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Identification and characterization of a novel intelectin in the digestive tract of *Xenopus laevis*

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ABSTRACT

The intelectin (Intl) family is a group of secretory lectins in chordates that serve multiple functions, including innate immunity, through Ca\(^{2+}\)-dependent recognition of carbohydrate chains. Although six Intl family lectins have so far been reported in *Xenopus laevis*, none have been identified in the intestine. Using a monoclonal antibody to the *Xenopus* embryonic epidermal lectin (XEEL or Intl-1), I identified cross-reactive proteins in the intestines. The proteins were purified by affinity chromatography on a galactose-Sepharose column and found to be oligomers consisting of N-glycosylated 39 kDa and 40.5 kDa subunit peptides. N-terminal amino acid sequencing of these peptides, followed by cDNA cloning, identified two novel Intls (designated Intl-3 and Intl-4) that showed 59–79% amino acid identities with known *Xenopus* Intl family proteins. From the amino acid sequence, immunoreactivity, and properties of the recombinant protein, Intl-3 was considered the intestinal lectin identified by the anti-XEEL antibody. The purified Intl-3 protein could potentially bind to *Escherichia coli* and its lipopolysaccharides (LPS), and to *Staphylococcus aureus* and its peptidoglycans, depending on Ca\(^{2+}\). In addition, the Intl-3 protein agglutinated *E. coli* cells in the presence of Ca\(^{2+}\)\(^{-}\). Intraperitoneal injection of LPS increased the intestinal and rectal contents of Intl-3 and XCL-1 (or 35K serum lectin) proteins within three days; however, unlike XCL-1, Intl-3 was detectable in neither the sera nor the other tissues regardless of LPS stimulation. Immunohistochemical analyses revealed accumulation of the Intl-3 protein in mucus secretory granules of intestinal goblet cells. The results of this study suggest that *Xenopus* Intl-3 is involved in the innate immune protection of the digestive tract against bacterial infections.

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1. Introduction

Lectins are structurally and functionally diverse proteins that recognize carbohydrates and which have attracted the attention of immunologists because of their roles as pathogen recognition molecules in the innate immune system [Sharon and Lis, 2004]. A group of Ca\(^{2+}\)-dependent lectins that have a conserved fibrinogen-like domain was designated as the intelectin (Intl) family, because the human lectin was found in intestinal epithelia (Lee et al., 2001; Tsuji et al., 2001). This group has also been referred to as the X-type lectin family, as the first lectin was reported in *Xenopus laevis* oocytes (Wyrick et al., 1974; Lee et al., 2004). Two or three closely related Intls have been identified in various mammalian tissues, including intestinal epithelia, vascular tissues, mesothelial mucus, and blood plasma (Lee et al., 2001; Tsuji et al., 2001; French et al., 2009; Tsuji et al., 2009; Washimi et al., 2012) and their expression is reportedly up-regulated in response to infestation with parasitic worms or allergen-induced inflammation (Pemberton et al., 2004; Datta et al., 2005; Gu et al., 2010). Human Intl-1, one of the most intensively characterized Intls, has been reported to have remarkable selectivity in the recognition of microbial over mammalian cell surface glycans (Wesener et al., 2015) and thereby enhance phagocytosis of bacteria by macrophages (Tsuji et al., 2009). In addition, a mouse strain with a genetic defect in Intl-2 production is unable to eliminate parasitic nematodes effectively, as compared to those having the wild type gene (Pemberton et al., 2004). Accordingly, the Intl family lectins are considered to act as microbial recognition molecules in the innate immune system.

