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Binding of the galactose-specific *Pseudomonas aeruginosa* lectin, PA-I, to glycosphingolipids and other glycoconjugates

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The carbohydrate-binding specificity of *Pseudomonas aeruginosa* lectin I (PA-I) in iodinated or biotinylated form was studied. A large number of glycosphingolipids, as well as some glycoproteins and neoglycoproteins were used as ligands. Also, inhibition by free saccharides of PA-I binding to glycosphingolipids was tested. It was found that the lectin binds most strongly to terminal and nonsubstituted Galα3Gal- or Galα4Gal-structures.

Keywords: glycoconjugate binding; neo-glycoprotein; saccharide inhibition

Abbreviations: PA-I, *Pseudomonas aeruginosa* lectin I; Cer, ceramide; lactosylceramide, Galβ4GlcβCer; iso globotriaosylceramide, Galα3Galβ4GlcβCer; globotriaosylceramide, Galα4Galβ4GlcβCer; globoside or globotetraosylceramide, GalNAcβ3Galα4Galβ4GlcβCer; Forssman glycolipid, GalNAcα3GalNAcβ3Galα4Galβ4GlcβCer; P1 glycolipid, Galα4Galβ4GlcNAcβ3Galβ4GlcβCer; lactoneotetraosylceramide, Galβ4GlcNAcβ3Galβ4GlcβCer; B5 glycolipid, Galα3Galβ4GlcNAcβ3Galβ4GlcβCer; gangliotetraosylceramide, Galβ3GalNAcβ4Galβ4GlcβCer; GM1, Galβ3GalNAcβ4(NeuAcα3)Galβ4GlcβCer; RBC, red blood cells; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; MS, mass spectrometry; FAB, fast-atom bombardment; EI, electron impact.

Introduction

Pseudomonas aeruginosa infections of the respiratory tract of patients with cystic fibrosis or immunosuppressed patients are serious because they are associated with high mortality rates. The bacterium binds to buccal cells, tracheal epithelium, and to tracheobronchial mucins [1–5]. *P. aeruginosa* successfully colonize the lungs of patients with cystic fibrosis [6, 7] which indicate that specific receptors can facilitate adherence of this organism to the host tissues [8]. Saccharide-binding molecules, lectins, on bacterial surfaces, often play an important role in mediating adhesion to surfaces colonized by the micro organism [9, 10].

Two distinct *P. aeruginosa* lectins have been isolated: PA-I, exhibiting a specificity for D-galactose [11] and its derivatives [12], and PA-II, which binds L-fucose, L-galactose, D-mannose and D-fructose [11]. The monomer molecular weight of PA-I is 13 000 Da and it is dependent on divalent cations for saccharide binding [11]. Although PA-I is mainly residing intracellularly [13] the lectin also appears on the bacterial surface, as antibodies directed to PA-I are able to agglutinate the bacterium [14, 15]. It has, therefore, been suggested that PA-I can act as an adhesin [16].

Although binding of PA-I to simple mono- or disaccharides or derivatives thereof has been studied [12], the binding to more complex glycosphingolipids has not been investigated. The purpose of the present study was mainly to characterize the carbohydrate-binding specificities of the galactose-specific *P. aeruginosa* PA-I lectin by using overlay of isolated labelled lectin on thin-layer plates with separated mixtures of glycolipids. Studies of PA-I binding to some glycoproteins and neoglycoproteins were also included. The results show that not only the terminal galactose is important for binding, but also neighbouring groups.

Materials and methods

Materials

PA-I lectin, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, α-D(+)-melibiose, and methyl-α-D-galactopyranoside were obtained from Sigma Chemical Co., USA, streptavidine-alkaline phosphatase, streptavidine-horseradish peroxidase, p-nitrophenyl phosphate (disodium salt), and NHS-LC-Biotin were obtained from Pierce

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Chemical Co., USA. The monoclonal antibodies, mouse pK002 and P001 were obtained from Accurate Chem. & Sci. Corp., USA, mouse anti Galα3Galβ4GlcNAc antibody (GAL-13) was a gift from Professor U. Galili, University of California, USA, and rabbit anti-mouse immunoglobulin antibodies were obtained from Dakopatts AB, Denmark. Neoglycoproteins were purchased from Accurate Chem. & Sci. Corp., USA.

