J. Biochem. **141,** 479–488 (2007)
doi:10.1093/jb/mvm048

Identification of Novel Members of the *Xenopus* Ca^{2+}-dependent Lectin Family and Analysis of Their Gene Expression During Tail Regeneration and Development

Tomoko Ishino[1,*], Takekazu Kunieda[2,*], Shunji Natori[3], Kazuhisa Sekimizu[1] and Takeo Kubo[2,†]

[1]Graduate School of Pharmaceutical Sciences, University of Tokyo; [2]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113-0033; and [3]National Institute of Agrobiological Sciences, an Independent Administrative Institution, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan

Received October 19, 2006; accepted January 20, 2007; published online February 13, 2007

We previously demonstrated that the gene for a member of the humoral C-type lectin family is transiently expressed in the regenerating legs of the American cockroach [Arai *et al.*, *Insect Biochem. Mol. Biol.* 28, 987–994 (1998)]. To identify candidate lectin(s) involved in tail regeneration in the *Xenopus laevis* tadpole, we isolated a 35-kDa Ca^{2+}-dependent lectin (XCL-1) from adult *Xenopus* serum and cloned its cDNA. Although XCL-1 gene expression was not induced in the regenerating tails, we isolated a cDNA for an XCL-1-related protein (XCL-2) by reverse transcription-polymerase chain reaction. In contrast to the XCL-1 gene, XCL-2 gene expression was significantly increased in the regenerating tails, suggesting its role in tail regeneration. Although both XCL-1 and XCL-2 belong to a recently identified *Xenopus* lectin family (X-lectins), XCL-1 and XCL-2 exhibit distinct developmental gene expression from two other known X-lectin members, both of which are expressed principally in the embryonic stage, whereas the XCL-1 and XCL-2 genes are predominantly expressed in the adult and middle/late tadpole stages, respectively, suggesting multiple functions of X-lectin family members. Thus, the presence of multifunctional Ca^{2+}-dependent lectin family and the induction of the member gene in regenerating organs are conserved among insects and vertebrates.

Key words: development, gene expression, regeneration, serum lectin, *Xenopus laevis*.

Abbreviations: EF-1α, elongation factor 1α; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB061238 and AB061239.

Many serum/hemolymph lectins have been identified from vertebrates and invertebrates (*1–10*). Although some of them are involved in innate immunity (*3*, *7*, *10–13*), the biologic function of the serum lectins remains obscure.

The hemolymph of the American cockroach *Periplaneta americana* contains multiple lectins (*14*, *15*). We previously purified four lectins, termed *Periplaneta* lectin, lipopolysaccharide (LPS)-binding protein, regenectin and 26-kDa lectin, all of which are C-type lectins with different sugar specificities, from the hemolymph of the American cockroach (*16–19*). Among them, *Periplaneta* lectin and LPS-binding protein function as defense proteins (*20*, *21*). In contrast, regenectin and 26-kDa lectin are suggested to be involved in leg regeneration (*18*, *19*, *22*). Cockroaches possess high ability to regenerate lost appendages such as legs, eyes and antennae in their nymphal stages (*23*). The regenectin gene is transiently activated in the newly formed epidermis in the late stage of leg regeneration, and regenectin protein accumulates around the young muscle cells (*22*, *24*). The 26-kDa lectin, however, appears transiently outside of the newly formed epidermis in the middle stage of leg regeneration (*19*). The stage- and tissue-specific localization of regenectin and 26-kDa lectin in regenerating legs suggests that they have different roles in leg regeneration. Furthermore, reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that there are many mRNAs encoding proteins structurally related to *Periplaneta* lectin in this insect and the above four lectins belong to the same protein family, which we termed the *Periplaneta* lectin-related protein family (*25*). These results suggest that the family contains two functionally distinct types of lectins: some participate in innate immunity and others in organ regeneration.

*The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
†To whom correspondence should be addressed. Tel. +81-3-5841-4446, Fax: +81-3-5841-4447, E-mail: stkubo@biol.s.u-tokyo.ac.jp

We hypothesized that a serum lectin(s) might also participate in organ regeneration in vertebrates. The African clawed frog *Xenopus laevis* has high ability to regenerate lost legs and tails at the tadpole stage (*26*, *27*). When a tadpole loses its appendage, wound healing occurs within 1 day, which is followed by the formation of a blastema (a small regenerating limb/tail bud) on the tip of the remaining appendage at an early stage and extension of the regenerating appendage continues for more than a week to complete regeneration. Although some genes are expressed transiently in the regenerating tails of the *Xenopus* tadpole (*28*, *29*) and adult *Xenopus* serum also contains a lectin (*4*), the involvement of serum lectin(s) in organ regeneration has not been examined in *X. laevis*.

In the present study, we attempted to isolate Ca^{2+}-dependent lectin(s) from adult *Xenopus* serum and analyse its possible involvement in tail regeneration. We identified two novel members (XCL-1 and XCL-2) of the *Xenopus* Ca^{2+}-dependent lectin family, previously called 'X-lectins' (*30*), and demonstrated their distinct gene expression patterns during tail regeneration and development.

MATERIALS AND METHODS

Animals and Manipulations—*Xenopus* tadpoles at stages 55–58 (*31*) were anaesthetized on ice and approximately two-thirds (~20 mm) of each tail was amputated with a surgical knife. The most anterior portions (~5 mm in length) of the removed tails were dissected immediately after amputation and stored at −80°C as control samples for normal tails. The tadpoles were kept in a plastic water tank at 21°C and fed boiled spinach. After 7 to 9 days, the regenerating tail tissues (blastemas) of ~2 mm in length that were swollen at the wound site were dissected. All the samples were stored frozen at −80°C until use. Embryos were bred in our laboratory.

Isolation of the Xenopus Serum Lectins—Twenty adult female *X. laevis* were anaesthetized on ice. Approximately 20 ml of blood was collected by a syringe from the hearts and incubated at room temperature for 1 h and then centrifuged for 15 min at 4°C to collect the serum. The serum was diluted 5-fold with Tris-buffered saline (TBS) (10 mM Tris/HCl pH 7.4, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) and then applied to a sucrose–Sepharose column (ø0.8 cm × 10 cm), which was prepared as described previously (*18*) and equilibrated with TBS. The column was then washed with TBS until the absorbance at 280 nm dropped below 0.001. The bound material was eluted with TBS containing 10 mM sucrose and then with TBS that contained 1 mM EDTA instead of 1 mM $CaCl_2$. Fractions (8 ml each) were collected. All procedures were performed at 4°C. Eluted proteins were subjected to 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie Brilliant Blue using the method of Fairbanks *et al*. (*32*).

