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Galactosides and sialylgalactosides in O-linked oligosaccharides of the primordial germ cells in *Xenopus* embryos

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The primordial germ cells (PGCs) are covered by surface glycoconjugates; some of them, like galactose residues recognized by peanut agglutinin (PNA), have been reported to be implicated in the PGC migration process. The aim of this work was the characterization of galactosides and sialylgalactosides in N- and O-linked oligosaccharides of Xenopus PGCs. Galactose(Gal)- and sialic acid(Neu5Ac)-binding lectin cytochemistry, in combination with chemical and enzymatic deglycosylation methods, were used. PGCs were slightly labeled with PNA, RCA-I and BSI-B_4, which suggests the presence of the sequences Gal(β1,4)GlcNAc and Gal(α1,3)Gal. Moreover, there was no labeling when β-elimination pretreatment was performed, suggesting that galactosides were in O-linked oligosaccharides. The strong staining with DSA was probably due to GlcNAc. Furthermore, sialylgalactosides with the sequence Neu5Ac(α2,3)Gal(β1,4)GlcNAc in O-linked oligosaccharides have been shown by means of MAA, PNA and RCA-I.

Keywords: glycoconjugates, lectins, cytochemistry, oligosaccharides, glycans

Introduction

In most animals, germ cells arise at early steps of embryo development as primordial germ cells (PGCs) located far away from the site of gonad formation. During development, PGCs migrate throughout the somatic tissues to the genital ridges [1,2]. In *Xenopus* embryos, PGCs originate from endodermal cells that contain the germ plasm. During neurulation, PGCs migrate to the dorsal mesentery and, then they move dorsally to the genital ridges, which lie on either side of the dorsal aorta [3].

Some glycoconjugates have been located at the surface of PGCs in many animals [1,2, 4–8], including *Xenopus* [9,10]. The best known are those of the mouse. In this animal, PGCs have surface carbohydrate antigens like stage-specific embryonic antigen-1 (SSEA-1, Lewis^x or Le^x), Lewis^y (Le^y), 4CP, SSEA-3 and the Forssman pentasaccharide [2]. The roles of these glycans remain unknown, although galactosides like SSEA-1 and Lewis oligosaccharides are implicated in several cell adhesion mechanisms [11].

Previous cytochemical works on *Xenopus* PGCs have shown that peanut agglutinin (PNA), a galactose(Gal)-binding lectin, strongly labels PGCs [9]. In addition, PNA injections in embryos inhibit PGC migration, suggesting that galactosides could be implicated in this process [10,12,13].

The aim of this work was the characterization of galactosides in N- and O-linked oligosaccharides of *Xenopus* PGCs by means of Gal-binding lectin cytochemistry in combination with chemical and enzymatic deglycosylation methods. As Gal residues can often be in a subterminal position in sialylgalactosides, acid hydrolysis, which removes sialic acid (Neu5Ac), and Neu5Ac-binding lectins were also employed.

Materials and methods

Sample preparation

Xenopus embryos were obtained by *in vitro* fertilization and reared in 1/10 normal amphibian medium (NAM) [14] at 18°C in a Selecta Prebatem refrigerated incubator (Izasa, Spain). Embryos at stage 46–47 [15] were anaesthetized with 0.1% (w/v) 3-aminobenzoic acid ethyl ester (MS-222, Sigma Química, Spain), and fixed with 2.5% (v/v) glutaraldehyde in

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Sorensen phosphate buffer at pH 7.4 for 2 h. After washing in the buffer, the embryos were immersed into 0.5 M $ClNH_4$ - to block free aldehyde groups of the glutaraldehyde -, dehydrated with ethanol solutions, and embedded in Epon. Finally, 0.5 μm-thick sections were obtained with an LKB Ultratome-III.

