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Glycoconjugate profiles of the lancelet (*Branchiostoma lanceolatum*) ovary: a lectin histochemical study by laser confocal microscopy

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Summary

The presence and the distribution of carbohydrate moieties in ripe lancelet (*Branchiostoma lanceolatum*) oocytes (mean diameter 130 μm) was studied by lectin histochemistry in combination with enzyme and chemical treatments. Binding sites for eight lectins with specificities towards different glycan moieties were studied on sections of the whole body of mature female lancelets. Only three of the lectins tested reacted positively. Concanavalin-A (ConA)-binding glycoconjugates were localized in the cytoplasm, namely in yolk granules, whereas *Artocarpus integrifolia* (AIA) and *Ricinus communis* (RCA) agglutinins bound strongly to extracellular coats of the oocyte identified as the jelly coat and vitelline layer. No other tissues of the lancelet body were found to be positive to any lectin tested, except gut enterocytes which reacted strongly with AIA. Reactivity to ConA was abolished by pretreatment of sections with N-glycosidase F but not by mild alkaline hydrolysis, confirming that the glycoconjugates were of the N-linked type. On the contrary, chemical removal of O-linked chains by mild alkaline hydrolysis abolished AIA and RCA reactivity but had no effect on ConA positivity.

Keywords: Amphioxus, Carbohydrates, Glycosylation, Lectins, Oocytes

Introduction

Carbohydrates have been shown to play a major role in the fertilization process in both vertebrates and invertebrates. The most crucial event is the interaction between glycoproteins of the extracellular matrix of the egg and cell surface receptors on the sperm (Wassarman & Litscher, 2001). The key role of carbohydrates as recognition signals in the sperm–egg interaction has been reported in species as diverse as algae (Callow *et al.*, 1985), sea urchins (Vacquier & Moy, 1997; Mengerink & Vacquier, 2001), molluscs (Focarelli & Rosati, 1995; Capone *et al.*, 1999), ascidians (Rosati, 1985; Baginski *et al.*, 1999), amphibians (Maturi *et al.*, 1998), mice (Wassarman & Litscher, 2001) and humans (Clark *et al.*, 1995).

The lancelet (= amphioxus) *Branchiostoma lanceolatum* is an animal of considerable phylogenetic importance among chordates (Wada & Satoh, 1994) and for more than a century it has been the subject of numerous comparative studies that have attempted to elucidate the evolutionary origins of vertebrates. The process of oogenesis in lancelets has been described in different species and subspecies, in light microscopy (Guraya, 1983; Fang & Qi, 1990) and transmission electron microscopy studies (Holland & Holland, 1989, 1991). Various organelles related to vitellogenesis have been described (Wang & Wang, 1987). Oocytes are known to undergo marked changes, accumulating many yolk granules. Carbohydrate components and their distribution during oocyte development have also been described in these animals (Fang & Welsch, 1995).

Its crucial position in the evolutionary history from invertebrates to vertebrates makes the lancelet an important model for studying the evolution of glycosylation. In this paper we report a preliminary study on oligosaccharide chains detected in whole animal sections by means of a panel of eight lectins. Except for a strong reactivity to *Artocarpus integrifolia*

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Table 1 Lectins used in this study and their specificity

Lectin		Specificity	References
Concanavalin A	ConA	N-glycan trimannosidic core	Debray *et al.* (1981)
Sambucus nigra	SNA	NeuAcα2,6Gal	Shibuya *et al.* (1987)
Arachis hypogea	PNA	Unsubstituted Galβ1,3GalNAc	Sastry *et al.* (1986)
Lotus tetragonolobus	LTA	Fucα1,3GlcNAc > Fucα1,6GlcNAc	Pereira & Kabat (1974)
Ulex europeus	UEA	Fucα1,3GlcNAc	Debray *et al.* (1981)
Pisum sativum	PSA	Complex N-glycans	Debray *et al.* (1981)
Artocarpus integrifolia	AIA	Substituted Galβ1,3GalNAc	Hortin (1990)
Ricinus communis	RCA	Galβ1,4GlcNAc	Debray *et al.* (1981)

agglutinin (AIA) in enterocytes, the only cell type with glycosylated epitopes recognized by the lectins used in this study was the oocyte. Yolk granules reacted to concanavalin A (ConA), and the egg vitelline coat and jelly layer to AIA and *Ricinus communis* agglutinin (RCA).