Determination of Partial Amino Acid Sequences of the 35-kDa Protein—Approximately 50 μg of the eluted proteins, which contained ~15 μg of the 35-kDa protein, was subjected to SDS–PAGE and then electrically blotted onto a nitrocellulose filter in a solution of 2.5 mM Tris base containing 19.2 mM glycine and 0.01% SDS. After staining with Ponceau S, the part of the filter corresponding to the 35-kDa protein band was excised and destained with 0.2 N NaOH for 1 min. The 35-kDa protein was then incubated with lysyl–endopeptidase (Lys-C) in 100 μl of 50 mM Tris/HCl buffer, pH 9.5, at an enzyme protein ratio of 1: 30 by weight overnight at 37°C. The mixture was then applied to a reverse phase high performance liquid chromatography (HPLC) Synchropak RP-P column (C_{18}, ø4.1 mm × 250 mm; Synchropak Inc., Leiden) connected to a Water's HPLC system. Peptides were eluted with a linear gradient of 0 to 70% solution B [0.09% (v/v) trifluoroacetic acid in 70% acetonitrile] in solution A (0.09% trifluoroacetic acid). The resulting peptides were applied to a Shimadzu PPSQ-10 protein sequencer (Shimadzu, Tokyo).

cDNA Cloning of the 35-kDa Protein (XCL-1)—RT-PCR was performed using total RNA extracted from adult *Xenopus* liver and degenerate primers, each corresponding to the partial amino acid sequences of the 35-kDa protein (XCL-1), AVPIVYD and EITEAAV, respectively, to obtain a cDNA fragment for the 35-kDa protein. The resulting 308-bp PCR product was cloned, labelled with [α-^{32}P] dCTP using a random primer labelling kit (BcaBEST; Takara, Tokyo), and used as a probe for screening a *Xenopus* liver tumour cDNA library (Uni-ZAP; Stratagene). Plaque hybridization was used to screen 5×10^5 clones. Positive clones were isolated, sequenced using an automatic DNA sequencer (ABI 373A, Applied Biosystems) with a *Taq* dye primer cycle sequencing kit (Applied Biosystems), and determined to be the same clones (pXCL-1). To obtain a cDNA fragment corresponding to the 5′-upstream region of pXCL-1, PCR was performed using a *Xenopus* liver tumour cDNA library. The initial PCR was performed with a gene-specific primer 1 corresponding to +167 to +185 of pXCL-1 and an adapter primer 1 (M13-20 primer), followed by a nested PCR with a gene-specific primer 2 corresponding to +31 to +54 of pXCL-1 and an adapter primer 2 (T7 primer). The resulting 301-bp PCR product was subcloned and sequenced. The nucleotide sequences of both strands were determined.

cDNA Cloning for a Subtype of XCL-1 (XCL-2)—To obtain a partial cDNA fragment for a new subtype of XCL-1, nested RT-PCR was performed with total RNA extracted from regenerating tails of *Xenopus* tadpoles and primers designed based on the amino acid sequences conserved among XCL-1, oocyte cortical granule lectin and mouse intelectin. They were sense primer 1: 5′-GA(TC)GGTAA(TC)TGGGC(ATCG)AA(TC)TA-3′, sense primer 2: 5′-(TA)(CG)(ATCG)GA(TC)GA(TC)TA(TC)AA(AG)AA(TC)CC-3′ and an antisense primer: 5′-ATAACC TCCTCC(ATCG)CC(AGT)AT(AG)CA-3′, each corresponding to DGNWANY, SDDYKNP and CIGGGG, respectively. The 402-bp PCR product (pXCL-2) was subcloned and sequenced. To obtain a cDNA fragment corresponding to the 5′-upstream and 3′-downstream regions of pXCL-2, 5′- and 3′-rapid amplification of the cDNA-end (RACE) was performed using total RNA

extracted from the regenerating *Xenopus* tadpole tails and a commercial kit (Marathon cDNA Amplification Kit, Clontech). For the 5′-RACE, the initial PCR was performed with gene-specific primer 1 corresponding to +79 to +99 of pXCL-2 and an adapter primer 1, followed by a nested PCR with gene-specific primer 2 corresponding to +19 to +45 of pXCL-2 and adapter primer 2. For the 3′-RACE, the initial PCR was performed with gene-specific primer 3 corresponding to +249 to +274 of pXCL-2 and adapter primer 3, followed by a nested PCR with gene-specific primer 4 corresponding to +347 to +373 of pXCL-2 and adapter primer 2. The respective 494 and 375-bp PCR products obtained were subcloned and sequenced, respectively.

RT-PCR—Total RNA was extracted from various tissues of *Xenopus* adults and embryos of various stages. Each microgram of RNA was reverse transcribed with or without 200 U of Superscript II (Gibco BRL) and 10 pmol of an oligo(dT) primer. PCR was performed in 20 μl of LA PCR buffer containing 1 U of LA-*Taq* polymerase (Takara), and 400 μM deoxynucleotides, 1/100 volume of the RT product and 400 nM of each gene-specific primer. The primers used were: +810/+830 and +1036/+1056 of the XCL-1 cDNA, +399/+418 and +669/+688 of the XCL-2 cDNA and +1104/+1123 and +1353/+1372 of the *Xenopus* EF1α cDNA. PCR conditions used were: (94°C × 30 s + 55°C × 30 s + 72°C × 1 min) × 24 to 28 cycles (for *XCL-1* and *XCL-2*) or 16 to 20 cycles (for *EF1α*). To confirm that the PCR products were amplified exponentially, aliquots were taken after every two PCR cycles and subjected to 2% agarose gel electrophoresis in each experiment. The gels were stained with ethidium bromide and examined using an FMBIO II Multi-View image analyzer (Takara). Photographs of the gels were taken at the PCR cycles where the DNA was amplified exponentially.

Quantitative Real-time PCR of XCL-2 in Regenerating Tadpole Tails—Regenerating tails were collected at 0, 1, 3 and 5 days after amputation. Six samples were prepared for each time-point. Real-time RT-PCR was performed with Light Cycler-DNA master hybridization probes (Roche) according to the manufacturer's protocol, using gene-specific primers (*XCL-2*; 5′-GGAGGAGGCTT CATACCAGA and 5′-TTAGGGACTTGGGGTTTAGG, *EF-1α*; 5′-GGAACGGTGACAACATGC and 5′-AGGCA GACGGAGAGGCTTA) and fluorescent probes (*XCL-2*; fluorescein probe, 5′-CAGCGGCCTCAGTAATAGCTTTA GTGCTGCTCCAT and LC-Red640 probe, 5′-CATACCCT GTCCCATATCCATTCCAGTCAAAGGCA, *EF-1α*; fluorescein probe, 5′-GGTGGCAAAATGCAGTCAAGAGCTT CCAGCA and LC-Red640 probe, 5′-GGTAGTTCCGCTG CCAGATCCCTCTTTACGTGT). The amount of *XCL-2* transcript was normalized with that of *EF-1α* and is shown as relative to the value of the sample at 0 day after amputation.

RESULTS

Isolation of Ca^{2+}-dependent Lectin(s) from Adult Xenopus Serum—We hypothesized that serum lectin(s) is also involved in organ regeneration in *X. laevis*. To test this, we first tried to isolate Ca^{2+}-dependent lectins from adult *Xenopus* serum by sucrose–Sepharose affinity chromatography, as this method is effective for isolating *Periplaneta* hemolymph lectins (*18*, *19*). The serum was applied to a sucrose–Sepharose column equilibrated with TBS containing 1 mM $CaCl_2$. After washing the column, almost no appreciable protein was detectable in the wash-fractions (Fig. 1). The bound material was eluted with TBS containing 10 mM sucrose and then with TBS containing 1 mM EDTA instead of $CaCl_2$, successively. When these fractions were subjected to SDS–PAGE, three bands with molecular masses of 70 and 72 kDa were detected in the 10-mM sucrose-fraction, whereas more than 12 bands were detectable in the 1-mM EDTA fraction (Fig. 1). Among them, we focussed on a 35-kDa protein in the 1-mM EDTA fraction, which was the most prominent component of the eluted fractions.