Other studies have identified and characterized Intl-1 as a lactoferrin receptor, a glycosylphosphatidyl inositol (GPI)-anchored iron transporter-binding protein on the membrane of the human intestinal brush border (Suzuki et al., 2001; Chatterton et al., 2013). In addition, human Intl-1 has been alternatively designated as...
omentin, because it has been characterized as one of the adipo-
tokines secreted from visceral adipose tissues (Kralisch et al., 2005).
Omentin has been reported to regulate the differentiation and
function of vascular endothelial cells (Tan et al., 2010; Maruyama
et al., 2012) and modify the action of insulin on target cells (Yang
et al., 2006). Moreover, human Intl-1 has been suggested to act as
tumor suppressor in prostate cells (Mogal et al., 2007) and as a
modulator of steroidogenesis in ovarian granulosa cells (Cloix et al.,
2014). Thus, despite their nomenclature, these studies suggest that
mammalian Intls are involved not only in intestinal innate immu-
ity but also in multiple physiological functions of various tissues.
Intl family proteins have also been reported in various tissues of
several chordate species, including ascidian plasma (Abe et al.,
1999), several tissues of amphioxus (Yan et al., 2013), and the
epidermal mucus of catfish (Tsutsui et al., 2011). In the amphibian,
X. laevis, expression of six distinct Intl family genes has so far been
reported in different adult or embryonic tissues (Nagata et al.,
2003; Lee et al., 2004; Ishino et al., 2007; Nagata et al., 2013).
Among them, the oocyte cortical granule lectins (XCGL1 and
XCGL2) are major components of oocyte cortical granules and upon
fertilization, are secreted to the extracellular space to form a
fertilization layer that prevents polyspermic fertilization and
pathogen invasion (Wyrick et al., 1974; Nishihara et al., 1986). The
Xenopus embryonic epidermal lectin (XEEl) is secreted from the
epidermis during a limited period of embryonic stages, while the
adaptive immune system is still immature, suggesting a possible
role in the protection of embryos against pathogenic microbes in
the environmental water (Nagata, 2005). Recently, XEEl is shown
to recognize bacterial surface glycans using its carbohydrate bind-
ing domain that has architecture highly conserved between XEEl
and human Intl-1 (Wangkanont et al., 2016). In addition, our recent
study demonstrated that XCl-1 (35K serum lectin) could bind to
extracellular carbohydrate components of Gram-negative and
Gram-positive bacteria, and that intraperitoneal injection of the
bacteria or their cell wall substances induced an acute increase of
the serum XCL-1 content (Nagata et al., 2013). The expression of
XCl-2 (Intl-1-like lectin) and XCL3 (Intl-2) genes has also been
reported in various adult tissues, although their physiological roles
remain unclear. Collectively, these findings suggest that amphibian
Intl family proteins play even broader physiological functions than
mammalian Intls. The present study reports two additional mem-
ers of the Xenopus Intl family, Intl-3 and Intl-4, both expressed in
the digestive tract. I also present data suggesting that amphibian
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2. Materials and methods

2.1. Bacteria, bacterial components, and sugars

Formaldehyde-fixed Staphylococcus aureus cells (Cat. No. S2014),
their lipoteichoic acids (LTA) (Cat. No. L2515), peptidoglycan (PGN)
(Cat. No. 77740), and protein A (PrA) (Cat. No. P6031), and LPS from
Escherichia coli 0111:B4 (Cat. No. L2630) were obtained from Sigma-
Aldrich (St. Louis, MO). Formaldehyde-fixed E. coli cells were pre-
pared as previously described (Nagata, 2005; Nagata et al., 2013).
The monosaccharides, disaccharides, and amino sugars were pur-
chased from Sigma-Aldrich and Wako Chemicals (Tokyo, Japan).