Labelling of the PA-I lectin and antibodies

PA-I lectin and antibodies were labelled with ^{125}I using 1,3,4,6-tetrachloro-3α, 6α-diphenyl glycoluril (Iodo-Gen, Pierce Chemical Co., USA) [17]. Labelling with Iodo-Gen was done as follows: the reagent (0.2 mg in chloroform) was evaporated in a reaction vessel and the film obtained was dried under a stream of nitrogen at room temperature. The protein (0.2 μg in 100 μl, 0.1 M KPO_4, pH 7.2) and 500 μCi $Na^{125}I$ were added to the reaction vessel and the reaction was allowed to proceed for 10 min at room temperature. The ^{125}I-labelled lectin was separated from $Na^{125}I$ by gel filtration on PD-10 prepacked columns (Pharmacia, Sweden) equilibrated in 0.015 M phosphate buffer, pH 7.4, 0.14 M NaCl, 5 mM KCl, PBS, and 2% BSA.

Biotinylated PA-I lectin was obtained using NHS-LC-Biotin according to Pierce Chemical Co. instructions. The lectin (0.2 mg), dissolved in PBS, was incubated with 0.1 mg NHS-LC-Biotin, at 0 °C for 2 h. The unreacted biotin was removed by centrifugation at 1000 × **g** for 15–30 min using a Microsep 10K microconcentrator (Filtron, Sweden). After centrifugation, the sample was diluted in PBS and this process was repeated twice.

Glycosphingolipids

The mixture of neutral or acid glycosphingolipids present in a number of organs of different animals, see Table 1, were prepared as described previously [18]. Further separation of the rabbit thymus glycolipids was performed by HPLC (Beckman Instruments Inc., USA) on a silica-gel column (22 mm i.d. × 300 mm, 10 μm, 120 Å pore-diameter, Yamamura Chem. Lab. Co. Ltd, Japan). The eluting gradient was chloroform:methanol:water, from 60:35:8 (by volume unless otherwise stated) to 10:10:3, 2 ml min^{-1}.

Glycolipid-binding assays

TLC was performed on alumina backed sheets, coated with silica gel 60, 0.2 mm thickness (HPTLC nanoplates, Merck, Germany) and the bulk composition of the mobile phase used was chloroform:methanol:water (60:35:8). The detection was accomplished by a chemical reagent, anisaldehyde [19] and by overlay with ^{125}I-labelled lectin or antibody, followed by autoradiography, using the method described previously [20].

α-Galactosidase treatment of glycolipids

The enzymatic treatment of glycolipids on the plates was done as described in [21]. After TLC separation of the glycolipids, the dried plates were dipped into a solution of 0.3% polyisobutylmethacrylate in hexane:diethyleter 1:3 and dried. The plates were then immersed in 0.05 M sodium citrate, pH 5.0, containing taurocholate (1 mg ml^{-1}) and 2.5 U α-galactosidase from coffee bean (Boehringer, Germany), incubated for 1 h at room temperature and for 3 h at 37 °C without shaking. Incubation continued at room temperature for 24 h with fresh enzyme (5 U) added. The plates were gently washed four to five times with 0.05 M Tris-HCl, pH 8.0, containing 0.1% sodium azide, 0.2 M NaCl and 3% BSA.

The enzymatic treatment of glycolipids in test tubes was done as follows: 120 μl of taurodeoxycholate in methanol (1 mg ml^{-1}) was added to the lipid extracts and the mixtures were evaporated to dryness. After that 120 μl of 0.05 M sodium citrate buffer, pH 5.0, were added and the samples were sonicated for 1 min. The solutions were then incubated with 3 μl α-galactosidase (0.25 U) for 16 h at 37 °C. Isolation of the glycolipids was done by Folch partitioning (chloroform:methanol:water 8:4:3) where the lower phase was washed three times with theoretical upper phase and dried.

