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Merging carbohydrate chemistry with lectin histochemistry to study inhibition of lectin binding by glycoclusters in the natural tissue context

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Running title: Lectin histochemistry enters glycocluster testing

Abstract

Recognition of glycans by lectins is a key route within the flow of biological information, leading for example to cell adhesion and growth regulation. Specificity and selectivity of this process are determined by carbohydrate structure (sequence and shape) and topology of its presentation on cells or in tissues. The chemical design of synthetic glycoconjugates with bi- to oligovalency (glycoclusters) affords tools to delineate structure-activity relationships experimentally by blocking lectin binding to an artificial matrix, often a glycoprotein, or to cultured cell lines. The drawback of these assays is that glycan presentation is different from the physiological situation of cells in tissues and from the natural glycocalyx. In order to approach the natural context we here introduce lectin histochemistry on fixed tissue sections to characterize the susceptibility of binding of two plant lectins, i.e. GSA-II and WGA, to a series of 10 glycoclusters. Besides valency, the panel further covers changes in the anomeric position (α/β) and the atom at the glycosidic linkage (O/S). Flanked by cell and solid-phase assays with human tumor lines and mucins, respectively, staining (intensity and profile) was analyzed in sections of murine jejunum, stomach and epididymis as a function of glycocluster presence. The marked and differential sensitivity of signal generation to structural aspects of the glycoclusters proves the applicability of this method. This enables comparisons between data sets obtained by using (neo)glycoconjugates, cells and the tissue context as test platforms. The special advantage of processing tissue sections is the monitoring of interference with lectin association at sites that are relevant for functionality. The introduction of glycoclusters to lectin histochemistry will especially be attractive in cases of multi-target recognition (glycans, proteins and lipids) by a lectin.

Keywords Agglutinin – Glycosylation – Glycoprotein – Mucins – Sugar Code

Introduction

The realization that glycan determinants of cellular glycoconjugates serve as platform to encode biochemical signals has given their enormous structural complexity and dynamic variability an active functional meaning (Abad-Rodríguez and Díez-Revuelta 2015; Corfield and Berry 2015; Gabius 2015; Hennet and Cabalzar 2015; Ledeen and Wu 2015; Patsos and Corfield 2009; Reuter and Gabius 1999; Schengrund 2015; Zuber and Roth 2009). In fact, information laid down in the sequence of glycans can enter cellular communication when being read and translated into post-binding effects by sugar receptors (lectins) (Gabius et al. 2011; Lis and Sharon 1998). Far from being a singular event, the stream of emerging insights into the ubiquity and physiological significance of glycan-lectin recognition has not only revealed its fundamental character as regulatory element in diverse cell activities. What's more, it also raised expectations to turn this knowledge into biomedical applications, e.g. by the design of optimized ligands to block or to target a distinct lectin. This work, and the hepatic asialoglycoprotein receptor became a landmark test case for synthetic bi- to oligovalent glycoconjugates (glycoclusters), taught the salient lesson that not the biochemical nature of the saccharide alone but the combination of structure with the topology of its presentation underlies the intriguing selectivity and specificity of lectin binding (Chabre and Roy 2009; Lee and Lee 1994; Murphy et al. 2013; Solís et al. 2015). Physiologically, only distinct glycoproteins or -lipids appear to qualify as productive counterreceptors for a lectin *in vivo*, for example the $\alpha_5\beta_1$ -integrin for galectin-1-dependent anoikis/apoptosis induction in carcinoma cells (Amano et al. 2012; Fischer et al. 2005; Sanchez-Ruderisch et al. 2011). Contributions by factors on six levels, starting with the key monosaccharide as contact site and then reaching the intricate glycoconjugate assembly in microdomains, are assumed to account for establishing counterreceptor properties (Gabius et al. 2015). Assays that determine glycocluster activity must thus take the possible influence of spatial parameters of ligand presentation into account. Challenge and opportunity at the same time, the closer the mode of glycan arrangement in assays comes to natural constellations, the more relevant will results be for predicting lectin blocking *in vivo*. Toward this aim, we here reveal the enormous potential of lectin histochemistry for assessing glycocluster potency on a proof-of-principle basis.

