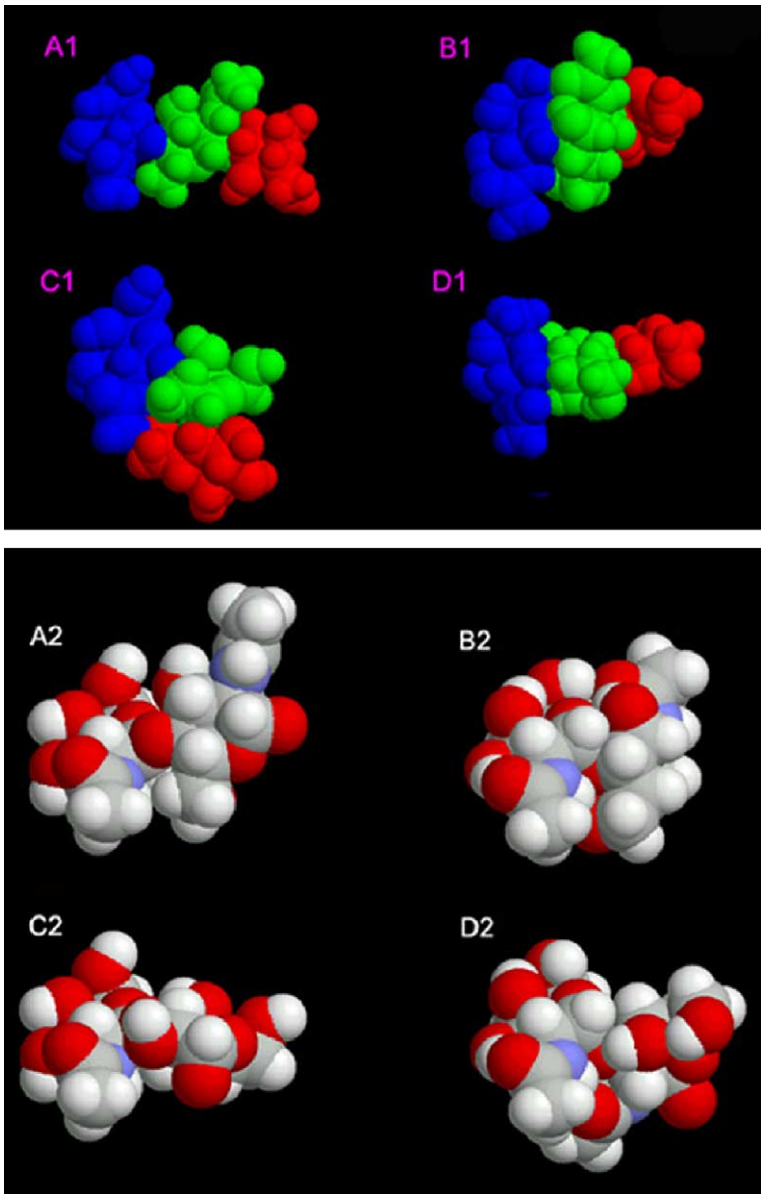

Fig. 3. Space-filling models corresponding to minimum energy conformations of terminal carbohydrate, using MM2 force field. Upper panel (1): colored terminal trisaccharides allowing easy monosaccharide identification. N4, GalNAcβ1-4GlcNAcβ1-3Manβ (A). N5a, GalNAcα1-4GalNAcβ1-4GlcNAcβ (B). Gb4, GalNAcβ1-3Galα1-4Galβ (C). Forssman, GalNAcα1-3GalNAcβ1-3Galα (D). Terminal GalNAc structure is shown in blue. Lower panel (2): terminal CPK disaccharides of the four structures.

to the hydrophobic domain of pNPβGalNAc, the best soluble ligand recognized by EML (Fig. 4). The position of this hydroxyl group and C6 hydroxyl residue from second Gal could interfere with binding to hydrophobic domain of adjacent GalNAc-binding site on EML, explaining the no interaction of EML with some terminal GalNAc. Subterminal GlcNAc of N4 has no hydroxyl group in a stereochemical position equivalent to C2 of Gal from Gb4; in the corresponding region it presents an aliphatic path formed by 5 and 6-carbon atoms of GlcNAc that could be recognized by hydrophobic loci adjacent to GalNAc-binding site of EML. Moreover, in N5a and Tn antigen, there is not a polar region in a topological loci equivalent to aromatic ring from pNPβGalNAc. Thus, the EML recognition to these glycolipids is possible, although with lower affinity constants in relation to EML-N4 interaction (Table 3). The hydroxyl stereochemical positions of C4 of subterminal GalNAc from Forssman or Gal from blood group A, are similar to that of C2 of subterminal Gal from Gb4, which is consistent with the lack of interaction of Gb4, Forssman, and blood group A glycolipids with EML.