Inhibition of PA-I lectin binding to TLC plates by preincubation with sugars

Inhibition of PA-I lectin binding was examined on TLC plates by the overlay assay with iodinated lectin in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05% Tween-20, 1% BSA, 0.2 M NaCl and 0.05 M sugar solution, (methyl-β-D-galactopyranoside, methyl-α-D-galactopyranoside, α-D(+)-melibiose, methyl-α-D-glucopyranoside, or lactose).

SDS-gel electrophoresis and binding to neoglycoproteins

Sodium dodecylsulphate-polyacrylamide gel electrophoresis was performed with neoglycoproteins and proteins, using a Phast System (Pharmacia-LKB, Sweden). Protein staining was accomplished by Coomassie Brilliant Blue. Separated proteins were electrophoretically blotted on to a nitro-cellulose membrane using Phast Transfer semi-dry electrotransfer kit (Pharmacia-LKB, Sweden) and the detection of the binding was done by overlay with iodinated lectin, using the method described before [22].

Mass spectrometry

Negative-FAB mass spectra of neutral glycolipids from rabbit thymus were obtained with a ZAB-2F/HF (VG Analytical, UK) sector instrument. Xe-atom bombardment, 8 kV, was used and the matrix was triethanolamine [23]. Positive-EI mass spectra were obtained on a Jeol SX102 (Jeol Ltd., Japan) sector instrument with 70 eV. Partially methylated alditol acetates [24–26] were analysed by

