Characterization of the Xenopus Galectin Family

THREE STRUCTURALLY DIFFERENT TYPES AS IN MAMMALS AND REGULATED EXPRESSION DURING EMBRYOGENESIS

We have isolated six novel galectin cDNAs from a Xenopus laevis kidney cDNA library. The newly identified X. laevis galectins (xgalectins) comprise one prototype (xgalectin-Vb), one chimera type (xgalectin-VIIa), and four tandem repeat types (xgalectin-Ilb, -Ilb, -Vla, and -VIIa). Thus, together with those mentioned in our previous work (Shoji, H., Nishi, N., Hirashima, M., and Nakamura, T. (2002) Glycobiology 12, 163–172), the 12 xgalectins are classified into three types based on their domain structures, as in mammals. The xgalectins whose counterparts in other species have not been identified (xgalectin-Iva, -Vb, and -Vla) were confirmed to possess lactose-binding activity by expression of their recombinant forms. This shows that they truly function as animal lectins. The protein purification study revealed that the major xgalectins in kidney are xgalectin-Ib, -Ila, -Ilb, -Ila, and -VIIa. The mRNA of xgalectin-Ib, -Ilb, -Vb, and -Vla were localized to specific adult tissues, whereas those of xgalectin-VIIa and -VIIa were broadly distributed. The temporal expression patterns of the mRNAs of the 12 xgalectins during embryogenesis were analyzed and categorized into three groups: 1) mRNA observed to exist throughout embryogenesis, i.e. maternal mRNA also exists (xgalectin-Ia, -IIa, -IIIa, -IIIb, -Vla, -VIIa, and -VIIa); 2) mRNA observed from the gastrula stage (xgalectin-Vla); and 3) mRNA observed from the tail bud or the tadpole stage (xgalectin-Ib, -Ilb, -Iva, and -Vb). The mRNA of the most abundant xgalectin in embryos, xgalectin-Vla, was localized to the surface layer of embryos, the epidermis, the cement gland, and various placentas. Xgalectin-VIIa protein was also observed to exist throughout embryogenesis by Western blot analysis with specific antiserum. These results show that the expression of each member is spatiotemporally regulated from eggs to adulthood, suggesting that galectins play multiple roles not only in adults, but also in development.

Galectins comprise a family of animal lectins that bind to β-galactoside-containing carbohydrate moieties of glycoconjugates (1, 2). Fourteen galectins have been isolated from mammals and are classified into the proto, chimera, and tandem repeat types based on their structures (3–8). Proto-type galectins contain one carbohydrate recognition domain (CRD), a structurally conserved domain that specifically recognizes a β-galactoside-containing carbohydrate; and chimera-type galectins consist of one CRD and an N-terminal elongating protein domain. Mammalian galectin-1 (proto type) and -3 (chimera type) are the most extensively studied galectins (9). They have been proposed to play roles in tissue organization, development, immunity, and cancer growth and metastasis by regulating such processes as cell adhesion and apoptosis. Tandem repeat-type galectins contain two CRDs covalently linked through a unique link peptide. Tandem repeat-type galectins are less well understood, but they have been shown to be expressed in a tissue-specific manner in adult animals as well as in mouse embryos. Also, recent studies revealed that they play roles in immunity, e.g. the chemoattractant activity of galectin-9 for eosinophils (10, 11) and activation of neutrophils by galectin-8.2 Thus, the galectin family seems to be significantly associated with development, immunity, and tumorigenesis, but the details of their functional mechanisms remain unclear.

Studies have been recently accumulating that indicate that the galectin family play roles in the development of vertebrate embryos. The expression of some members is temporally and spatially regulated during embryogenesis (13–16); galectin-1 regulates the axonal growth of neural cells in mouse and rat (17–19); a proto-type galectin of chicken affects the differentiation of cultured mesonephroi (20); a null mutant mouse of galectin-3 has subtle but significant defects in bones and inflammatory responses (21–23), etc. Other evidence that galectins might contribute to developmental regulation has been obtained for Xenopus. Milos and co-workers (24, 25) have extensively studied the endogenous β-galactoside-binding lectins in Xenopus embryos and have proposed that these lectins are important for the development of neural crest cells, craniofacial tissue, and heart, etc. Although the identity of these β-galactoside-binding lectins has yet been determined, they are very likely to be members of the galectin family.

Our purpose is to clarify the functions of the galectin family in animal development using the Xenopus system. However, when we started this project, only one proto-type galectin had been identified in Xenopus (26). Therefore, in a previous work (27), we reported the isolation and characterization of five
novel galectins from *Xenopus* liver, which seemed to be the most suitable organ for identifying novel galectins. In this study, we chose *Xenopus* kidneys to further identify novel galectins because they contain large amounts of tandem repeat-type galectins, including other protein(s) that are absent in the liver (27). We successfully identified six novel galectins, with all three types, i.e. the proto, chimera, and tandem repeat types, being represented. We have comprehensively analyzed the expression of all the galectins identified so far and have demonstrated that they are specifically regulated throughout, from eggs to adulthood.

