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We have identified members of the Xenopus cortical granule lectin (xCGL) family as candidate target glycoproteins of Xenopus galectin-VIIa (xgalectin-VIIa) in Xenopus embryos. In addition to the original xCGL, we also identified a novel member of the xCGL family, xCGL2. Expression of the mRNAs of xCGL and xCGL2, as well as that of xgalectin-VIIa, was observed throughout early embryogenesis. Two and three potential N-glycosylation sites were deduced from the amino acid sequences of xCGL and xCGL2, respectively, and xgalectin-VIIa recognizes N-glycans linked to a common site in xCGL and xCGL2 and also recognizes N-glycans linked to a site specific to xCGL2. However, interaction between xgalectin-Ia and xCGLs was not detectable. We also obtained consistent results on surface plasmon resonance analysis involving xCGLs as ligands and xgalectins as analytes. The $K_d$ value of the interaction between xgalectin-VIIa and xCGLs was calculated to be 35.9 nM. The structures of the N-glycans of xCGLs, which were recognized by xgalectin-VIIa, were analyzed by the two-dimensional sugar map method, and three kinds of N-acetyllactosamine type, biantennary N-glycans were identified as the major neutral N-glycans. The binding specificity of oligosaccharides for xgalectin-VIIa was analyzed by frontal affinity chromatography (FAC). The oligosaccharide specificity pattern of xgalectin-VIIa was similar to that of the human homolog galectin-3, and it was also shown that the N-acetyllactosamine type, biantennary N-glycans exhibit high affinity for xgalectin-VIIa ($K_d = 11 \mu M$). These results suggest that xgalectin-VIIa interacts with xCGLs through binding to N-acetyllactosamine type N-glycans and that this interaction might make it possible to organize a lectin network involving members of different lectin families.

Key words: cortical granule lectin/galectin/N-acetyllactosamine/N-glycan/Xenopus

Introduction

Galectins comprise a family of animal lectins that bind to the β-galactoside-containing carbohydrate moieties of glycoconjugates (Barondes et al., 1994a,b; Leffler et al., 2004). Fifteen galectins have been isolated from mammals and are classified into the proto-, chimera- and tandem-repeat-types based on their structures (Kasai and Hirabayashi, 1996; Cooper and Barondes, 1999; Dunphy et al., 2000; Visegrady et al., 2001; Yang et al., 2001; Dunphy et al., 2002). Tandem-repeat type galectins contain one carbohydrate-recognition domain (CRD), a structural conserved domain that specifically recognizes a β-galactoside-containing carbohydrate, and a chimera-type galectin consisting of one CRD and an N-terminal elongating protein domain. Tandem repeat-type galectins contain two CRDs covalently linked through a unique peptide link. Recently, studies have been accumulating that indicate that the galectin family is significantly associated with development, immunity, and tumorigenesis, but the details of the functional mechanisms remain unclear.

Among the galectins, mammalian galectin-3 (chimera-type) is one of the most extensively studied. It has been proposed to play roles in tissue organization, development, immunity, and cancer growth and metastasis by regulating such processes as cell adhesion and apoptosis (Perillo et al., 1998; Hughes, 2001). Regarding development, evidence has been accumulating that indicates that galectin-3 plays essential roles. This evidence includes such as the expression of mRNAs of mouse and human galectin-3 are temporally and spatially regulated during embryogenesis (Fowlis et al., 1995; Colnot et al., 1997; Gotz et al., 1997), a null mutant mouse of galectin-3 has subtle but significant defects in bone and inflammatory responses (Colnot et al., 1998; Hsu et al., 2000; Colnot et al., 2001), and galectin-3 has been reported to play a role in mammalian kidney development (Winyard et al., 1997; Bullock et al., 2001; Hughes, 2004).

We have introduced Xenopus laevis as a model system for studying the roles of galectins in vertebrate development and innate immunity. In the previous two articles, we reported the isolation and characterization of eleven novel Xenopus galectins (xgalectins-Ia-VIIa) including all three types, that is, the proto-, chimera- and tandem repeat-types (Shoji et al., 2002; Shoji et al., 2003). We have comprehensively analyzed the expression of all the xgalectins identified so far and have demonstrated that they are specifically regulated throughout the life cycle, from egg to adulthood.
Among them, the *Xenopus* homolog of galectin-3, xgalectin-VIIa, was found to be the most abundant in embryos and its mRNA was expressed in a remarkably tissue specific manner, it being localized to epidermal cells during early development.

