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Embryonic Epidermal Lectins in Three Amphibian Species, *Rana ornativentris*, *Bufo japonicus formosus*, and *Cynops pyrrhogaster*

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Intelectins (Itlns) are secretory lectins found in several chordate species that recognize carbohydrates on the bacterial cell surface depending on Ca^{2+}. In newly hatched larvae of *Rana ornativentris* (*R. orn*), *Bufo japonicus formosus* (*B. jpn*), and *Cynops pyrrhogaster* (*C. pyr*), an anti-Itln monoclonal antibody (mAb) labeled a subset of epidermal cells in whole-mount immunocytochemical assays. In western blot analyses, the mAb identified protein bands at approximately 33–37 kDa in the larval extracts and concentrated larval culture media. Using RT-PCR and RACE techniques, we isolated cDNAs from newly hatched larvae that encoded proteins of 343 (*R. orn*), 336 (*B. jpn*), and 337 (*C. pyr*) amino acids having 70%, 71%, and 60% identities with that of the *Xenopus laevis* embryonic epidermal lectin (XEEL), respectively. The proteins, designated REEL, BEEL, and CEEL, showed characteristics conserved among reported Itln proteins, and their amino acid sequences following the signal peptides were identical to those of the N-terminal peptides determined on Itln proteins in the respective larval extracts. Recombinant REEL (rREEL), rBEEL, and rCEEL proteins produced by HEK-293T cells were homo-oligomers of 34–37 kDa subunit peptides, which were similar to the Itlns found in the newly hatched larvae. The rEELs showed carbohydrate-binding specificities similar to that of XEEL and agglutinated *Escherichia coli* and *Staphylococcus aureus* cells depending on Ca^{2+}. These results suggest that REEL, BEEL, and CEEL are Itlns produced and secreted by epidermal cells of *R. orn*, *B. jpn*, and *C. pyr* larvae, respectively, and that Itlns have a conserved role as pathogen recognition molecules in the larval innate immune system.

Key words: intelectin, epidermal secretion, innate immunity, amphibian larva, host defense, bacterial infection

INTRODUCTION

Lectins are carbohydrate recognition proteins with a vast structural diversity. They are classified into several types or families based on their structural characteristics, carbohydrate recognition specificities, and tissue distribution (Dodd and Drickamer, 2001; Sharon and Lis, 2004). As most organisms have carbohydrate chains on their cell surface, lectins play important roles in cellular adhesion, recognition, and communication. In particular, some lectins are known to act as pathogen recognition molecules, signal mediators, and effectors of innate and adaptive immunity (Dam and Brewer, 2010).

Intelectins (Itlns), members of a newly identified lectin family, have been reported in several chordate species, including amphioxus (Yan et al., 2012), tunicates (Abe et al., 1999), and mammals (Komiya et al., 1998). They are usually homo-oligomers of subunit peptides containing a fibrinogen-like domain and unique carbohydrate recognition motif, are found mostly in body fluids or epithelial secretions, and selectively recognize structurally divergent carbohydrate chains of the bacterial cell surface over those of eukaryotes in a manner dependent on Ca^{2+} (Wesener et al., 2015; Wangkanont et al., 2016). The expression of mammalian Itlns increases in response to infestation by parasitic nematodes, bacterial infection, and allergen-induced inflammation (Pemberton et al., 2004; Takano et al., 2008; Yi et al., 2017). A mouse strain with a genetic defect in Itln-2 production was found to be susceptible to infestation by parasitic nematodes compared to wild-type mice (Pemberton et al., 2004). Although detailed molecular mechanisms of their action are unknown, some Itlns have been shown to facilitate phagocytosis of bacteria by macrophages (Tsuji et al., 2009; Nagata, 2018). Thus, Itlns have been implicated as pathogen recognition molecules of the innate immune system.

The first Itln discovered was the oocyte cortical granule lectin of *Xenopus laevis* (XCGL) that is reportedly involved in the prevention of polyspermic fertilization (Wyrick et al., 1974; Lee et al., 1997). Other studies have shown that mammalian Itlns act as a lactoferrin receptor on intestinal epithelia (Suzuki et al., 2001), adipocytokine regulating endothelial cell function (Maruyama et al., 2012), and modulator of steroidogenesis in ovarian granulosa cells (Cloix et al., 2014). Thus, despite their nomenclature, Itlns might play a role not

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only in the intestinal innate immunity but also in the diverse physiological functions of various tissues and developmental stages.

We have previously reported that *X. laevis* embryos and larvae secreted an Itln, XEEL (*Xenopus* embryonic epidermal lectin), from a major subset of epidermal cells during their limited developmental stage (Nagata et al., 2003; Nagata, 2005). The secretion levels increased significantly just before hatching and were maintained for several days, while the larval acquired immune system was still immature. XEEL is a hexameric protein consisting of N-glycosylated 43 kDa peptide subunits. Similar to the human Itln-1, it shows a higher affinity to pentoses, such as D-ribose (Rib) and D-xylose (Xyl), than hexoses and disaccharides, and selectively recognizes carbohydrates exposed on the bacterial surface to cause their agglutination (Tsuji et al., 2001; Nagata, 2005; Wesener et al., 2015; Wangkanont et al., 2016). Therefore, XEEL may protect newly hatched larvae against microbial pathogens in the environmental water. An Itln was also reported in the cutaneous secretions of an adult freshwater catfish and implicated in the anti-microbial host defense system (Tsutsui et al., 2011). However, besides *X. laevis*, no study has described Itln production in the cutaneous tissues of amphibian larvae, even though they adapt ecologically to similar aquatic life in the freshwater environment. We report here on embryonic epidermal lectins (EELs) in two frogs, *Rana ornativentris* (*R. orn*) and *Bufo japonicus formosus* (*B. jpn*), and a newt, *Cynops pyrrhogaster* (*C. pyr*) that are similar to XEEL in structure, tissue distribution, and carbohydrate specificities, suggesting a conserved role of Itlns in innate immunity of amphibian larvae with immature acquired immunity.