**EXPERIMENTAL PROCEDURES**

**Expression of Xenopus Galectins**

**Xenopus Tissues and Embryos**—Female frogs were used for the protein purification study and expression analysis of adult tissues, except for tests. To obtain embryos, mature eggs were collected after injection of 500 IU of human chorionic gonadotropin (GONATROPIN®, Toikoukouzi, Inc., Tokyo, Japan) into females. In vitro fertilized eggs were dejellied with 3% L-cysteine hydrochloride monohydrate (Nacalai Tesque, Inc., Kyoto, Japan) in Steinberg's solution and then cultured at 20 °C. Albinoid embryos were used only for whole-mount in *situ* hybridization. Embryos were staged according to Nieuwkoop and Faber (28).

**cDNA Cloning of Xgalectins**—A full-length cDNA clone (1080-bp insert) of xgalectin-VIIa was isolated from a Xenopus kidney cDNA library (Stratagene) using a cDNA fragment of xgalectin-IIa (nucleotides 1–786, GenBank™/EBI accession number AB060970) as a probe. Partial cDNA clones of xgalectin-IIIb were isolated by screening the kidney library using a cDNA fragment of xgalectin-IIIi (nucleotides 26–452, accession number AB060971) as a probe. Because all the clones lacked the 5'-region (even the clone with the longest insert) or lacked nucleotides 1–77, 5'-RACE-PCR was performed with a Marathon cDNA amplification kit (Clontech) and kidney mRNA. A specific primer with the following sequence was used: 5'-ATCCGTTGATGTT-ATTCCAAGTA-3'. As a result, a cDNA fragment of 422 bp covering the entire 5'-region of xgalectin-IIIb was obtained. The full-length sequence of the 5'-RACE-PCR product and that of the clone with the longest insert. A partial cDNA fragment of xgalectin-Vb was isolated by degenerate oligonucleotide-based PCR cloning as described (27). A sense primer (Gd4, 5'-CATTGAAGAAGC-3') and an antisense primer (Go rA, 5'-TTAATGAAGCGAG-3') corresponding to highly conserved amino acids in members of the galectin family, HNFPRF, and an antisense primer (Gd7, 5'-TCTTCTTCCC-3') were used. Partial cDNA clones of xgalectin-IIIb were isolated by screening a cDNA library (Stratagene) using a cDNA fragment of xgalectin-IIa (nucleotides 20–264) as a probe.

**Construction of Expression Vectors and Preparation of Recombinant Proteins**—Recombinant xgalectin-Ia, -Vb, and -VIIa proteins were expressed as fusion proteins with glutathione S-transferase (GST) using the GST fusion system (Amersham Biosciences). Each cDNA encoding an open reading frame was inserted into pGEX-4T-1, and the expression plasmids were introduced into *Escherichia coli* BL21. Expression of fusion proteins was carried out as described in our previous report (11).

The extracted recombinant proteins were purified by affinity chromatography on a lactosyl-agarose column (Seikagaku Co., Tokyo). The proteins bound to the lactosyl-agarose were eluted with buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM diethiothreitol, and 200 mM lactose and then analyzed by SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue R-250.

**Purification of Xgalectins and Amino Acid Sequence Analysis**—Purification of xgalectins and amino acid sequence analysis were performed as described in our previous work (27). Briefly, *Xenopus* kidneys obtained from adult females were homogenized in a 5-fold volume of 10 mM Tris-HCl (pH 7.2), 0.15 M NaCl, 1 mM EDTA, 1 mM diethiothreitol, 5 mM benzamidine HCl, 1 mM phenylmethylsulfonyl fluoride, and 1 mM diisopropylphosphofluoridate, and the homogenate was centrifuged. 0.1 volume of 4 M NaCl (final concentration of −0.5 M) and 0.01 volume of 1% CHAPS (Sigma) were added to the supernatant recovered, and then the mixture was applied to a lactosyl-agarose column (2 ml) and washed extensively with 10 mM Tris-HCl (pH 7.2), 0.5 M NaCl, 1 mM EDTA, 0.2 M diethiothreitol, and 0.01% CHAPS and then with the same buffer, except the NaCl concentration was 0.15 M. Proteins adsorbed to the affinity resin were eluted with buffer containing 200 mM lactose. All steps were performed at 4 °C. The protein mixture eluted from the lactosyl-agarose resin was separated into two fractions by anion-exchange chromatography on a Resource Q column (1 ml; Amersham Biosciences). The flow-through (30–36-kDa proteins) and adsorbed (14-kDa proteins) fractions were separately pooled to examine the amino acid composition.

The 14- and 36–kDa kidney xgalectins were treated with 1% SDS and directly dot-blotted onto polyvinylidine difluoride membranes. The blotted proteins were reduced and S-pyridylethylated on the membranes by the method of Iwamatsu and Yoshida-Kubomura (30). The denatured proteins were digested with trypsin (0.6 μg/50 μl) at 25 °C for 18 h, and the fragments liberated from the membranes were purified on a ρBondosphere 5C18 column (Waters Associates) in a reverse-phase high performance liquid chromatography system (Toho Co., Tokyo). The N-terminal 10 amino acids of each peptide were determined with an ABI 492 gas-phase sequencer (Applied Biosystems).