In this study, candidate target glycoproteins of xgalectin-VIIa were isolated. They were found to be members of another lectin family, the *Xenopus* cortical granule lectin family, xCGLs (Nishihara et al., 1986). We have identified a novel member of xCGL family, xCGL2, in addition to original xCGL, as a candidate target of xgalectin-VIIa. Both xCGL and xCGL2 are N-glycosylated, and xgalectin-VIIa recognizes their N-glycans. We have precisely analyzed the interaction and identified the structures of the N-glycans of xCGLs that are recognized by xgalectin-VIIa. Our results suggest that there might be a lectin network involving different lectin families.

**Results**

**Identification of Xenopus cortical granule lectins (xCGL and xCGL2) as candidate target glycoproteins of Xenopus galectin-VIIa**

To identify candidate targets of *Xenopus* galectin-VIIa (xgalectin-VIIa), affinity chromatography was performed on a column of recombinant xgalectin-VIIa. Soluble extracts of tail bud stage embryos were applied to the column, and, after extensive washing, the adsorbed glycoproteins were eluted with buffer containing lactose. The eluted glycoproteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a broad band of 40-46 kDa being obtained as the major protein (Figure 1A). The amino acid sequences of tryptic peptides of the protein were determined and some of them completely matched the sequence of the originally identified xCGL (Chang et al., 2004). However, the sequences of peptides 1, 4 and 7 only partially matched the xCGL sequence. Then we searched the GenBank for these sequences and identified several EST sequences encoding completely identical amino acid sequences to peptides 1, 4 and 7. On combining the EST sequences, it was revealed that peptides 1, 4 and 7 were derived from a novel member of the CGL family, which we named xCGL2. XCG2 was composed of 305 amino acids, without its signal peptide, and its molecular weight was calculated to be 33.7 kDa. The amino acid sequence of xCGL2 was 87.5% identical to that of xCGL (Figures 1B and C).

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to analyze the temporal expression patterns of the mRNAs of members of the *Xenopus* CGL family during embryogenesis (Figure 1D). The expression of the mRNAs of xCGL and xCGL2 was observed throughout early embryogenesis, as well as that of xgalectin-VIIa, which we reported in a previous paper (Shoji et al., 2003). The expression levels of xCGL and xCGL2 were both highest in unfertilized eggs and decreased with embryogenesis. On the other hand, the levels of the other members increased with embryogenesis. The mRNAs of XEEL and xSL2 were only observed after the gastrula stage.

Specific binding of xgalectin-VIIa to N-glycans of xCGLs

xCGL was originally identified and well characterized as a protein related to fertilization by Hedrick and colleagues (Nishihara et al., 1986). As they showed that xCGL is abundant in unfertilized eggs and that it can be easily purified from the supernatant of Ca<sup>2+</sup>-ionophore-stimulated eggs, we used xCGL purified from such eggs for further analysis. The purified xCGLs were adsorbed to a column of xgalectin-VIIa, just like xCGLs from embryonic extracts. However, they were not trapped on a column of proto-type galectin, xgalectin-Ia, showing the specific interaction between xgalectin-VIIa and xCGLs (Figure 2A).

The specific interaction was also observed on surface plasmon resonance analysis involving xCGLs as ligands. With xgalectin-VIIa as an analyte, concentration-dependent binding was observed, but only very limited binding was observed when xgalectin-Ia was used (Figure 2B). The *K<sub>d</sub>* value of the interaction between xgalectin-VIIa and xCGLs was calculated to be 35.9 nM.

As xCGL has shown to be N-glycosylated (Chamow and Hedrick, 1986), xCGLs were digested with N-glycanase to determine if deglycosylated xCGLs interact with xgalectin-VIIa. Figure 2C shows that when xCGLs were extensively digested with N-glycanase, a rather sharp single band (35kDa) was observed on SDS-PAGE. The deglycosylated xCGLs were applied to a xgalectin-VIIa column, but they were not adsorbed (Figure 2D).

**Determination of the N-glycosylation sites of xCGLs, where the N-glycans recognized by xgalectin are linked**

To determine where the N-glycans recognized by xgalectin are linked, purified xCGLs were first digested with lysyl-endopeptidase, and then the resulting glycopeptides were affinity purified using a xgalectin-VIIa column. The glycopeptides that interacted with xgalectin-VIIa were further purified by reversed-phase HPLC, two major peaks being obtained (Figure 3A). The amino acid sequence of peak 8 matched that of one of the common N-glycosylation potential sites in both xCGL and xCGL2, and the sequence of peak 9 matched that of the N-glycosylation site specific to xCGL2 (Figures 1C and 3B).