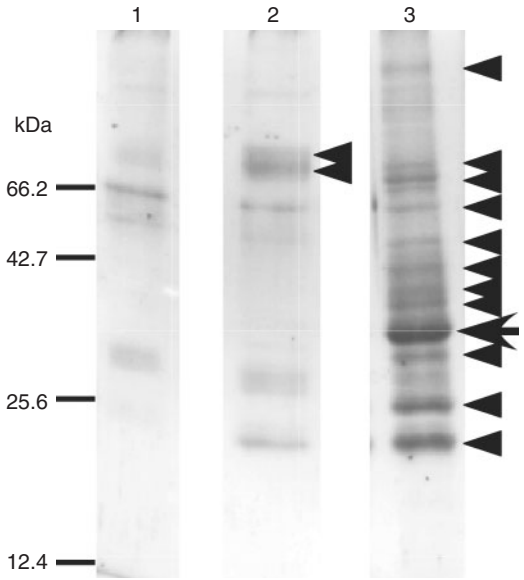

Fig. 1. **Isolation of Ca^{2+}-dependent lectins from adult *Xenopus* serum. Adult *Xenopus* serum (10 ml) was diluted with TBS and applied to a sucrose–Sepharose column.** Proteins eluted in 500 μl of the wash (lane 1), 10 mM sucrose (lane 2) and 1 mM EDTA fractions (lane 3) were subjected to SDS–polyacrylamide gel electrophoresis. The protein bands detected in each fraction are indicated by arrowheads. The band for the 35-kDa protein is indicated by an arrow. The molecular mass markers used were: bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), α-chymotrypsinogen (25.6 kDa) and cytochrome *c* (12.4 kDa).

To determine the N-terminal and partial amino acid sequences of the 35-kDa protein, we blotted the protein separated by SDS–PAGE onto a nitrocellulose filter. The part of the filter corresponding to the protein was excised and incubated with lysyl–endopeptidase. The resulting peptides were then applied to a reverse phase HPLC column and three partial amino acid sequences: TVGDRXTSQGN, DHGPAVPIVYDLGNPDLT and EITEAAVLLFY (where Xs indicate unidentified amino acid residues) were determined by subjecting the HPLC fractions to a protein sequencer. The N-terminal amino acid sequence, XDNFSLDQ, was determined by subjecting the filter corresponding to the 35-kDa protein directly to the protein sequencer.

Identification of the 35-kDa Protein (XCL-1)—To obtain a cDNA fragment of the 35-kDa protein, RT-PCR was performed using total RNA extracted from adult *Xenopus* liver and degenerate primers corresponding to two parts of the above partial amino acid sequences, AVPIVYD and EITEAAV. We used the liver total RNA, because a 69-kDa lectin, which was previously purified from the *Xenopus* serum, is synthesized in the liver (*4*). The resulting 308-bp PCR product was cloned and used to screen an adult *Xenopus* liver tumour cDNA library (5×10^5 clones) with plaque hybridization. Six positive clones were isolated and nucleotide sequencing revealed that they were the same clones (pXCL-1). As pXCL-1 lacked the sequence corresponding to the N-terminal amino acid sequence, nested PCR was performed using primers corresponding to the 5′ regions of pXCL-1 and the same cDNA library, and a 310-bp PCR product was isolated. The assembled cDNA was 1196-bp in length and contained an open reading frame (ORF) encoding 338 amino acid residues (Fig. 2A). An in-frame termination codon and a poly(A) addition signal were located in the 5′- and 3′-untranslated regions, respectively. All of the N-terminal and the three partial internal amino acid sequences determined were present in this sequence. Furthermore, the predicted molecular mass of the mature protein was 35.4 kDa, which corresponded well with that determined by SDS–PAGE (35 kDa), indicating that this is the cDNA for the 35-kDa protein. The 20 amino acid residues upstream of the N-terminus were hydrophobic and likely comprise a signal peptide.

A database search revealed that the 35-kDa protein had high sequence similarity with two *Xenopus* lectins: embryonic epidermal lectin [XEEL (*33*, *34*)] and oocyte cortical granule lectin [XCGL (*30*, *35*, *36*)], with overall sequence identities of 64 and 57%, respectively (Fig. 2B), indicating that these three lectins belong to the same family, which was previously called 'X-lectins' (*30*). The 35-kDa protein also had high sequence identities (~58–60%) with mouse and human intelectins (*37–40*) and lamprey serum lectin (47%, Genbank accession no. AB055981), as do XEEL and XCGL (*30*). In contrast to the *Periplaneta* lectin-related protein family, XCL-1 did not contain the carbohydrate-binding domain characteristic of the C-type lectins, but contained the fibrinogen-related domain, which is conserved among X-lectins (*30*; Fig. 2B). We termed this 35-kDa protein XCL-1 (*Xenopus* Ca^{2+}-dependent serum lectin-1).

Identification of a New Member (XCL-2) of the Xenopus Lectin Family—RT-PCR using gene-specific primers revealed that the XCL-1 gene was not induced in regenerating tadpole tails please see (Fig. 4A). By analogy with *Periplaneta* lectin-related proteins (*25*), we hypothesized that the gene(s) for other X-lectin member(s) might be induced in regenerating tadpole tails. To test this, we designed primers corresponding to the highly conserved amino acid sequences of XCL-1, XCGL and mouse intelectins, and performed nested RT-PCR using total RNA extracted from the regenerating tadpole tails. The resulting 420-bp PCR product was subcloned and sequenced. Among the 10 subclones sequenced, 1 coincided with the XCL-1 cDNA, whereas the other 9 (pXCL-2) had similar but distinct sequences with XCL-1, XEEL and XCGL cDNAs (60, 44 and 43% identities, respectively), suggesting that pXCL-2 encodes a novel X-lectin member.

To obtain a full-length XCL-2 cDNA, 5′- and 3′-RACE were performed. The assembled cDNA sequence was 1106 bp in length and contained an ORF consisting of 315 amino acid residues (Fig. 3A). The N-terminal sequence consisting of 19 amino acid residues starting from the putative first Met was hydrophobic and likely to be a signal peptide. XCL-2 had 59, 61 and 59% sequence identities with XCL-1, XEEL and XCGL, respectively, and one of the putative *N*-glycosylation sites was conserved among XCL-1 and XCL-2 (Fig. 3B). Furthermore, in addition to the fibrinogen-related domain, all eight cysteine residues conserved in the X-lectin family were also conserved in both XCL-1 and XCL-2 (Fig. 2B and 3B) (*36*). A phylogenetic analysis of four *Xenopus* lectins: XCL-1, XCL-2, XCGL (*35*) and XEEL (*33*); two intelectins from each of mouse and human (*37–40*); and lamprey serum lectin (Genbank accession no. AB055981) confirmed that these nine lectins belong to the same lectin super family and formed clusters according to their species (Fig. 3C).