MATERIALS AND METHODS

Materials

Usage of animals was performed under the regulations of the Experimental Animal Committee of Japan Women's University. Frogs and egg masses of *R. orn* and *B. jpn* were collected from the area around Lake Naguri, Saitama prefecture, Japan, and maintained at 20°C. Adult males and females of *C. pyr* were obtained from local dealers, maintained in aquaria at 20–22°C, and ovulated by injections of the human chorionic gonadotropin as described previously (Matsuda and Oya, 1977). The larvae at two days after hatching were used for the experiments. *Escherichia coli* (XL1 Blue MRF´) was grown in LB medium, fixed in 4% formaldehyde in PBS, and stored at 4°C. Formaldehyde-fixed *Staphylococcus aureus* (*S. aur*) cells were purchased from Sigma-Aldrich (Cat. No. S2014; St. Louis, Mo).

Monoclonal antibodies

The monoclonal antibody (mAb) 5G7 to XEEL has been described previously (Nagata, 2005). The mAb 3A8 (IgG1/κ) was produced using the standard mouse hybridoma technique and the synthetic peptide antigen with an amino acid sequence highly conserved among chordate Itln proteins (GATSDDYKNPGYYDI). Hybridoma ascites containing high titers of mAb were used in the present study.

Whole-mount immunocytochemistry

Larvae were fixed overnight with 4% paraformaldehyde in amphibian PBS (APBS; 70% PBS) and bleached overnight in 1% H_2O_2, 5% formamide, and 0.5 × SSC (Sive et al., 2000). The larvae were washed in APBS, blocked in 1% normal goat serum, and then incubated sequentially with the 3A8 mAb or a normal mouse serum (diluted 1:2000) and the horseradish peroxidase (HRPO)-labeled anti-mouse IgG goat antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactivities were visualized by color development in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris HCl, pH 7.4) containing 0.01% H_2O_2 and 0.5 mg/ml 3,3′-diaminobenzidine (H_2O_2/DAB). The stained larvae were observed and micrographs were taken under a dissecting microscope.

Western blotting and peptide sequencing

Extracts and their galactose (Gal)-Sepharose-binding fractions were prepared as described previously (Nagata, 2005). Larvae were cultured in plastic dishes at 50–100 individuals/5 ml deionized water for 6–8 hrs, and the culture media were harvested and concentrated to approximately 1/100 volume using a centrifugal filter device (Amicon Ultra-50K, Merck Millipore, Cork, Ireland). Proteins in the samples were measured with the Pierce 660 nm Protein Assay Reagent (Thermo Fischer Scientific, Rockford, IL), fractionated by SDS-PAGE under reducing or non-reducing conditions, and western blotting was performed using 3A8 mAb (1:5000) and alkaline phosphatase-conjugated anti-mouse IgG (1:3000; Sigma-Aldrich).

Gal-Sepharose-binding fractions of the larval extracts were fractionated by SDS-PAGE and the blots were prepared on a PVDF membrane. The bands at the sizes of major Itln immunoreactive proteins were excised from the membrane and sent to Nippi Inc. (Tokyo, Japan) for determination of the N-terminal amino acid sequences.

Isolation and characterization of cDNA clones

Total RNA fractions were isolated from larvae, the single strand cDNAs were synthesized and the cDNA fragments encoding EELs in *R. orn* (REEL), *B. jpn* (BEEL), and *C. pyr* (CEEL) were amplified by degenerate PCR as described previously (Nagata, 2016). Sequences of the degenerate oligonucleotide primers (Supplementary Table S1A online) were deduced from the amino acid sequences highly conserved among Itlns (WTLVASVH and DDYKNPG). Amplified cDNA fragments were cloned and their sequences were determined. Based on the sequence data, 5′ and 3′ RACE techniques were applied to obtain sequence data on full length cDNAs using a RACE kit (Takara Bio Inc., Kusatsu, Japan). The primers for RACE are listed in Supplementary Table S1B. The RACE products were cloned and ligated to obtain the cDNA clones containing complete open reading frames. Amino acid sequences of REEL, BEEL, and CEEL were deduced from the cDNAs, and aligned with XEEL using the GENETYX-MAC software (Ver. 19, Genetyx Corp., Osaka, Japan). A phylogenetic tree showing the relationship of REEL, BEEL, and CEEL to *X. laevis* Itlns was constructed by the neighbor-joining method based on comparison of whole amino acid sequences using multiple alignment and tree-making software (ClustalW, Ver. 2.1, http://clustalw.ddbj.nig.ac.jp/index.php?lang=en; Njprot, Ver 2.3, https://macdownload.informer.com/njplot/).

RT-PCR

For RT-PCR assays, total RNA fractions were isolated from various adult tissues or whole larvae and the single strand cDNAs were synthesized. Fragments of the REEL, BEEL, CEEL, and histone H4 transcripts were amplified by 25–30 cycle reactions using specific primers (Supplementary Table S1C).

Production and analyses of recombinant lectin proteins

cDNA fragments encoding REEL, BEEL, and CEEL proteins were amplified by RT-PCR using total RNA fractions of larvae and specific primers (Supplementary Table S1D) and cloned into the pCEP4 plasmid vector (Invitrogen, Carlsbad, CA) to create pCEP-*REEL*, pCEP-*BEEL*, and pCEP-*CEEL*. Recombinant plasmid DNAs were purified and used to transfect the human epithelial kidney cell line HEK-293T as described previously (Nagata, 2016). Culture

supernatants of the transfected cells were analyzed by western blotting using 3A8 mAb. The recombinant EEL (rEEL) proteins were partially purified by affinity chromatography on a Gal-Sepharose column.