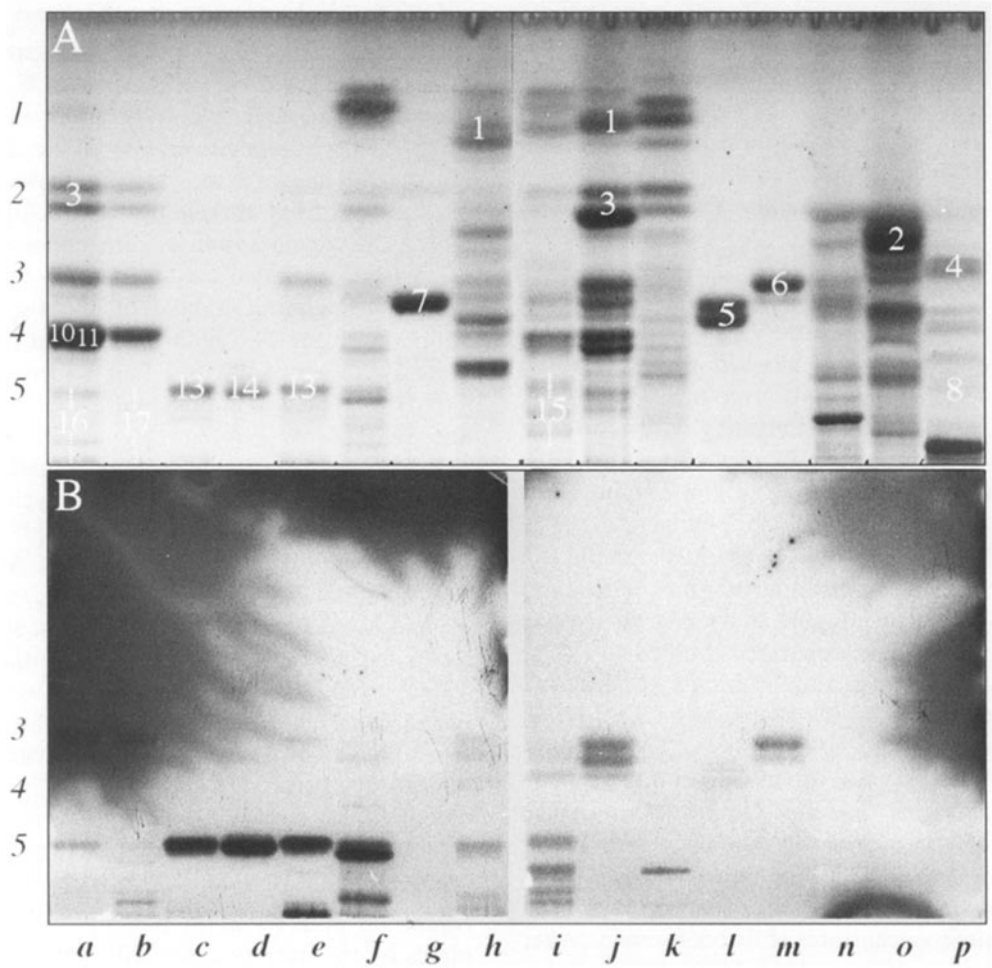

Figure 1. Binding of ^{125}I-labelled PA-I to glycolipids separated on thin-layer chromatograms as shown by autoradiography, B; and corresponding chemical staining (anisaldehyde) of the same panel of glycolipids, A. White labels refer to structures in Table 1, and numbers in the margin indicate the approximate number of sugars in the separated glycolipids. Lanes *a-b* and *e-k* contain neutral (N) and lanes *n-p* acid (A) glycolipid mixtures isolated from different human or animal tissues ($\leqslant$30 μg glycolipid per lane). Lanes *c-d* and *l-m* contain purified (P) glycolipids ($\leqslant$3 μg per lane, see Table 1 for structure). Lane ***a***, human A-erythrocyte (N); ***b***, human B-erythrocyte (N); ***c***, No. 13, B5, (P); ***d***, No. 14, P1 antigen (P); ***e***, rabbit erythrocyte (N); ***f***, rabbit thymus (N); ***g***, guinea-pig erythrocyte (N); ***h*** guinea-pig small intestine (N); ***i***, black/white rat, small intestine, non-epithelial tissue (N); ***j***, human stomach (N); ***k***, chicken erythrocyte (N); ***l***, No. 5, iso-globotriaosylceramide (P); ***m***, No. 6, globotriaosylceramide (P); ***n***, human erythrocyte (A); ***o***, human kidney (A); and ***p***, calf brain (A).

GC-MS (Hp 5830, and Trio-II, VG Masslab, UK). The capillary column DB-1, 0.25 mm × 15 m, 0.25 μm stationary-phase thickness (J&W Scientific, USA) was operated with He as carrier gas.