Materials and methods

Animals

Lancelets (*Branchiostoma lanceolatum*) were collected in June using a bottom dredge on sand bars near Banyuls-sur-mer (France). Female lancelet oocytes were considered to be fully ripe if their diameter was 130 ± 5 μm and the nucleus was situated at the animal pole.

Tissue preparation and staining

The whole animal was immersed for 24 h in freshly prepared Bouin fixative. After fixation specimens were cut into small blocks and tissue samples were dehydrated through a graded ethanol series, then embedded in paraffin. Blocks were sectioned serially (thickness 6 μm).

Lectin histochemistry

Deparaffinized sections were blocked with TBS (Tris-buffered saline; Tris-HCl 20 mM, NaCl 150 mM, pH 7.5) or PBS (phosphate-buffered saline; 50 mM K_2PO_4, 150 mM NaCl, pH 7.4) containing 2% bovine serum albumin (BSA) and then incubated for 1 h at room temperature in the same buffer containing biotin-labelled lectins (Sigma, St Louis, MO), at a final concentration of 25 μg/ml for all the lectins tested (Table 1) except ConA, which was used at a final concentration of 2.5 μg/ml (Table 1). Sections were then washed with TBS or PBS and incubated with avidin conjugated with fluorescein (FITC; Sigma, St Louis, MO) at a dilution of 1:30 for 1 h at room temperature. Slides were rinsed with PBS, mounted in PBS-glycerol and observed with a laser scanning confocal apparatus (Leica Lasertechnik, Heidelberg, Germany). The specificity of the staining was tested by incubating sections with avidin-FITC only, or by preincubation with 0.1 M of the corresponding hapten sugars.

Enzyme and chemical deglycosylation

N-linked sugar chains were released by treatment with N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (Boehringer, Mannheim, Germany). Briefly, deparaffinized sections were incubated for 10 min in TBS containing 1% BSA. After a brief washing in the buffer without BSA, the sections were incubated in the enzyme at a final concentration of 4 U/ml in 50 mM sodium phosphate buffer (pH 7.5). Deparaffinized sections were incubated in the buffer without enzyme as a control.

O-linked oligosaccharide chains were removed by mild alkaline hydrolysis (β-elimination). Briefly, deparaffinized sections were treated with 0.5 N sodium hydroxide in 70% ethanol at 4 °C for 4, 7, 10 and 14 days, according to Ono *et al.* (1983). As control, some sections were incubated for the same time in 70% ethanol.

Results

Five of the eight lectins used in this study (UEA, LTA, PNA, SNA and PSA) (Table 1) were found to be completely negative in all the lancelet tissues. Results with the other three lectins are described below.

Concanavalin A (ConA)

In female lancelets ConA reactivity was restricted to yolk granules of ripe oocytes (Fig. 1*A*), where it labelled the periphery of granules, leaving an unlabelled region at the center (Fig. 3*A*). This suggests that glycoconjugates are regionalized in yolk granules of amphioxus oocytes. When sections were pretreated with PNGase F, an enzyme which removes all types of N-linked chains, reactivity was completely abolished,

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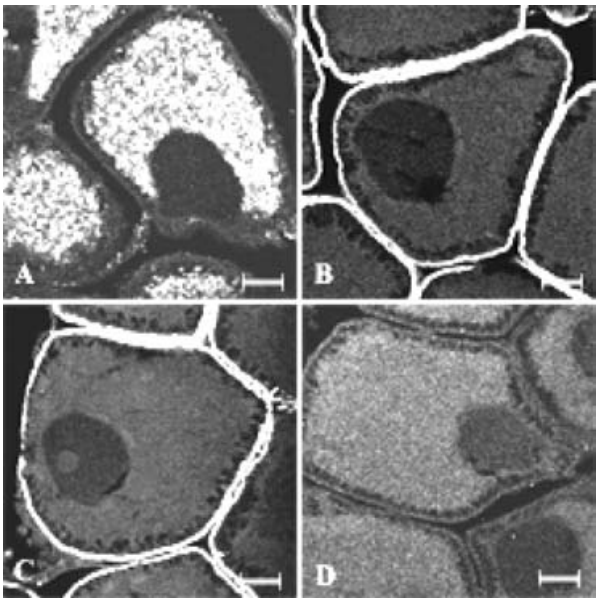

Figure 1 Laser confocal micrographs of *Branchiostoma lanceolatum* oocytes treated with the lectins ConA (*A*), AIA (*B*) and RCA (*C*) showing labelling associated with yolk granules (*A*) and the oocyte periphery (*B*, *C*). A control section obtained after incubation with AIA and 0.1 M galactose is shown in (*D*). Scale bars represent 30 μm.