**RT-PCR**—Total RNAs were extracted from adult tissues and embryos at various stages using Isogen (Nippon GENED, Tokyo). RT-PCR was performed using an RNA PCR kit (Applied Biosystems). A primer set was designed for each total RNA for each reaction. Synthesis of first-strand cDNA was performed according to the manufacturer's protocols. The primers used for PCRs were as follows: Ia, 5'-CAGCGAAGACAAATATAGC-3' (sense) and 5'-GCCCTTTGATGTGATTGTTG-3' (antisense); IIa and IIb, 5'-GGAGGCGACACCAAAAAAATT-3' (sense) and 5'-CTAAGAAGCAGCAGTACACT-3' (antisense); IIIa and IIIb, 5'-AAATACCATCACCAGTCCCACAT-3' (sense) and 5'-TAAGAACATTCTTTGGTTGGC-3' (antisense); IVa, 5'-CAGCGAACGAGCAGT-3' (sense) and 5'-TTAATGAAGCGAG-3' (antisense); Vfa and Vfb, 5'-TCAACACAGCAGCAGT-3' (sense) and 5'-TCAACACAGCAGCAGT-3' (antisense); Vfa, 5'-GGTTCTAGGTAGCGAGGCC-3' (sense) and 5'-TCAACACAGCAGCAGT-3' (antisense); VIa, 5'-GAATTTGTCAAAGAGAC-3' (sense) and 5'-GCAACAGTCCCAC-3' (antisense); VIIa, 5'-TATGCGACAGCAGT-3' (sense) and 5'-TCAAGAATTTGAAGAGAC-3' (antisense); VIIb, 5'-TTCATTGGCGCTCTGAC-3' (antisense); VIIIa, 5'-GAACCTCTCAACCTC-3' (sense) and 5'-TGATTCCGAGATCCT-3' (antisense); VIIIb, 5'-GTCGACGAC-3' (antisense); IXa, 5'-GAACCTCTCAACCTC-3' (sense) and 5'-TGATTCCGAGATCCT-3' (antisense); IXb, 5'-GTCGACGAC-3' (antisense); Xa, 5'-GTCGACGAC-3' (antisense); Xb, 5'-GTCGACGAC-3' (antisense). The primers for ornithine decarboxylase were prepared according to Yamada et al. (31). The reaction mixtures were as follows: (A) 10 μl, followed by thermal cycles of the following: 94 °C for 30 s, 60 °C for 15 s, and 72 °C for 1 min. For analysis of adult tissues, reactions were repeated for 30 cycles; and for embryos, the cycle numbers were as indicated in legend to Fig. 5. Ornithine decarboxylase or EF-1α (GenBank™/EBI accession number M25504) was amplified as an internal control. All PCR products were analyzed on 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Control experiments to show the specific amplification of xgalectin-IIa, -Vb, and -VIIa were performed using 1 ng of each plasmid clone as template DNA in each reaction. A control experiment to show the simultaneous and equal amplification of xgalectin-IIa and -Ib was performed by adding 0.1 pg of each plasmid clone used as template DNA (1 pg) to each reaction. A control experiment for xgalectin-Ia and -Ib was performed in the same way. Specific amplification with each primer pair was confirmed by a reaction without reverse transcriptase and also by sequence analysis of the PCR products (data not shown).

**Northern Hybridization**—Poly(A)⁺ RNAs were prepared from adult...
tissues. RNA (1 μg) from each sample was fractionated and blotted onto a nylon membrane. cDNA fragments with the following sequences were labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals) by PCR and then used as probes: xgalectin-VIa, nucleotides 693–1427; xgalectin-VIIa, nucleotides 1–1074; xgalectin-VIIIa, nucleotides 96–1043; and EF-1α, nucleotides 758–1378 (GenBankTM/EBI accession number M25504). Hybridization, washing, and detection were performed as described previously (32).

Whole-mount in Situ Hybridization—Whole-mount in situ hybridization was performed by the method described previously (33). Digoxigenin-labeled complementary RNA probes were synthesized with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals). A plasmid clone with a cDNA fragment (nucleotides 84–864) of xgalectin-VIIa was used as template DNA. A sense probe was used as a negative control.

Preparation of Anti-xgalectin-VIIa Serum—Preparation of recombinant xgalectin-VIIa was performed as described (11). A plasmid clone carrying the entire open reading frame was constructed on the pGEX-4T-2 vector (Amersham Biosciences). The protein was expressed as a fusion protein with GST. The purified recombinant protein was used to immunize Japanese white rabbits as described (27).