Results and discussion

PA-I binding to glycosphingolipids separated by TLC

The binding of radio-labelled PA-I lectin to glycolipids separated on thin-layer chromatograms was examined using a spectrum of reference glycolipids (Fig. 1, Table 1). The binding was controlled with biotinylated lectin using the same panel of glycolipids as used in Fig. 1, and the results showed that the biotinylated PA-I (data not shown) bound to the same glycolipids as the iodinated lectin.

The lectin binds most strongly to some glycolipids with five sugars (Fig. 1, B, lanes *c*, *d*, *e*, and *f*) although these glycolipids were applied in relatively small amounts (less than 3 μg per lane, Fig. 1, A). The binding glycolipid in lanes *c* and *e* is B5 glycolipid, Galα3Galβ4GlcNAcβ3Galβ4-GlcβCer of rabbit erythrocytes [27], and in lane *d*, the P1 antigen, Galα4Galβ4GlcNAcβ3Galβ4GlcβCer of human erythrocytes. The P1 antigen is also present in small amounts in human erythrocytes of blood group A (lane *a*).

The structure of the strongly binding double band, moving as a five-sugar glycolipid, in rabbit thymus (Fig. 1, lane *f*) was analysed after isolation by HPLC. Analysis with GC-MS after degradation to partially methylated alditol acetates showed the presence of a terminal galactose, two internal three-linked galactoses, one four-linked glucose and

Table 1. Binding of ^{125}I-labelled PA-I to glycosphingolipids on TLC plate. Galα epitope is indicated in italics.