Glycopeptidase F treatment

Purified rEEL fractions containing 100 ng protein were digested with glycopeptidase F (Takara Bio) according to the manufacturer's instruction manual. The digested samples were analyzed by western blotting.

Saccharide specificity assay

Saccharide binding assays were performed as described previously (Nagata, 2016). Gal-Sepharose beads were incubated in the culture supernatants of the HEK-293T cells containing rEEL proteins (and 1.8 mM $CaCl_2$ as a medium ingredient), washed four times with TBS containing 5 mM $CaCl_2$ (TBS-Ca), and resuspended in TBS containing 10 mM EGTA (TBS-EGTA) or TBS-Ca containing 100 mM of one of the following saccharides (all from Sigma-Aldrich): D-arabinose (Ara), Rib, Xyl, D-fructose (Fuc), D-Gal, D-glucose (Glc), D-mannose (Man), lactose (Lac), maltose (Mal), melibiose (Mel), N-acethyl-D-galactosamine (GalNAc), and N-acethyl-D-glucosamine (GlcNAc). After the mixtures were agitated for 10 min at room temperature, they were centrifuged, and eluted lectin proteins in the supernatants were examined by western blotting.

Bacterial binding and agglutination assays

These assays were performed as described previously (Nagata, 2016). Culture supernatants containing rEELs or their partially purified fractions (1.2 µg protein/ml) were used for bacterial binding assays and bacterial agglutination assays.

RESULTS

Identification and characterization of epidermal Itlns in newly hatched larvae

The anti-XEEL 5G7 mAb recognized neither rREEL, rBEEL, nor rCEEL in western blotting, whereas the anti-Itln peptide mAb 3A8 recognized all of them (Supplementary Fig. S1A). In whole-mount immunocytochemical assays, the 5G7 and 3A8 mAbs labeled similar subsets of epidermal cells in the *X. laevis* larvae (Supplementary Figure S1B). The immunoreactivity of 3A8 mAb was completely blocked by preincubation with the Itln peptide, proving the specific immunoreactivity of mAb to Itln. Labeled spots on the epidermis found in the control larvae were due to cross reactivity of the HRPO-labeled second antibody to the cilia. The 3A8 mAb immunolabeled a subset of larval epidermal cells in *R. orn*, *B. jpn*, and *C. pyr* (Fig. 1), but dissection of the stained larvae revealed no immunoreactivities to any other tissues. In *R. orn*, and *B. jpn* larvae, immunoreactive cells formed a cellular meshwork around non-reactive ciliated or nonciliated cells, whereas those in *C. pyr* larvae filled the epidermal areas without ciliated cells. Nonspecific labeling of epidermal cilia was also