Western Blot Analysis—For late tadpole stage samples (stages 57–59), whole protein was extracted from whole embryos or epidermis removed from embryos by homogenization in a 5-fold volume of 80% methanol and 20% Tris-buffered saline (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl). The extracts were centrifuged, and the precipitates were dissolved in the general sample buffer for SDS-PAGE. Proteins derived from 1 mg of wet tissues were applied to each lane. At first, the same protocol was used for younger embryos; but in this case, the huge amount of vitellogenin disturbed the SDS-PAGE. To overcome this, unfertilized eggs and embryos (gastrula/early tadpoles) were homogenized in buffer comprising 20 mM Tris-HCl (pH 7.5), 100 mM lactose, 2 mM EDTA, and 5 mM benzamidine HCl and then centrifuged. Under these conditions, most of the vitellogenin precipitate and xgalectin-VIIa are recovered in the supernatant. Proteins in supernatants were precipitated by adding a 9-fold volume of methanol and then dissolved in the SDS-PAGE sample buffer. The protein amount for unfertilized eggs and embryos (gastrula/early tadpoles) subjected to SDS-PAGE was standardized as to the number of embryos, and protein equivalent to 0.5 embryos was applied to each lane. Western blotting was performed in the same way as described in our previous work (27), except that anti-xgalectin-VIIa serum was used at a dilution of 1:500.

RESULTS
cDNA Cloning of Six Novel Galectins from a X. laevis kidney cDNA library—We have isolated and named six novel xgalectin cDNAs. As described in our previous report (27), a Roman numeral and letter were assigned to each Xenopus galectin not according to the number of mammalian galectins, but according to the order of their discovery, because complete correspondence of the members of the Xenopus and mammalian galectin families was impossible.

A novel proto type (xgalectin-Vb) was structurally most similar to Xenopus skin 16-kDa galectin (78% amino acid identity) (Fig. 1A), which was previously described by Marschal et al. (26). As these two xgalectins are structurally similar and also exhibit similar expression patterns, being abundant in adult skin (to be described below), we propose the designations xgalectin-Va for the skin 16-kDa galectin and xgalectin-Vb for the newly identified galectin. Mammalian counterparts of xgalectin-Va and -Vb have not been identified.

One chimera-type galectin, xgalectin-VIIa, was cloned, and its complete sequence was determined. In the course of this study, we found nine Xenopus EST clones related to xgalectin-Va (78% nucleotide sequence identity), which were previously described by Marschal et al. (26). As these two xgalectins are structurally similar and also exhibit similar expression patterns, being abundant in adult skin (to be described below), we propose the designations xgalectin-Va for the skin 16-kDa galectin and xgalectin-Vb for the newly identified galectin. Mammalian counterparts of xgalectin-Va and -Vb have not been identified.

One chimera-type galectin, xgalectin-VIIa, was cloned, and its complete sequence was determined. In the course of this study, we found nine Xenopus EST clones related to galectin-3, a mammalian chimera type, and the full coding sequence was reconstructed by combining them. The amino acid sequence deduced from the consensus nucleotide sequence is identical to that of xgalectin-VIIa (Fig. 1B). Thus, the xgalectin-VIIa and EST clones must have originated from the same gene. As the amino acid sequence of xgalectin-VIIa is highly similar to that of mammalian galectin-3 (Table I), xgalectin-VIIa seems to be a Xenopus homolog of mammalian galectin-3.

Four tandem repeat-type galectins, xgalectin-IIb, -IIIb, -VIIa, and -VIIIa, were newly identified. Two of them, xgalectin-IIb and -IIIb, are structurally very similar to xgalectin-IIa and -IIIa, respectively (Fig. 2, A and B), which we described in a previous work (27). The amino acid sequences of the CRDs in xgalectin-IIa and -IIb or in xgalectin-IIIa and -IIIb are 88–90% identical. The major structural difference between each pair of isoforms is the length of the link peptides. The link peptide of xgalectin-IIa is longer by 12 amino acids compared with that of xgalectin-IIb, and the link peptide of xgalectin-IIIa is 35 amino acids longer than that of xgalectin-IIIb (Fig. 2, A and B).

Based on the sequence similarity, both xgalectin-IIa and -IIb seem to be Xenopus homologs of mammalian galectin-4, and both xgalectin-IIIa and -IIIb seem to be Xenopus homologs of mammalian galectin-9 (Table I).

There are three Xenopus EST clones related to xgalectin-Va, and the amino acid sequence deduced from the consensus nucleotide sequence completely matches that of part of the N-terminal CRD of xgalectin-Va (Fig. 2C). Thus, the xgalectin-Va and EST clones must have originated from the same gene. A mammalian counterpart of xgalectin-Va has not been identified. One EST clone related to xgalectin-VIIa was found. Although the partial amino acid sequence encoded by the EST sequence is only 88% identical to that of xgalectin-VIIa, the entire nucleotide sequence of the EST clone matches the cDNA sequence of xgalectin-VIIa (96% identity), even though most of the compared region corresponds to the 3′-noncoding sequence (Fig. 2D). Thus, the disagreement between the sequences of xgalectin-VIIa and the EST clone may be due to allelic differences and/or sequence errors, and the xgalectin-VIIa and EST clones may have originated from the same gene. Xgalectin-VIIa seems to be a Xenopus homolog of mammalian galectin-8 because its amino acid sequence is highly similar to that of mammalian galectin-8 (53% identical to human galectin-8) (Table I).