Expression of the XCL-1 and XCL-2 Genes in Regenerating Tadpole Tails—Next, to test whether XCL-1 and/or XCL-2 is involved in tail regeneration in *Xenopus* tadpoles, we examined whether expression of the XCL-1 and XCL-2 genes was induced in regenerating tadpole tails. RT-PCR was performed using gene-specific primers for XCL-1 and XCL-2 and total RNA extracted from the blastemas of ~100 tadpoles 7 to 9 days after amputation. The intensity of the band for the XCL-2 mRNA was ~1.6-fold stronger in the blastemas than that in the normal tails, whereas band intensities for the XCL-1 and *Xenopus* EF1α mRNAs were almost the same between the blastema and normal tails (Fig. 4A and B). For further analysis of the inducible expression of XCL2 during tail regeneration, we performed quantitative expression analysis using real-time PCR and total RNAs extracted from regenerating tadpole tails at 0, 1, 3 and 5 days after amputation ($N = 6$ for each sample). The XCL-2 gene expression gradually increased in the early phase of regeneration and the level reached ~2-fold by day 3 compared to day 0, which was a statistically significant increase ($p \sim 0.036$). The expression level decreased by day 5 to that comparable to day 0 (Fig. 4C). These results indicated that expression of the XCL-2 gene, but not the XCL-1 gene, is transiently induced in regenerating tails.

Developmental Stage-specific Expression of the XCL-1 and XCL-2 Genes—X-lectin member genes so far identified are expressed only in the early developmental stages; the XCGL gene is expressed from oogenesis to embryo (*35*, *36*), and the XEEL gene is expressed from gastrula to early tadpole (*33*, *34*). Therefore, we examined the expression of the XCL-1 and XCL-2 genes during the development of *X. laevis* by RT-PCR. The XCL-1 gene was strongly expressed in the adult stage (Fig. 5A), which is consistent with the fact that XCL-1 was isolated from adult serum. We also examined the tissue-specific expression of the XCL-1 gene in the adult *Xenopus laevis* by RT-PCR and found that the gene was

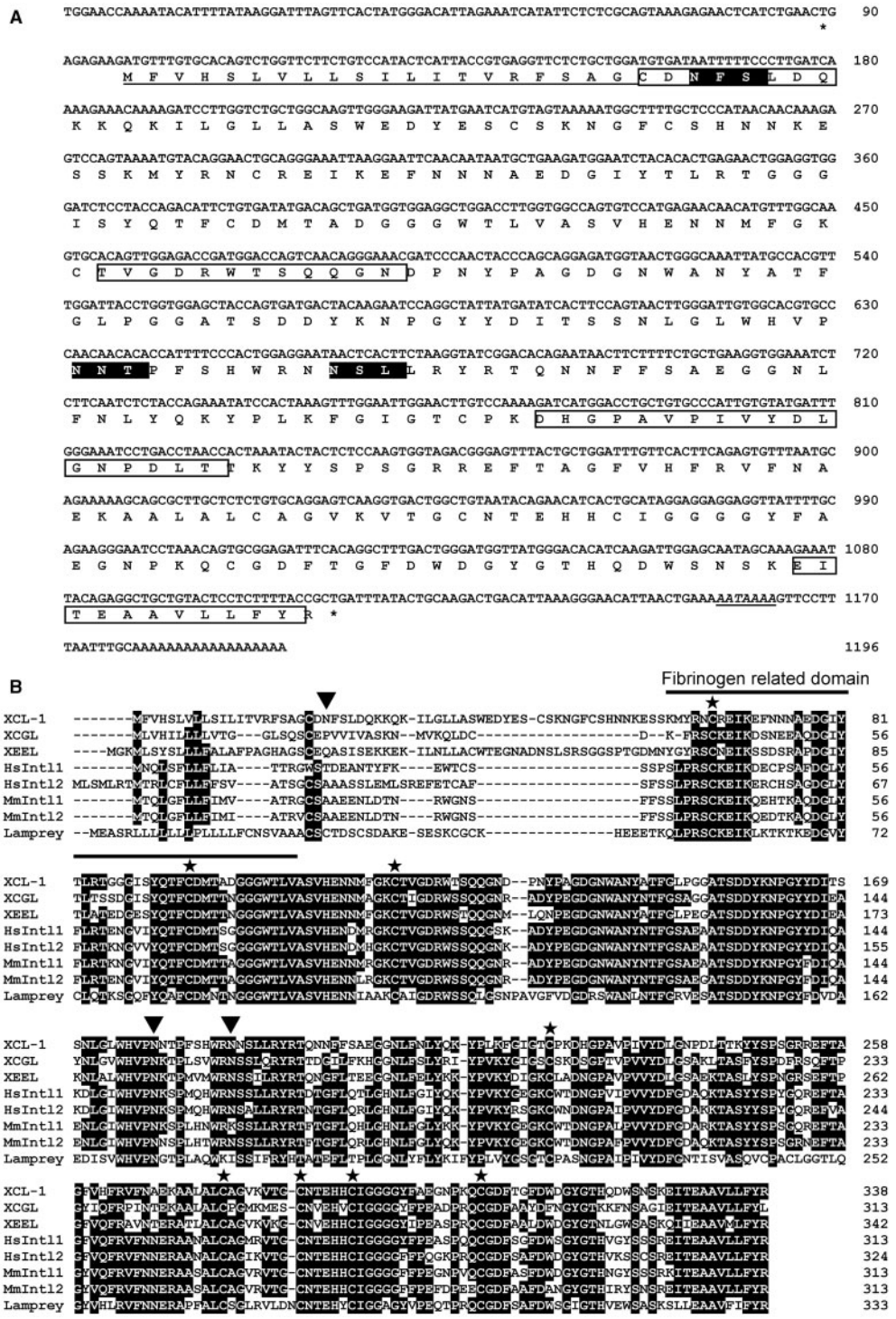

Fig. 2. **Nucleotide and amino acid sequences of the 35-kDa protein (XCL-1) and its alignment with the other X-lectin super family.** (A) Nucleotide and deduced amino acid sequence of the XCL-1 cDNA. The deduced amino acid sequence is shown under the nucleotide sequence. Numbers on the right represent nucleotide positions starting from the 5′-end. N-terminal and partial amino acid sequences of XCL-1 are boxed. The putative signal sequence is underlined. Three possible N-glycosylation sites are indicated by inverted boxes. The termination codons and possible poly(A) addition signals are shown by asterisks and underlined italics, respectively. (B) Comparison of amino acid sequences of XCL-1, XCGL, XEEL, human intelectin1, 2 (HsIntl1, HsIntl2), mouse intelectin1, 2 (MmIntl1, MmIntl2) and lamprey serum lectin (Lamprey). Numbers indicate the positions of amino acid residues starting with the first methionine of each putative protein. Amino acid residues conserved in more than half of these proteins are indicated by inverted box. The conserved fibrinogen-related domain is shown by solid line. Three possible *N*-glycosylation sites are indicated by arrowheads. The first site is found only in XCL-1, while the second site is highly conserved in all of these lectins and the last site is also highly conserved. Stars indicate eight cysteine residues conserved in all family members.