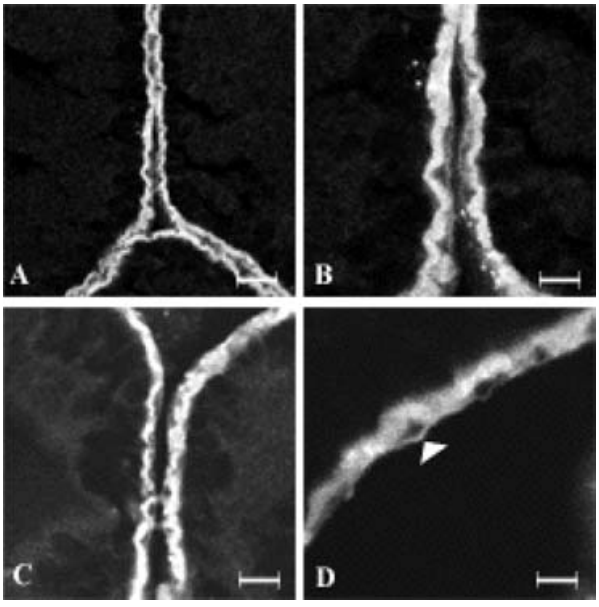

Figure 2 Higher magnifications of lancelet oocytes treated with AIA (*A*, *B*) and RCA (*C*, *D*) lectins. Fluorescence of the jelly coat and vitelline layer is clearly visible. No reactivity is present in the blood sinus. Background signal is detectable on epidermal cells (arrowhead). Scale bars represent 9 μm (*A*, *C*) and 4 μm (*B*, *D*).

suggesting that it was not linked to polysaccharides but to glycoconjugates bearing a typical GlcNAc-Asn linkage.

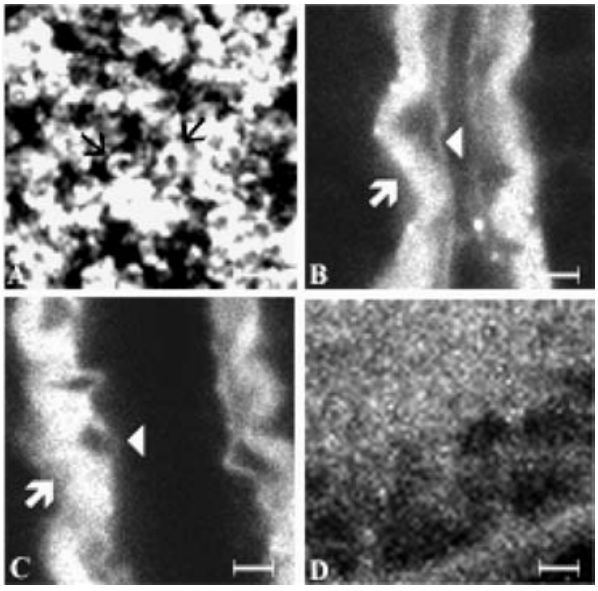

Figure 3 Further magnification of ConA-positive yolk granules (*A*) with labelling at the periphery of granules and an unlabelled region at the centre (black arrows). Further magnification of the periphery of oocytes treated with AIA (*B*) and RCA (*C*) lectins with labelling of the jelly coat and vitelline layer (arrows) and a very faint reactivity of the epidermal cells (arrowheads). A section treated with PNGase F after incubation with ConA is shown in (*D*). Scale bars represent 1.5 μm (*A*, *B*, *C*) and 7 μm (*D*).

Artocarpus integrifolia agglutinin (AIA)

Interestingly, AIA reactivity had exactly the same localization as that of RCA (Fig. 1*B*), strongly labelling oocyte extracellular coats and leaving blood sinus and oocyte cytoplasm unstained (Figs 2*A*, *B*; 3*B*). Strong reactivity to AIA was also observed in enterocytes of the digestive tract (data not shown).

In all cases, inhibition assay with the corresponding hapten completely abolished lectin reactivity. An example of such inhibition experiments for AIA is shown in Fig. 1*D*.