Lectin histochemistry

The lectins binding Neu5Ac and Gal that were employed are shown in Table 1. WGA, a glucosamine(GlcNAc)-binding lectin was also used to test whether DSA labeling was caused by Gal or GlcNAc. Lectin binding patterns were established on semithin sections, after removal of epon resin with a saturated solution of KOH in ethanol, a method that does not degrade glycans nor remove O-linked residues by β-elimination [16]. Lectin histochemistry was performed using horseradish peroxidase (HRP)- and digoxigenin (DIG)-conjugated lectins as previously reported [17]. For the DIG-conjugated lectins, an anti-DIG/HRP-labeled goat antibody (Boeheringer Mannheim, Spain) was also used. For each lectin, the histochemical procedure was repeated in three ways: 1) without previous treatment; 2) after chemical deglycosylation (β-elimination), a procedure that removes the O-linked oligosaccharides [18]; and 3) after enzymatic deglycosylation with Endoglycosidase F/Peptide N glycosidase F (PNGase F, Boeheringer Mannheim, Spain), which removes the N-linked oligosaccharides [19]. Wheat germ agglutinin (WGA), a lectin that recognizes both GlcNAc and Neu5Ac, was also combined with acid hydrolysis pre-treatment, which removes Neu5Ac. After this treatment staining was only attributable to GlcNAc. Moreover, when labeling with Gal-binding lectins was weak, the treatments were repeated after acid hydrolysis, to localize Gal moieties in a sub-terminal position to Neu5Ac.

The staining intensity in the PGCs was classified by two independent observers into four arbitrary categories: no labeling (0), weak (1), moderate (2) and strong (3). This allowed us to evaluate the effects of the pre-treatments.

Deglycosylation pre-treatments

Two chemical pre-treatments were performed: β-elimination and acid hydrolysis. β-elimination to remove O-linked oligosaccharides, was performed with NaOH as previously reported [17,18]. Acid hydrolysis, which removes Neu5Ac residues, was performed by immersing the sections in 0.1 M hydrochloric acid for 2–3 h at 82°C [20,21].

PNGase F pre-treatment to remove N-linked oligosaccharides was performed as described [17,19].

Controls

The following controls were used: 1) substitution of the lectins and the antibody anti-DIG by the buffer alone; 2) preincubation of the lectins with the corresponding hapten sugar inhibitor (GlcNAc for WGA and DSA; Neu5Ac for LFA; lactosamine for DSA and RCA-I; Gal for PNA and BSI-B_4; α2,3sialyllactosamine for MAA and α2,6sialyllactosamine for SNA, from Sigma Química, Spain) at a concentration of 0.2 M; 3) preabsortion of the antibody with the corresponding antigen; and 4) staining of sections of other tissues of known altered binding pattern for each of the chemical and enzymatic pre-treatments [17,20, 22–24].

Results

The results are shown in Table 2. Most of the lectins labeled the PGCs and the surrounding tissues, so none of the lectins employed in the present work can be used as markers for PGCs.

Concerning the lectins that bind Neu5Ac, both LFA and MAA labeled the PGCs with all the pre-treatments employed, although MAA staining was faint (Figure 1). However, PGCs were always negative for SNA.

Most of the lectins that preferentially bound Gal were slightly positive in *Xenopus* PGCs (Figures 2, 3a). The exception was DSA, which strongly labeled PGCs, with any of the procedures used. The positivity with PNA, RCA-I and BSI-B_4 disappeared after β-elimination. In addition, acid

Table 1. Neu5Ac- and Gal-binding lectins employed, with indication of their working dilution and major carbohydrate binding specificity

Lectin	*Abbreviation*	*Marker**	*Supplier*	*Working dilution*	*Carbohydrate binding specifictiy*
Limax flavus	LFA	HRP	EY	25 μg/ml	Neu5Ac
Maackia amurensis	MAA	DIG	Roche	10 μg/ml	Neu5Ac(α2,3)Gal(β1,4)GlcNAc
Sambucus nigra	SNA	DIG	Roche	30 μg/ml	Neu5Ac(α2,6)Gal/GalNAc
Triticum vulgaris (wheat germ)	WGA	HRP	Sigma	10 μg/ml	$(GlcNAc)_n$ > Neu5Ac
Arachis hypogea (peanut)	PNA	HRP	Sigma	50 μg/ml	Gal(β1,3)GalNAc > Gal(β1,4)GlcNAc
Datura stramonium	DSA	DIG	Roche	10 μg/ml	Gal(β1,4)GlcNAc > $(GlcNAc)_n$
Ricinus communis-I	RCA-I	HRP	Sigma	3 μg/ml	Gal(β1,4)GlcNAc > Gal(β1,3)GalNAc
Bandeiraea simplicifolia-I-B_4	BSI-B_4	HRP	Sigma	0.9 μg/ml	Gal(α1,3)Gal

*HRP: horseradish peroxidase, DIG: digoxigenin.