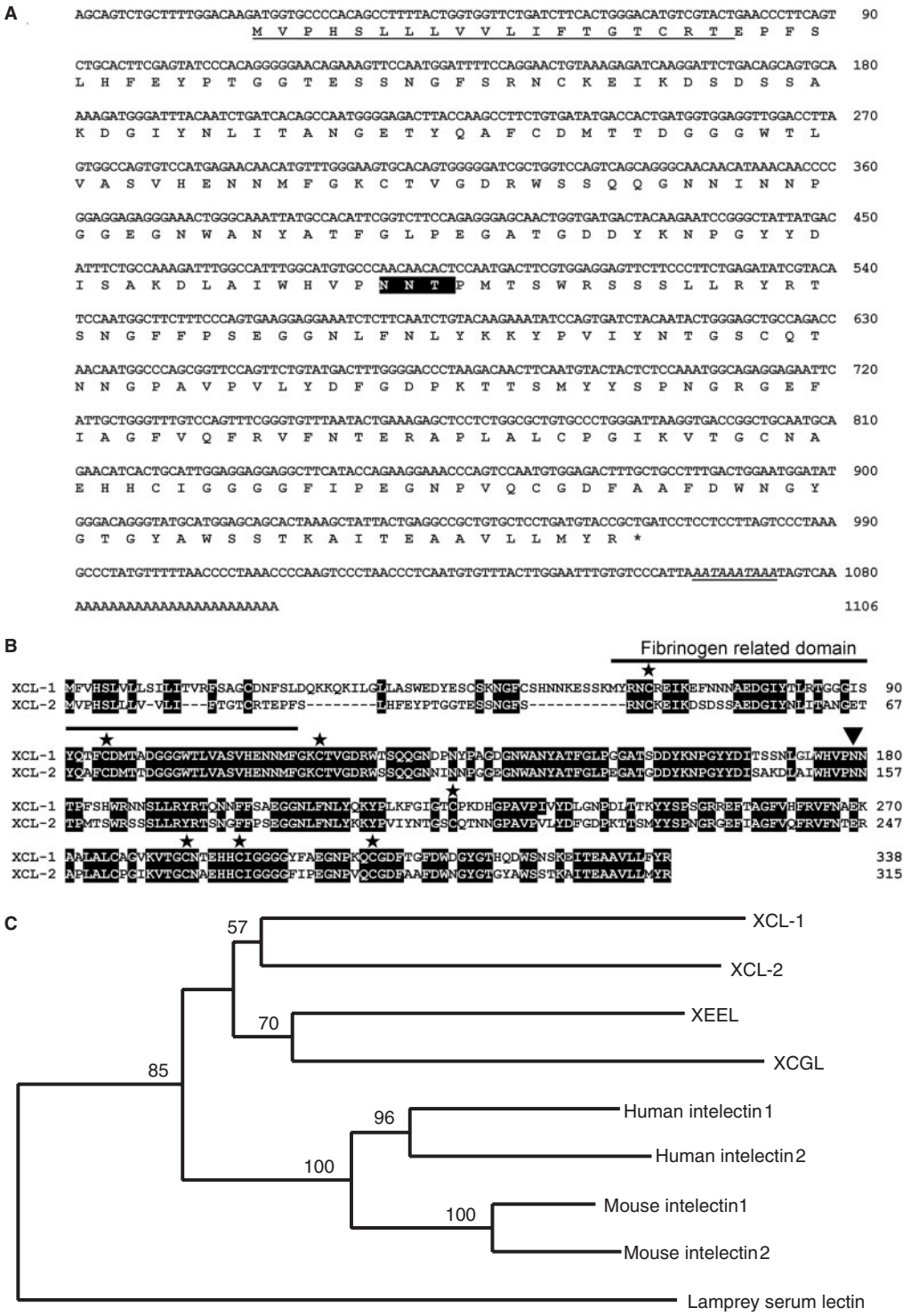

Fig. 3. **Nucleotide and amino acid sequences of the XCL-2 and alignment with the other X-lectin super family members.** (A) Nucleotide and deduced amino acid sequence of the XCL-2 cDNA. The deduced amino acid sequence is shown under the nucleotide sequence. Numbers on the right correspond to nucleotide positions starting from the 5′-end. The putative signal sequence is underlined. The termination codon and possible poly(A) addition signals are shown by an asterisk and underlined italics, respectively. XCL2 contains only one possible *N*-glycosylation site indicated by inverted box. (B) Comparison of amino acid sequences of XCL-1 and XCL-2. Numbers indicate the positions of amino acid residues starting with the first methionine of each putative protein. Amino acid residues conserved among these two proteins are indicated by inverted boxes. A possible *N*-glycosylation site conserved among XCL-1 and XCL-2 is indicated by an arrowhead. Eight conserved cysteine residues are indicated by stars. (C) Phylogenetic tree analysis of XCL-1, XCL-2, XCGL, XEEL, human and mouse intelectins and lamprey serum lectin using the neighbour-joining method (*56*) with DNASIS software (Hitachi Software Engineering Co., Ltd). The numbers at each node represent bootstrap values as a percentage of 1000 trials.

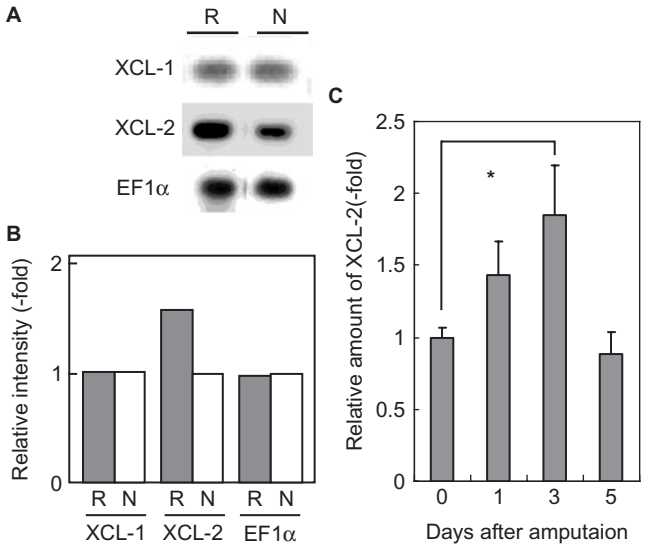

Fig. 4. **RT-PCR analysis of XCL-1 and XCL-2 mRNAs in regenerating tadpole tails.** (A) RT-PCR was performed using gene-specific primers for XCL-1 (upper panel), XCL-2 (middle panel), *Xenopus* EF1α (lower panel), total RNA extracted from tail blastemas (lane 'R') and normal tail parts (lane 'N') of ~100 tadpoles 7 to 9 days after amputation. (B) Summary of the results in (A). The relative intensity of the bands for XCL-1, XCL-2 and EF1α mRNAs in the blastemas (R) was compared to those for the normal tails (N) as 1. (C) Quantitative analysis of the XCL-2 transcript during tail regeneration by real-time PCR using specific primers and fluorescent probes at 0, 1, 3 and 5 days after amputation. The expression amount of *XCL-2* was divided by that of *EF-1α* and is shown as relative to the mean value at 0 day after amputation. Asterisk indicates statistical significance ($p \sim 0.036$; Student's *t*-test). Quantitative data are shown as the means ± SEM ($n = 6$).