2.2. Animals and antibodies

Purchase, care and usage of X. laevis were performed with standard protocols (Sive et al., 2000) under the regulations of the Experimental Animal Committee of Japan Women’s University. Frogs aged 1.5—2 years were injected intraperitoneally with LPS in
amphibian phosphate-buffered saline APBS (70% PBS) at a dose of
4 μg/g body weight; on day 3 following injection, they were
anesthetized, blood was collected by cardiac puncture, and sera
were isolated by centrifugation at 3000 × g for 10 min. The resected
tissues of frogs were either frozen in liquid nitrogen and stored
at −80°C for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immersed in an RNA conservation medium
(RNAlater; Qiagen, Tokyo, Japan) and stored at 4°C for RNA iso-
lation, or fixed with 4% paraformaldehyde in 0.1 M 3-(N-morpholino)
propanesulfonic acid (MOPS) buffer (pH 7.4) for further preparation of
frozen sections. As previously described, bacterial recombinant
proteins were used as immunogens to prepare the monoclonal antibodies (mAbs) 5G7 to XEEl, 4C8 to XCl-1, and 4G12 to all known Xenopus Intl family proteins (Nagata, 2005; Nagata et al.,
2013).

2.3. Purification of Intls

Intestines of 20—28 LPS-stimulated frogs (total wet weight, 5.1—6.8 g) were homogenized on ice in 30 mL Tris-buffered saline (TBS) (100 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 1% Triton
X-100, 0.5 mM phenylmethylsulfonyl fluoride, and the ethyleneglycol
betaine (EB) (EDTA)-free protease inhibitor cocktail (GE Healthcare Japan, Tokyo, Japan), using a Polytron homogenizer (Kinematica Japan, Tokyo). The homogenates were centrifuged
for 60 min at 20,600 × g. The supernatants were supplemented with
300 μL of 1 M CaCl2 and placed on ice for 15 min. The tubes were
centrifuged again for 60 min at 20,600 × g and the supernatants were mixed with the same volume of TBS containing 5 mM CaCl2
(TBS-Ca) and fractionated on a galactose (Gal)—Sepharose column as previously described (Nagata et al., 2013). The fractions eluted
with TBS containing 5 mM EDTA (TBS-EDTA) were pooled and centri-
fuged on a Centricon YM 100 centrifugal filter device (Merck Millipore, Darmstadt, Germany) to enrich high molecular weight
(>100K) proteins. Protein concentrations of the samples were determined using a Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA).

2.4. SDS-PAGE, western blotting, and peptide sequencing

Samples were fractionated by SDS-PAGE and the proteins in the
gel were visualized using a silver staining kit (Kanto Chemicals,
Tokyo, Japan) or Coomassie brilliant blue. In western blot analyses,
the proteins in gels were transferred to a polyvinylidene fluoride
(PVDF) membrane (Bio-Rad) and the lectins on the blots were
visualized with the mAbs, as previously described (Nagata, 2005).
Chemiluminescence signals of the western blots were quantified as
previously described (Nagata et al., 2013), and the data were pre-
sented as mean ± S.D. of each group and values of p < 0.05 in the
Student's unpaired t-test were accepted as statistically significant.
Protein bands on the blot were excised from the PVDF mem-
brane and sent to Nippi Inc. (Tokyo, Japan) for determination of the
N-terminal amino acid sequences.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) and
cDNA cloning

The total RNA fraction free of contaminant genomic DNA was
isolated from the intestines using an RNAeasy Plus Total RNA
Isolation Kit (Qiagen) and single strand cDNAs were synthesized using a PrimeScript 1st strand cDNA synthesis kit (Takara Biotechnologies,
Tokyo, Japan). Using the cDNAs as templates, cDNA fragments
encoding novel Intls were amplified with the PrimeSTAR Max DNA
polymerase (Takara) and degenerate oligonucleotide primers
(Supplementary Table S1). The sequences of primers were deduced

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from the highly conserved amino acid sequences among IntI family proteins (WTIVASVH and DDYKNPGY). The amplified cDNA fragments were cloned into the pGEM plasmid vector, and the sequences were determined and analyzed with the GENETYX-MAC software (Genetyx Co., Osaka, Japan). From the sequence data obtained, specific primers were designed (Supplementary Table S2) and applied to the rapid amplification of cDNA ends (RACE) procedure using the Smarter RACE 5’/3’ kit (Clontech Laboratory, Mountain View, CA). The amplified DNA fragments were cloned and ligated to obtain the amplified cDNA clones containing complete open reading frames.