Table 2. Lectin labeling pattern of the PGCs of *Xenopus* embryos. Staining intensity: 0 negative, 1 weak, 2 moderate, 3 strong

Lectin	*Without pre-treatment*	*β-elimination*	*PNGase F*	*Acid hydrolysis*	*Acid hyd. + β-elimination*	*Acid hyd. + PNGase F*
LFA	2	2	2			
MAA	1	1	1			
SNA	0	0	0			
PNA	1	0	0/1	2	0	2
DSA	3	2	2	2		
RCA-I	0/1	0	0	2	0	2
BSI-B_4	1	0	1	1	0	0
WGA	3	0/1	1	2		

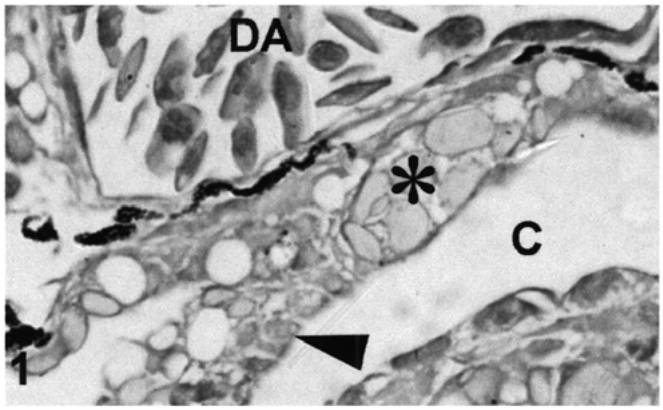

Figure 1. Localization of sialic acid in *Xenopus* PGCs by MAA histochemistry. The PGCs (asterisk) of *Xenopus* embryos are located at the dorsal mesentery (arrowhead), under the dorsal aorta (DA). The cytoplasm and surface of PGCs are slightly positive. C: coelom. ×1075.

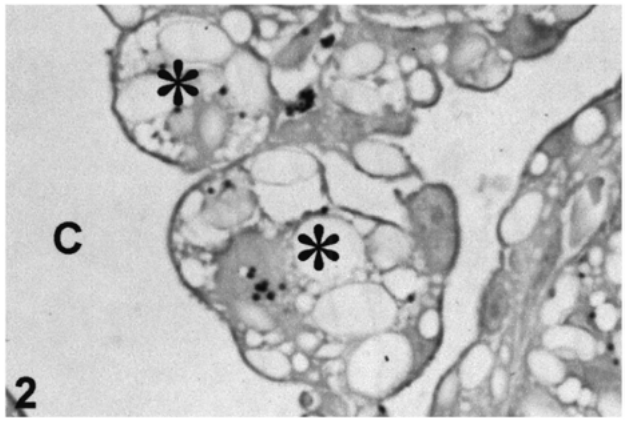

Figure 2. Localization of β-galactosides in *Xenopus* PGCs by PNA histochemistry. The PGCs (asterisk) are scarcely positive. C: coelom. ×1300.

hydrolysis increased PNA and RCA-I staining; for both lectins, the labeling disappeared when acid hydrolysis was combined with β-elimination, and remained when it was combined with PNGase F (Figure 3b). The PGCs were negative for BSI-B_4 when acid hydrolysis was combined either with β-elimination or PNGase F pre-treatments.

WGA alone caused a strong staining of the PGCs. The WGA labeling diminished with acid hydrolysis pre-treatment and disappeared after β-elimination.

Discussion

In the present work, Neu5Ac was localized in both N- and O-linked oligosaccharides in PGCs of *Xenopus* embryos with LFA and MAA, because PGCs were positive for both lectins after removal of O- and N-linked glycans by means of β-elimination and PNGase F pre-treatments, respectively. However, PGCs were negative for SNA. LFA binds to Neu5Ac in any linkage and in any position in a glycoconjugate, while MAA bound preferentially to the trisaccharide Neu5Ac-(α2,3)Gal(β1,4)GlcNAc [25]; thus, these results suggest the presence of the sequence Neu5Ac(α2,3)Gal(β1,4)GlcNAc in both N- and O-linked oligosaccharides. In addition, as SNA requires the disaccharide Neu5Ac(α2,6)Gal/GalNAc [25,26], our results suggest the absence of this sequence in *Xenopus* PGCs.