The specificity of lectins to distinct carbohydrates (mono-, di- or oligosaccharides) makes it possible to obtain information on their distribution in routinely processed tissue sections (Damjanov 1987; Roth 1978, 2011; Schrével et al. 1981), from the sites of synthesis and processing in the cell to routes of secretion and the final destinations, e.g. in the “sugary coating of cells”, termed glycocalyx (Bennett 1963), or the membrane (Danguy et al. 1994; Roth et al. 1983; Roth 1996, 2011; Spicer and Schulte 1992). What can hardly be designed artificially, i.e. these forms of natural glycan presentation including glycom complexity, is at hand in tissue sections. Interestingly, the rather obvious inherent potential of this material for biorelevant activity assessment of glycoclusters has so far not been deliberately examined. Extending the common specificity control of lectin binding from cognate mono- or disaccharides to glycoclusters thus appears as an attractive concept, warranting a pilot study.

To do so we built on our recent work with a panel of 10 synthetic probes using hydroquinone, resorcinol, benzene-1,3,5-triol and tetra(4-hydroxyphenyl)ethylene as scaffolds for bi-, tri-, and tetravalency (2015a; André et al. 2015b). Spaced azide derivatives of GlcNAc and scaffold alkynes had been used for conjugation to prepare the probes, which differ in structural aspects of the glycosidic linkage (bridging by S- or O-atoms, anomeric position in α or β) and in valency (for overview on structures, please see Fig. 1; for detailed structures, please see Fig. 2) (André et al. 2015a). GlcNAc-specific binding of wheat germ agglutinin (WGA) and *Griffonia (Bandeiraea) simplicifolia* agglutinin-II (GSA-II) to a matrix of (neo)glycoproteins had therein been shown to be inhibited by the glycoclusters. Measurements with the Lec8 mutant cell line of the Chinese hamster ovary (CHO) system, which has a high-level surface presentation of GlcNAc by truncated N-glycans, backed the result of varying degrees of inhibitory activity among the glycoclusters (André et al. 2015a). In order to take testing of the synthetic products to the histochemical level, suited organs based on lectin binding must first be identified.

Natural ligands for these two lectins encompass core 2 (branched) O-glycans with an α -GlcNAc terminus, which are found in mucins from mammalian gastric mucus such as hog gastric mucin (type II/III) (Hanisch et al. 2014; Kochetkov et al. 1976; Rossez et al. 2012; van Halbeek et al. 1983). Matching the expression of the α 1,4-N-acetylglucosaminyltransferase, the enzyme completing the synthesis of these O-glycans

in stomach (Nakayama et al. 1999), fundic gland cells had been described to strongly bind GSA-II, in intermediate and *trans* Golgi cisternae and mucous granules intracellularly, reactivity also seen in duodenum, colon, pancreas and gallbladder (Ihida et al. 1988; Nakamura et al. 1998; Oinuma et al. 1994; Suzaki and Kataoka 1992). In addition to these O-glycans, WGA is special to interact with GlcNAc present in the core region of N-glycans (Ardá et al. 2013; Goldstein and Poretz 1986). This aspect of WGA specificity enables histochemical staining without concomitant GSA-II reactivity, for example in epididymal epithelium of mouse (Lee and Damjanov 1984). Based on this experience we selected murine gastrointestinal tract (jejunum and stomach with GSA-II/WGA reactivity) and epididymis (WGA reactivity) for lectin histochemical study.

In this report, we first take glycocluster testing of WGA from the CHO mutant cells to the level of human tumor cells *in vitro* and then map the panel's inhibitor activity on mucin binding by GSA-II and WGA in a solid-phase assay. The ensuing histochemical monitoring, assessed semi-quantitatively and by fully automated morphometry, provides clear evidence for a versatile applicability of lectin histochemistry in glycocluster testing.

Materials and methods

Lectins and glycoclusters

WGA was purified by affinity chromatography, checked for purity by one- and two-dimensional gel electrophoresis and labeled under activity-preserving conditions using the N-hydroxysuccinimide ester derivative of biotin (Sigma, Munich, Germany) (Debray et al. 1981; Gabius et al. 1992; Lotan et al. 1973). Biotinylated GSA-II was purchased from Vector Laboratories (distributed by Enzo Life Sciences, Lörrach, Germany). The panel of the 10 GlcNAc-presenting bi- to tetravalent glycoclusters and the bivalent GalNAc-carrying compound used as specificity control were synthesized, analyzed and rigorously controlled for purity as described (2015a; André et al. 2015b).