**Structural analysis of the N-glycans on xCGLs that are recognized by xgalectin-VIIa**

Total N-glycans of xCGLs were first chemically liberated by hydrazinolysis and then pyridylaminated (PA). The PA-N-glycans were applied to a column of xgalectin-VIIa. As a result, a large amount of the PA-N-glycans passed through the column, but some of them were actually adsorbed on the column, which were eluted with the buffer containing lactose, but not with the buffer containing sucrose (Figure 4A). The ratio of the peak areas of the flow-through (FT in Figure 4A) and eluted (E in Figure 4A) fractions was calculated to be 83:17. The eluted PA-N-glycans were first fractionated by ion-exchange HPLC on a MonoQ HR5/5 column (Amersham Biosciences, Piscataway, NJ) and then separated into one neutral fraction and four negatively
**Fig. 1.** Identification of *Xenopus* cortical granule lectins (xCGL and xCGL2) as candidate target glycoproteins of xgalectin-VIIa. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) profile of the eluted fraction on affinity chromatography, which was performed by applying a soluble fraction of tail bud stage embryos on a recombinant xgalectin-VIIa column. (B). Amino acid sequences of the tryptic peptides derived from the 40–46 kDa protein in Figure 1A. (C) Comparison of the amino acid sequences of the original xCGL and novel member xCGL2. The full-length amino acid sequence of xCGL2 was deduced by combining EST sequences (GenBank accession numbers: BF232570, BU900172). Light gray shading indicates the amino acid sequences identified in Figure 1B and dark gray shading indicates those of the glycopeptides that were bound with xgalectin-VIIa (Figure 4). Asterisks indicate potential N-glycosylation sites. The underline with arrowheads indicates signal peptides. (D) Reverse transcriptase-polymerase chain reaction (RT–PCR) analysis of the temporal expression patterns of mRNAs of members of the *Xenopus* CGL family during embryogenesis. Ornithine decarboxylase (ODC) was amplified as an internal control. The length of the cDNA region amplified with each primer set is as follows, xCGL, 608 bp; xCGL2, 626 bp; XEEL, 367 bp; xSL, 373 bp; xSL2, 640 bp. Abbreviations and references for nucleotide sequences: xCGL, *Xenopus* cortical granule lectin (Chang et al., 2004); xCGL2 (GenBank accession numbers, BF232570, BU900172); XEEL, *Xenopus* embryonic epidermal lectin (Nagata et al., 2003); xSL, *Xenopus laevis* 35 kDa serum lectin (GenBank accession number, AB061238); and xSL2, *Xenopus laevis* lectin type 2 (GenBank accession number, AB061239).
charged fractions (Figure 4B). The origin of the negative charge has not been determined, because they were highly resistant to general sialidase treatment and chemical conditions for removing sialic acids or sulfates (data not shown).

The structures of PA-N-glycans in the neutral fraction were analyzed by the two-dimensional-sugar map method, which utilizes a combination of two kinds of HPLC columns (Hase, 1994; Fujimoto et al., 1999). The neutral PA-N-glycans were first fractionated by reversed-phase HPLC. Although many peaks were obtained, we found that three of the major peaks (peaks 10–12 in Figure 5A) were retained on the column at the same positions as three kinds of standard N-glycans, S1, S2, and S3 (Figure 5A; all of the standard N-glycans are derivatives of N-acetyllactosamine type, biantennary N-glycans, as shown in Figure 6). Peak materials 10–12 were purified and each of them was next analyzed by size-fractionation HPLC. In this case, PA-isomaltooligosaccharides (PA-glucose oligomer) were used as elution scale. The glucose unit (GU) of each PA-N-glycan matched that of each standard N-glycan as summarized, in Figure 5B. Each N-glycan, purified by size-fractionation HPLC, was confirmed again to be eluted at the same retention time as each standard N-glycan on reversed-phase HPLC.

Also, digestion of PA-N-glycans with β-galactosidase reduced the glucose units of every N-glycan by 1.4–1.5 GU, as expected (Figure 5B). From these results, we concluded that the structures of PA-N-glycans in peaks 10–12 are derivatives of N-acetyllactosamine type, biantennary N-glycans, as shown in Figure 6.

