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Forssman disaccharide is the specific ligand of a galectin from the sponge *Geodia cydonium* but does not mediate its binding to nuclear protein np56

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The galectin from *Geodia cydonium* (GCA) had previously been shown to be involved in regulatory mechanisms of cell sorting and adhesion during reaggregation of allogeneic sponge cells. In this contribution the binding specificity of GCA was established to be GalNAcα1–3GalNAcβ as structural component of Forssman pentasaccharide. Crossreactivities of terminal structural elements were revealed in the order GalNAcα1–3GalNAcβ > GalNAcα1–3(Fucα1–2)Galβ ≫ Galβ1–3GlcNAcβ > Galβ1–4Glc. Lectin binding to the Forssman antigen (K_i range 10^{-7}) or to blood group A-trisaccharide exceeded that to lactose (K_i range $10^{-3}/10^{-2}$) by three to four orders of magnitude. Cytochemical staining of eukaryotic cells on the light and electron microscopic level revealed lectin binding in the cytosol and in the nucleus (nucleoli), which was inhibitable with the soluble high affinity ligands. The nuclear binding of GCA could be ascribed to affinity-isolated 56 kDa protein (np56) in the nucleoplasm and was shown to be mediated by the peptide conformation of the ligand. Although GCA–np56 interaction was inhibitable with Forssman glycolipid or globopentaose, the carbohydrate binding site of the lectin is not involved due to the lack of competition by Forssman-specific lectins HPA or DBA. Since anti-CBP70 was immunologically cross-reactive to np56, it is concluded that the galectin GCA binds to np56 via similar mechanisms as reported previously for the interaction of CBP-35 (galectin-3) and CBP-70. Thus, GCA resembles galectin-3 in its binding characteristics but is likewise related to galectin-1 by sequence homology of its primary structure and by the molecular mass of its subunits.

Key words: CBP35/Forssman antigen/galectin/*Geodia cydonium* agglutinin/nuclear protein

Introduction

Since the first report on hemagglutinating activity in the hemolymph of *Limulus polyphemus* by Noguchi in 1903 (Noguchi, 1903), lectins have been found in nearly all phyla, classes and subclasses of invertebrates. While functional aspects of most plant lectins have remained obscure, the lectins of invertebrates have been assigned a series of essential biological roles (Rögener and Uhlenbruck, 1984), in particular, in carbohydrate mediated cell recognition or aggregation of sponge cells (Müller *et al.*, 1979), the recognition and removal of aged or altered glycoconjugates (Baldo and Uhlenbruck, 1975) or defense mechanisms protecting invertebrates against foreign invaders (Prokop *et al.*, 1968).

The soluble agglutinin from the marine sponge *Geodia cydonium* (GCA) is involved in the sorting-out of cells during reaggregation of allogeneic cells (Müller, 1982). GCA has recently been sequenced on the protein and DNA levels (Pfeifer *et al.*, 1993) and shown to exhibit striking sequence homology to the carbohydrate recognition domain characteristic for mammalian galectin-1. It can also be classified as galectin-1-related, since the tri- or tetrameric lectin is composed of subunits with apparent molecular masses M_r 15 (corresponding to the LEC1 gene) and M_r 13 or 16, respectively, (corresponding to the LEC2 gene) (Müller *et al.*, 1983; Pfeifer *et al.*, 1993). The subunits show isoform distribution in the pI range from 4.8 to 5.8 (M_r 15 and 16) or from 6.3 to 7.5 (M_r 13) (Hanisch *et al.*, 1984). Two subunits of the oligomeric lectin are linked via disulfide bridges (Müller *et al.*, 1983). The lectin molecule binds two moles of lactose per mole of native lectin in a calcium independent manner (Müller *et al.*, 1983) and shows a rather broad binding specificity for β-galactosides of various linkage types in inhibiton studies of hemagglutination and quantitative precipitation (Hanisch *et al.*, 1984). On the other hand, histochemical studies on human tissue sections have revealed that GCA binds to blood group A, B expressing epithelial and endothelial cells more strongly than to blood group O specimen and that blood group dependent binding cannot be inhibited by even high concentrations of lactose (Vierbuchen *et al.*, 1991). In the present contribution we demonstrate (1) that GCA binds with high affinity to the blood group A-trisaccharide and even more strongly to the Forssman di- and pentasaccharides, and (2) that nuclear binding of the lectin is mediated by protein–protein interactions with a 56 kDa nucleoplasmic component (np56).

Results

Purity of the lectin preparation

The lectin was isolated by affinity chromatography on immobilized lactose and was analyzed by SDS–polyacrylamide gel electrophoresis (Figure 1). In the presence of 0.1% SDS at room temperature the native lectin exhibited an apparent molecular mass of 60 kDa (Figure 1, lanes 6 and 8). Under denaturating conditions (1% SDS, 90°C, 10 min) protein bands were stained at 29, 16, 15, and

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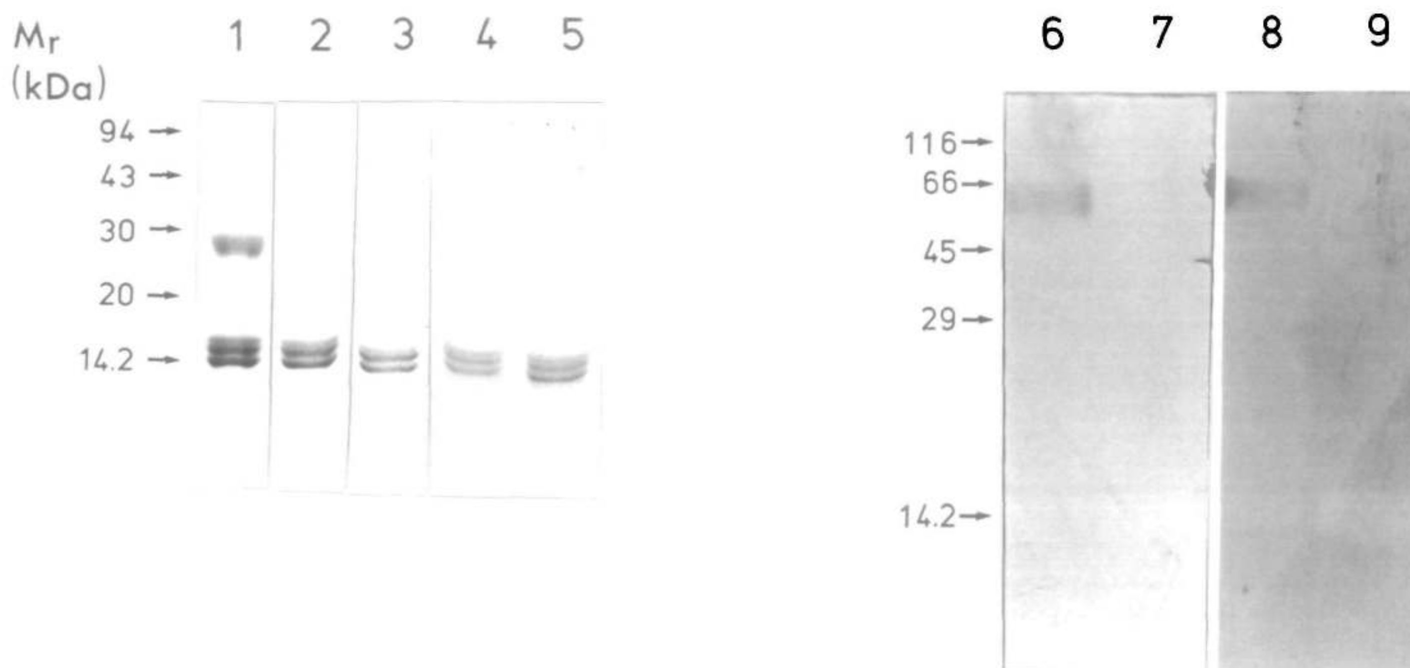

Fig. 1. SDS-gel electrophoresis of GCA and lectin subunit preparations SDS-gel electrophoresis was performed in 15% slab gels. GCA was affinity-isolated on lactosyl-agarose and analyzed after heating in sample buffer under non-reducing (lane 1) or reducing conditions (lane 2). The protein band at 29 kDa (refer to lane 1) was eluted from the gel and rerun as above under reducing conditions (lane 3). The lectin preparation from affinity chromatography on lactosyl-agarose was further purified by chromatography on immobilized Forssman disaccharide (lane 4) or precipitated with FS-di-PA (lane 5) and analyzed under reducing conditions. GCA from lactosyl-agarose was solubilized in sample buffer containing 0.1% SDS and incubated under nonreducing conditions at 25°C for 10 min (lanes 6 and 8). The same lectin preparation in sample buffer containing 1% SDS was pretreated under reducing conditions at 90°C for 10 min (lanes 7 and 9). Western blots on immobilon were stained with biotinylated Lac-BSA (lanes 6 and 7), or with biotinylated FS-di-PA (lanes 8 and 9) followed by avidine–phosphatase and were developed with 5- bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium.

13 kDa, indicating that the native lectin may represent a tetramer composed of subunits with apparent molecular masses 16, 15, or 13 kDa, respectively, and that two of these subunits could be linked covalently (Figure 1, lane 1). To demonstrate that two subunits of the oligomeric lectin are linked via disulfide bridges the 29 kDa protein was eluted from the gel and rerun after reduction in the presence of 2-mercaptoethanol (Figure 1, lane 3). Again three protein bands appeared at 16, 15, and 13 kDa demonstrating that the dimeric unit of the lectin has more than one subunit configuration. It is concluded from the above summarized data that the 29 kDa protein band is composed of isomeric heterodimers of the 15 and 16 kDa or of the 15 and 13 kDa subunits, respectively, and that the position of the non-covalently linked subunits within the tetramer are occupied by various combinations of the 16, 15, and 13 kDa species. Consequently, the LEC1 and LEC2 gene products are integral components of a structural and functional unit which exists in form of several isomeric variants. While isoforms of the subunits have previously been revealed by isoelectric focusing (Hanisch *et al.*, 1984) the isomeric variants of the native oligomeric lectin could not be separated as distinct species (data not shown).