Cell assay

Lectin binding to the surface of human colon adenocarcinoma (CaCo-2), pancreas adenocarcinoma (Capan-1) and glioblastoma (U87) cells was determined cytofluorimetrically after an incubation step for 30 min at 4 °C using 5×10^4 cells

suspended in Dulbecco's phosphate-buffered saline with biotinylated lectin (in the absence or presence of inhibitor), as done previously with the Lec8 mutant cells (André et al. 2015a). Besides the tumor lines, the CHO wild-type line and its Lec2 mutant (reduced degree of sialylation), kindly provided by P. Stanley (Albert Einstein College of Medicine, Bronx, USA), as well as U87 cells treated with 150 μ M 1-deoxymannojirimycin (Calbiochem, Darmstadt, Germany) for 24 h to reduce presence of complex-type glycosylation and confirmed to present increased extent of mannose on their surface by labeling with concanavalin A, as described (André et al. 2009b) <<2009a), were used. Signal detection in FACScan analysis was based on the reporter conjugate R-phycoerythrin/streptavidin (1:40; Sigma), leading to quantitative data on lectin binding in terms of mean fluorescence intensity/percentage of positive cells. Experimental series with a cell line were routinely done with aliquots of the same cell suspension at the same day, with at least four independent titrations per compound and cell line. Controls included monitoring of viability and assessment of lectin-independent staining. Standard deviations after normalization of data did not exceed 11.9%.

Solid-phase assay

Solutions of the three types of mucin (hog gastric mucin, types II/III from Sigma; bovine submaxillary mucin (BSM) prepared as described (Reuter et al. 1983)) in phosphate-buffered saline (PBS; 50 μ l) were applied to coat the surface of microtiter plate wells with glycoprotein overnight at 4 °C. Remaining sites free for non-specifically binding protein were then saturated by an incubation step with 100 μ l PBS containing 1 % (w/v) carbohydrate-free bovine serum albumin for 1 h at 37 °C. The following incubation with lectin-containing solution in the absence or presence of inhibitors was set to 1 h at 37 °C, and spectrophometric signal assessment at 490 nm was facilitated by stepwisely applying commercial streptavidin/horseradish peroxidase conjugate (0.5 μ g/ml; Sigma) and the chromogenic substrates (1 μ g/ml *o*-phenylenediamine and 1 μ l/ml hydrogen peroxide) as described (André et al. 2008; Zeng et al. 1993). Titrations with free sugar or glycoclusters were routinely performed in duplicates with up to seven independent series, the standard deviation after data normalization not exceeding 13.7%.

Lectin histochemistry

Jejunum, stomach and epididymis from four six-week-old C57BL/6 mice were cut in small pieces, which were immediately fixed in methanol with 30% acetic acid, 4%

buffered paraformaldehyde or Bouin's solution, dehydrated by stepwise passage through a series of solutions with increasing ethanol content, then isopropanol, finally xylene, and embedded in paraffin wax at 61 °C. Sections (about 5 µm) were mounted on Superfrost® plus glass slides (Menzel, Braunschweig, Germany) and taken through the series of incubation steps for visualization of the staining profile of the labeled lectin, starting with a washing step using 10 mM Hepes buffer (pH 7.5) and blocking sites for unspecific binding of protein by an incubation with buffer containing 1% (w/v) carbohydrate-free bovine serum albumin as described (Lohr et al. 2010). The incubation with lectin-containing solution was done overnight at 4 °C in a humid chamber, systematically testing various concentrations, first covering a broad range, the range thereafter between 0.125 µg/ml and 1 µg/ml, to obtain optimal signal-to-noise ratio. Bound lectin was visualized using Vectastain® ABC-Kit alkaline phosphatase standard AK-5000 reagents and then the Vector® Red-Kit alkaline phosphatase substrate SK-5100 (Enzo Life Sciences) used for 30 min in the dark in all comparative analyses. Counterstaining with Mayer's haemalaun, dehydration and mounting in Eukitt® (Kindler, Freiburg, Germany) completed the histochemical processing. Omission of the incubation step with lectin-containing solution was performed to detect any lectin-independent staining. Recording of the images was done with an AxioImagerM1 microscope (C. Zeiss MicroImaging, Göttingen, Germany) equipped with an AxioCam MRc3 digital camera and the software AxioVision version 4.6. Semi-quantitative analysis by two independent observers was based on examining at least ten high-power fields per section and at least three sections per organ of each of the four animals. The histomorphometric analysis based on digitalized images was performed, as described in detail previously (Toegel et al. 2014). The percentage of stained area at each experimental condition in a series was then plotted against the concentration of sugar using the curve-fitting tool of the GraphPad Prism Software version 5.0 (GraphPad Prism Software Inc., La Jolla, USA).