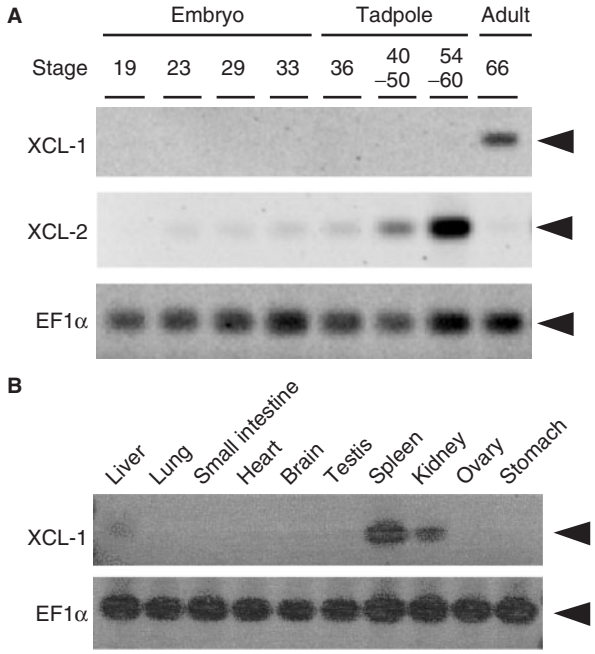

Fig. 5. **RT-PCR analysis of XCL-1 and XCL-2 mRNAs during *Xenopus* development.** (A) Developmental stage-specific expression of the XCL-1 and XCL-2 genes. RT-PCR was performed using gene-specific primers for XCL-1 (upper panel), XCL-2 (middle panel) and EF1α (lower panel) and total RNA extracted from whole *Xenopus* of various developmental stages. (B) Tissue-specific expression of the XCL-1 gene in the adult *Xenopus*. RT-PCR was performed using gene-specific primers for XCL-1 (upper panel) and EF1α (lower panel) and total RNA extracted from various adult *Xenopus* tissues. The positions of the bands are indicated by the arrowheads on the right.

preferentially expressed in the spleen, kidney and liver (Fig. 5B), which is also consistent with our supposition that XCL-1, a serum lectin, is synthesized at least in the liver. Significant expression of the XCL-1 gene in the spleen and kidney may reflect their additional roles in these organs. In contrast, expression of the XCL-2 gene began at stage 23 (neurula) embryo and reached a maximum level at the late tadpole stages 54 to 60 (Fig. 5A). No significant XCL-2 gene expression was detected in the adult stage. These results demonstrated that these two lectins have distinct gene expression patterns and that the XCL-1 gene is expressed in a tissue-specific manner in the adult *Xenopus*.

DISCUSSION

In the present article, we identified XCL-1 and XCL-2 as novel members of a *Xenopus* lectin family, previously called X-lectins (*30*). Phylogenetic tree analysis revealed that the X-lectin family members, XCL-1, XCL-2, XCGL and XEEL, are more closely related to each other than human, mouse or fish homologues (Fig. 3C), suggesting that these four lectin genes originally expanded in *X. laevis*. Both XCL-1 and XCL-2 also share fibrinogen-related domains, which are suggested to be saccharide-binding motifs for X-lectins (*30*, *41*). Furthermore, all X-lectin family members, including XCL-1 and XCL-2, have eight cysteine residues at conserved positions (Fig. 2B and 3B) (*36*), strongly suggesting that the tertiary structure, which is needed for the sugar-binding activity of X-lectins, are also conserved in XCL-1 and XCL-2. Although we did not succeed to detect obvious saccharide-binding activities using recombinant XCL-1 and XCL-2 expressed in *Escherichia coli* (data not shown), the fact that XCL-1 bound to the sucrose–Sepharose column and was eluted by EDTA suggests that, like XCGL and XEEL (*34*, *42*), XCL-1 also has Ca^{2+}-dependent saccharide-binding property (Fig. 1). The 10-mM sucrose and 1-mM EDTA fractions from the sucrose–Sepharose column yielded many proteins, suggesting that there are many saccharide-binding proteins other than XCL-1 in adult *Xenopus* serum (Fig. 1). Previously, Roberson *et al*. (*4*) isolated a 69-kDa galactoside-binding lectin from adult *Xenopus* serum (*4*). It is plausible that the 70–72 kDa proteins detected in the 10-mM sucrose fraction correspond to the 69-kDa lectin, although the nucleotide sequences of both of these proteins have not been determined.

Among the X-lectin members, XCGL is localized in the oocyte cortical granules, released, and participates in the formation of the fertilization envelope (*43*). XCGL gene expression continues during the embryonic stages, suggesting that it has another role in embryogenesis (*35*, *36*). XEEL is localized in embryonic epidermal cell

granules and secreted to extra embryonic environments, and the XEEL gene is expressed from the gastrula to early tadpole stage (*33*, *34*). In contrast, the XCL-1 and XCL-2 genes exhibited distinct developmental-stage-specific expression patterns: XCL-1 is an adult serum protein and the gene is expressed specifically in the adult spleen, kidney and liver, whereas the XCL-2 gene expression is most prominent at the late tadpole stages 50–60, indicating their functional specialization among the X-lectin members (Fig. 5). Nagata *et al*. (*33*) also examined XCL-1 and XCL-2 gene expression in the early embryonic stages (from stage 1 to 35) based on the nucleotide sequences, which we had deposited in the database. Although they detected XCL-1 and XCL-2 transcripts by RT-PCR as early as stage 20 or stage 10, respectively (*33*), our report is the first demonstration of XCL-1 and XCL-2 gene expression in the whole *Xenopus* developmental stages and clearly shows that the primary expressions are observed at stage 66 (adult) for the XCL-1 gene and at stages 54–60 (middle/late-stage tadpole) for the XCL-2 gene, respectively. RT-PCR is highly sensitive and a lesser amount of XCL-1 and XCL-2 transcripts might have been detected in the previous report (*33*). Recent large-scale survey for thyroid hormone (TH)-regulated genes in *X. laevis* revealed that XCL-2 gene expression was induced in 24 h and down to the basal level in 48 h after TH-treatment, whereas XCL-1 gene expression was not significantly affected (*44*). Therefore, high XCL-2 gene expression in late tadpole stages may partly be due to increasing TH titre during metamorphosis. TH-induced XCL-2 expression was observed in every organ examined, including tails, brains and limb-buds (*44*), suggesting that XCL-2 is expressed in various tissues in the late tadpole stage, irrespective of whether the organ was larval (tails) or adult (limb-buds) origin.

XCL-2 gene expression increased transiently in the regenerating tadpole tails by ~2-fold (Fig. 4C). Although the genes for fibroblast growth factor (FGF) (*45*, *46*), FGF receptor (*47*), homeobox proteins (*29*, *48–50*), collagens (*51*, *52*) and neuronal pentraxin I (*28*) are induced in the regenerating organs of the newt and *X. laevis*, to our knowledge, this is the first report of lectin gene induction in regenerating organs in vertebrates. By analogy to regenectin and 26-kDa lectin in *P. americana*, it is possible that XCL-2 has a role as a cell-cementing substance in *X. laevis*, although we could not determine the tissue that expresses the XCL-2 gene in the regenerating tails by *in situ* hybridization (data not shown). Extracellular environments have important roles in proper cell differentiation or maintenance of the cell differentiated state (*53*, *54*). It is possible that XCL-2 is secreted to the extracellular spaces to bind to sugar moieties that are exposed on the surface of regenerating cells and/or the extracellular matrix, to provide the proper extracellular environment. It is also possible that XCL-2, like *Periplaneta* lectin and LPS-binding lectin, functions as a defense protein, acting as an opsonin (*20*, *21*). Even in this case, XCL-2 is unique in that it is *de novo* synthesized in regenerating tadpole tails.