The same subunit composition was revealed for GCA preparations affinity-isolated on immobilized lactose or Forssman disaccharide (Figure1, lanes 2 or 4, respectively) and for precipitates of the lectin with FS-di-PA (polyacrylamide-conjugated Forssman disaccharide) (Figure 1, lane 5). While lectin protein was quantitatively precipitated with FS-di-PA , no precipitate was formed in the presence of Core5-di-PA (polyacrylamide-conjugated core 5 disaccharide GalNAcα1–3GalNAcα) (not shown). On Western blots (Figure 1, lanes 6–9) the three lectin subunits obtained after heating in the presence of 2-mercaptoethanol were demonstrated to be non-reactive to Lac-BSA (bovine serum albumin-conjugated α-lactose) (lane 7) or to FS-di-PA (lane 9) which stained, however, the native oligomeric lectin (Figure 1, lanes 6 and 8). Attempts to separate the lectin subunits by avoiding heating and by using high concentrations of urea (8 M) or guanidinium hydrochloride (4 M) or of ionic (1% CHAPS) or nonionic detergents (1% octylglucosid) were unsuccessful.

Blood group dependency of lectin–erythrocyte binding

The lectin from *Geodia cydonium* agglutinates native and desialylated human red cells in the presence and absence of calcium ions. Hemagglutination titers measured in the absence or presence of calcium ions are dependent on the A, B, O blood group status of the donors in contradiction to previously published results (Vaith *et al.*, 1979). Human asialoerythrocytes were agglutinated according to their blood groups in the order A > B > O (Table I). Blood group dependent agglutination of asialoerythrocytes was strongly inhibited by the blood group A-trisaccharide, but only weakly by lactose (Table I). In the same way the agglutination of asialoerythrocytes was inhibited by the serum albumin conjugate of blood group A-trisaccharide, but not by the corresponding blood group B- or H-oligosaccharide conjugates or Lac-BSA. Distinct agglutination pro-

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Table I. Hemagglutination and inhibition studies of GCA-binding to human erythrocytes

[a]Erythrocytes from donors of blood group	Lectin concentration (μg/ml) causing agglutination of			
	Asialoerythrocytes	Native erythrocytes		
	GCA	GCA	DBA	HPA
A	0.24	31	250	<0.24
B	8	31	>1000	>1000
0	31	250	>1000	>1000
[b]Asialoerythrocytes from donors of blood group	Inhibitor concentration (mM) causing 50% inhibition of hemagglutination by GCA			
	A-Trisaccharide	Lactose	GalNAc	
A	0.02	17.0	>33	
B	0.01	9	>33	
0	0.01	4	>33	
[b]Asialoerytrocytes from donors of blood group	Inhibitor concentration (nM) causing 50% inhibition of hemagglutination by GCA			
	A-tri-HSA	B-tri-HSA	H-di-HSA	Lac-BSA
A	128	>128	>128	>128
B	4 (8)	>128	>128	>128
0	8 (32)	>128	>128	>128

[a]The abbreviations GCA, HPA, or DBA refer to the lectins from *Geodia cydonium, Helix pomatia,* or *Dolichos biflorus*, respectively. Parallel assays were performed in Tris (50 mM), NaCl (0.15 M), $CaCl_2$ (5 mM), pH 7.2 (TBS), or phosphate (50 mM) NaCl (0.135 M), pH 7.2 (PBS) with blood group defined cells from three to five individual donors. Titers measured in TBS or PBS were found to be identical and are given as average (mean) values.
[b]The titers given were measured in PBS. Titers given in brackets refer to parallel assays in TBS (see above), where deviations were observed from titers read in PBS.

files of blood group-defined asialo-erythrocytes were observed for lectins from *Helix pomatia* and *Dolichos biflorus* (Table I).

High-affinity binding of GCA to GalNAcα1–3Gal(NAc)β

The lectin from *Geodia cydonium* binds with high affinity to the Forssman and blood group A-oligosaccharides. This was demonstrated by using glycolipids of the globoseries and neoglycolipids or glycoproteins with conjugated blood group A, B, H active oligosaccharides and blood group precursor oligosaccharides (Tα, Galβ1–3GalNAcα; Tβ, Galβ1–3GalNAcβ; type 1, Galβ1–3GlcNAcβ, or type 2, Galβ1–4GlcNAcβ, backbone disaccharides) or milk oligosaccharides (LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; lactose) in binding studies with the biotinylated lectin (Figure 2a,b). It could be shown that GCA binding affinity to the A-trisaccharide exceeds that to the B-trisaccharide by a factor of approximately 500, whereas the HSA-conjugated blood group H disaccharide does not bind lectin in detectable quantities. Within the series of β-galactosides tested in this study, GCA preferentially binds to neoglycoproteins in the order Tβ-disaccharide: Galβ(1–3)GalNAcβ, type 1 chain disaccharide: Galβ1–3GlcNAcβ, Tα-disaccharide: Galβ1–3GalNAcα and lactose: Galβ1–4Glcα, while the binding to the type 2 chain disaccharide: Galβ1–4GlcNAcβ, is significantly weaker (Figure 2a). The same order of binding affinities has previously been observed in quantitative precipitin inhibition assays (Hanisch *et al.*, 1984). The binding of GCA to the blood group A-trisaccharide is distinct from other lectins with a related specificity like the agglutinins from *Dolichos biflorus* or *Helix pomatia* which unlike GCA are cross-reactive to O-linked α-GalNAc (Tn-antigen) on desialylated ovine submaxillary mucin (data not shown). Similarly, GCA is not reactive to the mucin-type core structure GalNAcα1–3GalNAcα (core 5) linked to polyacrylamide as demonstrated by quantitative precipitation (not shown).

GCA exhibits, however, strong reactivity to blood group A-related oligosaccharides on (neo)glycolipids (Figure 2b), in particular, to the Forssman glycolipid (GalNAcα1–3GalNAcβ1–3-Galα1–4Galβ1–4Glcβ1-Cer) or to GalNAcα1-OCH_2-CHO (linked to dipalmitoylphosphatidylethanolamine) derived from the disaccharide sequence GalNAcα1–6GalNAc on bovine submaxillary mucin (Figure 2b). No binding of GCA to lactosylceramide or to globoside was observed.

The binding affinities of GCA to β-galactosides or to the Forssman and blood group A antigens were compared by quantitative inhibition studies (Figure 3 and Table II). Forssman pentasaccharide was found throughout to be the most potent inhibitor of GCA binding with K_i values in the range of 10^{-4} to 2×10^{-4} mM when assaying its inhibitory potential on different immobilized ligands. While GCA binding to A-tri-HSA (Figure 3a) was specifically inhibited by 50% in the presence of 2.8×10^{-3} mM A-trisaccharide, the K_i for lactose was measured at 4.4 mM. Similarly, on immobilized β-galactoside ligands (Lac-BSA) the inhibitory potential of A-trisaccharide (K_i 0.25×10^{-3} mM) exceeded that of lactose by three orders of magnitude (Figure 3b and Table II). Since the observed inhibitory potency of A-tri and lactose on GCA binding to the respective immobilized ligands, A-tri-HSA or Lac-BSA, was not re-

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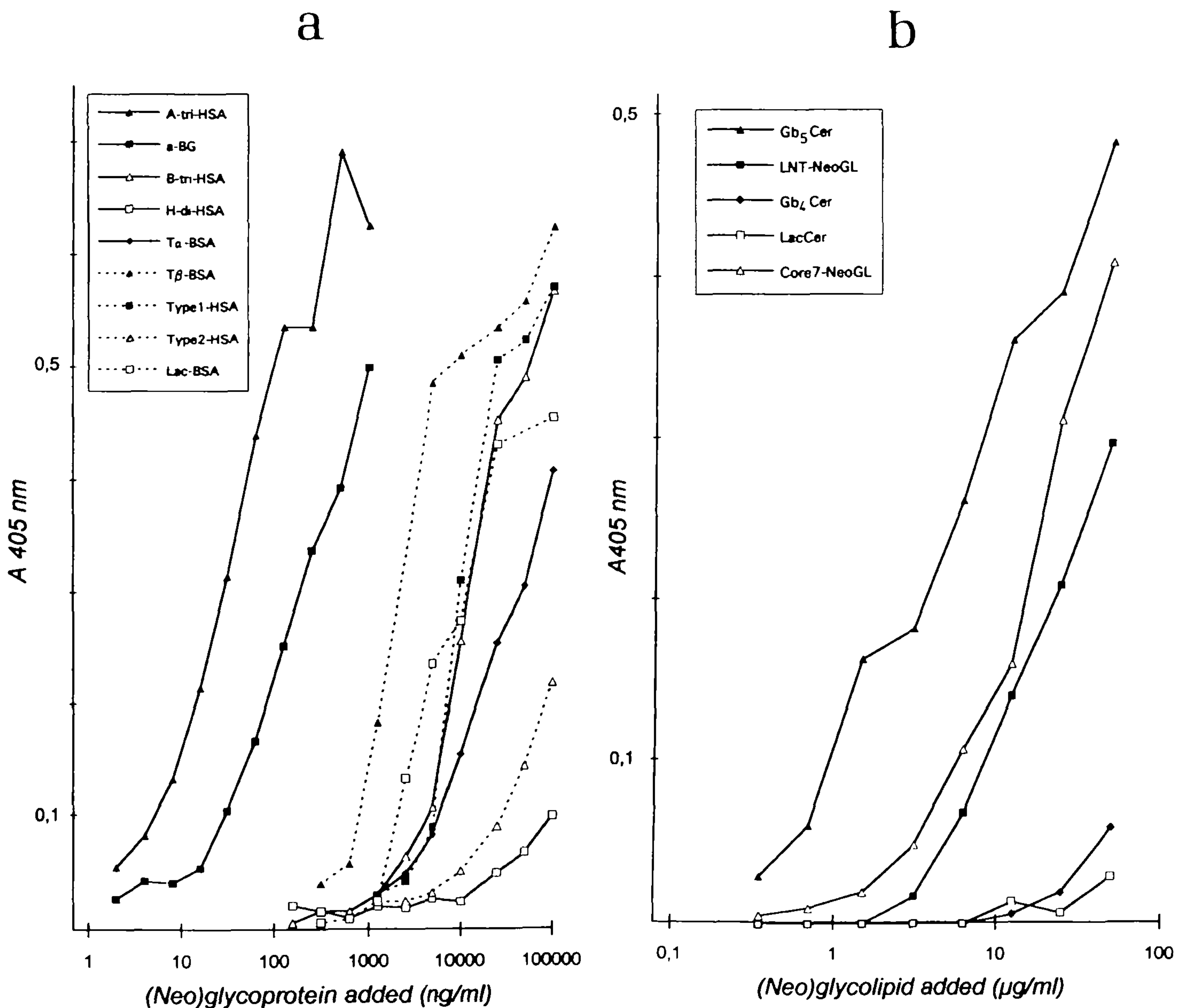