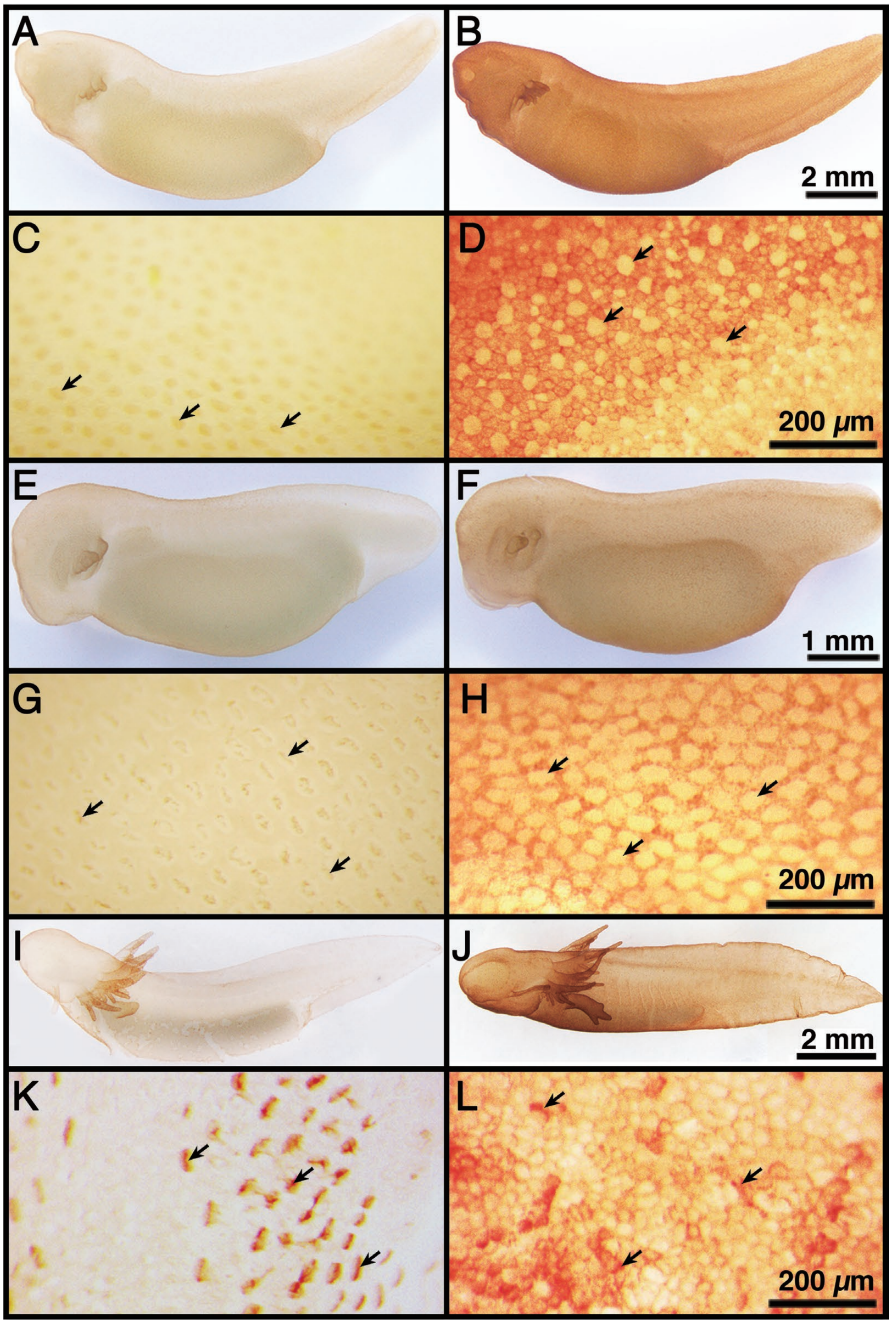

Fig. 1. Whole-mount immunocytochemical staining of Itlns in larvae. *R. orn* **(A–D)**, *B. jpn* **(E–H)**, and *C. pyr* **(I–L)** larvae were fixed, bleached, and then incubated with the anti-Itln 3A8 mAb **(B, D, F, H, J, L)** or a control mouse serum **(A, C, E, G, I, K)**. The immunoreactivities were visualized with anti-mouse IgG-HRPO conjugate and H_2O_2/DAB. The micrographs are lateral views of the larvae with their head to the left. The arrows indicate individual ciliated cells.

found in the control larvae, most remarkably of those in *C. pyr*.

In western blot analyses, the 3A8 mAb recognized proteins of a major 36 kDa and a minor 33 kDa band (*R. orn*), a major 37 kDa and a minor 34 kDa band (*B. jpn*), and a single 35 kDa band (*C. pyr*) in the larval extracts (Fig. 2, left panel). To examine whether these proteins were secreted from the larvae, the concentrated larval culture media were assayed by western blotting (Fig. 2, central panel). The 3A8 mAb visualized diffuse protein bands at 32 kDa to 35 kDa in the culture media of *R. orn* and *B. jpn* larvae, and a major 37 kDa band and multiple smaller bands in that of *C. pyr* larvae, suggesting that the immunoreactive proteins were present as partially degraded forms in the embryo culture media. In SDS-PAGE under non-reducing conditions, immunoreactive proteins in the Gal-Sepharose-binding fraction of the larval extracts were fractionated to major bands at 240 kDa and 60 kDa (*R. orn*), 220 kDa and larger than 400 kDa (*B. jpn*), and 55 kDa and multiple bands larger than 100 kDa (*C. pyr*), indicating that the immunoreactive proteins had multiple oligomeric forms consisting of 33 kDa to 35 kDa subunit proteins (Fig. 2, right panel). Therefore, a subset of epidermal cells

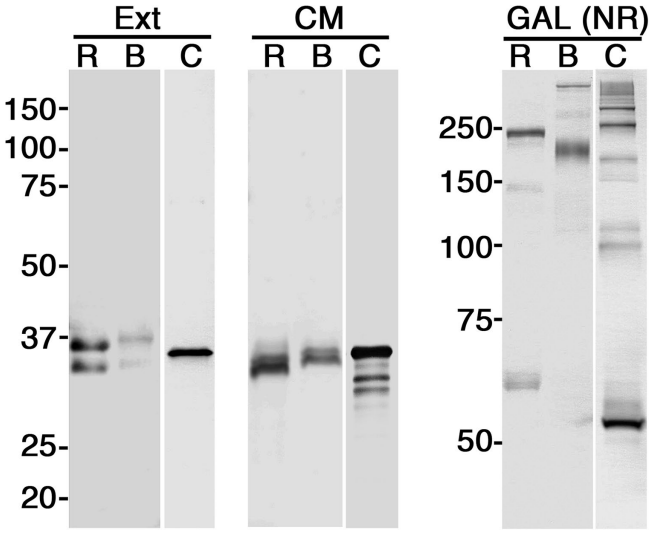

Fig. 2. Western blot analyses of Itlns in *R. orn*, *B. jpn*, and *C. pyr* larvae. Extracts (Ext; 50 μg protein/lane) of *R. orn* (R), *B. jpn* (B), and *C. pyr* (C) larvae were examined by western blotting using 3A8 mAb (left panel). The larval culture media (CM) were concentrated and assayed by western blotting (central panel). Larval extracts were incubated with Gal-Sepharose in TBS-Ca and the bound proteins were fractionated by SDS-PAGE under non-reducing conditions (GAL (NR); right panel). Numbers on the left of the blots are the sizes (kDa) of the marker proteins.

Table 1. Comparison of N-terminal amino acid sequences of Itlns purified from larvae and those of REEL, BEEL, and CEEL deduced from isolated cDNA.

Protein	Determined sequence*	Deduced from cDNA
R. orn Itln	NKXEYASLPE	NKCEYASLPE
B. jpn Itln	KXHDASIXX(K/L)	KCHDASISEK
C. pyr Itln	ADEXDAND(L/N)P	ADECDANDLP

*Cysteine residues were not determined. X, undetectable.