Results and discussion

Inhibition of lectin binding to human tumor cells

Because the glycan display on surfaces sets the structural prerequisites for lectin activity on cells, this type of platform for an inhibition assay of lectin binding can grade efficacy of blocking by glycoclusters with functional relevance. Previously, GSA-II

binding to β -GlcNAc termini of truncated N-glycans, abundantly expressed on CHO Lec8 mutant cells due to their impairment in galactosylation capacity, had been documented (André et al. 2015a). They establish a second form of ligands besides O-glycans of mucins with α 1,4-linked GlcNAc at branch-end positions. The CHO wild-type cells with their α 2,3-sialylgalactose termini were not reactive with GSA-II, confirming its binding specificity to β -GlcNAc at truncated branch ends, but they were with WGA (André et al. 2015a). In the case of WGA, which has dual specificity to GlcNAc and sialic acid (Goldstein and Poretz 1986), theoretically possible to the sialic acid and the core GlcNAc moieties. In principle another CHO glycosylation mutant, i.e. Lec2 with defective sialic acid transport into the Golgi (Patnaik and Stanley 2006), can help clarify the nature of the primary contact site. Binding of WGA was slightly reduced for the Lec2 mutant line (not shown), pointing to a predominant reactivity of the N-glycan core to WGA. This appears to be in line with NMR spectroscopy-based identification of the core as exclusive contact site for WGA in a sialylated biantennary glycopeptide (Ardá et al. 2013). However, shifts in glycoprotein profiles, caused by altered glycosylation recently seen for a growth factor receptor in a related CHO mutant (Gabiús et al. 2011), and the reactivity of truncated (GlcNAc-free) O-glycans of ovine submaxillary (asialo)mucin (sialyl T_n and T_n), measured by precipitation analysis (Peters et al. 1979), compromise reliability of extrapolations between wild-type and mutant cells. For GSA-II, the galactosylation of GlcNAc at branch ends in Lec2 cells completely switched off reactivity (not shown), as was the case for the wild-type cells (André et al. 2015a). In order to obtain data on glycocluster activity with human cells we processed tumor lines.

When monitoring human tumor lines with GSA-II and WGA, reactivity was invariably found to be confined to WGA so that glycocluster testing could only be performed for this lectin. The presence of free cognate sugar was effective to inhibit binding (Fig. 3A,C,E), non-cognate GalNAc was not active underscoring specificity of the association (not shown). Grading of blocking activity in the three tested lines resembled that measured previously with the glycoprotein asialofetuin as ligand (André et al. 2015a), as exemplarily documented in Fig. 3B,D,F. That cell binding was not markedly dependent on N-glycan sialylation was demonstrated by using an inhibitor of N-glycan processing, i.e. 1-deoxymannojirimycin, which acts on mannosidase I that initiates the conversion of the Man₉GlcNAc₂ precursor to complex-type N-glycans. When the N-glycan profile was hereby shifted to high-mannose-type glycosylation, as controlled by an increase of cell

binding of the mannose-specific lectin concanavalin A, extent of binding was not significantly affected for the glioblastoma (U87) cells (not shown). This result further backed the importance of the (GlcNAc)₂ core of N-glycans for WGA binding. As consequence, spatial accessibility of this inner part in close vicinity to the protein surface will be a major factor for lectin binding and then for the competition with glycoclusters. The impact of this factor can most likely only be delineated in a natural context, for cells in tissues and their glycocalyx.

In essence, the assays on human tumor cells provided insights into the inhibitor activity of glycoclusters on WGA binding to (most likely) the N-glycan core, extending the reported data on CHO wild-type and Lec8 cells, the latter presenting branch-end β-GlcNAc as docking site in the periphery (André et al. 2015a). Naturally, not only N-glycans requiring an abundance of terminal β-GlcNAc but primarily O-glycans of mucins, and here for example on secretory mucin-6, are known as ligands primarily for GSA-II that readily accommodates the terminal α1,4-GlcNAc moiety, as outlined in the introduction. In such a case of counterreceptor secretion or of its presence in the extracellular matrix, limits for the assay using cells in suspension become apparent. Because lectin binding to a mucin can for example be relevant for its organization into a structural network used as protective layer or for cell association to the glycoprotein, glycocluster assays need to be adapted accordingly to avoid missing this aspect of lectin recognition. We therefore first performed solid-phase assays for both lectins with hog gastric mucins (type II and III), already successfully tested as matrix for WGA (Maierhofer et al. 2007; Zanini and Roy 1997), added BSM for WGA assays, and then proceeded to the lectin histochemical approach.