Ricinus communis agglutinin (RCA)

RCA strongly labelled the periphery of all ripe oocytes (Fig. 1*C*). At higher magnifications (Figs 2*C*, *D*; 3*C*) it was evident that it specifically stains the jelly coat and the vitelline layer. The blood sinus was completely negative and the epithelium lining the primary ovarian cavity showed only very faint fluorescence. No reactivity at all was observed in the oocyte cytoplasm (Fig. 1*C*) or in any other region of the lancelet body.

Deglycosylation procedures

N-linked removal did not affect AIA and RCA reactivity (data not shown) but abolished ConA reactivity

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in yolk granules (Fig. 3*D*). On the contrary, removal of O-linked chains had no effect on ConA reactivity but completely abolished AIA and RCA reactivity of the oocyte extracellular coat. Loss of reactivity was complete after 10 days of incubation in 0.5 N sodium hydroxide (data not shown).

Discussion

The evolution of glycan structures through non-vertebrate pluricellular metazoans is of great interest, but has been the subject of very few studies. The pathway along which the biosynthesis and function of protein-associated oligosaccharides has evolved is therefore still unclear. Most studies have been concerned with the role of glycans in sperm–egg interactions at fertilization, especially in the mollusc bivalve *Unio elongatulus* (Focarelli *et al.*, 2001), in ascidians (Rosati & De Santis, 1980; Mengerink & Vacquier, 2001) and in echinoderms (Mengerink & Vacquier, 2001 and references therein).

The peculiar and fundamental position of cephalochordates in the evolutionary tree makes them a key link for the study of the evolution of glycan structures. In this preliminary investigation of the glycobiology of lancelet tissues by laser confocal lectin histochemistry, we found that with the exception of gut enterocytes, only ripe oocytes were positive to some of the eight lectins tested (ConA, RCA and AIA).

ConA is specific for the trimannosyl core of N-glycans. It binds to all classes of N-glycans except tri- and tetra-antennary ones. RCA recognizes terminal galactose residues, preferentially those bound to N-acetylglucosamine with a β1,4 linkage. AIA binds specifically to the Core 1 structure (Galβ1,3GalNAc) of O-linked oligosaccharide chains containing glycoconjugates even if it is substituted by other monosaccharides (Hortin, 1990).

ConA strongly labelled cytoplasmic yolk granules of *Branchiostoma lanceolatum* oocytes, while AIA and RCA bound the jelly coat and vitelline layer. AIA also strongly labelled gut enterocytes.

Enzyme and chemical removal of N- and O-linked oligosaccharide chains confirmed that ConA reactivity was associated with N-linked chains and suggested that AIA and RCA bind to carbohydrate determinants linked via O-glycosyl linkages to the polypeptide portion of a glycoprotein. O-linked oligosaccharides may therefore be present in the glycoconjugates of the extracellular coats, presenting a Core 1 type structure with one or more extensions of the N-acetyl-lactosamine type. These results are particularly interesting since in most vertebrate and invertebrate species, sperm–egg recognition at fertilization is mediated by O-linked oligosaccharide chains on the oocyte extracellular coats and a corresponding lectin-like receptor on sperm (Focarelli *et al.*, 2001 and references therein).

To our knowledge only one paper has been published on the presence and distribution of glycoconjugates in the lancelet (Fang & Welsch, 1995). In this study, the distribution of binding sites of several lectins was studied during oocyte maturation, with particular emphasis on nuclear and perinuclear material. Our results only partially agree with those of Fang & Welsch, namely in the strong labelling of yolk granules and the presence of RCA reactivity on the generic oocyte surface. However, the two studies concern different species and use different technical approaches.

Although preliminary, our results are intriguing in the light of the crucial position of lancelets in the evolutionary pathway from invertebrates to vertebrates. Indeed they suggest that cephalochordates lack any fully developed pathways for the biosynthesis of terminal elaborations (Drickamer & Taylor, 1998), being restricted to the production of relatively 'simple' N- and O-glycan chains. Furthermore, as already postulated, the absence of reactivity to SNA and PSA suggests that (with the possible exception of some Insecta) sialic acids and core-fucosylated, complex-type N-glycans could represent evolutionary novelties related to the appearance of vertebrates (Varki *et al.*, 1999). Further biochemical studies, in particular of the extracellular coat of the lancelet egg, are needed to verify these hypotheses.

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