Fig. 2. Binding studies of *Geodia cydonium* lectin on (neo)glycoproteins and (neo)glycolipids Quantitative binding analyses were performed by enzyme immunoassay on polystyrene-immobilized glycoprotein (**a**) or glycolipid (**b**) ligands using biotinylated GCA (3 µg/ml) and avidine-phosphatase for detection. Explanation of abbreviations: (a) A-tri-HSA, B-tri-HSA or H-di-HSA, neoglycoproteins of blood group A, B, H oligosaccharides linked to human serum albumin; Tα- or Tβ-BSA, Thomsen-Friedenreich disaccharide linked α or β to bovine serum albumin; Lac-BSA, α-lactose linked to bovine serum albumin; type 1- or type 2-HSA, Galβ1-3GlcNAcβ or Galβ1-4GlcNAcβ linked to human serum albumin; aBG, asialo bovine glycophorin; (b) Gb_5Cer, globopentaosylceramide (Forssman glycolipid); LNT-neoGL; lacto-N-tetraose neoglycolipid; Gb_4Cer, globosid; Lac-Cer, lactosylceramide; Core7-neoGL, GalNAcα-O-CH_2-CHO derived neoglycolipid

ciprocal, a bispecificity of the oligomeric lectin can be excluded. N-Acetylgalactosamine was less effective compared to lactose by a factor of approximately 25–100. Using glycoprotein or neoglycoproteins as inhibitors the same order of inhibitory potential was revealed (Figure 3c). 50% Inhibition of GCA-binding to desialylated bovine glycophorin was measured at <1 µg/ml for A-tri-HSA, 2 µg/ml for asialo bovine glycophorin or >30 µg/ml for Lac-BSA, respectively. Inhibition of GCA-binding to Forssman glycolipid by globopentaose (Figure 3d) confirmed the specificity of the lectin–carbohydrate interaction and revealed further evidence for the high inhibitory potential of this oligosaccharide exceeding that of lactose by more than four orders of magnitude (Table II).

Intracytoplasmic and nuclear cell staining by GCA

Permeabilized human mammary carcinoma cells (T47D) or isolated nuclei were stained with biotinylated GCA prior to or after enzyme treatments (DNase I, RNase A) and in the presence or absence of sugar or glycoprotein inhibitors (Table III, Figure 4). Microscopic inspection re-

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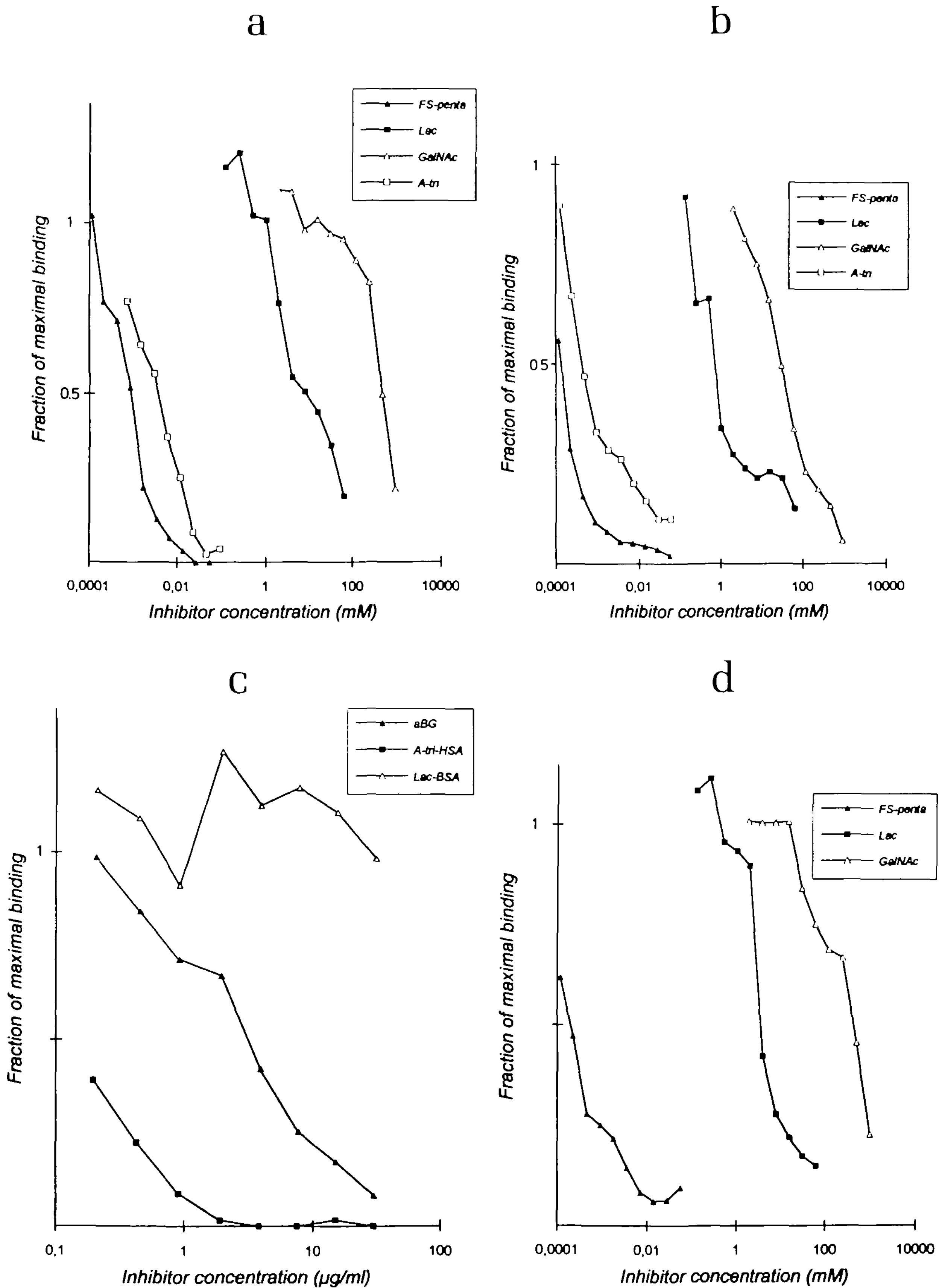

Fig. 3. Binding inhibition studies of *Geodia cydonium* lectin on high or low affinity ligands. Quantitative binding analyses in the presence of sugar or glycoprotein inhibitors were performed by enzyme immunoassay on polystyrene- immobilized neoglycoproteins A-tri-HSA, 0.5 µg/ml (**a**), Lac-BSA, 5 µg/ml (**b**), asialo bovine glycophorin, 0.5 µg/ml (**c**), or Forssman glycolipid, 2.5 µg/ml (**d**) using biotinylated GCA (3 µg/ml) and avidin–phosphatase for detection. Inhibitors used were: FS-penta, globopentaose (Forssman pentasaccharide); A-tri, blood group A trisaccharide; Lac, a-lactose; GalNAc, N-acetylgalactosamine.

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Table II. Inhibition of GCA-binding to high or low affinity ligands

Immobilized ligand[a]	Inhibitor[a]	Ki (mM)[b]	Ki(lactose)/Ki[b]
Lac-BSA	GalNAc	28.8×10^{-3}	4.1×10^{-2}
	Lac	0.88	1.0
	A-tri	0.25×10^{-3}	3320
	FS-penta	0.1×10^{-3}	7545
Tβ-HSA	GAlNAc	28.88	1.5×10^{-2}
	Lac	0.44	1.0
	FS-penta	0.1×10^{-3}	4000
A-tri-HSA	GalNAc	400.0	1.1×10^{-2}
	Lac	4.4	1.0
	A-tri	2.8×10^{-3}	1543
	FS-penta	0.8×10^{-3}	5500
Forssman-glycolipid	GalNAc	400.0	0.8×10^{-2}
	Lac	3.0	1.0
	A-tri	3.2×10^{-3}	938
	FS-penta	0.2×10^{-3}	15000

[a]Abbreviations and structures of ligands or inhibitors: Lac, lactose, Galβ1-4Glcα; Tβ, Thomsen- Friedenreich β antigen, Galβ1-3GalNAcβ; A-tri, blood group A-trisaccharide, GalNAcα1p3(Fucα1-2)Galβ; LNT, lacto-N-tetraose, Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ; FS-penta, Forssman pentasaccaride, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ.
[b]The inhibition constant Ki corresponds to the inhibitor concentration at which half maximum binding is achieved. It was calculated using the formula: fraction of maximal binding = Ki/(Ki + [c]).

vealed a diffuse intracytoplasmic staining with GCA of all cells (Table III) and intranuclear staining of a varying fraction of the total cells (Table III) or isolated nuclei (Figure 4a,b). Specificity of lectin binding was demonstrated by inhibition with non-labeled GCA (Figure 4f). No staining was observed, if the lectin was omitted or replaced by biotinylated BSA (Table III). On coincubation with the high affinity ligands blood group A trisaccharide or Forssman pentasaccharide lectin binding to nuclei was inhibited (Table III, Figure 4e). On the other hand, the low affinity ligand lactose was able to inhibit lectin staining only at high concentration (100 mM) (Table III, Figure 4d). Similarly, the nuclear and cytoplasmic binding of GCA was prevented on coincubation of the lectin with desialylated bovine glycophorin and A-tri-HSA, but not with the corresponding blood group B or H conjugates or with Lac-BSA at the indicated concentrations (Table III). While the nuclear staining was unaffected by removal of chromatin from the nuclear residue (Table III), RNase A digestion abolished the intranuclear lectin binding without reduction of the intracytoplasmic staining intensity (Figure 4c).

Ultrastructural investigation of the nucleus of mammary carcinoma cells displayed one or more nucleoli in most nuclei with a high concentration of lectin binding sites (Figure 5a,b). Moreover, some scattered particles could be detected over the remaining karyoplasm. The nuclear membrane was almost unlabelled, but sometimes a small cytoplasmic rim of electron dense particles could be ob-

Table III. Intracellular staining by GCA of permeabilized eukaryotic cells[a]

Cellular treatments and inhibitors	Microscopic inspection of intracellular GCA-staining in the phase contrast microscope at 400× magnification		Electron microscope at 14,000× magnification
	Cytosol	Nucleus	Number of particles bound to nucleolus vs. karyoplasm[b]
Positive control (biotinylated GCA)	+ (E)	+ (E)	88/12
Negative control (absence of GCA)	− (E)	− (E)	
Negative control (biotinylated BSA, absence of GCA)	−	−	
Enzyme treated cells			
RNase A	+	−	
DNase I	+	+	
Inhibitors coincubated with GCA			
Lactose (0.1M)	+/−	− (E)	
A-trisaccharide (0.2 mM)	−	− (E)	
FS-pentasaccharide (0.1 mM)	−	− (E)	7/3
A-tri-HSA (15 μg/ml)	−	− (E)	9/5
B-tri-HSA (15 μg/ml)	+	+ (E)	
H-di-HSA (15 μg/ml)	+	+ (E)	
Lac-BSA (15 μg/ml)	+/−	− (E)	9/4
Bovine glycophorin (15 μg/ml)	−	− (E)	
Non-labeled GCA (50 μg/ml)	(+)	− (E)	62/10

[a]Intracellular staining by GCA of permeabilized eukaryotic cells was performed with synchronized mammary carcinoma cells T47D after 16 h growth in the presence of fetal calf serum. The cells were stained on multitest slides with biotinylated GCA (3 μg/ml) and avidine–peroxidase. Staining was examined by microscopic inspection and confirmed by electronmicroscopy when indicated (E).
[b]Electronmicroscopic samples were fotographed at 14,000× magnification and evaluated by counting the numbers of particles bound to 10 cm^2 of 20 different nucleoli vs. surrounding karyoplasm. (+) indicates that GCA-binding is significantly reduced, but still above background binding. +/− indicates staining on light microscopic/electron microscopic inspection of nuclei or cytosol.