Inhibition of lectin binding to mucins

The mucins were adsorbed to the plastic surface of microtiter plate wells, systematically testing solutions of different concentrations. The extent of lectin binding was dependent on the surface density of the glycoprotein and of the lectin concentration, up to reaching a plateau level. These two parameters (concentration of mucin for coating and of lectin in solution) were selected to reach an OD-response within the linear range. This setting ensures a nearly complete blocking of lectin binding by free cognate sugar. As illustrated exemplarily by the presented courses of the signal reduction (% bound lectin) in the presence of increasing concentrations of inhibitor, the free sugar GlcNAc and

glycoclusters with GlcNAc headgroups were found to be differentially active to inhibit lectin binding (Fig. 4). The concentration, at which the measured OD-signal is reduced to 50% of the control (inhibitory concentration to give a 50% reduction: IC₅₀), could be determined from these curves. It is a relative measure of inhibitory activity. These data are compiled in Table 1.

Reflecting the biochemical similarity of the two gastric mucin preparations, only small differences were seen in assays with GSA-II. Specificity of binding was ascertained by running test series using GalNAc as headgroup in the bivalent conjugate **11**. The tetravalent compound **7** was the most potent inhibitor, and the preference of GSA-II to the α -anomeric linkage of GlcNAc in the glycoclusters was clear (Table 1). For WGA, the inhibition profile with by far strongest effect by compound **7** and rather equal activity among the other compounds, as seen in Table 1, resembled the pattern assessed previously when using a high-density GlcNAc presentation by adsorbing a corresponding neoglycoprotein to the plate well surface (André et al. 2015a). Providing further evidence for the importance of α -GlcNAc presence in the glycan structure for GSA-II binding, running the assays with BSM as matrix constituent resulted in background OD-value, in full agreement with a recent report (Hanisch et al. 2014). BSM contains the sialyl T_n disaccharide (Neu5Ac/Gc α 2,6GalNAc) and the branched core 3 trisaccharide (GlcNAc β 1,3[Neu5Ac/Gc α 2,6]GalNAc) (Savage et al. 1990; Tsuji and Osawa 1986; Yamada et al. 2007). Considering the already mentioned WGA reactivity to the GlcNAc-free ovine submaxillary mucin with its high density of sialyl T_n (Hill et al. 1977; Peters et al. 1979), the β 1,3-linked GlcNAc and the sialic acid can both associate with WGA. In comparison to the inhibition profile of the glycoclusters in assays with the gastric mucin as ligand, binding of WGA to BSM by the synthetic compounds was diminished with a different grading, compounds **2** and **6** now on top (Table 1). The measured profile is similar to the previously obtained results of respective measurements with the glycoprotein asialofetuin (André et al. 2015a). Crystallographical analysis of complexes of WGA with di- and tetravalent compounds attributed their inhibitory capacity to an intramolecular bridging of two contact sites (13-14 Å distance) up to complete saturation of the hevein domains (Schwefel et al. 2010).

The combined results of the cell and solid-phase assays, and these with several types of matrix, i.e. β -GlcNAc in a neoglycoprotein, in BSM and on Lec8 CHO cells, (GlcNAc)₂ in the N-glycan core of asialofetuin and glycoproteins on human tumor cells and α -GlcNAc in hog gastric mucin, reveal occurrence of differences in relative inhibitory capacity when changing the matrix. That IC₅₀-values of spacers of GlcNAc derivatives and three bi- to trivalent compounds had also been determined to be considerably lower when using a surface with covalently attached GlcNAc residues (Maierhofer et al. 2007) is in line with the herein reported differences between data sets obtained with neoglycoprotein of high GlcNAc density as ligand relative to using asialofetuin (André et al. 2015a). Even the choice of the glycoprotein as matrix, when varying the number of antennae in N-glycans, can have an impact, shown for human galectins (André et al. 2009a) <<<=2009b>>>. These observations give further incentive to probe into inhibition of WGA binding in a tissue context, along with experiments on GSA-II, as a proof-of-principle experiment.