Two lectins, PNA and RCA-I, were negative after β-elimination pre-treatment, suggesting that Gal is in O-linked glycans. Both PNA and RCA-I lectins show similar binding properties. PNA showed a strong reactivity with the disaccharide Gal(β1,3)GalNAc, and in a minor degree with Gal(β1,4)GlcNAc [27]. RCA-I bound Gal(β1,4)GlcNAc, preferentially in biantennary oligosaccharides [27], but some works suggest that this lectin can react with the disaccharide Gal(β1,3)GalNAc, although showing a low affinity [28,29]. Thus, our results offer three possible explanations: 1) The sequences Gal(β1,3)GalNAc and Gal(β1,4)GlcNAc are both present in O-linked glycans of PGCs; 2) Only the disaccharide Gal(β1,3)GalNAc is in O-linked oligosaccharides, and is labeled by both lectins; and 3) The unique galactoside of

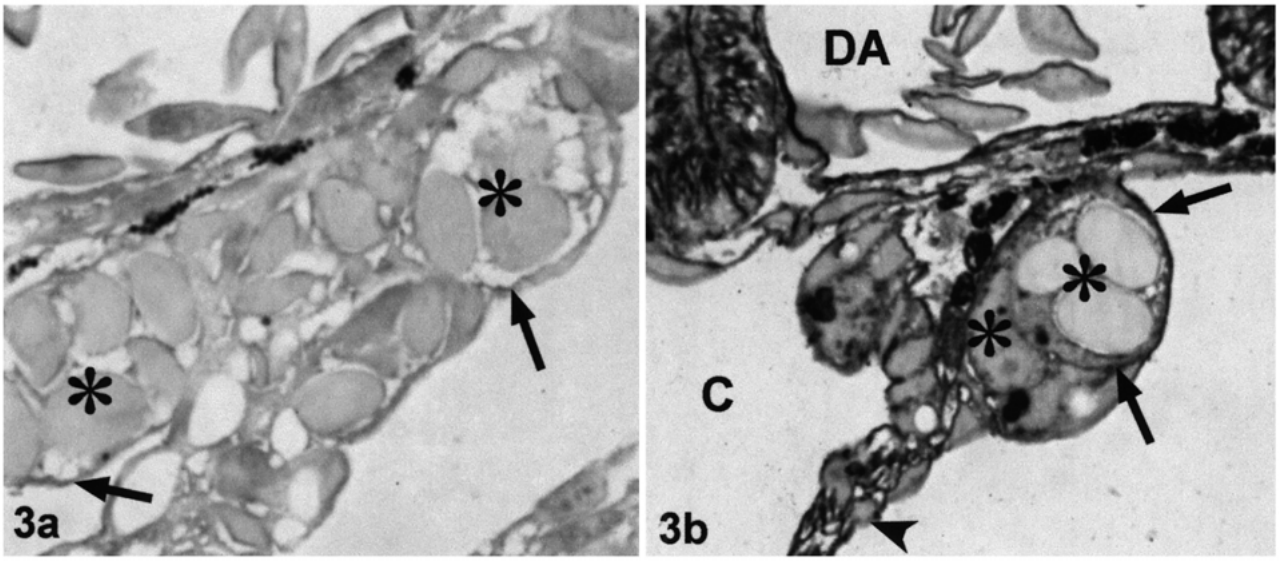

Figure 3. Characterization of galactosides and sialylgalactosides in *Xenopus* PGCs by RCA-I histochemistry. After RCA-I staining (a), weak labeling is present in the cytoplasm and the cell surface (arrows) of the PGCs (asterisk), suggesting the presence of β-galactosides. After acid hydrolysis performed in addition with PNGase F pre-treatment (b), PGCs are strongly labeled (arrows), which suggests that β-Gal in subterminal position to Neu5Ac is in O-linked oligosaccharides. DA: dorsal aorta, C: coelom. a ×1800, b ×1300.

PGCs labeled by PNA and RCA-I lectins is Gal(β1,4)GlcNAc in O-linked sugar chains.