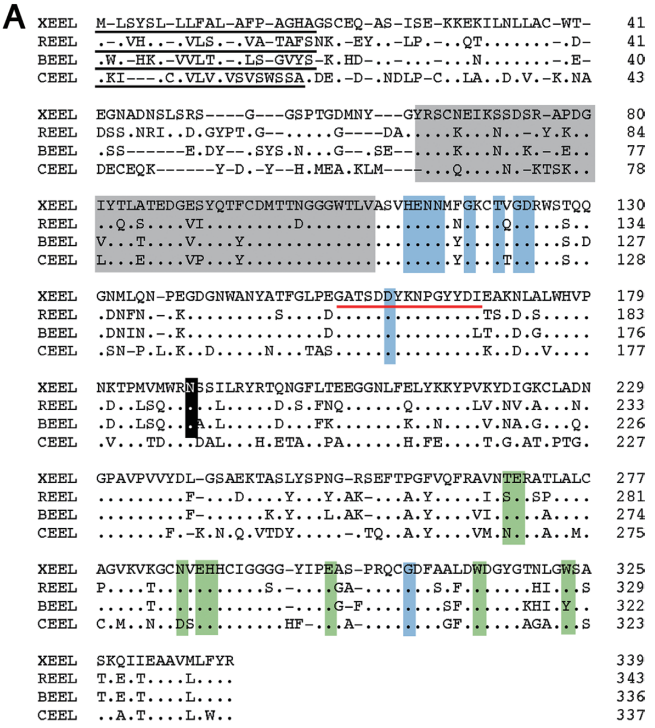

Fig. 3. Characterization of REEL, BEEL, and CEEL proteins. **(A)** Alignment of amino acid sequences of XEEL, REEL, BEEL, and CEEL proteins. Amino acid sequences deduced from the cDNAs isolated from *R. orn* (R), *B. jpn* (B), and *C. pyr* (C) larvae were compared to that of XEEL. Identical residues, gaps, and potential N-glycosylation residues are shown as dots, dashes, and reverse color, respectively. Signal peptides (black underlines), fibrinogen-like domains (gray boxes), residues in the structural calcium coordination (blue boxes), and ligand-binding sites (green boxes) are highlighted. The red underline indicates the sequence of the synthetic peptide used to generate the 3A8 mAb. **(B)** Phylogenetic tree showing the relationship of REEL, BEEL, and CEEL to *X. laevis* Itlns. The tree was constructed by the neighbor-joining method using whole amino acid sequences, with the scale bar indicating the number of substitutions per residue. Numbers on the branches represent the bootstrap values. Nucleotide and amino acid sequences of the REEL, BEEL, and CEEL cDNAs and proteins are available from the DNA database with accession numbers, LC360281, LC360280, and LC506622, respectively. References for the sequence data: XEEL (Nagata et al., 2003), XCGL1 and XCGL2 (Shoji et al., 2005), XCL-1 and XCL-2 (Ishino et al., 2007), XCL-3 (accession no: NM001092293.1), and XItln-3 and XItln-4 (Nagata, 2016).

produced and extraembryonically secreted Itlns in the newly hatched larvae of the three amphibian species.

The Itlns were partially purified from newly hatched larvae, and N-terminal amino acid sequences of the major Itln proteins were determined by Edman degradation. The sequences of Itln proteins from *R. orn*, *B. jpn*, and *C. pyr* larvae were consistent with those of the REEL, BEEL, and CEEL proteins, respectively (Table 1).

Isolation and characterization of Itln cDNAs

To clone the cDNAs encoding larval epidermal Itlns, RT-PCR/RACE procedures were applied using total RNA fractions isolated from the larvae of *R. orn*, *B. jpn*, and *C. pyr*. RT-PCR assays using degenerate oligonucleotide primers (Supplementary Table S1A) amplified 179 bp cDNA fragments from all the examined species. Sequences of the independent 8–16 cDNA fragments amplified in each species were identical and showed 74%–81% nucleotide identities with that of XEEL, suggesting that the fragments were those of a predominant Itln transcript in the newly hatched larvae. Specific oligonucleotide primers (Supplementary Table S1B) were designed from these sequences and used for 5´ and 3´ RACE. Sequence analyses of the RACE products and reconstruction of cDNAs revealed encoding of proteins of 343 (*R. orn*), 336 (*B. jpn*), and 337 (*C. pyr*) amino acids. Their overall amino acid identities with XEEL were 70% (similarity, 91%), 77% (similarity, 94%), and 60% (similarity, 90%), respectively, with a possible signal peptide and fibrinogen-like domain (Fig. 3A). In addition, the residues involved in Ca^{2+} coordination were perfectly conserved and those involved in carbohydrate binding had limited amino acid substitutions that possibly did not collapse the carbohydrate recognition structure (Wesener et al., 2015; Wangkanont et al., 2016). The proteins of *R. orn*, *B. jpn*, and *C. pyr* were designated as REEL, BEEL, and CEEL, respectively. REEL and BEEL had a potential

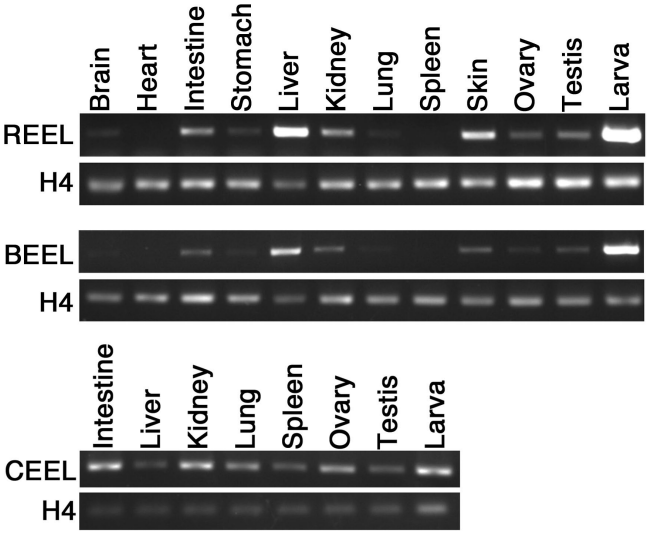

Fig. 4. Tissue distribution of REEL, BEEL, and CEEL transcripts. RT-PCR assays were performed using the total RNA fractions isolated from the adult tissues indicated and the whole larvae of *R. orn*, *B. jpn*, and *C. pyr*. Histone H4 (H4) was a reverse transcription control.