Oligosaccharide specificity of xgalectin-VIIa analyzed by frontal affinity chromatography

The binding specificity of oligosaccharides favored by xgalectin-VIIa was analyzed by frontal affinity chromatography (FAC) using recombinant xgalectin-VIIa (Figure 7).
The oligosaccharide specificity pattern of xgalectin-VIIa was similar to that of human galectin-3, as described in a previous report (Hirabayashi et al., 2002a), and the next two points were more remarkably observed for xgalectin-VIIa than for human galectin-3: (1) xgalectin-VIIa preferred linearly repeated N-acetyllactosamine structures to branching structures (sugar number 42 showed higher affinity than sugar numbers 01, 02, or 04, see Figure 7); (2) xgalectin-VIIa preferred β1–4 to β1–3 (sugar number 26 showed higher affinity than sugar numbers 01, 02, or 04).
Fig. 7. Oligosaccharide specificity of xgalectin-VIIa analyzed by frontal affinity chromatography (FAC). (A) Bar graph representation of affinity constants ($K_a$) between xgalectin-VIIa and 42 PA-oligosaccharides. (B) Schematic representation of PA-oligosaccharides used for FAC analysis. Thick and thin bars represent $\alpha$ and $\beta$ bonds, respectively.
affinity than sugar number 27, see Figure 7). However, the overall affinity was lower for xgalectin-VIIa and PA-oligosaccharides than that for human galectin-3 by 2–20 times. The $K_d$ value for the interaction between xgalectin-VIIa and $N$-acytllactosamine type, biantennary $N$-glycan was determined to be 11 $\mu$M.

**Discussion**

As the first step for functional analysis of *Xenopus* galectins during embryogenesis, we searched for glycoproteins that interact with *Xenopus* galectin-VIIa (xgalectin-VIIa) by affinity chromatography on a xgalectin-VIIa column. In this study, we have identified members of the xCG family as candidate target glycoproteins of *Xenopus* galectin-VIIa (xgalectin-VIIa) from a soluble extract of tail bud stage embryos. To our knowledge, this is the first article reporting not only the identification of a candidate target of galectins from natural sources, but also the identification of the structures and the linking sites of galectin-binding $N$-glycans. Although, there were several minor and huge proteins in the eluted fraction, we have not identified yet, amino acid sequence analysis and searching of the EST data base revealed that the major component of the eluted fraction is a mixture of the original xCGL and a novel member, xCGL2. This was also true for the xCGLs purified from cortical granules of unfertilized eggs, which we used as the xCGL-source of further analysis, because we identified a xCGL2-specific glycopeptide as a target of xgalectin-VIIa among the peptides of the purified xCGLs. The lectin activity of xCGLs is similar to that of galectins in that they recognize galactosides, but they are different in that xCGLs bind monosaccharides galactose, whereas galectins do not.

As it was revealed that xgalectin-VIIa recognizes the $N$-glycans of other sites than those we detected are $N$-glicosylated. One is $154^{\text{NKT}}156$ (Figure 1C) and the other is $217^{\text{NLT}}219$, which is a specific site in another polymorphic protein (An et al., 2003), but they did not succeed in detecting $N$-glycosylation at the sites we detected. Although, this seems to be inconsistent with our results, our explanation is as follows. We have only analyzed the $N$-glycosylation sites that are glycosylated with the $N$-glycans recognized by xgalectin-VIIa, so $N$-glycans linked to other sites might not interact with xgalectin-VIIa. Regarding their method, they were only looking at the glycopeptides that were successfully identified with their mass system, so there is no evidence that the sites we detected are not glycosylated. We have evidence supporting this idea. We have analyzed structures of not only $N$-glycans recognized by xgalectin-VIIa, but also total N-glycans of xCGLs (data not shown). Among them, there were a lot of high mannose type $N$-glycans, which were reported to be linked to $154^{\text{NKT}}156$ and $217^{\text{NLT}}219$ by An et al. Also, we have observed that high mannose type $N$-glycans are eluted in the flow-through fraction on affinity chromatography on a xgalectin-VIIa column. Therefore, depending on the protein molecules, some may be glycosylated with complex type $N$-glycans at the sites we detected and some may be glycosylated with high mannose type $N$-glycans at the sites they detected.

On affinity chromatography of xCGL proteins, all proteins were adsorbed on the xgalectin-VIIa column, but on affinity purification of PA-$N$-glycans derived from xCGLs, only 17% of the $N$-glycans were adsorbed on the column (Figure 4A). This inconsistency may be explained by the fact that xCGLs form oligomers consisted of 10–12 monomers (Chamow and Hedrick, 1986). Therefore, even if some molecules are not glycosylated with galectin-interacting $N$-glycans, they may be trapped on the xgalectin-VIIa column with other molecules containing galectin-interacting $N$-glycans as a huge complex.