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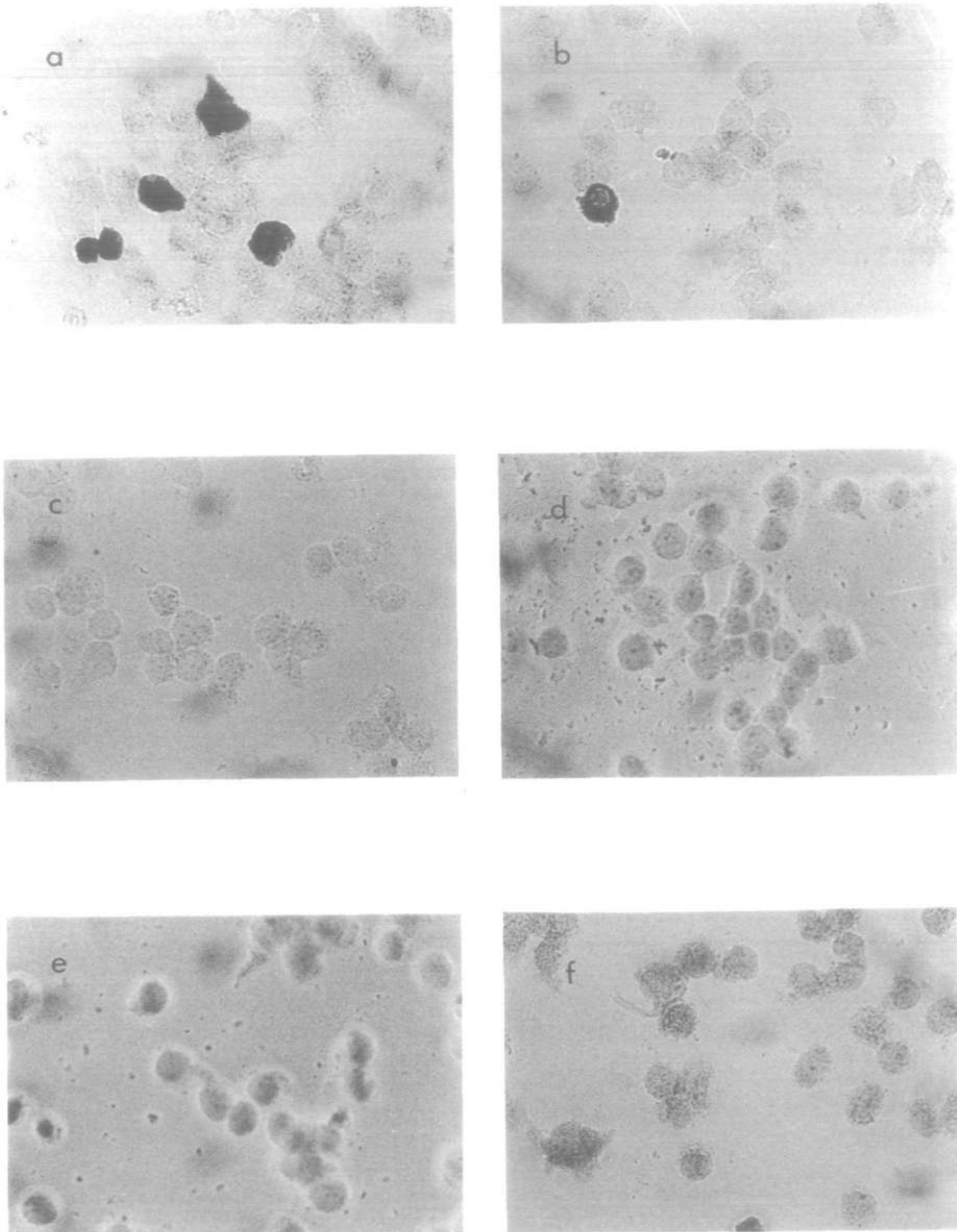

Fig. 4. Light microscopy of nuclear staining by GCA isolated nuclei of nonsynchronized T47D cells were stained on multitest slides with biotinylated GCA (3 μg/ml) and avidin–peroxidase. Staining was examined by light microscopic inspection at 400× magnification (refer also to Table III). Different preparations of cell nuclei stained with biotinylated GCA (**a, b**), or with biotinylated GCA after treatment with RNase A (**c**), or in the presence of 0.1 M lactose (**d**), 0.1 mM FS-penta (**e**), or 50 μg/ml of nonlabeled GCA (**f**).

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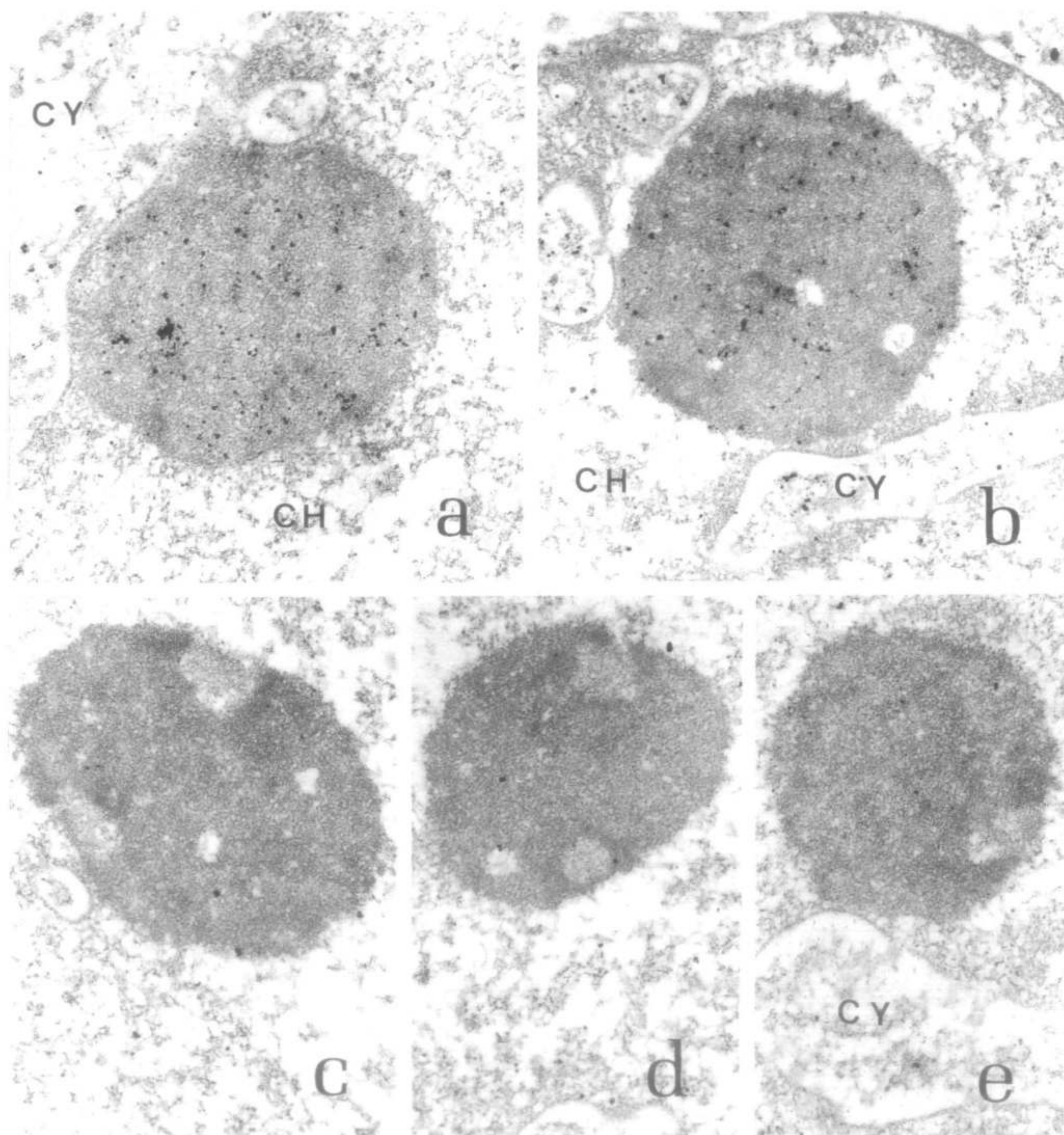

Fig. 5. Ultrastructural study of the nuclear lectin binding sites in mammary carcinoma cells (**a**, **b**) Nucleus with a high concentration of gold particles over the nucleolus beneath some scattered elements over the chromatin. In the adjacent cytoplasm, GCA binding sites are also detected. Inhibition of GCA by A-tri-HSA (**c**), FS-penta (**d**), and Lac-BSA (**e**) results in a marked reduction of gold grains (refer also to Table III). CH, chromatin; CY,cytoplasm; (a–e) 28,000×.

served near this structure (Figure 5b). Semiquantitative data were obtained by counting of particles bound to nucleoli or to the surrounding karyoplasm. On the average 88 particles were bound to nucleoli (10 cm^2 at 28,000 ×magnification) compared to 12 particles counted on a surrounding area of the same size.

After coincubation of GCA with bovine glycophorin (not shown), Forssman pentasaccharide (Figure 5d) or neoglycoprotein carrying high-affinity ligand carbohydrates (A-tri-HSA, Figure 5c), particles over the nucleoli were reduced to some scattered elements, showing a diffuse distribution pattern with densities of the nucleoplasmic background (less than 10 particles per 10 cm^2). Contrasting to the observations in light microscopy Lac-BSA (Figure 5e) was able to inhibit lectin binding to the nucleoli at concentrations given in Table III. The labeled lectin was

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only partially displaced by nonlabeled GCA (not shown). Controls by eliminating the step of lectin binding gave negative results.