After acid hydrolysis, the staining of PGCs with both PNA and RCA-I was increased and, in addition, after combination of acid hydrolysis with β-elimination there was no labeling. These results suggest the presence of sialyl-galactosides whose sequence, a priori, could offer the same three above explained possibilities as for galactosides. However, it should be noted that, as is shown above by MAA, the sequence Neu5Ac-(α2,3)Gal(β1,4)GlcNAc has been localized on PGCs. Therefore, we can speculate about the possibility that both PNA and RCA-I combined with acid hydrolysis could label this oligosaccharide. Thus, as the presence of Neu5Ac in α2,3 linkage totally inhibits the binding of RCA-I lectin [27], we could suppose that galactosides and sialyl(α2,3) galactosides with the sequence Gal(β1,4)GlcNAc are both in O-linked oligosaccharides of *Xenopus* PGCs. However, we cannot dismiss the presence of terminal and sub-terminal Gal(β1,3)GalNAc.

The results with another Gal-binding lectin, DSA, were apparently contradictory. Two differences with PNA and RCA-I labeling were as follows: 1) labeling was stronger, and 2) labeling was not modified after acid hydrolysis. This lectin shows high affinity to Gal(β1,4)GlcNAc in biantennary oligosaccharides [26,27], but binding to GlcNAc in non-reducing or reducing position has also been reported [26,27,30,31]. Thus, DSA can label Gal(β1,4)GlcNAc, like PNA and RCA-I, or GlcNAc. To resolve this dilemma, PGCs were stained with WGA, a lectin that binds to GlcNAc and Neu5Ac. As shown in Table 2, WGA alone and after acid hydrolysis strongly labeled PGCs, suggesting that the WGA staining was due to GlcNAc. Moreover, since the DSA-binding pattern resembles that of WGA, the binding of DSA to GlcNAc in the PGCs can be suggested as the simplest explanation.

PGCs were slightly positive for BSI-B_4, but there was no labeling after β-elimination, which removes O-linked oligosaccharides. Staining was not modified when PNGase F or acid hydrolysis pre-treatments were performed. BSI-B_4 binds to Gal, preferentially linked by α1,3 to Gal [26,27], so it can be inferred that the galactoside Gal(α1,3)Gal occurs in O-linked glycans of *Xenopus* PGCs.

What could be the role and significance of the glycoconjugates identified by lectin histochemistry in this work? The answer can be inferred by comparing with glycans localized in PGCs from other vertebrate species: previous works have shown a high variability in the lectin-labeling pattern of these cells [6,8,32], even in phylogenetically-related species, like rat and mouse [7]. Monoclonal antibodies that recognize carbohydrate chains have been used to detect carbohydrate epitopes in PGCs of some animals. For example, the sequence Gal(β1,4)[Fuc(α1,3)]GlcNAc has been detected as the EMA-1, SSEA-1 or Le^x antigens in PGCs of mouse and chick [2,4,6,33,34]. This oligosaccharide sequence is partially coincident with the sequence Gal(β1,4)GlcNAc localized in the present work in *Xenopus* PGCs by means of PNA and RCA-I. Interestingly, PNA and RCA-I also label chick PGCs, showing that β-Gal is in glycans of these cells. In addition, in chick a β-galactose-binding lectin has been detected [6,35,36], suggesting a role of the interaction β-Gal/lectin in the mechanism of PGCs adhesion and migration. No α-Gal is localized in chick PGCs, where the BSI-B_4 binding is not

evident [6]. However, α-Gal has been detected in mouse PGCs by means of antibodies against the ECMA-2 antigen [37], and the sequence Gal(α1,3)Gal is overexpressed in human germ cell tumors [38]. It is now well established that glycan-glycan and glycan-protein interactions play critical roles in cell migration, adhesion, and signalling [39–42]. In conclusion, although further demonstration is required, the glycans shown in this work could be implied in the adhesive and migratory properties of the PGCs during embryo development [1,2,4,6,43].

In summary, sialylgalactosides with the sequence Neu5Ac-(α2,3)Gal(β1,4)GlcNAc in O-linked oligosaccharides have been shown in *Xenopus* PGCs. In addition, galactosides in O-linked oligosaccharides have also been detected. The results suggest that these glycan chains contain the sequences Gal(β1,4)GlcNAc and Gal(α1,3)Gal. These data coincide with those of mouse and chick showing β-galactosides in PGCs, and suggest a possible common role in PGC adhesion and migration.

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