The affinity-purified $N$-glycans were next fractionated by ion-exchange HPLC on MonoQ HR5/5. As a result, we obtained a neutral fraction and four negatively charged fractions (Figure 4B). The acidic substances, which we have not identified yet, seemed to have nothing to do with the recognition of $N$-glycans by xgalectin-VIIa, because acid-removed $N$-glycans were still adsorbed on the xgalectin-VIIa column (data not shown). Although the acidic substances were not removed under general conditions for removing sialic acid (25 mM HCl, 80°C, 1 hr), they were removed under more severe conditions (25 mM HCl, 100°C, 1 hr). Therefore, in the case of the $N$-glycans in the negatively charged fractions, their neutral backbones seem to interact with xgalectin-VIIa.

As the neutral $N$-glycans were one of the major components of galectin-interacting sugars and it was easier to carry out structural analysis, we chose them as the first to be analyzed by the two-dimensional sugar map method. We observed a lot of peaks on the first fractionation by reversed-phase HPLC, and we identified three major peaks that match known $N$-glycans (Figure 5A). However, we failed to map the $N$-glycans of other peaks to spots of
known N-glycans. This suggests that there are N-glycans with a variety of unknown structures on xCGLs that participate in the interaction with xgalectin-VIIa.

Although the $K_d$ value of the interaction between xgalectin-VIIa and whole xCGL proteins was calculated to be 35.9 nM from the results of surface plasmon resonance analysis, the $K_d$ value of the interaction between xgalectin-VIIa and N-acetyllactosamine type, biantennary N-glycans was determined to be 11 μM on frontal affinity chromatography. The affinity was about 300 times higher for the whole glycoproteins than for each individual N-glycan. We have two possible explanations for the difference in affinity. (1) There might be unidentified N-glycans exhibiting higher affinity with xgalectin-VIIa. (2) There may be a “clustering effect” (Lee et al., 1984, 1989, 1990, 1991; East and Isacke, 2002; Kilpatrick, 2002; Weigel and Yik, 2002) in the whole glycoprotein. XCGLs might form sugar chain clusters, which exhibit especially high density, because xCGLs have shown to undergo oligomerization (Chamow and Hedrick, 1986).

Xenopus cortical granule lectin was originally identified as a protein released from cortical granules upon fertilization and participates in the formation of the fertilization membrane, which prevents polyspermy (Nishihara et al., 1986). However, the expression analysis revealed that the mRNAs of both xCGl and xCGL2 are expressed throughout embryogenesis, as well as xgalectin-VIIa. It has also been shown by Chang et al. that xCGL proteins also exist in early embryos (Chang et al., 2004). We have examined if xgalectin-VIIa and xCGLs would be co-localized during fertilization events, but we failed to detect their co-localization during the events. We are now planning to analyze the co-localization sites in early embryos, including the co-localization of xgalectin-VIIa and other members of the xCGL family that are also expressed in embryos.

Although the developmental functions of members of the xCGL family are unknown, studies have been accumulating that suggest that they play roles during Xenopus development. A member of the xCGL family has been reported to be involved in Ca$^{2+}$-dependent cell adhesion in Xenopus blastulas (Nomura et al., 1998). The mRNA of XEEL has been found to be specifically expressed in nonciliated epidermal cells of tail bud embryos (Nagata et al., 2003). Barondes and colleagues have reported the presence of a galactose-binding lectin at sites of active morphogenesis, such as the extracellular matrix around the blastopore, on the roof of the blastocoel, and in the developing neural fold (Outenreath et al., 1988). The identity of their galactose-binding lectin has not been determined, but its molecular properties indicate that it is very likely a member of the xCGL family. Regarding galactose-binding lectins, Milos et al. has proposed that endogenous β-galactoside-binding lectins are important for the development of neural crest cells, craniofacial tissue, the heart, and so on (Fruchak et al., 1993; Evanson and Milos, 1996).

The results of this study suggest that xgalectin-VIIa interacts with xCGLs through binding to N-acetyllactosamine type N-glycans and that this interaction might make it possible to organize a lectin network involving members of different lectin families. Taking the studies mentioned above also into consideration, such a lectin network may play roles in animal development. Moreover, such a network might have potential roles in modulating the development of the immune system, because galectins have been shown to play a key role in adaptive and innate immune responses, and the immune system in Xenopus develops during rather early stages (Du Pasquier et al., 2000).