GCA binds to a nuclear and cytoplasmic 56 kDa protein

Synchronized and metabolically labeled carcinoma cells T47D were homogenized and the isolated nuclei were ruptured by sonication prior to enrichment of the nucleoplasm by ultracentrifugation on a discontinuous sucrose gradient. Affinity chromatography of nucleoplasm or cytoplasm on immobilized GCA was performed in the absence or presence of 0.1 M lactose to suppress low affinity binding of β-galactosides (Figure 6). Lectin-reactive protein was demonstrated to be present in nucleoplasm and in cytoplasm, but less protein was bound in the presence of lactose. The registration of incorporated ^{3}H-uridine revealed that RNA was not associated with the lectin reactive protein (Figure 6).

Affinity-isolated nuclear and cytoplasmic proteins were concentrated by ultrafiltration prior to SDS–polyacrylamide gel electrophoresis (Figure 6). Silver staining revealed the presence of one major band corresponding to a protein with 56 kDa apparent molecular mass (np56). Irrespective of the starting material (nucleoplasm or cytoplasm) used for affinity chromatography this protein was the only component within the affinity-isolated fraction (Figure 6).

Binding of GCA to nuclear protein np56 is not mediated by carbohydrate ligands

To demonstrate a possible involvement of carbohydrates in the binding of nuclear protein np56 to the lectin, the affinity-isolated material was treated with periodate or α-N-acetylgalactosaminidase. Both treatments did not reduce GCA binding acitivity of np56 (Table IV, Figure 7a). However, control experiments performed in parallel with A-tri-HSA (Figure 7b) and Forssman glycolipid (not shown) demonstrated that under the same conditions a strong reduction of GCA binding activity was observed after exoglycosidase treatment. On the contrary, proteolytic digestion by pronase, trypsin or α-chymotrypsin destroyed GCA-binding activity. No effects were observed on treatment with the Glu-C-specific *Staph. aureus* V8 protease or the Gly-C-specific papaya protease IV. The involvement of peptides was further corroborated by the heat lability of the GCA-binding active nuclear component (Table IV). Incubation at 60°C for 10 min destroyed approximately 50% of the GCA binding activity.

The contents and compositions of carbohydrates present in affinity-isolated np56 were estimated on the basis of quantitative monosaccharide analyses in gas-liquid chromatography-mass spectrometry. The gas chromatographic profiles registered by single ion monitoring at m/z 173, 204, 217, and 298 for the trimethylsilylated 1–O-methylglycosides (derived from 100 μg of Lowry-protein) revealed the absence of N-acetyl-D-hexosamines and sialic acid on the subnanomol level and the presence of trace amounts of D-galactose and D-glucose in all np56 preparations, irrespective of the chromatographic conditions used for affinity isolation with regard to the presence or absence of lactose. D-Xylose was detected in trace amounts only in nucleoplasmic preparations of np56.

Despite the absence of potential carbohydrate ligands on affinity-isolated nuclear proteins their binding to GCA was inhibited by Forssman glycolipid (K_i 8 μM) the free Forssman pentasaccharide (K_i 100 μM) or lactose (K_i 100 μM) (Figure 8a), suggesting the involvement of the carbohydrate binding site of the lectin. The difference in the slopes of binding inhibition curves measured for the free and for the lipid linked Forssman pentasaccharide should result from the multifunctionality of the micellar Gb_5Cer in aqueous solution and its higher avidity compared to the monomolecular sugar inhibitor. Control experiments with Forssman-specific lectins (HPA, DBA) as competitive inhibitors of GCA-binding to np56 did not support the assumption that the lectin interacts with carbohydrate ligands on the nuclear protein.

The above summarized findings suggest a possible relationship of np56 to CBP70 which has been shown to bind via protein–protein interaction to CBP35 (galectin-3) (Seve *et al.*, 1993). In accordance with this assumption np56 was demonstrated to be immunologically cross-reactive with CBP70 by binding to rabbit anti-CBP70 (Figure 8b).

Discussion

In this study the galectin from the marine sponge *Geodia cydonium* is shown to exhibit an unusual binding specificity compared to galectin-1 by its high affinity to Forssman and blood group A-related carbohydrates of the general sequence GalNAcα1–3Gal(NAc)β. The lectin is demonstrated, moreover, to exhibit high binding affinity to a 56 kDa protein abundant in the nuclei of eukaryotic cells. Lectin binding is demonstrated to be unrelated to glycosylation of the nuclear protein, and to result from protein–protein interactions without direct involvement of the carbohydrate binding site.

Detailed aspects of the structural requirements for GCA–interaction with oligosaccharide ligands were inferred from binding and inhibition studies. From the order of preferred oligosaccharide sequences (Table V) it was concluded that a terminal α-GalNAc1–3 and a subterminal β-Gal(NAc) motif are essential prerequisits for high affinity binding of the lectin. In accordance with this, sequences with terminal β-GalNAc1–3/4 (Gb_4Cer, Gg_3Cer) or β-Gal1–3 (Tβ-disaccharide) and the corresponding epimer β-GlcNAc1–3 (degalactosylated lacto-N-tetraose-neoglycolipid) are binding inactive or of reduced binding affinity. The β-anomeric configuration of the subterminal sugar residue is essential, due to the finding that the α-anomer corresponding to the Core 5-disaccharide (GalNAcα1–3GalNAcα) was not precipitated by GCA as polyvalent polyacrylamid conjugate. The N-acetamido group of the terminal nonreducing α-GalNAc obviously contributes to the high affinity recognition by GCA, since the blood group A-trisaccharide is strongly preferred over the blood group B-trisaccharide. On the other hand, the N-acetamido group of the subterminal 'reducing' β-GalNAc of Forssman disaccharide can be substituted by α-Fuc1–2 as in the A-trisaccharide without significant reduction in the binding affinity.

In a conformation, which corresponds to the lowest energy for Forssman disaccharide in the isolated state (80°,

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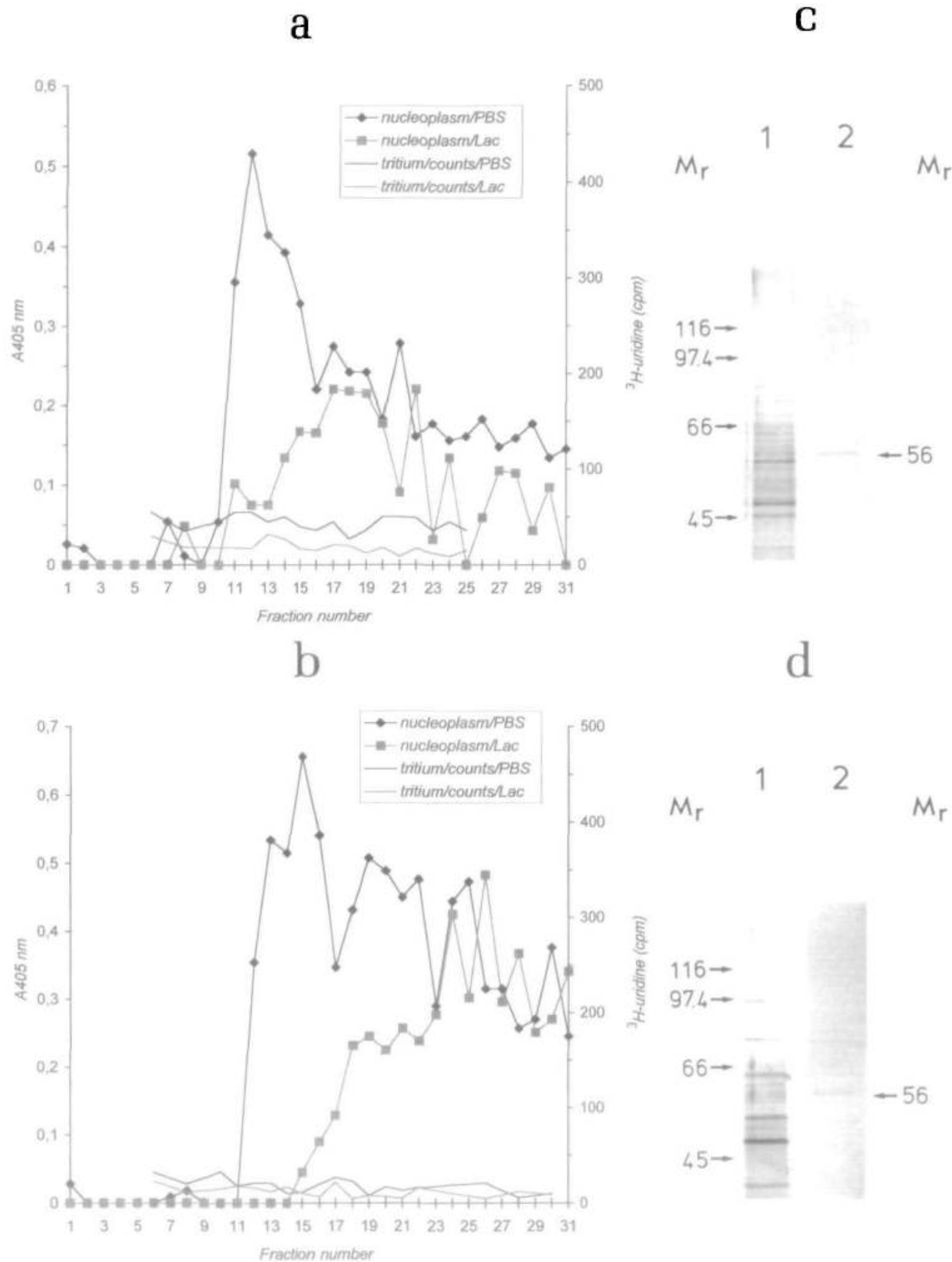

Fig. 6. Isolation of nuclear protein np56 by affinity chromatography on immobilized GCA Nucleoplasm (**a**) or cytoplasm (**b**) prepared from mammary carcinoma cells T47D were chromatographed on GCA-Sepharose 4 B in the presence or absence of 0.1 M lactose. Aliquots (50 μl) of 1 ml fractions eluted at low pH were analyzed after immobilization by drying at 37°C on polystyrene microtitration plates and assaying with biotinylated GCA (3 μg/ml) by enzyme immunoassay. A second aliquot (50 μl) was analyzed by liquid scintillation counting for the presence of RNA (^{3}H-uridine). SDS-gel electrophoresis in 10% polyacrylamid slab gels was performed with cellular fractions and affinity-isolated protein from nucleoplasm (**c**) or from cytoplasm (**d**). (**c**) Lane 1, nucleoplasm; lane 2, nucleoplasmic fraction isolated on immobilized GCA in the absence of lactose and corresponding to fractions 10–20 in (a); (d) lane 1, cytoplasm; lane 2, cytoplasmic fraction isolated on immobilized GCA in the absence of lactose and corresponding to fractions 12–22 in (b).