Inhibition of lectin binding to tissue sections

To identify conditions with optimal signal-to-noise ratio the concentrations of both lectins were systematically varied and resulting staining patterns examined. The concentration of 0.5 μ g/ml was found to give relatively best results for both lectins. Specimens fixed in Bouin's solution consistently presented the most preferable degree of tissue preservation. Controls in the absence of lectin and in the presence of compound **11** with GalNAc instead of GlcNAc as headgroup ascertained the dependence of staining on lectin presence and the lack of inhibitory capacity of a tested scaffold with a non-cognate sugar, as is the case in solid-phase and cell assays.

The profiles of binding of GSA-II and WGA in sections of murine jejunum were rather similar. Due to the abundant presence of O-glycans of different structure the profile of ligand selection of the two lectins may not yet necessarily be identical. In detail, *crypts of Lieberkuhn* and epithelial cells of villi were positive, the staining of cells seen in cytoplasm never in nuclei, whereas goblet cells were invariably devoid of a signal, (Fig. 5A, 6A). In both cases, titrations with GlcNAc caused stepwise decreases of signal intensity (Fig. 5B-F, 6B-D), down to complete abolishment (Fig. 5F). The absolute IC₅₀-values based on semi-quantitative assessments were different between the two lectins (Table 2). When testing the panel of glycoclusters, the staining intensity was sensitive to

their presence in a graded structure-dependent manner, enabling to determine IC₅₀-values (Fig. 5G-P, Fig. 6E-I; Table 2). In general, positivity was affected to a similar extent throughout the sections. Overall, compound **7** stood out from the group as most active, as seen in the mucin-based solid-phase assays (Table 1). Its application at a concentration far above the IC₅₀-value will thus trace any carbohydrate-independent staining. Of note, fully automated histomorphometric analysis came up with a similar grading (Table 2). The IC₅₀-values of GlcNAc (Fig. 7A: 48.4 μM), compound **6** (Fig. 7B: 14.2 μM) and compound **7** (Fig. 7C: 0.21 μM) compared very well with respective data from visual inspection at 50 μM (GlcNAc), 10 μM (**6**) and 0.25 μM (**7**) (Table 2).

Solidifying these results by analysis of sections of a different organ, analysis of lectin binding in murine stomach, here to the glands of the corpus (Fig. 5Q-T), gave an IC₅₀-based grading with rather few differences compared to staining in jejunum for both lectins. As in jejunum, they shared sites of positivity (glands in fundus and corpus regions, here mucous neck cells, mucus-secreting cells and surface mucous cells lining foveolae). In contrast, chief and parietal cells were intensely stained only by WGA. Inhibition reaching 50% required about 500 μM GlcNAc for GSA-II, 10 μM for compound **5** and a respectively lowered concentration for compounds **7** (Fig. 5R-T). In the case of WGA, susceptibility to blocking was decreased for GlcNAc to an IC₅₀-value of 400 mM, for compound **7** to 2.5 μM, thus maintaining its high potency (not shown). The fact that this glycocluster consistently reached highest inhibitory activity establishes an equivalence to the results on interference of WGA binding to hog gastric mucin (Table 1, Table 2). To next study an organ, where WGA positivity is not accompanied by GSA-II reactivity, we had selected murine epididymis.

The monitoring with WGA showed the epithelial lining of the ductus epididymidis to be strongly positive in the corpus region. GSA-II, applied in an up to 20fold increased concentration (at 10 μg/ml), did not bind specifically to any tissue part. The staining of cytoplasm of principal cells, especially in the supranuclear region, and of basal cells as well as of luminal spermatozoa was sensitive to presence of GlcNAc (Fig. 8A-E). Titrations with glycoclusters enabled determination of the IC₅₀-values, which revealed strongest activity for tetravalent compound **7** (Fig. 8F-H, Table 2). Thus, and this with marked superiority, the tetravalent reagent blocked WGA binding also to the sections of this organ.