The mammalian homologues of X-lectins, intelectins, are expressed predominantly in intestinal tissues (*37*, *39*, *40*), and human intelectin1 has ability to recognize arabinogalactan of bacterial cell wall (*38*). In addition, the expression of mouse intelectin2 was induced by nematode infection in BALB/c and 129/SvEv strains of mice (*40*). Thus, mammalian intelectins are likely to participate in innate immunity, especially in intestinal tissues. Similarly, XCGL and XEEL are also thought to be involved in innate immunity (*30*, *34*). In this respect, it is also possible that XCL-1 functions as a serum defense protein that is specific to the adult stage, whereas XCGL, XEEL and XCL-2 function as defense proteins that are specific to the embryonic and/or tadpole stages in *X. laevis*.

In summary, our present study identified two novel members of X-lectins, and demonstrated their unique developmental stage-specific and regenerating organ-specific gene expression patterns. Although X-lectins and *Periplaneta* lectin-related protein families represent different protein families in that the former proteins have fibrinogen-related domains, whereas the latter represent typical C-type lectins, the presence of multifunctional serum lectins and the possible involvement of lectin family members in organ regeneration are commonly observed in insects and vertebrates. Functional analysis of X-lectins and the mammalian intelectin family in organ regeneration using reverse genetic methods (*55*) will be important in our future research.

REFERENCES

1. Marchalonis, J.J. and Edelman, G.M. (1968) Isolation and characterization of a hemagglutinin from *Limulus polyphemus*. *J. Mol. Biol.* **32**, 453–465
2. Hammarström, S. and Kabat, E.A. (1969) Purification and characterization of a blood-group A reactive hemagglutinin from the snail *Helix pomatia* and a study of its binding site. *Biochemistry* **8**, 2629–2705
3. Komano, H., Mizuno, D., and Natori, S. (1980) Purification of a lectin induced in the hemolymph of *Sarcophaga peregrina* on injury. *J. Biol. Chem.* **255**, 2919–2924
4. Roberson, M.M., Wolffe, A.P., Tata, J.R., and Barondes, S.H. (1985) Galactoside-binding serum lectin *of Xenopus laevis*. *J. Biol. Chem.* **260**, 11027–11032
5. Giga, Y., Sutoh, K., and Ikai, A. (1985) A new multimeric hemaggulutinin from the coelomic fluid of the sea urchin *Anthocidaris carassispina*. *Biochemistry* **8**, 2629–2705
6. Haq, S., Kubo, T., Kurata, S., Kobayashi, A., and Natori, S. (1996) Purification, characterization, and cDNA cloning of a galactose-specific lectin from *Drosophila melanogaster*. *J. Biol. Chem.* **271**, 20213–20218
7. Kawasaki, T. (1999) Structure and biology of mannan-binding protein, MBP, an important component of innate immunity. *Biochim. Biophys. Acta* **1473**, 186–195
8. Gokudan, S., Muta, T., Tsuda, R., Koori, K., Kawahara, T., Seki, N., Mizunoe, Y., Wai, S.N., Iwanaga, S., and Kawabata, S. (1999) Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. *Proc. Natl. Acad. Sci. USA* **96**, 10086–10091
9. Richards, R.C., Hudson, D.M., Thibault, P., and Ewart, K.V. (2003) Molecular characterization of the Atlantic salmon serum lectin, a long-from C-type lectin expressed in kidney. *Biochim. Biophys. Acta* **1621**, 110–115
10. Takahashi, K., Ip, W.K.E., Michelow, I.C., and Ezekowitz, A.B. (2006) The mannose-binding lectin: a prototypic pattern recognition protein. *Curr. Opin. Immunol.* **18**, 16–23