**Materials and methods**

**Identification of xCGLs as candidate targets of Xenopus galectin-VIIa**

To identify candidate target proteins of xgalectin-VIIa, we performed affinity chromatography by using a column of recombinant xgalectin-VIIa. The recombinant xgalectin-VIIa protein was prepared using a pET system (Stratagene, La Jolla, CA) and *Escherichia coli* strain BL21 and purified on a lactosyl-agarose column as described (Matsushita et al., 2000; Shoji et al., 2003). The purified recombinant xgalectin-VIIa (1.3 mg) was immobilized on a HiTrap N-hydroxysuccinimide (NHS) activated HP column (1 mL; Amersham Biosciences) according to the manufacturer’s instructions. *Xenopus* embryos were obtained as described (Shoji et al., 2003). Embryos at the tail bud stage were collected and homogenized in a threefold volume of extraction buffer (20 mM Tris–HCl [pH 7.5]/0.15 M NaCl/2 mM EDTA/1 mM benzamidine-HCl/1 mM PMSF). The extract was centrifuged and the supernatant (soluble fraction) was applied to the column. The column was extensively washed with 20 mM Tris–HCl (pH 7.5)/0.15 M NaCl/2 mM benzamidine-HCl/1 mM PMSF. The elution was fractionated by SDS–PAGE and eluted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore), and the amino acid sequences of tryptic peptide fragments of the major protein (40–46 kDa) were analyzed. Western blotting and amino acid sequence analysis were performed as previously described (Shoji et al., 2002).

**Reverse transcriptase-polymerase chain reaction**

Total RNAs were extracted from embryos at the stages indicated in Figure 1D, using Isogen (Nippon Gene, Tokyo, Japan). RT–PCR was performed with an RNA PCR Kit (Applied Biosystems, Tokyo, Japan) and 1 μg of each total RNA for each reaction. The synthesis of first strand cDNA was performed according to the manufacturer’s protocol. The primers used for PCR were as follows:

- xCGL sense: 5′-TCAGTCTTTGTAACCGTGGT-3′
- xCGL antisense: 5′-TTTACGGCTTCTCAGTGGT-3′
- xCGL2 sense: 5′-GCCATGACGAGCTATCGTG-3′
- xCGL2 antisense: 5′-AATTCGACCTTCAAGATCG-3′
- XEEL sense: 5′-GTGGTCTTGGTGAACGACG-3′
- XEEL antisense: 5′-ACTTCCGGATGGTACGCC-3′
- xSL sense: 5′-ACCGTGAGGATCCTGACG-3′
- xSL antisense: 5′-CTGCTCAGGAGTTGAGCTG-3′
- xSL2 sense: 5′-TTCAGGGATCTGACG-3′
- xSL2 antisense: 5′-AGTAATGGGATCTGACG-3′
- ODC sense: 5′-GTCATGAGTGGATGGATG-3′
- ODC antisense: 5′-TCCATTCCGCTCTCAGACG-3′

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The abbreviations and references for nucleotide sequences are as follows: xCGL, *Xenopus* cortical granule lectin (Chang et al., 2004); xCGL2 (GenBank accession numbers, BF232570, BU900172); XEEL, *Xenopus* embryonic epidermal lectin (Nagata et al., 2003); XSL, *Xenopus laevis* 35 kDa serum lectin (GenBank accession number, AB061238); xSL2, *Xenopus laevis* lectin type 2 (GenBank accession number, AB061239); and ODC, ornithine decarboxylase, (Yamada et al., 1999). The reaction mixtures were preincubated for 2 min at 94°C, followed by the following thermal cycle: 94°C for 30 sec, 60°C for 15 sec, and 72°C for 1 min. The cycle number for each reaction is indicated in Figure 1D. All PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Specific amplification with each primer pair was confirmed by a reaction without reverse transcriptase and also by restriction enzyme mapping of the PCR products (data not shown).

**Purification of xCGLs from eggs**

As xCGL has been shown to be abundant in unfertilized eggs and to be easily purified from a supernatant of Ca²⁺-ionophore-stimulated eggs (Nishihara et al., 1986; Lee et al., 1997), we used xCGL purified from such eggs for biochemical analyses of the glycopeptides and their N-glycans.

*Xenopus* eggs were prepared as described (Shoji et al., 2003). XCGLs were harvested in the supernatant of Ca²⁺-ionophore-stimulated eggs (A23187; Calbiochem, San Diego, CA), followed by affinity purification on a melibiose-agarose column (Seikagaku Co., Tokyo) as follows (Lee et al., 1997). The egg extract was centrifuged and the supernatant was directly applied to the melibiose-agarose column (1 mL). The column was extensively washed with TBS/0.03% CHAPS, and, because xCGLs are Ca²⁺-dependent lectins, the proteins adsorbed to the column were eluted with TBS/5 mM EDTA.

**Recombinant protein and affinity column of xgalectin-Ia**

The recombinant xgalectin-Ia protein was expressed as a fusion protein with glutathione S-transferase (GST) using a GST fusion system (Amersham Biosciences) and *E. coli* strain BL21 and purified on a lactosyl-agarose column as described (Shoji et al., 2003). To prepare an affinity column, 1 mg of the purified recombinant xgalectin-Ia was immobilized on a HiTrap NHS-activated HP column (1 mL; Amersham Biosciences) according to the manufacturer's instructions. The purified GST-xgalectin-Ia was also used for surface plasmon resonance analysis.