No.	*Glycolipid structure*	*Binding*[a]	*Lane in Fig. 1*	*Reference*
1	Galβ1Cer	—	*f h i j*	[38]
2	$SO_3$3Galβ1Cer	—	*o*	[39, 40]
3	Galβ4Glcβ1Cer	—	*a b f j*	[38, 41]
4	NeuAcα3Galβ4Glcβ1Cer	—	*	
5	*Galα*3Galβ4Glcβ1Cer	+	*h l*	Isolated from dog intestine [42]
6	*Galα*4Galβ4Glcβ1Cer	++	*a b e f j m*	Isolated from human erythrocytes [38]
7	GalNAcβ4Galβ4Glcβ1Cer	—	*g*	[43]
8	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	—	*p*	[41]
9	Galβ3GalNAcβ4Galβ4Glcβ1Cer	—	*	
10	GalNAcβ3*Galα*4Galβ4Glcβ1Cer	—	*a b i j*	[38, 41]
11	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	—	*a j*	[38]
12	GalNAcα3GalNAcβ3*Galα*4Galβ4Glcβ1Cer	—	*h*	[44]
13	*Galα*3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	++	*c e f*	[27, 28]
14	*Galα*4Galβ4GlcNAcβ3Galβ4Glcβ1Cer	++	*a d*	[43]
15	*Galα*3[*Galα*3]$_{1-4}$*Galα*4Galβ4Glcβ1Cer	+	*i*	[31]
16	GalNAcα3(Fucα2)Galβ4-x-Galβ4Glcβ1Cer	—	*a*	Human RBC blood group A [38]
17	*Galα*3(Fucα2)Galβ4-x-Galβ4Glcβ1Cer	—	*b*	Human RBC blood group B [38]

[a] ++, Strong binding; +, moderate binding; —, no or very weak binding.
* Data not shown.

one four-linked GlcNAc. Since only one terminal sugar was detected the structure must be linear. Negative FAB-MS analysis (Fig. 2, A) indicated the sequence HexHexHexNAcHexHexCer. Two ceramide species were found; dihydrosphingosine combined with either 16 or 24 carbon atom fatty acid. The positive EI spectrum of methylated glycolipid (Fig. 2, B) has a molecular ion at *m/z* 1640 which corresponds to five sugars (four Hex and one HexNAc) and a ceramide with dihydrosphingosine and 16 carbon fatty acid. Fragments from terminal hexose (*m/z* 219 − 32 = 187), $Hex_2HexNAc$ (*m/z* 668 and 668 − 32 = 636), and from $Hex_3HexNAc$ (*m/z* 872) are very prominent in the spectrum (compare with formula). Furthermore, the glycolipid, on TLC plates, was incubated with antibodies specific for terminal Galα3- and Galα4-structures. These investigations unequivocally identified the terminal as Galα3 (not shown). Treatment with α-galactosidase of a TLC plate with separated rabbit-thymus glycolipid mixture (not shown) eliminated the binding capability of this five-sugar glycolipid. The glycolipid was, therefore, identified as B5. The binding of PA-I to this compound and B5 isolated from rabbit erythrocytes was equally strong (Fig. 1, lanes *c* and *f*). The presence of B5 in rabbit thymus was recently reported by He *et al.* [28].

The weakly binding and slow-moving band from rabbit thymus (Fig. 1, lane *f*) was analysed by FAB-MS and found to contain at least seven sugars (not shown). After treatment of the HPLC-isolated fraction with α-galactosidase the binding of PA-I lectin was destroyed. Investigation with Galα3- and Galα4-specific antibodies showed the same behaviour as for the five-sugar glycolipid, which implies that it carries the same terminal saccharide(s) as the five-sugar structure discussed above. This is in accordance with the recent analysis of rabbit thymus glycolipids [28].

In addition to the weakly binding band of rabbit thymus, additional weak binding was observed in lanes *a*, *b*, *h*, *i*, *j*, *k*, and *m* (Fig. 1). Globotriaosylceramide (Galα4Galβ4Glcβ1Cer) is present in lanes *a*, *b*, *h*, and *j* [29] and as a reference in lane *m* and binds in all cases while the isomer isoglobotriaosylceramide, Galα3Galβ4Glcβ1Cer (lane *l*) always binds more weakly. Galα4Gal placed directly on ceramide, as in galabiose (Galα4Galβ1Cer) is present in chicken erythrocytes, lane *k* (B.-M. Olsson *et al.*, unpublished), but showed no binding.