77°) (Imberty *et al.*, 1994) and which is stabilized by hydrogen bonding between the O4 oxygen of the reducing residue and the NH hydrogen of the other one, both N-acetamido groups are orientated in opposite directions. Referring to the low or medium affinities of β-galactosides the combining site of GCA may recognize two motifs of an oligosaccharide chain as previously discussed for galectin-3 (Feizi *et al.*, 1994). While β-Gal of lactose or other β-galactosides and β-GalNAc of Forssman disaccharide may occupy identical positions within the combining site of the lectin, the terminal prolongation by α-GalNAc creates a motif that should interact with an extension of the combining site characteristic for galectin-3. However, contrasting to galectin-3 which prefers extended sequences the combining site of GCA comprizes a disaccharide.

The galectins, generally, bind β-galactosides in a Ca^{2+}-independent manner and have been found in many different species as extra- and intracellular components which

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Table IV. Biochemical characterization of GCA–np56 interaction

Treatment of np56	Remaining GCA-binding activity (%)
Heat treatment (10 min)[a]	
30°C	100
40°C	80
50°C	67
60°C	47
70°C	25
80°C	4
90°C	14
100°C	9
Protease digestion (15 min)[b]	
Pronase (0.8 DMC-U/ml)	27
Trypsin (3.1 U/ml)	34
α-chymotrypsin (4.5 U/ml)	48
Staph. aureus V8 protease (50 U/ml)	95
Papaya protease IV (6 U/ml)	104
Periodate oxidation (1 mM, 60 min, 25°C)[b]	91
Coincubation with lectin[c] (0.1 mg/ml TBS)	
HPA	96
DBA	102

The assays were performed in duplicate (a, c) or triplicate (b) on polystyrene microtitration plates after treatment in solution (a) or *in situ* treatment of the immobilized ligands (b). The lectins were coincubated with GCA in the presence of 0.5 mM $CaCl_2$ (c).

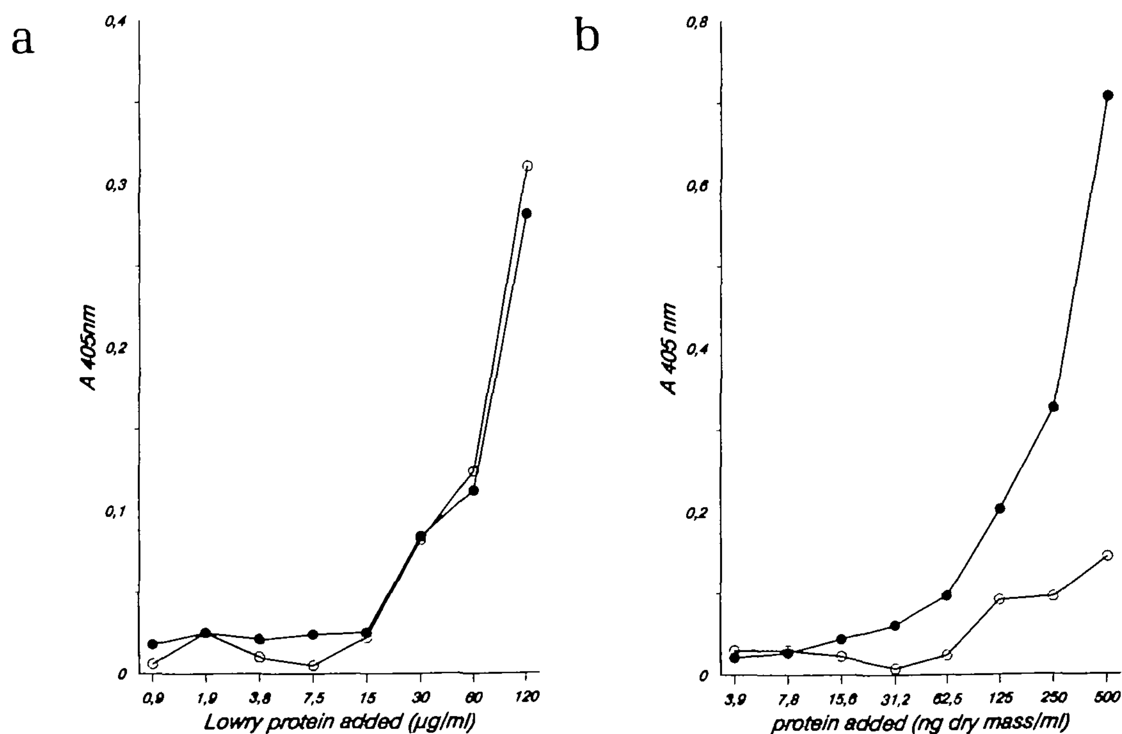

Fig. 7. GCA-binding to affinity-isolated nuclear protein np56 or to A-tri-HSA after treatment with exoglycosidase (**a**) Affinity-isolated nuclear protein np56 was immobilized to polystyrene microtitration plates, treated with 100 mU/ml α-N-acetylgalactosaminidase from chicken liver (O) or incubated with buffer (●) and assayed for GCA-binding activity. (**b**) Neoglycoprotein A-tri-HSA was immobilized and treated with enzyme as above (O) or buffer (●) prior to enzyme immunoassay of GCA-binding activity.

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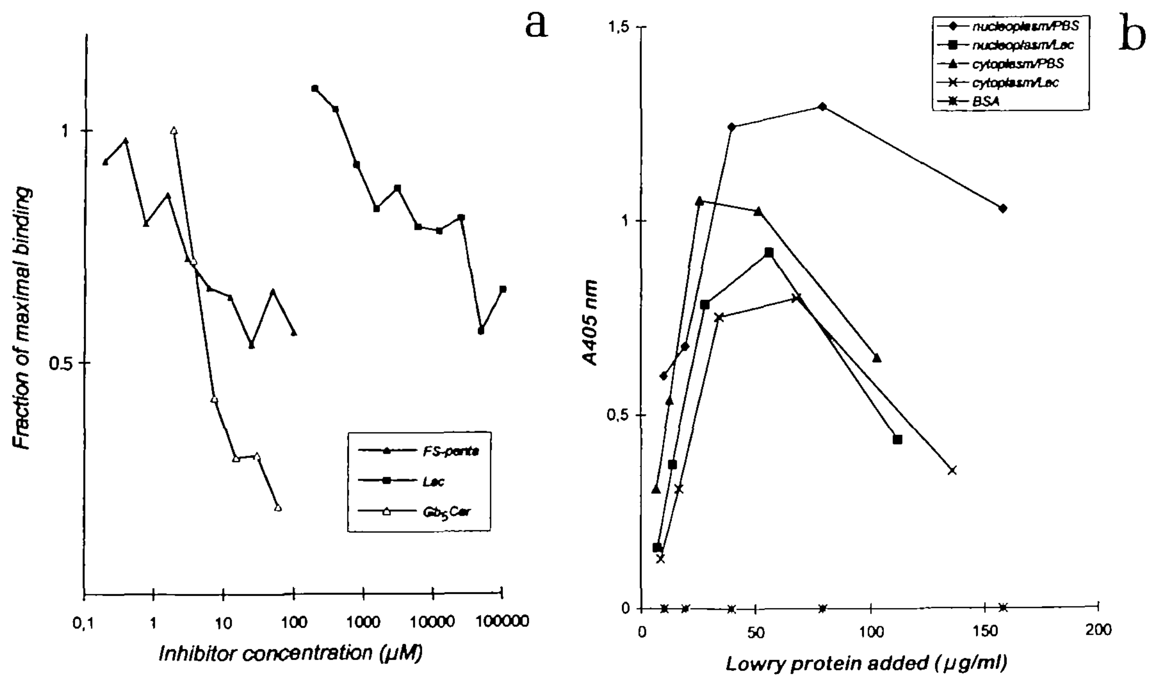

Fig. 8. Evidence for np56-CBP70 relationship (**a**) Inhibition of GCA-np56 binding: affinity-isolated nuclear protein np56 from mammary carcinoma cells T47D was immobilized to polystyrene microtitration wells and assayed for its binding capacity to GCA in the presence of inhibitors: Gb_5Cer, globopentaosylceramide (Forssman glycolipid); FS-penta, globopentaose; Lac, α-lactose. (**b**) Immunological cross-reactivity of np56 and CBP70: nuclear protein np56 was immobilized to polystyrene microtitration wells as serial twofold dilution. Bovine serum albumin was used as a control. After blocking the wells were incubated with 1:500 diluted rabbit antiserum to CBP70 for 1 h at 37°C and developed with 1:100 diluted swine anti-rabbit immunoglobulin-phosphatase (1 h, 37°C).

Table V. Oligosaccharide sequences tested in this study ordered according to decreasing binding affinity of GCA

Designation	Structure	Binding affinity
FS-penta[a,c]	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc	
FS-di[f]	GalNAcα1-3GalNAcβ	High
A-tri[a,d]	GalNAcα1-3(Fucα1-2)Galβ	
Tβ[d]	Galβ1-3GalNAcβ	
type 1-di[d]	Galβ1-3GlcNAcβ	Medium
B-tri[a,d]	Galα1-3(Fucα1-2)Galβ	
LNT[b]	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ	
Tα[d]	Galβ1-3GalNAcα	
Lac[a,c,d]	Galβ1-4Glcα	Low
type 2-di[b,d]	Galβ1-4GlcNAcβ	
aGal-LNT[b]	GlcNAcβ1-3Galβ1-4Glc	
Gb_4[c]	GalNAcβ1-3Galα1-4Galβ1-4Glc	
Gb_3[c]	Galα1-4Galβ1-4Glcβ	
Gg_3[c]	GalNacβ1-4Galβ1-4Glc	Inactive
Core 5-di[f]	GalNAcα1-3GalNAcα	
H-di[d]	Fucα1-2Galβ	
Tn[e]	GalNAcα	

[a]Free oligosaccharide.
[b]As neoglycolipid.
[c]As ceramide.
[d]As neoglycoprotein.
[e]On glycoprotein.
[f]As polyacrylamide conjugate.