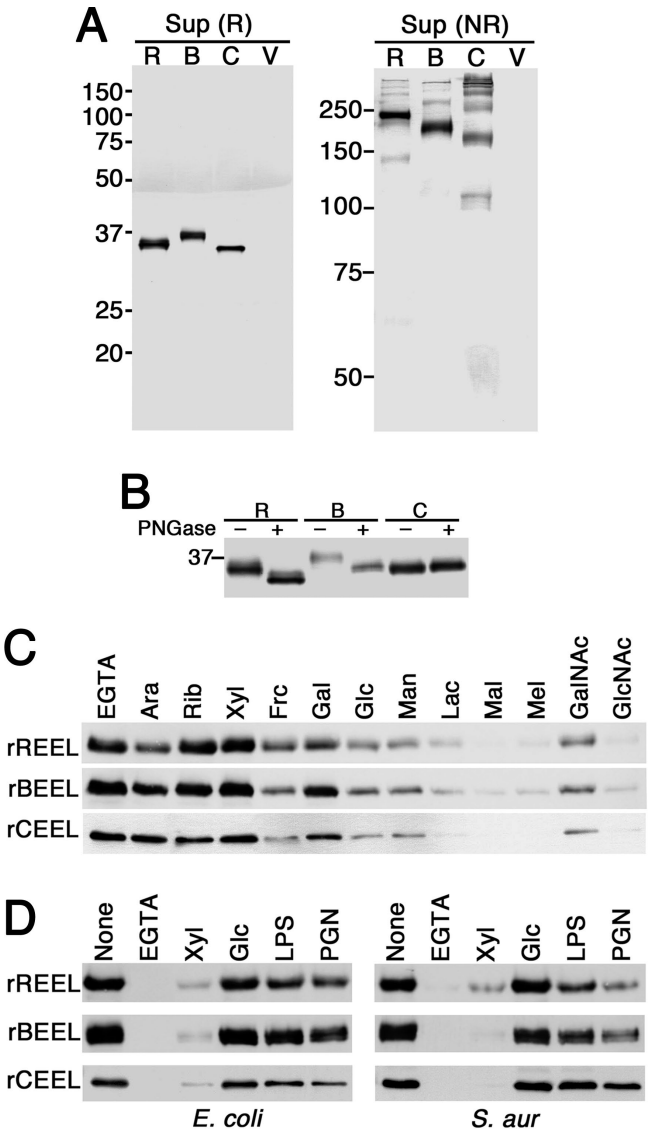

Fig. 5. Production and characterization of rREEL, rBEEL, and rCEEL. **(A)** Examination of rREEL, rBEEL, and rCEEL in the culture supernatants of HEK-293T cells. The culture supernatants (Sup) of the cells transfected with the pCEP-*REEL* (R), pCEP-*BEEL* (B), pCEP-*CEEL* (C), or control pCEP vector (V) were fractionated by SDS-PAGE under reducing (R; left panel) or nonreducing (NR; right panel) conditions and analyzed by western botting using the 3A8 mAb. **(B)** Glycopeptidase F treatment of rREEL, rBEEL, and rCEEL. The culture supernatants containing rREEL (R), rBEEL (B), and rCEEL (C) were incubated with (+) or without (–) glycopeptidase F (PNGase) and examined by western blotting. **(C)** Carbohydrate specificities of rREEL, rBEEL ,and rCEEL. Gal-Sepharose beads were incubated with the culture supernatants containing rREEL, rBEEL, or rCEEL, and after washing were reincubated in TBS-EGTA (EGTA) or TBS-Ca plus 100 mM saccharides or amino sugars as indicated. rREEL, rBEEL, and rCEEL in the eluates were examined by western blotting. **(D)** Bacterial binding assays of rREEL, rBEEL, and rCEEL. Fixed *E. coli* or *S. aureus* cells were incubated with the culture supernatants containing rREEL, rBEEL, or rCEEL in TBS (None), TBS-EGTA (EGTA), or TBS-Ca containing either Xyl, Glc, LPS, or PGN. After washing, the bound rREEL, rBEEL, and rCEEL were examined by western blotting.

N-glycosylation residue, however, CEEL did not. The phylogenetic tree constructed by the neighbor joining method showed that REEL and BEEL were located at the closest branches to XEEL (Fig. 3B).

RT-PCR assays revealed transcripts of REEL, BEEL, and CEEL in the larvae of *R. orn*, *B. jpn* and *C. pyr*, respectively (Fig. 4). In the adult tissues examined, the transcripts were found rather ubiquitously, showing relatively higher levels in the intestine, liver, kidney, and skin for REEL and BEEL, and the intestine, kidney, and ovary for CEEL.

Production of rEEL proteins

For further characterization of REEL, BEEL, and CEEL, the expression vectors were transfected to HEK-293T cells and the culture supernatants were analyzed by western blotting. The 3A8 mAb identified single protein bands at 36 kDa, 37 kDa, and 35 kDa in the culture supernatants of cells transfected with pCEP-*REEL*, pCEP-*BEEL*, and pCEP-*CEEL*, respectively (Fig. 5A). Under nonreducing conditions, the rEEL proteins showed major bands at approximately 230 kDa (rREEL), 200 kDa (rBEEL), and 180 kDa (rCEEL) with multiple minor bands (Fig. 5A). Glycopeptidase F treatment of the rREEL and rBEEL proteins reduced the apparent sizes from 36 kDa to 34 kDa and 37 kDa to 35 kDa, respectively, whereas the treatment did not alter the size of the rCEEL protein (Fig. 5B). Therefore, the rREEL and rBEEL proteins were produced and secreted by the transfected HEK-293T cells as multiple oligomers of the N-glycosylated peptide subunits, whereas the rCEEL protein was without N-glycosylation.