For RT-PCR assays, total RNA fractions were isolated from various tissues or whole embryos and the single strand cDNAs were synthesized as described above. The following transcripts were amplified using a HotStarTaq DNA polymerase (Qiagen) and specific primers (Supplementary Table S3): XCL-1, XCL-2, XCL3a/b, XEEL, and ornithine decarboxylase (ODC). Amplification was performed by 25–30 cycle reactions, the products were electrophoresed on agarose gels and visualized with ethidium bromide stain.

2.6. Recombinant Intls

The cDNA fragments encoding XEEL, XCL-1, XCL-2, XCL-3a, IntI-3, and IntI-4 proteins were amplified using cDNA clones as templates and primer pairs specific to each lectin (Supplementary Table S4), and inserted into the pCEP4 plasmid vector (Clontech). The recombinant vectors were transfected to the human epithelial kidney cell line 293T and the culture supernatants or cell lysates were obtained as previously described (Nagata et al., 2013). Recombinant lectins were purified by affinity chromatography on a Gal-Sepharose column as described above.

2.7. IntI-3 binding assay

Lectin binding assays were performed as previously described (Nagata et al., 2013). The purified IntI-3 protein was mixed with a suspension of formaldehyde-fixed *E. coli* or *S. aureus* cells, LPS-Sepharose, PGN-Sepharose, or Gal-Sepharose in TBS-Ca and incubated for 30 min at 24 °C with continuous rotation. In the competitive binding assays, various saccharides, amino sugars, or bacterial cell surface substances were added to the IntI-3 binding reaction. Following incubation, the bound IntI-3 was examined by western blotting. In the competitive elution assays, Gal-Sepharose beads with bound IntI-3 were incubated with various concentrations of competitors in TBS-Ca and the eluted lectin was examined by western blotting.

Lectin-mediated bacterial agglutination assays were performed using recombinant IntI-3 (rlntI-3) and the IntI-3 protein fraction purified from frog intestines. GFP-fluorescent *E. coli* (GFP-E. coli) was generated by transformation of XL1 MRF’ strain *E. coli* with the plasmid pAcGFP (Clontech) and fixed in paraformaldehyde in TBS. The same volume of GFP-E. coli suspension (OD600 = 0.5) in TBS-Ca and lectin solutions (20 μg/ml) were mixed in wells of a microplate, vibrated for 30 min on a microplate mixer (Sanko Junyaku, Tokyo, Japan) and then incubated for 3 h at room temperature. After incubation, the suspensions were observed under the fluorescent microscope.

2.8. Immunohistochemistry

Digestive tracts were removed from anesthetized frogs, dissected into five parts, i.e., stomach; proximal, intermediate, and distal small intestines; and rectum, and fixed overnight in 0.1 M MOPS buffer containing 4% paraformaldehyde. The specimens were embedded in a resin (OCT compound, Sakura Finetek, Tokyo, Japan) and the frozen sections were made using a cryomicrotome. The sections were treated in methanol containing 3% H2O2 for 5 min to inhibit endogenous peroxidase activities, and were incubated with the primary mAbs (1:2000), anti-XEEL 5G7, anti-XCL1 4C8, or anti-Xenopus T cell XT-1 (control). In some experiments, the 5G7 mAb was preabsorbed with rlntI-3 before usage. Gal-Sepharose beads were incubated for 30 min with the culture supernatant of 293T cells transfected with the vacant vector or the recombinant vector inserted with IntI-3 cDNA. After washing in TBS-Ca, the beads were reincubated with the 5G7 mAb solution (1:1000), centrifuged for 1 min at 500 × g, and the supernatants were used as the preabsorbed control mAbs. After incubation with the primary mAbs, the sections were washed in PBS, incubated with goat anti-mouse IgG-horseradish peroxidase (HRPO) conjugate (Santa Cruz Biotechnologies, Dallas, TX) as a secondary antibody, and color development was conducted in diaminobenzidine (DAB)/H2O2 solution. Some sections were incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibody as a secondary antibody.