**Affinity chromatography of purified xCGLs**

Using columns of xgalectins-Ia and -VIIa prepared as described above, the interaction with xCGLs purified from eggs was examined. The xCGLs eluted from the melibiose-agarose column was directly applied to the xgalectin columns (100 µg of xCGLs for each column). The columns were extensively washed with TBS/0.03% CHAPS and then the proteins adsorbed to the columns were eluted with TBS/200 mM lactose. The eluted proteins, as well as the flow-through fraction on each chromatography, were analyzed by SDS–PAGE. The proteins were stained with Coomassie brilliant blue-R250.

**Deglycosylation of xCGLs and their interaction with xgalectin-VIIa**

The purified xCGLs were digested with Glycopeptidase F (Peptide: N-glycosidase F; Takara Bio, Tokyo, Japan) under the native conditions according to the manufacturer's instructions. For partial digestion, 20 µg of xCGLs were digested with 2.5 µM of Glycopeptidase F in 0.1 M Tris–HCl (pH 8.6) at 37°C for 1 hour. For extensive digestion, 40 µg of xCGLs were digested with 16 µM of Glycopeptidase F in 0.1 M Tris–HCl (pH 8.6) at 37°C for 15 hours. The digested products, deglycosylated xCGLs, were directly applied to a column of recombinant xgalectin-VIIa, and then it was examined if they were adsorbed on the affinity column. The affinity chromatography was performed as described in the above paragraph.

**Surface plasmon resonance analysis**

Surface plasmon resonance analysis was performed with a Biacore 2000 (Biacore, Neuchatel, Switzerland). The purified xCGLs were immobilized on a Sensor Chip CM5 (Biacore) using an Amine Coupling Kit (Biacore) according to the manufacturer's instructions. The interaction of xgalectins and xCGLs was examined using degassed HBS-EP (Biacore; 10 mM HEPES [pH 7.4]/0.15 M NaCl/3 mM EDTA/0.005% Surfactant P20) as the running buffer, and by using recombinant xgalectin-Ia or –VIIa as a ligand at the concentrations indicated in Figure 2B. To determine the *K*ₐ value of the interaction, the following concentrations of xgalectin-VIIa were used, 62.5 nM, 31.25 nM, 15.6 nM, and 7.8 nM, because the curves for lower ligand-concentrations showed better fitting on kinetics analysis with BIAevaluation 3.0 (software by Biacore).

**Determination of the N-glycosylation sites where the N-glycans recognized by xgalectin-VIIa are linked** (Hirabayashi et al., 2002b)

The purified xCGLs were first concentrated by methanol precipitation. The precipitate was dissolved in 4 M urea and then digested with lysyl-endopeptidase (Wako, Tokyo, Japan) at 30°C for 24 hours in a reaction volume of 120 µL with the following final concentrations of the reagents, 17 mM Tris–HCl (pH 9.0), 3.3 M urea, 10 mg/mL xCGLs, and 200 µg/mL lysyl-endopeptidase. After digestion, to inactivate the proteinase, 2 mM diisopropylphosphofluoridate (DFP) was added, followed by standing for 1 hour and then heating at 70°C for 10 min. The digested and proteinase-inactivated product was diluted to 1 mL with buffer comprising Tris–HCl (pH 7.5) and NaCl at the final concentrations of 20 mM and 150 mM, respectively. The diluted peptide solution was applied to a column of xgalectin-VIIa, and the glycopeptides that interacted with xgalectin-VIIa were purified by affinity chromatography as described above. The fraction eluted on the affinity chromatography was further purified by reversed-phase HPLC, and the N-terminal ten amino acids of each glycopeptide were determined as described previously (Shoji et al., 2002).
Preparation of pyridylaminated-N-glycans recognized by xgalectin-VIIa for structural analysis

To determine the structures of the N-glycans on xCGLs that are recognized by xgalectin-VIIa, the total N-glycans of the purified xCGLs were chemically liberated by hydrazinolysis, followed by N-acetylation, both by the standard procedures of Hase et al. (1984). Briefly, 1 mg of freeze-dried xCGLs were heated with 0.2 mL of anhydrous hydrazine at 100°C for 10 hours. After evaporation of excess hydrazine, 0.2 mL of a saturated-sodium bicarbonate solution and 8 μL of acetic anhydride were added. After 5 min on ice, another 0.2 mL of the saturated-sodium bicarbonate solution and 8 μL of acetic anhydride were added. The reaction mixture was left to stand for 30 min on ice and then desalted with Dowex 50WX2 (H+, 100–200 mesh).