Lactose-binding Activity of Xgalectins Whose Counterparts in Other Species Have Not Been Identified—Of the 12 xgalectins we have identified so far, there are four xgalectins whose coun-
terparts in other species have not been identified, xgalectin-IVa, -Va, -Vb, and -VIa (Table I). As described above, xgalectin-Va has been well characterized as skin 16-kDa galectin by Marschal et al. (26) and has been shown to bind lactose with high affinity. To clarify whether xgalectin-IVa, -Vb, and -VIa truly act as lectins, we tested the lactose-binding activity of their recombinant forms. Recombinant xgalectin-IVa, -Vb, and -VIa proteins were expressed as GST fusion proteins and purified by affinity chromatography on a lactosyl-agarose column. As shown in Fig. 3, recombinant xgalectin-IVa, -Vb, and -VIa proteins were successfully recovered from the adsorbed fractions of each E. coli extract on the lactosyl-agarose column.

**Protein Purification Study**—To identify the major xgalectins in kidney, we determined the amino acid sequences of tryptic peptides of xgalectins purified from kidney. Xgalectins were purified by an affinity chromatography method using lactosyl-agarose resin, and the eluted proteins were further fractionated into 14-kDa protein-rich and 30–36-kDa protein-rich fractions by anion-exchange chromatography. From the 14-kDa protein-rich fraction, only the sequence of xgalectin-Ib or the

| Type          | Xenopus                  | Mammalian       | Amino acid sequence identity |
|---------------|--------------------------|-----------------|-----------------------------|
| Proto         | Xgalectin-Ia and -Ib     | Galectin-1      | 49                          |
| Tandem repeat | Xgalectin-IIa and -Iib   | Galectin-4 (galectin-6) | 50–51 |
| Tandem repeat | Xgalectin-IIIa and -Iib  | Galectin-9      | 42                          |
| Tandem repeat | Xgalectin-IVa            | Unknown         |                             |
| Proto         | Xgalectin-Va and -Vb     | Unknown         |                             |
| Tandem repeat | Xgalectin-Vla            | Unknown         |                             |
| Chimera       | Xgalectin-VIIa           | Galectin-3      | 42                          |
| Tandem repeat | Xgalectin-VIIa           | Galectin-8      | 53                          |

* Full sequences of human and Xenopus homologues were compared.

* Galectin-6 is closely related to galectin-4 but has been identified only in mouse.
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common sequence of xgalectin-Ia and -Ib was obtained, and no particular peptide for xgalectin-Ia was recovered (Table II). From the 30–36-kDa protein-rich fraction, three sequences of xgalectin-IIa, four sequences of xgalectin-IIb, two common sequences of xgalectin-IIa and -Ib, three sequences of xgalectin-IIIa, and six sequences of xgalectin-VIIa were identified (Table II). There was no particular peptide for xgalectin-IIb. Thus, as summarized in Table III, the dominant xgalectins in kidney are xgalectin-Ib, -Ia, -IIb, -IIIa, and -VIIa, and they are quite different from those in liver, which we reported in a previous study (27).

**Distribution of mRNAs of Novel Xgalectins in Adult Tissues**—Fig. 4 shows the expression profiles of the newly identified xgalectin mRNAs in adult tissues. Expression of the mRNAs of xgalectin-Ib, -IIb, and -Vb was analyzed by RT-PCR to distinctly detect the structurally similar isoforms (Fig. 4A). To distinguish the mRNAs of xgalectin-IIa and -Ib, we used a primer pair that recognizes the sequences of both xgalectin-IIa and -Ib and that amplifies the coding region, including link peptides. In this way, cDNAs derived from xgalectin-IIa and -Ib could be detected as different sized DNA bands because of the different lengths of the link peptides. A control experiment involving this primer set is presented in Fig. 5, which shows that, with the same amount of template DNA, the two bands exhibited the same intensity. The distribution of xgalectin-Ib mRNA was tissue-specific, and the major tissues producing xgalectin-Ib mRNA were stomach, kidney, and testes, whereas xgalectin-IIa mRNA was abundant in liver, stomach, intestine, and kidney (Fig. 4A) (27). The mRNAs of xgalectin-IIa and -Ib were distinctly detected by the same method used for xgalectin-IIa and -Ib. The mRNA of xgalectin-IIb was detected only in intestine, kidney, and testes, whereas that of xgalectin-IIIa was broadly distributed. The mRNAs of xgalectin-Va and -Vb were distinctly detected using a specific primer pair for each. The control experiments in Fig. 5 show the specific amplification by the primers used. The mRNAs of both xgalectin-Va and -Vb were extremely abundant in skin, and their expression in other tissues was quite low. The quantitative differences in the mRNAs of both xgalectin-Va and -Vb between skin and other tissues were more conspicuously observed by Northern blot analysis (data not shown).

Expression of the mRNAs of xgalectin-Vla, -Vla, and -VIIa was analyzed by Northern hybridization (Fig. 4B). The mRNA of xgalectin-Vla was detected only in the tissues directly in contact with the outside of the body, i.e. lung and skin. The mRNA of xgalectin-VIIa was broadly distributed and was particularly abundant in ovary, consistent with the fact that a large amount of xgalectin-VIIa mRNA and protein exists in eggs, as described below. The mRNA of xgalectin-VIIa was also distributed broadly, but uniformly, in tissues other than heart, stomach, and muscle.