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fall mainly into two distinct groups, based on the approximate molecular masses of their subunits (Wang *et al.*, 1991; Drickamer and Taylor, 1993) or can be classified according to the primary structures of the lectin proteins (Barondes *et al.*, 1994). Among the many functions which have been assigned to galectin-1 distinct functions in cellular differentiation have been postulated (Cooper *et al.*, 1990). For the related galectin-3 of mouse fibroblasts (CBP-35), where it is located predominantly in the nucleus as part of a ribonucleoprotein complex, a possible role in RNA-processing has been suggested (Wang *et al.*, 1992). The molecular basis of this function may involve interactions of CBP-35 with O-linked sugars on proteins of transcription and nuclear pore complexes (Wang *et al.*, 1992). However, O-linked oligosaccharides other than N-acetylglucosamine have not been described to occur in the nucleus (Hart *et al.*, 1989) on a chemical basis. Lectin binding studies have revealed evidence that more complex carbohydrates containing mannose, galactose, fucose, and N-acetylhexosamines may exist on the cytoplasmic surfaces of isolated nuclei (Nicolson *et al.*, 1972; Hart *et al.*, 1989). Lectin ligands on nuclear matrix and nuclear pore components were demonstrated for WGA at the electron microscopic level (Seve *et al.*, 1984) or for UEA I and ConA-ligands in the histone fraction of the protozoan *Tetrahymena thermophyla* (Levy-Wilson *et al.*, 1983).

As shown above, the galectin from the sponge *Geodia cydonium* defining a partial structure of Forssman and blood group A antigens is also reactive to components in the cell nucleus, indicating the possible presence of these complex blood group active carbohydrates. Also previous observations of Wang *et al.* (1992) have pointed to a possible involvement of blood group A-related structures in nuclear spliceosomes and their functional role in RNA processing. However, as shown in this contribution, the nuclear staining by GCA results from high affinity binding of the lectin to a protein ligand without involvement of the carbohydrate binding site. No chemical or enzymatic evidence was obtained in this study for the presence of high affinity carbohydrate ligands in the GCA-binding fraction of nuclear proteins. The interaction of GCA and np56 resembles a recently published mechanism of nuclear lectin–lectin interaction and its regulation via binding of the specific carbohydrate ligand (Seve *et al.*, 1993, 1994). In the same way as the complex of CBP-70 and CBP-35 is dissociated by conformational alterations of CBP-35 on binding of lactose, the GCA-np56-interaction is inhibited noncompetitively by the specific lectin ligands Forssman glycolipid and Forssman pentasaccharide. A relationship of nuclear protein np56 from mammary carcinoma cells to CBP70 is indicated by their immunological cross-reactivity. The galectin of *Geodia cydonium*, on the other hand, exhibits an obvious relationship to the galectin-3 with reference to both its binding specificity and its nuclear binding. Evidence obtained by Leffler and Barondes for the galectin-3 supports the assumption of related binding specificities within this class of lectins, since the authors were able to show that binding of the lectins to lactose derivatives was strongly enhanced by the incorporation of αGalNAc at position 3 of the βGal moiety (Leffler and Barondes, 1986; Sparrow *et al.*, 1987). According to the peptide sequence of GCA, its monomeric molecular mass and its binding characteristics the sponge lectin cannot be grouped unequivocally to one of the galectin classes (Barondes *et al.*, 1994). It shares properties of galectin-1 and galectin-3 indicating that GCA may be regarded as an ancestral form of the galectin family which later has diverged into a series of subfamilies with distinct structural properties and binding specificities.

Little is known on the role of GCA in the cellular biology of the sponge The established function of GCA in cell-sorting mechanisms might not be mediated by high-affinity ligands of the lectin. Accordingly, no immunochemical evidence for the expression of blood group A or Forssman antigens could be obtained for the plasma membranes of sponge cells in histochemistry using monoclonal antibodies (unpublished results). However, two classes of binding sites of different affinities were distinguished in a previous study which both could be inhibited by 20 mM lactose (Conrad *et al.*, 1984). Glycoconjugates in the extracellular matrix of *Geodia cydonium* which are involved in species-specific cell aggregation were analyzed to lack N-acetylgalactosamine, but to contain approximately 10-fold more galactose than any other neutral or amino sugar detected (Bretting *et al.*, 1981). There is also evidence that GCA interacts (even in the absence of Ca^{2+}) with itself (Conrad *et al.*, 1984). This interaction is, however, not mediated by covalently linked carbohydrate, since the reported sugar compositions of the lectin (Bretting *et al.*, 1981; Müller *et al.*, 1983) could be attributed to copurifying glycoconjugates (Hanisch *et al.*, 1984, and unpublished results).

It remains to be established whether intracellular galectin function is mediated by sugar binding specificity and, if so, which are the natural intracellular ligands of lectins with Forssman specificity and in which cellular processes are these lectin–carbohydrate interactions involved. Recent studies on the glycosylation of cytokeratins have revealed evidence that except the well-established O-GlcNAc substitution a novel type of posttranslational modification occurs on these cytoplasmic proteins which was identified as αGalNAc-containing glycans (Goletz,S., Hanisch, F.-G. and Karsten, U., unpublished observations).

Materials and methods

Neoglycoproteins, neoglycolipids, and oligosaccharides

Neoglycoproteins with different spacers were used. Those from Bio-Carb (Lund, Sweden): GalNAcα(Fucα1–2)Galβ-(APE)-HSA, Galα1–3(Fucα1–2)Galβ-(APE)-HSA, Galβ1–3GlcNAcβ1–3Galβ1–4(Glc)-APD-HSA, Galβ1–4GlcNAcβ1–3Galβ1–4Glc-(APE)-HSA have aminophenylethyl (APE) or acetylphenylenediamine (APD) as spacer arms and carry 10–20 (claimed) or 20–23 (analyzed) mol sugar hapten per mol of protein carrier. Another series of neoglycoproteins: Galβ(EAO)-HSA, Fucα1–2Galβ(EAO)-HSA, Galβ1–3GalNAcα(EAO)-BSA and Galβ1–3GalNAcβ-(EAO)-HSA was prepared by Dr. Kolar from Behringwerke AG (Marburg, Germany) and have in common an ethyl-N-acetamido-octanoic acid spacer (EAO) and carry 30 (claimed) or 29–34 (analyzed) mol sugar hapten per mol of protein carrier. The Sigma products (Sigma, Munich, Gemany): Galβ1–4Glc-(PIT)-BSA, Galβ1–3GlcNAcβ (CETE)-BSA, Galβ1–4GlcNAcβ(CETE)-BSA, and Galβ1–3GalNAcβ (CETE)-BSA are characterized by phenylisothiocyanate (PIT) or carboxyethylthioethyl (CETE) as spacer arms and carry 15–20 (claimed) or 18–22 (analyzed) mol sugar hapten per mol of protein carrier. Forssman glycolipid, globosid, globotrihexosylceramide, lactosylceramide, and the ganglioside GM2 were bought from Sigma, while neoglycolipids of milk oligosaccharides (lacto-N-tetraose and its enzymatically degalactosylated

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derivative) or mucin-derived alditols were synthetized according to Stoll *et al.* (1990). The synthetic conjugates of GalNAcα1-3GalNAcα (Core 5-di-PA), GalNAcα1-3GalNAcβ (FS-di-PA), GalNAcα1-3(Fucα1-2)Galβ (A-tri-PA), or Glcβ (Glcβ-PA) linked via a propyl spacer to poly [N-(2-hydroxy-ethyl)acrylamide] were purchased from Syntesome (München, Germany). Oligosaccharides used as inhibitors of lectin binding were GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc or GalNAcα1-3(Fucα1-2)Galβ (Accurate Chemical and Scientific Corp., Westbury, USA), Galβ1-4Glcα or GalNAc (Serva, Heidelberg, Germany). Bovine glycophorin was isolated from erythrocyte ghosts by extraction from a suspension in physiological saline with an equal volume of 90% phenol (15 min, 60°C). The aqueous layer was extensively dialyzed against distilled water and dried. A further enrichment was achieved by ethanol precipitation from a solution in 1% potassium acetate and collection of the fraction sedimenting at 40–50% ethanol.

Lectin extraction and purification

The siliceous sponge *Geodia cydonium* (Jam.) was collected near Rovinj (Mediterranean Sea) and a crude extract, which was kindly provided by Prof. W.E.G.Müller, University of Mainz, Germany, was prepared by solubilization on Ca^{2+}-free and Mg^{2+}-free artificial sea water as described previously (Vaith *et al.*, 1979). The lectin was isolated by affinity chromatography on immobilized lactose conjugated via divinyl sulfone to an agarose matrix (Medac, Hamburg, Germany) (Hanisch *et al.*, 1984). Aliquots of the lectin solution in PBS (7 mg/ml) were applied on 0.5 ml columns of Synsorb conjugated with Forssman disaccharide (Chembiomed, Edmonton, Canada) and chromatographed in the presence of 0.05% Tween 20 according to the manufacturer's instructions. The lectin was precipitated from solution (PBS) by incubating 70 μg of GCA (affinity-isolated on lactosyl-agarose) and 20 μg of glycoconjugates (Core5-di-PA, FS-di-PA) for 24 h at 4°C. The precipitate was pelleted by centifugation at 12,000 g and washed threefold prior to analysis of Lowry-protein and SDS-gel electrophoresis.

The purity of the lectin preparation was confirmed by polyacrylamide gel electrophoresis in the presence of SDS. The samples (10–100 μg protein) were analyzed in 15% polyacrylamide slab gels buffered with Tris-glycine, pH 8.9, (0.1% SDS). Lectin protein was dissolved in a sample buffer system containing 1% SDS (w/v) with or without 1% 2-mercaptoethanol in Tris-glycine and was loaded on top of a 3% stacking gel. Electrophoresis was performed in a Bio-Rad apparatus at 35 mA/gel for 3 h. Proteins were fixed and stained with Serva Blue G (Serva, Heidelberg, Germany) or with silver. For Western blot analysis the proteins were electrophoretically transferred to nitrocellulose in a semi-dry blot apparatus (Biotech, Denmark) and stained after blocking of the membrane with 5% BSA/PBS (1 h) with Forssman disaccharide conjugated to biotinylated polyacrylamide or with biotinylated lactosyl-BSA (20 μg/ml) by overnight incubation at 4°C.

Immunochemical methods

Hemagglutinating activity of the lectin was tested in Cook microtiter plates using 2% cell suspensions (25 μl) of native or *Vibrio cholerae* sialidase-treated human erythrocytes (10 mU/ml, 1 h, 37°C) from blood group A, B and O individuals and 1 mg/ml solutions of lectin (25 μl) in Tris–HCl (20 mM), NaCl (150 mM), $CaCl_2$ (0.5 mM), pH 7.2 (TBS), or sodium phosphate (50 mM), NaCl (0.135 M), pH 7.2 (PBS). The minimum concentrations of lectin that gave hemagglutination were read after 30 min incubation at room temperature by macroscopic inspection. For inhibition studies, serial twofold dilutions of sugar or glycoproteins (25 μl) were added to the wells and preincubated with the fourfold minimum concentration of lectin that gave hemagglutination of erythrocytes of blood groups A, B or O.