11. Lackie, A.M. (1980) Invertebrate immunity. *Parasitology* **80**, 393–412
12. Natori, S., Shiraishi, H., Hori, S., and Kobayashi, A. (1999) The roles of *Sarcophaga* defense molecules in immunity and metamorphosis. *Dev. Comp. Immunol.* **23**, 317–328
13. Kawabata, S. and Iwanaga, S. (1999) Role of lectins in the innate immunity of horseshoe crab. *Dev. Comp. Immunol.* **23**, 391–400
14. Scott, M.T. (1971) A naturally occurring hemagglutinin in the hemolymph of the American cockroach. *Arch. Zool. Exp. Gen.* **112**, 73–80
15. Lackie, A.M. (1980) The specificity of the serum agglutinins of *Periplaneta americana* and *Schistocerca gregaria* and its relationship to the insect's immune response. *J. Insect Physiol.* **27**, 139–143
16. Kubo, T. and Natori, S. (1987) Purification and some properties of a lectin from the hemolymph of *Periplaneta americana* (American cockroach). *Eur. J. Biochem.* **168**, 75–82
17. Jomori, T., Kubo, T., and Natori, S. (1990) Purification and characterization of lipopolysaccharide-binding protein from the hemolymph of American cockroach (*Periplaneta americana*). *Eur. J. Biochem.* **190**, 201–206
18. Kubo, T., Kawasaki, K., and Natori, S. (1990) Sucrose-binding lectin in regenerating cockroach (*Periplaneta americana*) legs: its purification from adlut hemolymph. *Insect Biochem.* **20**, 585–591
19. Kubo, T., Kawasaki, K., and Natori, S. (1993) Transient appearance and localization of a 26-kDa lectin, a novel member of the *Periplaneta* lectin family, in regenerating cockroach leg. *Dev. Biol.* **156**, 381–390
20. Jomori, Y. and Natori, S. (1992) Function of the lipopolysaccharide-binding protein of *Periplaneta americana* as an opsonin. *FEBS Lett.* **296**, 283–286
21. Kawasaki, K., Kubo, T., and Natori, S. (1993) A novel role of *Periplaneta* lectin as an opsonin to recognize 2-keto-3-deoxy octonate residues of bacterial lipopolysaccharide. *Comp. Biochem. Physiol.* **106B**, 675–680
22. Kubo, T., Kawasaki, K., Nonomura, Y., and Natori, S. (1991) Localization of regenectin in regenerates of American cockroach (*Periplaneta americana*) legs. *Int. J. Dev. Biol.* **35**, 83–90
23. Kunkel, J.G. (1981) Regeneration in *The American Cockroach* (Bell, W.J. and Adiyodi, K.D., eds.) pp. 425–443, Chapman and Hall, London
24. Arai, T., Kawasaki, K., Kubo, T., and Natori, S. (1998) Cloning of cDNA for regenectin, a humoral C-type lectin of *Periplaneta americana*, and expression of the regenectin gene during leg regeneration. *Insect Biochem. Mol. Biol.* **28**, 987–994
25. Kawasaki, K., Kubo, T., and Natori, S. (1996) Presence of the Periplaneta lectin-related protein family in the American cockroach *Periplaneta Americana*. *Insect Biochem. Mol. Biol.* **26**, 355–364
26. Deuchar, E.M. (1975) Regeneration of the tail bud in *Xenopus* embryos. *J. Exp. Zool.* **192**, 381–390
27. Warren, R.H. (1981) Myoblasts are aligned with collagen fibrils in regenerating frog tadpole tails. *Tissue Cell* **13**, 773–785
28. Ishino, T., Shirai, M., Kunieda, T., Sekimizu, K., Natori, S., and Kubo, T. (2003) Identification of genes induced in regenerating *Xenopus* tadpole tails by using the differential display method. *Dev. Dyn.* **226**(2), 317–325
29. Christen, B., Beck, C.W., Lombardo, A., and Slack, J.M. (2003) Regeneration-specific expression pattern of three posterior Hox genes. *Dev. Dyn.* **226 (2)**, 349–355
30. Lee, J.K., Baum, L.G., Moremen, K., and Pierce, M. (2004) The X-lectins: a new family with homology to the *Xenopus laevis* oocyte lectin XL-35. *Glycoconj. J.* **21**, 443–450
31. Nieuwkoop, P.D. and Faber, J. (1956) In *Normal Table of Xenopus laevis.* Daudin, North-Holland, Amsterdam
32. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Electrophoretic analysis of the major polymeptodes of the erythrocyte membrane. *Biochemistry* **10**, 2606–2617
33. Nagata, S., Nakanishi, M., Nanba, R., and Fujita, N. (2003) Developmental expression of XEEL, a novel molecule of the *Xenopus* oocyte cortical granule lectin family. *Dev. Genes Evol.* **213**, 368–370
34. Nagata, S. (2005) Isolation, characterization, and extra-embryonic secretion of the *Xenopus laevis* embryonic epidermal lectin, XEEL. *Glycobiology* **15**, 281–290
35. Lee, J.K., Buckhaults, P., Wilkes, C., Teilhet, M., King, M.L., Moremen, K.W., and Pierce, M. (1997) Cloning and expression of a *Xenopus laevis* oocyte lectin and characterization of its mRNA levels during early development. *Glycobiology* **7**, 367–372
36. Chang, B.Y., Peavy, R.R., Wardrip, N.J., and Hedrick, J.L. (2004) The *Xenopus laevis* cortical granule lectin: cDNA cloning, developmental expression, and identification of the eglectin family of lectins. *Comp. Biochem. Physiol. Part A* **137**, 115–129
37. Komiya, T., Tanigawa, Y., and Hirohashi, S. (1998) Cloning of the novel gene intelectin, which is expressed in intestinal paneth cells in mice. *Biochem. Biophys. Res. Commun.* **251**, 759–762
38. Tsuji, S., Uehori, J., Matsumoto, M., Suzuki, Y., Matsuhisa, A., Toyoshima, K., and Seya, T. (2001) Human intelectin is a novel soluble lectin that recognizes galacto-furanose in carbohydrate chains of bacterial cell wall. *J. Biol. Chem.* **276**, 23456–22463
39. Lee, J.K., Schnee, J., Pang, M., Wolfert, M., Baum, L.G., Moremen, K.W., and Pierce, M. (2001) Human homologs of the *Xenopus* oocyte cortical granule lectin XL35. *Glycobiology* **11**, 65–73
40. Pemberton, A.D., Knight, P.A., Gamble, J., Colledge, W.H., Lee, J.K., Pierce, M., and Miller, H.R. (2004) Innate BALB/c enteric epithelial responses to *Trichinella spiralis*: inducible expression of a novel goblet cell lectin, intelectin-2, and its natural deletion in C57BL/10 mice. *J. Immunol.* **173**, 1894–1901
41. Matsushita, M., Endo, Y., Taira, S., Sato, Y., Fujita, T., Ichikawa, N., Nakata, M., and Mizuochi, T. (1996) A novel human serum lectin with collagen- and fibrinogen-like domains that functions as an opsonin. *J. Biol. Chem.* **271**, 2448–2454
42. Roberson, M.M. and Barondes, S.H. (1982) Lectin from embryos and oocytes of *Xenopus laevis*. *J. Biol. Chem.* **257**, 7520–7524
43. Nishihara, T., Wyrick, R.E., Working, P.K., Chen, Y.H., and Hedrick, J.L. (1986) Isolation and characterization of a lectin from the cortical granules of *Xenopus laevis* eggs. *Biochemistry* **25**, 6013–6020
44. Das, B., Cai, L., Carter, M.G., Piao, Y.-L., Sharov, A.A., Ko, M.S.H., and Brown, D.D. (2006) Gene expression changes at metamorphosis induced by thyroid hormone in *Xenopus laevis* tadpoles. *Dev. Biol.* **291**, 342–355
45. Christen, B. and Slack, J.M. (1997) FGF-8 is associated with anteroposterior patterning and limb regeneration in *Xenopus*. *Dev. Biol.* **192**, 455–466
46. Yokoyama, H., Yonei-Tamura, S., Endo, T., Belmonte, J.C.I., Tamura, K., and Ide, H. (2000) Mesenchyme with *fgf*-10 expression is responsible for regenerative capacity in *Xenopus* limb buds. *Dev. Biol.* **219**, 18–29
47. Poulin, M.L., Patrie, K.M., Botelho, M.J., Tassava, R.A., and Chiu, I.M. (1993) Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (*Notophthalmus viridescens*). *Development* **119**, 353–361
48. Simon, H.G. and Tabin, C.J. (1993) Analysis of Hox-4.5 and Hox-3.6 expression during newt limb

regeneration: differential regulation of paralogous Hox genes suggest different roles for members of different Hox clusters. *Development* **117**, 1397–1407
49. Simon, H.G., Kittappa, R., Khan, P.A., Tsilfidis, C., Liversage, R.A., and Oppenheimer, S. (1997) A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate genes involved in vertebrate forelimb/hindlimb patterning. *Development* **124**, 1355–1366
50. Endo, T., Tamura, K., and Ide, H. (2000) Analysis of gene expressions during *Xenopus* forelimb regeneration. *Dev. Biol.* **220**, 296–306
51. Wei, Y., Yang, E.V., Klatt, K.P., and Tassava, R.A. (1995) Monoclonal antibody MT2 identifies the urodele alpha 1 chain of type XII collagen, a developmentally regulated extracellular matrix protein in regenerating newt limbs. *Dev. Biol.* **168**, 503–513
52. Asahina, K., Obara, M., and Yoshizato, K. (1999) Expression of genes of type I and type II collagen in the formation and development of the blastema of regenerating newt limb. *Dev. Dyn.* **216**, 59–71
53. Lukashev, M.E. and Werb, Z. (1998) ECM signaling: orchestrating cell behaviour andmisbehaviour. *Trends. Cell Biol.* **8**(11), 437–441
54. Boudreau, N. and Bissell, M.J. (1998) Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr. Opin. Cell Biol.* **10**(5), 640–646
55. Kroll, K.L. and Amaya, E. (1996) Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173–3183
56. Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425