**Temporal Expression Patterns of mRNAs from the Xenopus Galectin Family during Embryogenesis—**RT-PCR was performed to analyze the temporal expression patterns of mRNAs from all members of the Xenopus galectin family identified so far during embryogenesis (Fig. 5). The mRNAs of sets of xgalectins (Ia/Ib, Ia/Ib, IIa/Ib, IIIa/Iib, and Va/Vb) were distinctly detected as shown in control experiments. The expression patterns of the 12 xgalectins can be categorized into three groups: 1) mRNA observed to exist throughout embryogenesis, i.e. maternal mRNA also exists (xgalectin-Ia, -Ib, -IIa, -IIb, -Vb); 2) mRNA observed from the gastrula stage (xgalectin-Vla); and 3) mRNA observed from the tail bud or tadpole stage (xgalectin-Ib, -IVa, -Vb, and -Ib). Among these members, xgalectin-VIIa seems to be the most abundant xgalectin in embryos because it was sufficiently detected even with

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**Table II**

| Tryptic peptide sequences of xgalectins from kidney |
|---------------------------------------------------|
| xgal, xgalectin.                                  |
| 14-kDa Xgalectin-rich fraction                    |
| 30–36-kDa Xgalectin-rich fraction                 |
| 1  65EENSWGETQR14 (xgal-Ib)                       |
| 2  184NSMGNN180 (xgal-VIIa)                      |
| 3  25THYDLPQPN159 (xgal-VIIa)                    |
| 4  25LQSXW426 (xgal-Ib)                          |
| 5  184VPGQPEPP123 (xgal-VIIa)                    |
| 6  152TVEVQPEPP161 (xgal-VIIa)                   |
| 7  125KGDEKMXD133 (xgal-Ib)                      |
| 8  195HILEPQPEPP177 (xgal-VIIa)                  |
| 9  225KHVQPEPP197 (xgal-VIIa)                    |
| 10 425YSPQPEPP245 (xgal-VIIa)                    |
| 11 405GDPQPEPP225 (xgal-VIIa)                    |
| 12 425HHNQPEPP245 (xgal-VIIa)                    |
| 13 425DNPQPEPP245 (xgal-VIIa)                    |
| 14 425QHPQPEPP245 (xgal-VIIa)                    |
| 15 425QHPQPEPP245 (xgal-VIIa)                    |

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**Fig. 3. Lactose-binding activities of recombinant xgalectins**—IVa, -Vb, and -VIIa. Recombinant xgalectin-IVa, -Vb, and -VIIa proteins were expressed as GST fusions in _E. coli_ carrying each expression plasmid and were collected by affinity chromatography on lactosylagarose columns. The adsorbed fractions on the lactosyl-agarose columns were analyzed by SDS-PAGE (12% acrylamide gel). The protein bands were visualized by Comassie Brilliant Blue R-250 staining. Two minor bands observed in the GST/xgalectin (xgal)-VIIa column (~42.7 kDa) are degraded products of the fusion protein. The calculated molecular masses of the fusion proteins are as follows: GST/xgalectin-IVa, 62.8 kDa; GST/xgalectin-Vb, 41.4 kDa; and GST/xgalectin-Vla, 61.4 kDa.

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25 PCR cycles and because it was remarkably detected by Northern analysis, which did not allow comparison with other members (data not shown).

mRNA of Xgalectin-VIIa Is Localized to the Surface of Embryos—To clarify the distribution of the mRNA of xgalectin-VIIa during embryogenesis, whole-mount in situ hybridization was performed in the neurula to early tadpole stages. Xgalectin-VIIa mRNA was localized to various placodes, the cement gland, and the epidermis throughout the developmental stages (Fig. 6). At the early neurula stage, a hybridization signal was observed in the neural fold region (which would give rise to placodes such as the lens and otic placodes), in the cement gland anlage, and in the epidermal ectoderm surrounding the embryo, but it was absent in the neural plate. The negative region disappeared with development because, during neurulation, the neural ectoderm was missing from the surface layer of the embryo. At the tail bud stage, the entire surface of embryos was positively stained, except for the proctodeum. This expression pattern persisted at least to the early tadpole stage.

Western Blot Analysis of Expression of Xgalectin-VIIa in Embryos—To confirm the protein expression of xgalectin-VIIa, Western blot analysis was performed using a specific rabbit antiserum raised against recombinant xgalectin-VIIa. Xgalectin-VIIa protein existed from unfertilized eggs to the early tadpole stages at almost comparable levels (Fig. 7). Furthermore, we prepared an extract of epidermis removed from late tadpoles and compared it with a whole-embryo extract. As a
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DISCUSSION

Together with those mentioned in our previous work (27), 12 Xenopus laevis galectins have been identified. Also, with the identification of chimera-type xgalectin-VIIa in this study, three structurally different types have been found. As summarized in Table IV, a galectin family comparable to that in mammals exists in Xenopus.