Carbohydrate specificity of rEELs

To examine carbohydrate specificities, rEELs in the culture supernatants were bound to Gal-Sepharose beads, the beads were then incubated with EGTA, various saccharides, or amino sugars, and the eluted rEELs were examined by western blot analyses. The incubation with EGTA effectively eluted the bound rREEL, rBEEL, and rCEEL (Fig. 5C). The pentoses, Rib and Xyl, eluted the rEELs as effectively as EGTA, whereas Ara and Gal (hexoses) were less effective in the elution and the other hexoses, disaccharides, and amino sugars were even less effective. Therefore, the carbohydrate specificity profiles of rREEL, rBEEL, and rCEEL were similar to those of XEEL and human Itln-1 (Tsuji et al., 2001; Nagata, 2005).

Bacterial binding and agglutinating activities of rEELs

The culture supernatants of HEK 293T cells containing rEELs were incubated with fixed *E. coli* or *S. aureus* cells in TBS-Ca, TBS containing 10 mM EGTA, or TBS-Ca containing various competitor sugars. After washing the bacteria,

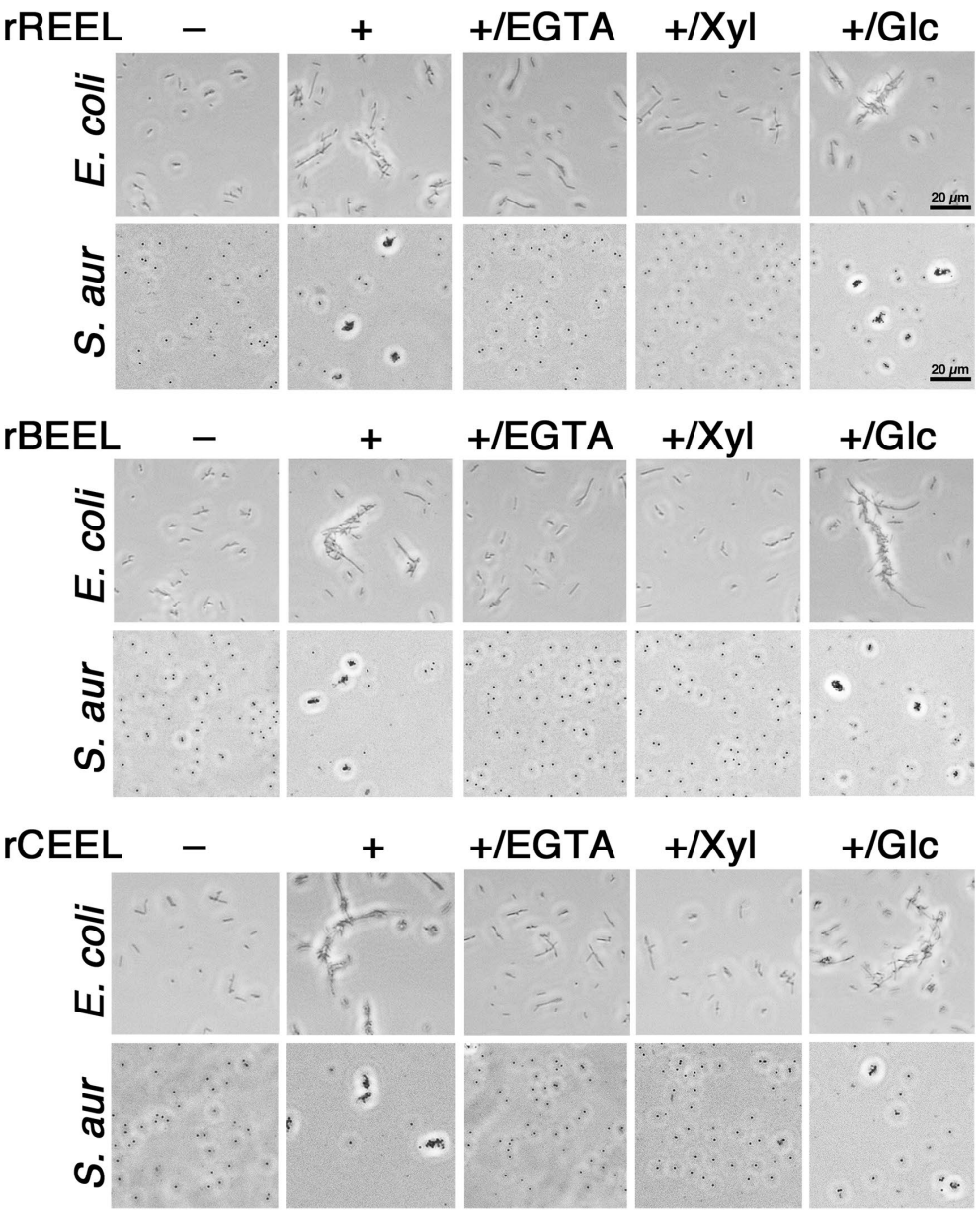

Fig. 6. Agglutination of bacteria with rREEL, rBEEL, and rCEEL. Fixed *E. coli* or *S. aur* cells were incubated in TBS, TBS-Ca, or TBS-Ca containing either EGTA, Xyl, or Glc. After incubation, the bacteria were observed under a microscope with phase contrast optics.