The prepared N-glycans were automatically PA with GlycoTag (Takara Bio) (Hase et al., 1978; Fujimoto et al., 1999). The PA-N-glycans were dissolved in H2O followed by purification by the following procedure. The PA-sugar solution was extracted twice with an equal volume of H2O-saturated phenol: chloroform (1:1 v/v), once with an equal volume of chloroform and once with an equal volume of diethyl ether, and then freeze-dried.

The PA-N-glycans were dissolved in TBS and then applied to a column of xgalectin-VIIa (flow rate, 0.25 mL/min). The column was washed with TBS, followed by washing with 10 mM Tris-HCl (pH 7.5)/20 mM sucrose. The N-glycans adsorbed on the column were eluted with 10 mM Tris-HCl (pH 7.5)/20 mM lactose. This affinity chromatography was monitored with a fluorescence detector (Waters), with an excitation wavelength of 310 nm and an emission wavelength of 380 nm. The chromatography was carried out at room temperature.

The eluted fraction was directly subjected to ion-exchange HPLC (MonoQ HR5/5; Amersham Biosciences), being separated into one neutral fraction and four acidic fractions. The initial solvent consisted of H2O adjusted to pH 9.0 with an ammonia solution, and the final solvent was 0.5 M ammonium acetate buffer (pH 9.0). After the injection of a sample, the ratio of the final solvent was increased linearly to 12% in 3 min and to 40% in 17 min, 100% being reached in 22 min. The chromatography was carried out at room temperature and the PA-N-glycans were detected at the excitation wavelength of 310 nm and the emission wavelength of 380 nm (Fujimoto et al., 1999). The N-glycans in the neutral fraction were analyzed by the two-dimensional sugar map method.

The two-dimensional sugar mapping

Reversed-phase HPLC was carried out on a Cosmosil 5C18 AR-300 column (4.6 x 150 mm) at a flow rate of 1.5 mL/min at room temperature. Solvent A was 20 mM acetic acid (pH 4.0; adjusted with ammonia solution) and solvent B comprised solvent A containing 1% 1-butanol. The column was equilibrated with a mixture of solvents A and B (ratio 97:3). After the injection of a sample, the ratio of solvent B was increased linearly to 40% in 45 min. The PA-N-glycans were detected at the excitation wavelength of 320 nm and the emission wavelength of 400 nm (Yanagida et al., 1998).

Size-fractionation HPLC was carried out on a Shodex Asahipak NH2P-50 column (4.6 x 50 mm) at a flow rate of 0.6 mL/min at room temperature. Solvent C consisted of 93% acetonitrile and 0.3% acetic acid (pH 7.0; adjusted with an ammonia solution), and solvent D consisted of 20% acetonitrile and 0.3% acetic acid (pH 7.0). The column was equilibrated with a mixture of solvents C and D (ratio 97:3). After injection of a sample, the ratio of solvent D was increased linearly to 33% in 3 min and 71% in 35 min. The PA-N-glycans were detected at the excitation wavelength of 310 nm and the emission wavelength of 380 nm (Yanagida et al., 1998).

Standard PA-sugars are purchased from Takara Bio (PA-Glucose Oligomer) and (S1, S2, S3). PA-glucose oligomer was used as the elution scale for size-fractionation HPLC. β-Galactosidase treatment was performed using β-galactosidase from Jack bean (Seikagaku Co., Tokyo, Japan) according to the manufacturer's instructions (37°C, 12 hours). The digestion products were heated at 100°C for 5 min to inactivate β-galactosidase and then subjected to size-fractionation HPLC.

Frontal affinity chromatography

The purified recombinant xgalectin-VIIa (1.3 mg) was immobilized on a HiTrap NHS-activated HP column (1 mL; Amersham Biosciences) according to the manufacturer's instructions. Reinforced FAC was performed as described previously (Hirabayashi et al., 2002a).

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Abbreviations

bp, base pairs; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)propanesulfonic acid; CRD, carbohydrate recognition domain; E. coli, Escherichia coli; EDTA, ethylenediamine tetra-acetic acid; EST, expressed sequence tags; FAC, frontal affinity chromatography; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; PA, pyridylaminated; PCR, polymerase chain reaction; PMSF, phenylmethane sulfonyl fluoride; RT–PCR, reverse transcription-PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; xCGL, Xenopus
cortical granule lectin; xgalectin; xSL galexin, Xenopus laevis galexin.

References