Conclusions

Having realized the broad significance of glycan-lectin interactions, the study of their molecular details is making clear that the topological modes of glycotope and of carbohydrate-binding site presentation matters. In order to delineate the rules that determine the specificity and selectivity of the recognition process synthetic carbohydrate chemistry is capable to generate any glycan derivative, in terms of structural aspects such as anomeric linkage type or of valency, as shown in Fig. 1. The produced glycoclusters are versatile tools acting like molecular rulers or sensors, with enormous potential for copying distinct aspects of the natural glycome complexity. The thereby possible stepwise in-depth studies can cover the range from individual glycan chains to cell surface-like constellations. For example, clustered glycan assembly on glycodendrimersomes resembling exosomes or vesicles in size and shape can disclose functional differences between structurally disparate lectins (originating from engineering or natural polymorphisms) in aggregation assays (2015a; Zhang et al. 2015b). Ideally, the activity of glycoclusters to block lectin binding should be measured in the natural context with its particular glycan arrangement.

Although cells in culture offer a convenient test system, one should be aware of the fact that it can not cover all aspects of physiological ligand presence in tissues. We have thus postulated lectin histochemistry on fixed tissue sections as a step toward monitoring lectin reactivity to glycoclusters in a natural context. Admittedly, fixation excludes any movements, and the treatment with organic solvents will extract hydrophobic compounds such as lipids. This loss can even completely abolish lectin reactivity, as documented for the human C-type lectin L-selectin (a lymphocyte homing molecule; CD62L) in frozen sections of rat kidney fixed with 0.5% glutaraldehyde or acetone/methanol (1:1, ice cold, 10 min) (Celie et al. 2005). In our test case, strong positivity in sections and thus the possibility to perform comparative analyses on a proof-of-principle level are documented. Relative to GSA-II, WGA staining is likely due to reactivity to a broader profile of glycoproteins, i.e. distinct mucins with α/β -GlcNAc at branch termini and to fully elaborated and truncated N-glycans. GSA-II staining is more restricted to α -GlcNAc and a high density of terminal β -GlcNAc. The obtained data attest the validity of the assumption on applicability of the lectin histochemical method. They

reveal differential responses within the glycocluster panel and a distinct preference of compound **7** for the two lectins. This proves the sensitivity of the method. A comparison of results on different types of test platform, i.e. (neo)glycoproteins, cell surfaces and the tissue context, is hereby made possible. As it turned out, the exceptionally high potency of compound **7** on WGA binding reaches a level of activity as in solid-phase assays on gastric mucin or neoglycoprotein but not on asialofetuin (N-glycan) binding. Thus, the histochemical data provide clear instructions on which glycocluster to apply for blocking tissue binding that may not reliably be derived from a solid-phase setting.

With respect to the targets of the two plant lectins used in this model study, it is worth mentioning that these sugar determinants can harbor physiological significance. Mammalian lectins, especially human trefoil factor 2 (originally referred to as pancreatic spasmolytic peptide), which may play a role in the suppressor activity of the α 1,4-linked GlcNAc glycotope in the gastrointestinal tract (Nakayama 2014), and the liver and lymph node sinusoidal endothelial cell C-type lectin (LSEctin, CLEC4G) with its GlcNAc β 1,2Man specificity involved in cell interactions, are candidates for their *in situ* recognition (Dominguez-Soto et al. 2007; Hoffmann 2015; Pipirou et al. 2011; Tang et al. 2010). Of course, tissue lectins can be tested as done for plant agglutinins, with immediate functional implications for even subtle changes in the glycomic profile, e.g. in effector T or tumor cell growth regulation (Amano et al. 2012; Wu et al. 2011). Glycocalyx staining, e.g. seen for zona pellucida and human galectins (Habermann et al. 2011), makes obvious that the histochemical procedure will be well suited to study an impact of glycoclusters.

When performing this analysis with endogenous lectins, the possibility should be considered that more than one site on the protein can be operative. If a tissue lectin is multifunctional and can target sites of different biochemical nature in the section, as known from galectins-1 and -3 and their reactivity with glycans of glycoproteins and -lipids, peptide motifs and even lipids (Dawson et al. 2013; Gabius et al. 1991; Plzák et al. 2004), then the inhibition pattern may not be uniform. In that case, potent clusters, along with blocking by antibodies against lectin-reactive proteins, can be helpful to assign distinct aspects of the staining profile to respective determinants, e.g. a glycan on the surface and farnesylated oncogenic H-ras in the cells in the case of human galectin-1 (Smetana et al. 2013). In summary, merging lectin histochemistry with carbohydrate

chemistry opens the door to study topological aspects of lectin specificity in a natural context, with the perspective to segregate staining profiles of multifunctional lectins into reactivity to different targets by using high-affinity glycoclusters.

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