The three newly identified xgalectins, xgalectin-IIb, -IIIb, and -IVb, are structurally very similar to the known xgalectins, xgalectin-IIa, -IIla, and -Va, respectively. However, they are not alleles from different individuals, but rather distinct genes. For the proto-type xgalectins, xgalectin-Va and -Vb, the similarity of the cDNA sequence in the 3′-noncoding region is quite low; and furthermore, the expression patterns of their mRNAs differ in early embryogenesis. For two sets of tandem repeat-type xgalectins, xgalectin-IIa and -IIb and xgalectin-IIla and -IIlb, we obtained two lines of strong evidence. First, there is a significant difference in the lengths of the link peptides. Second, as shown in Figs. 4 and 5, their expression is differently regulated in both adult tissues and whole embryos.

There are several EST clones related to xgalectins identified in either the previous (27) or present study. Analysis of their cDNA sequences revealed that they originated from the xgalectin-VIIa, -VIIla, or -VIIlb gene, and there are no apparent sequences of isoforms.

The relationship of Xenopus and mammalian galectins is summarized in Table I. Our proposal of a homologous relationship is based not only on structural similarities, but also on their expression patterns. Xgalectin-IIa and -IIb and mammalian galectin-4 are apparently abundant in the digestive tract, although the xgalectins have some other expression sites (15). The expression patterns of xgalectin-IIla and -IIlb share common characteristics with that of the mammalian counterpart, galectin-9. Three isoforms of mammalian galectin-9, galectin-9S, -9M, and -9L (29, 34), are produced through alternative splicing of exons coding the link peptide, resulting in the production of proteins with link peptides of different lengths. The major isoform of galectin-9, galectin-9M, is broadly distributed in adult tissues; but another isoform, galectin-9L, has been shown to be localized in small intestine in mouse (29). Almost the same is true for xgalectin-IIla and -IIlb, viz. xgalectin-IIla is broadly distributed in adult tissues, whereas the major expression site of xgalectin-IIlb is restricted to intestine, although minor expression is observed in kidney and testis.

Other xgalectins for which mammalian counterparts have been identified (xgalectin-1a, -1b, -VIIa, and -VIIla) are broadly distributed in adult tissues, and the same is true for their mammalian counterparts (galectin-1, -3, and -8) (9, 35, 36). On the other hand, mammalian counterparts of xgalectin-IVa, -Va, -Vb, and -Vla have not been identified. Xgalectin-Va and/or -Vb is the most abundant protein in skin (5% of the total protein) (26). In mammals, a proto-type galectin, galectin-7, is abundantly expressed in skin keratinocytes (12, 37, 38), but its amino acid sequence is dissimilar from that of either xgalectin-Va or -Vb. Thus, galectin(s) distributed in skin may have evolved independently in various animal species. Xgalectin-VIIa exhibits relatively high structural similarity to mammalian galectin-4 (49% amino acid sequence identity to human galectin-4). However, their expression patterns are completely different. The mRNA of xgalectin-VIIa is localized to skin and lung, whereas that of mammalian galectin-4 is localized to the digestive tract (15). Therefore, xgalectin-IIa and -IIb are more suitable as Xenopus homologs of galectin-4. No mammalian galectin that is localized to skin and lung has been identified.

Because homologous proteins for xgalectin-IVa, -Va, -Vb, and -Vla have not been identified in any other species, not only in mammals, they must be demonstrated to bind β-galactoside to be categorized into the galectin family. Xgalectin-Va has been shown to bind lactose by Marschal et al. (26), and we have demonstrated the lactose-binding activities of xgalectin-IVa, -Vb, and -Vla in this study. Furthermore, we performed a preliminary analysis using antisera raised against the recombinant C-terminal CRD of xgalectin-VIa. As a result, we detected native xgalectin-VIa protein in the lactose-binding fraction of the adult lung extract, where the xgalectin-VIa mRNA exists abundantly (data not shown). Therefore, all four galectins identified only in Xenopus have been shown to truly act as lectin proteins.

The protein purification study revealed that the dominant xgalectins in kidney are xgalectin-IIa, -IIb, -IIla, and -Vla, which are quite different from those in liver, which we reported...
TABLE IV

Expression of Xenopus Galectins

| Proto-type | Chimera-type | Tandem repeat-type |
|------------|--------------|-------------------|
| CRD -14 kDa | -30 kDa     | -36 kDa            |

**Classification of mammalian and Xenopus galectins**

- **mammalian**
  - galectin-1, 2, 5, 7, 10, 11, 13 & 14
  - galectin-3
  - galectin-6, 8, 9 & 12

- **Xenopus**
  - xgalectin-Ia, Ib, Va & Vb
  - xgalectin-VIIa
  - xgalectin-Ila, Iib, IIIa, IIIb, IVa, VIa & VIIa

in a previous study (27). The point we should emphasize here is that the dominant proto-type galectin is different in liver and kidney, i.e. xgalectin-Ia in liver, but xgalectin-Ib in kidney. This shows that even though xgalectin-Ia and -Ib are structurally very similar (91% amino acid sequence identity and 86% identity in cDNA sequence, including noncoding regions), their expression is differentially regulated, suggesting that they have distinct roles in each organ.