Enzyme immunoassays of lectin binding and binding inhibition were performed on 96 well, flat bottom polystyrene microtitration plates (Nunc, Wiesbaden, Germany). Glycoproteins or neoglycoproteins (0.1–10 μg/ml) were immobilized to the plates by drying 50 μl solutions in Na_2CO_3/$NaHCO_3$ buffer (0.1 M), pH 9.6 overnight at 37°C. Neoglycolipids or glycolipids were similarly dried down from solutions in methanol. Over a range of concentrations tested (0.025–5 μg/well) about 60–80% of neutral glycolipids and 70–90% of neoglycoproteins added were bound to the plastic surface (refer also to Dreßen *et al.*, 1992). Active surface remaining after coating was blocked with 200 μl solutions of 5% bovine serum albumin (BSA) in PBS (1 h, 37°C). Lectin (3 μg/ml PBS containing 0.5% BSA) which had been biotinylated with biotinamidocaproate N-hydroxysuccinimide ester (0.2 mg/ml and mg lectin for 4 h at ambient temperature) was incubated with the immobilized glycoproteins for 1 h at 37°C, followed after washing with 0.5% BSA/PBS for three times by addition of 50 μl streptavidine–alkaline phosphatase (Boehringer Mannheim, Germany) diluted 1/5000 in PBS/0.5% BSA. After 30 min at room temperature and four washing steps the wells were developed with p-nitrophenyl phosphate in diethanolamine buffer, pH 9.3 and analyzed photometrically at 405 nm. Inhibition studies were performed after immobilization of ligand by coincubation of sugar or glycoprotein inhibitors with the biotinylated lectin.

Immobilized ligands (Forssman glycolipid, A-tri-HSA or affinity isolated nuclear protein) were treated with periodate according to Woodward (1985) or solutions of α-N-acetylgalactosaminidase from chicken liver (EC. 3.2.1.49) in 0.1 M sodium acetate, pH 3.65 (100 mU/ml) for 24 h at 37°C. Proteolytic digestions of affinity isolated nuclear protein was performed in 0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.0 containing 0.1 mg/ml of the proteases: pronase E (8.6 DMC-U/mg), TPCK-treated trypsin (31 U/mg), and α-chymotrypsin (45 U/mg) (Serva, Heidelberg, Germany), *Staph. aureus* V8 protease (500 U/mg) (Sigma) or Papaya protease IV (60 U/mg) (Novabiochem, Bad Soden, Germany) for varying times (5–60 min) at 37°C prior to binding assays using biotinylated GCA.

Cytochemical studies

Light microscopic studies

Mammary carcinoma cells T47D (American Type Culture Collection, Rockville, MD, USA) or mouse fibrosarcoma cells L1LM12 (Hanisch *et al.*, 1990) were grown on multitest slides (10 wells/slide, ICN-Flow, Meckenheim, Germany) for 24 h. The medium was removed and the cells were air-dried and stored frozen over liquid nitrogen until used for cytochemical studies. Nuclei were prepared according to Crowles *et al.* (Crowles *et al.*, 1993), air-dried on mulitest slides, and fixed with 1% formaldehyde/PBS for 5 min. Cells were permeabilized after fixation as above by incubation in PBS containing 6 μg/mg digitonin (Karsten *et al.*, 1993). Alternatively, cells were permeabilized in 20 mM Tris, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride, pH 7.2 (TKM), buffer containing 1 mM vanadyl adenosine, 250 mM ammonium sulfate, and 0.5% Triton X-100 for 30 min at 4°C (Laing and Wang, 1988). The buffer (30 μl) was removed and the nuclei or cells were washed three times in TKM buffer prior to enzyme treatments or lectin binding studies. Treatments of nuclei or permeabilized cells with DNase I (Sigma) or RNase A (type XII A from bovine pancreas, essentially pure and protease-free, Sigma), respectively, were performed according to Laing and Wang (Laing and Wang, 1988). Nuclei or cells were washed in Tris-buffered saline (TBS; 20 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.5% bovine serum albumin (BSA). Aliquots of 30 μl lectin dilution (3 μg biotinylated GCA/ml) in TBS/0.5% BSA were added to the wells and incubated for 60 min at ambient temperature. For inhibition of lectin binding the high affinity ligands Forssman pentasaccharide (0.1 mM) or blood group A trisaccharide (0.2 mM), the low affinity ligand lactose (100 mM) or neoglycoproteins (15 μg/ml) were coincubated with GCA. Nuclei or cells were washed three times in TBS/0.5% BSA and incubated with 30 μl of streptavidine–peroxidase conjugate (1:300; Dako, Hamburg, Germany) or fluorescein-labeled streptavidine for 30 min at ambient temperature. After three washing steps the bound streptavidine–peroxidase was visualized by incubation in 0.05 M acetate buffer, pH 5.0, containing 0.02% 3-amino-9-ethylcarbazole and 0.5% H_2O_2 for 30 min at ambient temperature. The multitest slides were washed in TBS for 5 min and mounted in glycerol-gelatine prior to microscopic inspection using a 40 × objective lens.

Electron microscopic studies

Tissue processing: mammary carcinoma cells were washed in PBS and fixed in an aldehyde solution (2% paraformaldehyde, 0.5% glutardialdehyde) in PBS buffer (pH 7.4) for 6 h at room temperature. After quick rinsing in the same buffer pellets were exposed to 50 mM NH_4Cl in PBS for 60 min at room temperature and stored in PBS until embedding.

The embedding procedure was performed in a low temperature embedding apparatus (CSauto, Reichert, Vienna, Austria) using Bioacryl (British BioCell International, Cardiff, England) as resin. Small pieces of the pellet were dehydrated in graded ethanol series while reducing the temperature progressively to −20°C and then infiltrated with Bioacryl.

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Infiltrated samples were transferred into resin filled gelatin capsules and polymerised in the same apparatus by illumination with UV light at −10°C.

Labeling protocol: *Geodia cydonium* agglutinin (GCA) was digoxigenylated according to the manufacturer's recommendations using the DIG-Antibody Labeling Kit (Boehringer Mannheim, Biochemica).

Ultrathin sections were mounted on Pioloform coated nickel grids. All labeling steps were carried out at room temperature. Grids were preincubated with PBS (pH 7.4) containing 1% BSA, 0.1% Tween 20, and 0.1% Triton X-100 (buffer 1) for 10 min, followed by two h lasting incubation with GCA diluted to a final concentration of 30 μg/ml in buffer 1. After washing with PBS grids were incubated with anti-digoxigenin gold (average diameter about 0.8 nm) diluted 1:20 with the same buffer, supplemented by 0.1% Triton X-100 (buffer 2) for 1 h, followed by rinsing in PBS and double distilled water. The gold labeling was enhanced by a photochemical silver reaction. Counterstaining was performed both with saturated uranyl acetate and lead citrate. Grids were examined with a Zeiss EM 902A.

The extent of nonspecific background binding by the detection system was checked by omitting the lectin incubation step. Binding specificity was controlled by preincubation of the digoxigenin-labeled lectin with non-labeled lectin, with the oligosaccharide inhibitors α-lactose (0.1 M), Forssman pentasaccharide (0.1 mM), or with a series of neoglycoproteins and bovine glycophorin (15 μg/ml) for 2 h at room temperature.

Affinity isolation of nuclear protein np56

Mammary carcinoma cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 1% glutamine (200 mM), 2% penicillin (5000 IU/ml)-streptomycin (5000 UG/ml) solution, and 0.04% insulin (40 IU/ml). The cells were synchronized by serum starvation for 48 h followed by addition of fetal serum for 16 h prior to radiolabeling of RNA by incubation of the exponentially growing cells in the presence of ^{3}H-uridine (10 μCi/ml) for 3 h. The nucleoplasma and cytoplasma from approximately 10^{8} cells were prepared according to Crowles *et al.* (Crowles *et al.*, 1993) and the purity of the nucleoplasmic fraction was revealed by the lack of lactate dehydrogenase activity measured according to (Kaplan and Kahn, 1962). The cytoplasmic fraction exhibited lactate dehydrogenase activity of 31 U/ml. Both fractions were diluted 1:4 with phosphate (50 mM), NaCl (0.135M), pH 7.2 (PBS) and cycled in the presence or absence of 0.1 M lactose over a 5 ml column of GCA coupled to cyanogenbromide-activated Sepharose 4B (approximately 1 mg lectin/ml gel) for at least three times at 4°C and a flow rate of 10 ml/h. The column was washed with 0.1 M lactose/PBS (25 ml) and/or PBS (25 ml) and bound material was eluted with 0.1 M glycine-HCl, pH 2.5. Fractions of 1 ml were collected and mixed with 0.1 ml of 1 M Tris–HCl, pH 8.5. Aliquots of each fraction (50 μl) were analyzed for Lowry protein, their contents of GCA-reactive components by enzyme immunoassay or for their RNA contents by scintillation counting.

Analysis of carbohydrates by gas chromatography-mass spectrometry

The content and composition of carbohydrates on affinity-isolated protein np56 was measured by quantitative analysis of per-O-trimethylsilylated 1-O-methylglycosides in gas-chromatography/mass-spectrometry using conditions described by Merkle and Poppe (1994). The sugar derivatives were separated on a 15 m capillary column wall-coated with OV1 or DB5, respectively, using a temperature gradient from 100°C to 250°C (10°C/min). In the scan mode sugars were identified by their mass spectra registered in cyclic scans at 70 eV, by single ion monitoring they were measured at m/z 204, 217 (neutral sugars), 173 (amino sugars), or 298 (sialic acids), respectively.

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Abbreviations

aBG, asialo bovine glycophorine; A-tri, blood group A trisaccharide; B-tri, blood group B trisaccharide; BSA, bovine serum albumin; core 5-di, GalNAcα1-3GalNAcα; DBA, Dolichos biflorus agglutinin; FS-di/penta, Forssman di- or pentasaccharides; Gb_3Cer, globotrihexosylceramide; Gb_5Cer, globopentaosyl ceramide; GCA, Geodia cydonium agglutinin; H-di, blood group H disaccharide; HPA, Helix pomatia agglutinin; HSA, human serum albumin; Lac, α-lactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; PA, polyacrylamide; Tα/β, Thomsen-Friedenreich α or β disaccharides.

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