There is an interesting difference in the inhibitory effect of GalNAc vs. GlcNAc on carbohydrate-binding ability of EML and *Helix pomatia* agglutinin (HPA)

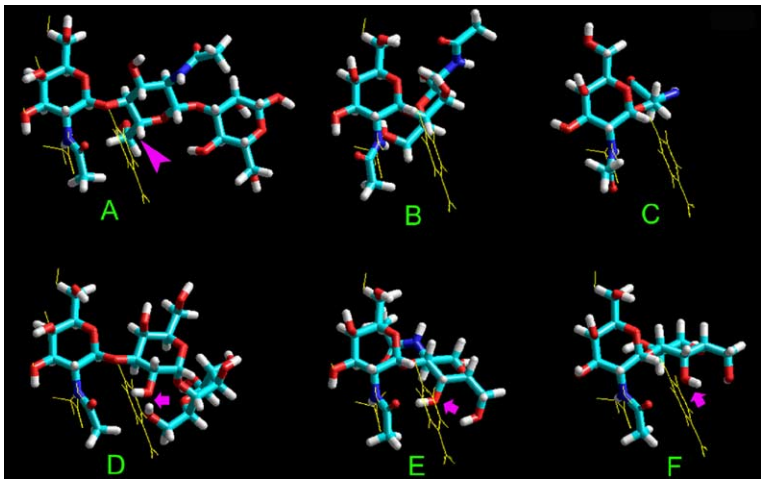

Fig. 4. Overlapping of pNPβGalNAc with terminal GalNAc glycoconjugate structures. Minimum energy conformation of pNPβGalNAc is shown in yellow sticks, whereas glycoconjugates are CPK colors in tube form. Overlapping structures of pNPβGalNAc with terminal GalNAc residue of: (A) N4 (GalNAcβ1-4GlcNAcβ1-3Manβ), (B) N5a (GalNAcα1-4GalNAcβ), (C) Tn antigen (GalNAcα1-Ser), (D) Gb4 (GalNAcβ1-3Galα1-4Galβ), (E) Forssman (GalNAcα1-3GalNAcβ), and (F) A blood group (GalNAcα1-3Galβ). Arrows show polar region yielded by hydroxyl residues from structures nonrecognized by EML. These stereochemical positions are equivalent to hydrophobic loci of major ligand (pNPβGalNAc) recognized by EML. Head arrow reveals a C5–C6 hydrophobic path of GlcNAc from N4 in complementary topological position to aromatic ring of pNPβGalNAc.

[33,34]. Both sugars were effective inhibitors of HPA, but GalNAc was 10 times stronger than GlcNAc, indicating that C4 hydroxyl position is less important for HPA than for EML recognition. The presence of *N*-acetamido on C2 of Gal (GalNAc) enhanced the inhibitory effect of Gal on HPA interaction 1000-fold, but had only a 20-fold corresponding effect on EML interaction. Thus, the *N*-acetamido residue of GalNAc is more significant in ligand binding of HPA than EML. HPA preferentially recognizes GalNAc through an additional hydrophobic aglycon in α-anomeric position, whereas EML binds GalNAc mainly through a β-linked hydrophobic residue.

Carbohydrate-binding specificity of soy bean (*Glycine max*) agglutinin (SBA) is closer to that of EML than HPA [33,34]. The axial position of C4 hydroxyl group of GalNAc is crucial, since Glc and GlcNAc do not inhibit the SBA or EML binding. The relative inhibitory