The distribution of the newly identified xgalectin mRNAs in adult tissues was determined by RT-PCR and Northern hybridization. Here we found other paradigms of isoform expression in adult tissues was determined by RT-PCR and Northern hybridization. This shows that even though xgalectin-Ia and -Ib are structurally very similar (91% amino acid sequence identity and 86% identity in cDNA sequence, including noncoding regions), their expression is differentially regulated, suggesting that they have distinct roles in each organ.

The expression patterns of the Xenopus galectin family during embryogenesis. As a result, we can categorize the 12 members into three groups: 1) mRNA observed to exist throughout embryogenesis, i.e. maternal mRNA also exists (xgalectin-IIa and -IIb and xgalectin-IIIa and -IIb. Xgalectin-IIb and -IIb might have specific functions in the digestive tract; and to perform these functions, shorter link peptides than those in xgalectin-IIa and -IIa, respectively, might be required. The mRNA of xgalectin-VIa was localized to skin and lung. Xenopus skin and lung are rich in mucosa and are in direct contact with the outside of the body. Another tissue with the same characteristics, i.e. mucosa-rich and in contact with the outside, is the digestive tract. From this point of view, many xgalectins are abundant and/or localized to tissues that are mucosa-rich and in contact with the outside. This suggests that xgalectins play roles in immunity, especially in innate immunity concerned with the function of the mucosa.

Comprehensive analysis was performed on the temporal expression patterns of the Xenopus galectin family during embryogenesis. As a result, we can categorize the 12 members into three groups: 1) mRNA observed to exist throughout embryogenesis, i.e. maternal mRNA also exists (xgalectin-IIa and -IIa, -IIb, -Va, -VIa, and -VIIa); 2) mRNA observed from the gastrula stage (xgalectin-VIa); and 3) mRNA observed from the tail bud or tadpole stage (xgalectin-Ib, -Ib, -IVa, and -Vb). This shows that most xgalectins exist in embryos and that their expression is specifically regulated not only in adult tissues, but also in embryos. Xgalectins whose mRNAs exist as maternal mRNAs may be required for the maintenance of unfertilized eggs and/or in early development until zygotic gene expression is initiated at the mid-blastula stage (mid-blastula transition). Furthermore, expression of these xgalectins persists even after the mid-blastula transition. Therefore, they seem to play roles throughout development. The expression of xgalectin-VIa, whose mRNA begins to be detectable at the gastrula stage, might be initiated at the mid-blastula transition, although more precise analysis is required. According to our preliminary results, xgalectin-VIa is highly expressed in the cement gland throughout its formation (data not shown).

These facts suggest that xgalectin-VIa plays a role in early development, especially in the transient formation of the cement gland. Xgalectins whose expression starts at the tadpole stage may play roles in the organization of various tissues because the formation of various organs is actively underway at this stage.

Among these xgalectins, we chose a chimera type, xgalectin-VIIa, as the first to be analyzed precisely in early embryos because it was judged to be the most abundant in embryos. Furthermore, it is valuable to examine its expression pattern to estimate the common role(s) of chimera-type galectins in vertebrate development because mammalian galectin-3 is the only chimera type that has been studied extensively in mammalian development and immune systems. Whole-mount in situ hybridization was performed and revealed that the mRNA of xgalectin-VIIa is localized to surface tissues of embryos, the epidermis, placodes, and the cement gland. Furthermore, Western blot analysis revealed that xgalectin-VIIa protein exists throughout embryogenesis from unfertilized eggs and is still abundant in the epidermis of late stage tadpoles. These results suggest that xgalectin-VIIa plays a role in the organization of tissues expressing xgalectin-VIIa and/or in embryonic self-defense. Murine galectin-3, a homolog of xgalectin-VIIa, has also been shown to be expressed in the epidermis of embryos, but at rather later stages, from 13.5 days postcoitus. In Xenopus, xgalectin-VIIa is expressed in epidermal cells at least at the neurula stage, suggesting that Xenopus requires galectin expression for organization of the embryonic epidermis at an earlier stage than mammals. Another suggestion is that galectins play roles in embryonic immunity and that Xenopus must establish an immune system at an earlier stage than mammals because Xenopus is oviparous, whereas mammals are viviparous. The expression of murine galectin-3 was also observed in the notochord and cartilage primordia. However, we could not find significant hybridization signals in tissues other than the epidermis, placodes, and the cement gland, even upon sectional analysis of whole-mount in situ hybridization specimens. In Xenopus, the roles of galectins in the notochord and cartilage primordia may be compensated for by some other members, including unidentified ones.

We are currently performing comprehensive analysis of the expression of other xgalectin mRNAs in early embryos by in situ hybridization and screening of the target molecule(s) of xgalectins. Identification of the targets of xgalectins in each tissue is essential for elucidating their functions. We recently isolated candidate 40–46-kDa protein(s) for xgalectin-VIIa from embryos. Information on the structure and gene expression of this candidate molecule will be useful in determining the role of xgalectin-VIIa in Xenopus embryogenesis.

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