A weakly binding unidentified band with >five sugars was found in glycolipids from chicken erythrocytes (lane *k*). This glycolipid mixture contains Galα4Gal-structures, as shown by binding by P-fimbriated *Escherichia coli* [30] to several bands in this region (B.-M. Olsson, personal communication). The glycolipids from rat intestine contain several glycolipids terminating with Galα3Gal, from the four-sugar region to the region with very long glycolipids [31]. This could explain the multiple binding observed to slow moving bands of rat intestine (lane *i*).

Several non-binding isomers included in the overlay experiments are worth mentioning. Terminal Galβ3- as presented in GM1 (Table 1, No. 8 and Fig. 1, lane *p*) and gangliotetraosylceramide (Table 1, No. 9) does not bind. Neither does terminal Galβ4- which is present in Fig. 1, lanes *a* and *j* as lactoneotetraosylceramide (Table 1,

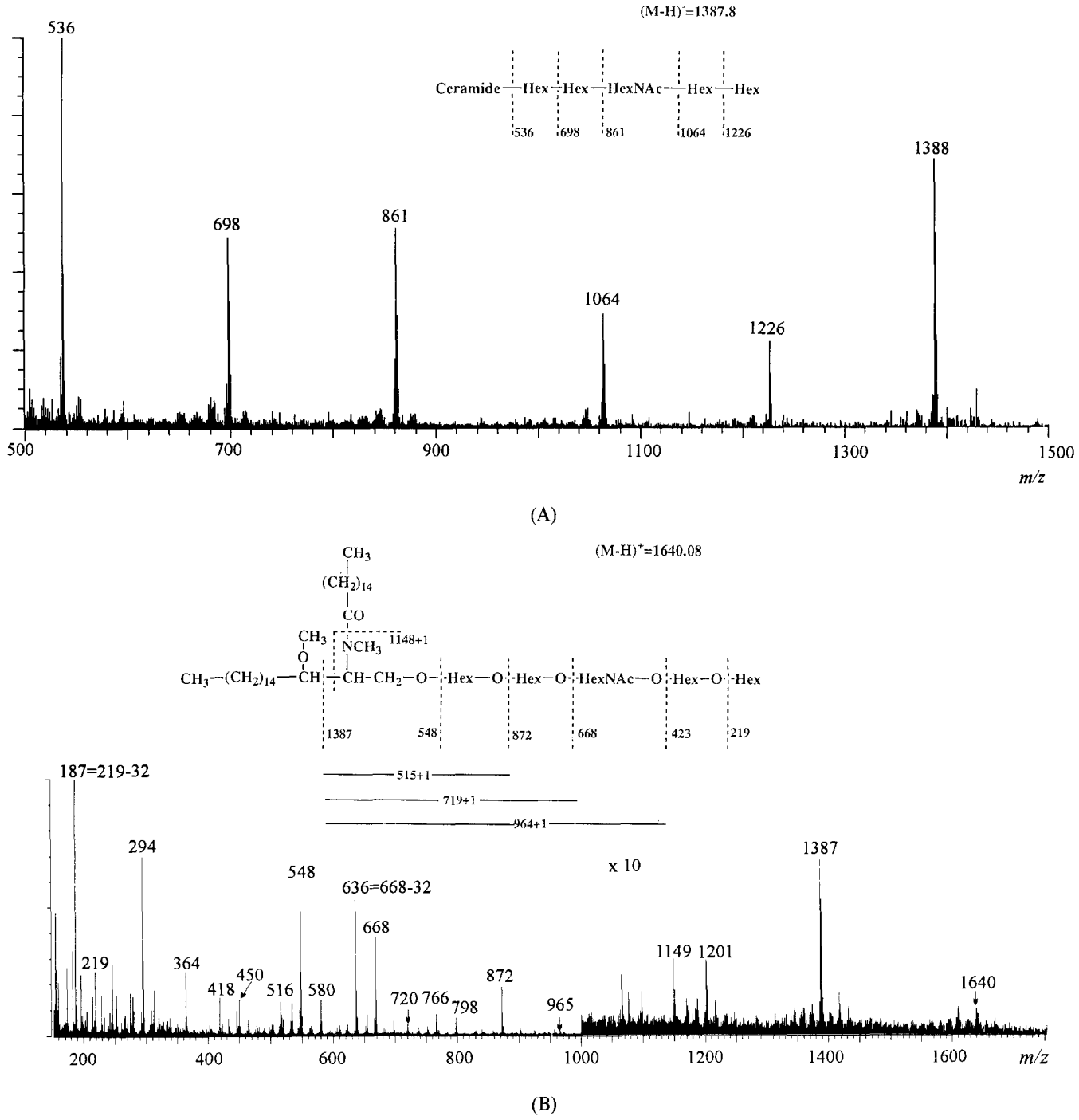

Figure 2. A, Negative-FAB mass spectrum; B, positive-EI spectrum and proposed fragmentations of a glycosphingolipid isolated from rabbit thymus. The dominating ceramide species in both spectra was dihydrosphingosine (18 carbon atoms) combined with a saturated 16-carbon fatty acid.

No. 11) or in most lanes as lactosylceramide (Table 1, No. 3). Terminal GalNAcα3- is present in blood group A glycolipids (Fig. 1, lane *a*, Table 1, No. 16) and the Forssman antigen (Table 1, No. 12), GalNAcβ3- in globoside (Fig. 1, lanes *a*, *b*, *i*, and *j*, Table 1, No. 10), and GalNAcβ4- in gangliotriaosylceramide (Fig. 1, lane *g*, Table 1, No. 7). Neither of these structures was bound by PA-I. As there was no binding to the six-sugar region of human RBC, blood-group B (Fig. 1, lane *b*), fucose substitution on the penultimate galactose of B6 prevents PA-I binding. The isolated lectin requires that the Galα3/4Gal-structures are placed at the non-reducing end of the glycolipids, while some other bacterial adhesions in addition can recognize binding epitopes located internally [32].