the bound rEELs were detected by western blotting (Fig. 5D). rREEL, rBEEL, and rCEEL bound to the bacteria in TBS-Ca and the binding was completely blocked by EGTA. The high-affinity saccharide Xyl effectively blocked the binding, whereas the low-affinity saccharide Glc did not. LPS from *E. coli* and peptidoglycans (PGN) from *S. aureus* also partially blocked the binding. To examine whether the binding of rEELs caused agglutination of the bacteria, the partially purified rREEL, rBEEL, and rCEEL were incubated with fixed *E. coli* and *S. aureus* cells in TBS-Ca. The controls without addition of rEELs showed no agglutination of *E. coli* or *S. aureus* cells, whereas the addition of the rREEL, rBEEL, or rCEEL caused agglutination of the bacteria (Fig. 6). The agglutination was abolished by EGTA and Xyl, but was not affected by Glc.

DISCUSSION

Using a mAb that recognized the peptide conserved among all known Itlns, we showed Itln immunoreactivities in a subset of epidermal cells of *R. orn*, *B. jpn*, and *C. pyr* larvae by whole-mount immunocytochemistry. The mAb identified proteins of 33–37 kDa in size in the extracts and culture media of larvae by western blotting. The two protein bands identified in the extracts of *R. orn* and *B. jpn* might represent products of a single gene generated by alternative RNA splicing or differential posttranscriptional modification that is often observed in Itlns (Lee et al., 2004; Nagata et al., 2013). Alternatively, these proteins could be distinct Itlns in each species, since *R. orn* and *B. jpn* probably have multiple Itlns, similarly to most chordate species examined. We isolated cDNA clones encoding Itlns, REEL, BEEL, and CEEL from *R. orn*, *B. jpn*, and *C. pyr* larvae, respectively. We think that REEL, BEEL, and CEEL are the Itlns identified in larvae using the anti-Itln mAb, although these amphibian species may have multiple Itlns (Lee et al., 2004; Nagata, 2016). This conclusion is based on our findings that, first, the amino acid sequences of REEL, BEEL, and CEEL were highly homologous to that of XEEL. Second, the N-terminal amino acid sequences of larval Itln proteins showed no inconsistency with those of the mature REEL, BEEL, and CEEL proteins, respectively. It has been shown that the N-terminal regions of all known Itlns are highly divergent (Lee et al., 2004). Finally, the molecular sizes of the subunit peptides and the oligomeric forms of rREEL, rBEEL, and rCEEL were similar to those of the major protein bands identified in the larvae of each species using the anti-Itln mAb.

The RT-PCR assays showed rather ubiquitous expression of REEL, BEEL, and CEEL in adult tissues, which contrasted with our previous results that XEEL expression was found specifically in epidermal cells in *X. laevis* (Nagata et al., 2003; Nagata, 2016). This difference may be due to the unique genomic structure of *X. laevis* that is thought to have arisen from two closely related progenitor species by hybridization and chromosomal tetraploidization (Session et al., 2016). Thus, the *X. laevis* genome consists of two subgenomes that retain many pairs of homeologous genes derived from two ancestral frogs. The XEEL gene (name; *itln-1.L*, position; Chr7L:31554575..31571611 (– strand)) appears to be the homeolog of the Itln-3 gene (name; LOC108697931, position; Chr7S:24348275..24360608 (+ strand)) whose transcripts are detectable rather ubiquitously in adult tissues, although the Itln-3 protein is produced particularly abundantly in adult intestinal goblet cells (Nagata, 2016). The XEEL and Itln-3 genes might have diverged in spatiotemporal expression patterns and physiological functions in *X. laevis*, whereas the REEL, BEEL, and CEEL genes might have retained the expression patterns and physiological functions of the ancestral gene that had the combined roles of EEL and Itln-3. In catfish, the skin mucus Itln is reportedly also expressed in other adult tissues, including kidney renal tubules (Tsutsui et al., 2011).

The characteristics of REEL, BEEL, and CEEL proteins, including the molecular structure, extraembryonic secretion in newly hatched larvae, carbohydrate specificities, and bacterial agglutination, were comparable to those of XEEL. These EELs contain regularly positioned, highly conserved amino acid residues in the carbohydrate recognition domain that directly coordinate Ca^{2+} and recognize the exocyclic 1,2-diols of synthetic saccharides or microbial glycans (Wangkanont et al., 2016). Thus, as a previous study suggested regarding *X. laevis* XEEL (Nagata, 2005), REEL, BEEL, and CEEL would also play a role in protecting the larvae of each species against microbial pathogens in the environmental water even though their acquired immune systems are immature. Because these EELs can bind and agglutinate bacteria, they immobilize pathogenic microorganisms on the epidermal surface as a component of the mucus-like layer (Dubaissi et al., 2014) or in its vicinity to prevent their invasion. Alternatively, upon bacterial invasion into subepidermal tissues, EELs might prevent the expansion of microbial infection by activating the phagocytosis of leukocytes, which have been shown to be distributed in subcutaneous tissues over the entire larval body of *Xenopus* (Costa et al., 2008; Paredes et al., 2015). *Xenopus* and human serum Itln promote the clearance of bacteria by facilitating phagocytosis by macrophages (Tsuji et al., 2009; Nagata, 2018). Whatever the mechanisms of action, the present study suggests that EELs are involved in a host defense system of newly hatched larvae that is broadly conserved in amphibians.

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COMPETING INTERESTS

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

SN designed and performed molecular and biochemical aspects of the study, and wrote the manuscript. MT performed immunocytochemistry and illustration works.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs190150)

Supplementary Figure S1. Immunoreactivities of the anti-Itln mAb 3A8.

Supplementary Table S1. Primers used in the present study.

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