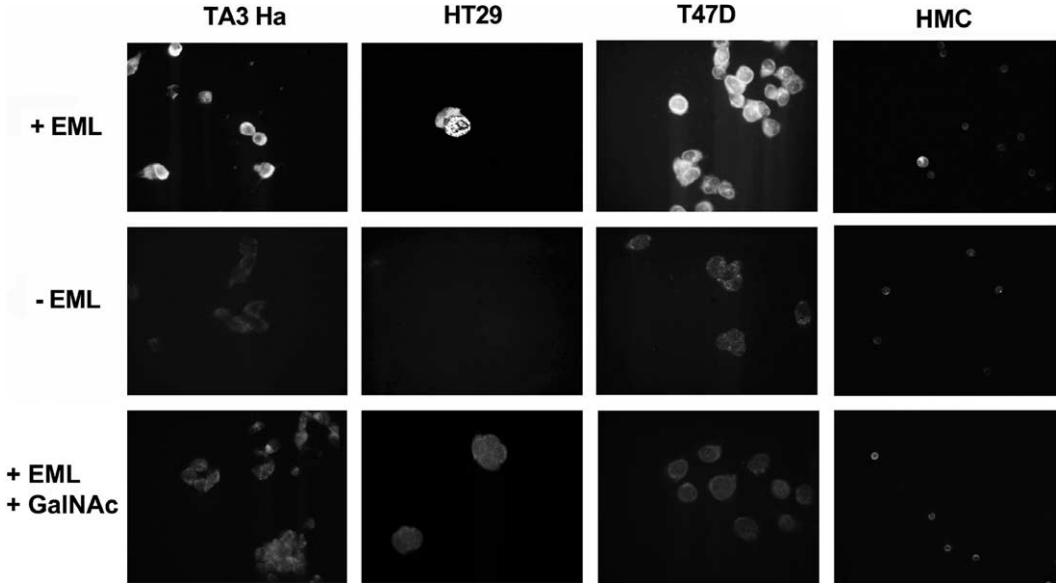

Fig. 5. Fluorescence staining of epithelial tumor cells using lectin probe. Lectin-cytological staining of three epithelial tumor cell lines (TA3 Ha, HT29, and T47D) and human mononuclear cells (HMC) were assayed without or with 0.5 μg/ml biotin-EML in PBS for 2 h at RT. For inhibition of cell-lectin staining, the biotin-EML sample was previously incubated with GalNAc (100 mM) in PBS during 1 h at RT. Samples were washed five times with PBS, and incubated with FITC-streptavidin (1 μg/ml) for 1 h at RT. Stained fluorescent cells were examined with a Zeis fluorescence microscope equipped with FITC-interference filters and 75 W xenon lamp, using epi-illumination.

potency (RIP) of GalNAc vs. Gal is ~3-fold higher for SBA than for EML, indicating the importance of *N*-acetamido group in SBA interaction. In contrast, the RIP of pNPβGalNAc vs. GalNAc is three times higher for EML than SBA, showing that β-hydrophobic locus adjacent to GalNAc-binding site is more important for EML than SBA recognition.

Glycans expressed on epithelial tumor cells are recognized by EML

EML has useful application as a probe to study glycoconjugates expressed on various epithelial tumor cells. We observed direct binding of EML to T47D, HT29, and TA3 Ha tumor cell lines, which express related GalNAc glycoconjugates [35–37], whereas human lymphocytes cells do not show significant EML recognition by fluorescent microscopy (Fig. 5). In addition, this figure shows a positive staining of human neutrophil cells (HMC sample) by using EML probe, in agreement with neutrophil migration and aggregation effect induced by EML [13]. GalNAc had important inhibitory effect on cell–EML interaction. Also, CellELLA shows EML interaction with cancer cells (data not shown). Competitive CellELLA and ELLA (CELLA), using sugars as inhibitors of EML interaction, reveal similar inhibitory profiles for the different cell lines. The great similarity of carbohydrate RIP between the two competitive assays is indicator of Tn (GalNAcα-Ser/Thr) antigen recognition by EML on cancer cells. Aberrant mucin-type O-glycosylation and related Tn antigen expression in epithelial tumor cells is well documented [9]. These truncated O-glycan chains are part of the tumor ''strategy'' leading to immunosuppression, metastasis, and reduced host survival [38,39].

In conclusion, carbohydrate recognition by EML involves mainly terminal GalNAc residue, whose presence in the EML ligand is a necessary but not sufficient requirement for binding. Degree of ligand–EML interaction also depends on stereochemistry of the subterminal region adjacent to GalNAc. EML is able to bind epithelial tumor cells, and thus provides a potentially useful tool for the study of over-expressed terminal GalNAc glycoconjugates, with possible extension to inflammation research.

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