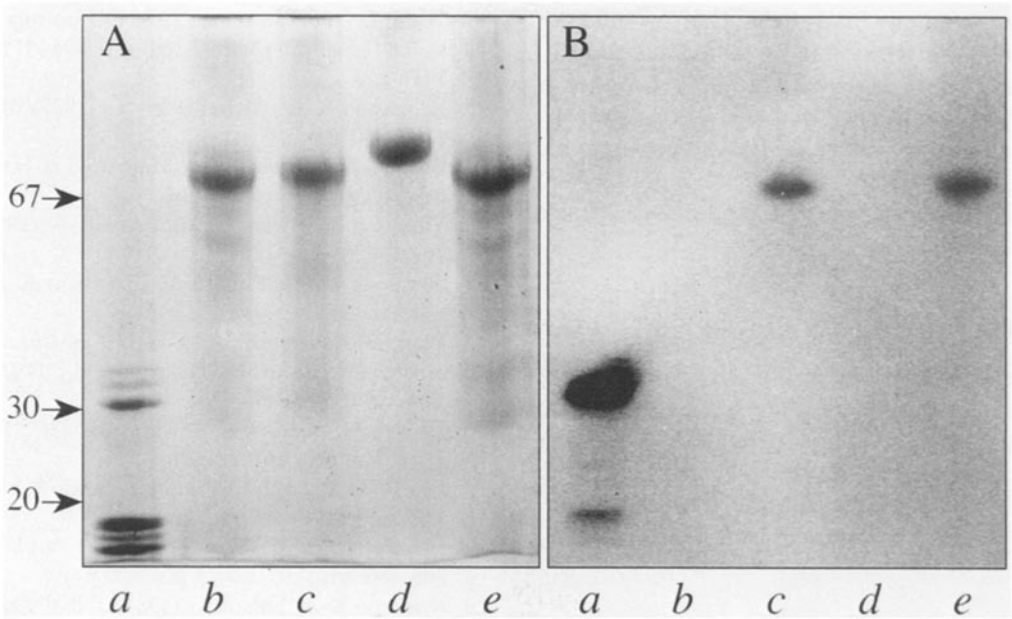

Figure 3. Binding of ^{125}I-labelled PA-I to blots of glycoproteins separated with SDS-PAGE. A, chemically stained (Coomassie brilliant blue) polyacrylamide gel, and B, corresponding autoradiogram of blot after overlay with ^{125}I-labelled PA-I. Lane ***a***, SDS-extract from chicken erythrocyte ghosts; ***b***, GalNAcβ3Galα4Galβ4Glcβ-BSA; ***c***, Galα4Galβ4Glcβ-BSA; ***d***, Galβ4Glcβ-BSA; and ***e***, Galα4Galβ-BSA. The figures to the left indicate approximate molecular masses (kDa).

Binding of PA-I to neoglycoproteins and glycoproteins

Demonstration that the PA-I binding is dependent on terminal Galα residues was also done using neoglycoproteins and glycoproteins. As can be seen in Fig. 3, the PA-I lectin showed affinity for Galα4Galβ-BSA (Fig. 3, B, lane *e*) and Galα4Galβ4Glcβ-BSA (Fig. 3, B, lane *c*), which both contain terminal Galα4 residues, but no binding was observed to internal Galα4 (GalNAcβ3Galα4Galβ4Glcβ-BSA, Fig. 3, B, lane *b*) or terminal Galβ4 (Galβ4Glcβ-BSA, Fig. 3, B, lane *d*). A strong binding was seen to chicken RBC glycoproteins (Fig. 3, B, lane *a*). These glycoproteins do not bind antibodies directed against terminal Galα4Gal structures (Zhantao Yang, personal communication).

Inhibition of PA-I binding to glycolipids on TLC by incubation with free saccharides

The role of galactose residues for the lectin binding was also investigated by inhibition of PA-I binding to the panel of glycolipids used for Fig. 1. The lectin was preincubated with five different mono- or disaccharides (Table 2) before overlaying on the TLC. Methyl-α and -β galactopyranoside as well as α-melibiose (Galα6Glc) completely abolished the lectin binding, while lactose inhibited it to a small extent. Methyl-α-glucopyranoside did not affect the binding at all. Galactose in this form is thus bound by the lectin irrespective of the C(1) anomeri, in contrast to the results from binding to glycolipids.

Table 2. Inhibition of PA-I binding to glycosphingolipids on TLC plates by free saccharides.

Saccharide	*Inhibition*[a]
Methyl-α-D-glucopyranoside	–
Methyl-β-D-galactopyranoside	++
Methyl-α-D-galactopyranoside	++
α-D-melibiose (Galα6Glc)	++
Lactose (Galβ4Glc)	+

[a] ++, strong inhibition; +, moderate inhibition; – no or very weak inhibition, saccharide concentration 50 mM.

Conclusions

Terminally placed Galα3Gal- or Galα4Gal-structures in glycolipids and glycoproteins bind the PA-I lectin. The binding is lost if the penultimate galactose is substituted with e.g. fucose. The binding is strongest when the Galα3/4Gal-structure is placed at least three sugars away from the ceramide; the binding to the Galα3Gal-structure is especially sensitive to a closely placed ceramide. When linked to BSA with a spacer, the two-sugar sequence Galα4Galβ is bound by PA-I (Fig. 3, lane *e*). From our data we cannot tell if carbohydrates or other chemical groups adjacent to the terminal Galα are directly bound by PA-I, or if they influence the binding by induction of conformational changes.

Apparently, the isolated PA-I lectin has a different binding pattern than those reported for different strains of the whole bacterium or an isolated adhesin, exoenzyme S [33]. They bind to lactosylceramide [33–35], gangliotetraosyl- and gangliotriaosylceramide [33–36], neuraminic acid [34, 36], Galβ3/4GlcNAcβ3Galβ4Glc [35] and Galβ3/4GlcNAc [37]. Neither of these binding specificities is shown by isolated PA-I.

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