3. Results

3.1. Isolation and characterization of IntI protein

Preliminary western blot analyses of intestinal extracts of frogs showed that the anti-XEEL mAb 5G7 identified protein bands at about 40 kDa, which are typical sizes of the IntI family lectins. Hence, an attempt to purify the proteins from intestinal extracts by affinity chromatography on a Gal-Sepharose column was performed, according to the methods previously outlined for the isolation of the XEEL and XCL-1 proteins (Nagata, 2005; Nagata et al., 2013). Extracts from 20 to 28 LPS-stimulated frogs were applied on the column and yielded 20.2 ± 32.9 μg of the protein sample. In an SDS-PAGE analysis under reducing conditions, the purified lectin protein exhibited two major bands at 39 and 40.5 kDa, both of which were identified by western blotting using

![Fig. 1. Purification of Intl proteins. Intl proteins (Intls) were purified from the intestinal extracts by affinity chromatography and fractionated along with the extract (Ext) by SDS-PAGE under reducing conditions (R) or fractionated alone under non-reducing conditions (NR). Proteins in the gels were visualized by silver staining (Prt), and the Intls, by western blotting using the 5G7 mAb (W). The numbers on the left of gels represent protein sizes in kDa. The triangles on the right panel indicate major bands at about 420 kDa, 210 kDa, 70 kDa, 35.5 kDa, and 34 kDa.](image)
the 5G7 mAb (Fig. 1). Analysis of the protein under non-reducing conditions showed a major protein band at about 420 kDa and minor bands at 210, 70, 34, and 35.5 kDa that reacted with the 5G7 mAb. This finding suggests that the major form of the lectin protein is a dodecamer (12-mer) of subunit peptides that exhibit apparent sizes of 34 and 35.5 kDa under non-reducing conditions. The differences in apparent sizes of the subunit peptides under reducing and non-reducing conditions were probably due to the protein folding involving intra-polypeptide disulfide bridges.

Strips of the 39 and 40.5 kDa peptide bands were cut out of the PVDF membrane blot and the N-terminal amino acid sequences were determined. The resulting sequences of two peptides were identical, REXDQASVSE, where X was an unidentified residue. Using this amino acid sequence as a query, a protein search was performed with the Basic Local Alignment Search Tool (BLAST) on the *X. laevis* protein database of the National Center for Biotechnology Information (NCBI) webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The search returned several similar sequences, including XEEL and other Intl family proteins but no identical matches, suggesting novelty of the purified lectin protein.

3.2. Cloning and analysis of Intl cDNA

To clone the cDNA encoding the novel lectin protein in frog intestines, RT-PCR/RACE procedures were applied using degenerate oligonucleotide primers, the sequences of which were determined based on the highly conserved amino acid sequences among known

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Xenopus (Wesener et al., 2015). The Intl-3 and Intl-4 translates showed possibly did not alter the basic carbohydrate recognition structure. 

**Fig. 3.** Characterization of Intl-3 protein. (A) SDS-PAGE and Western blot analyses of rIntl-3. The purified rIntl-3 fraction was separated by SDS-PAGE under reducing (5% 2-ME), partially reducing (0.1% 2-ME), or non-reducing (0% 2-ME) conditions and the proteins were visualized by silver staining (Prt), and the rIntl-3, by western blotting (W). The triangles indicate the rIntl-3 protein and the asterisks, the non-relevant proteins. (B) Intl-3 is the predominant Intl in Xenopus intestines. Intestine extracts and rXEL, rXCL-1, rXCL-2, and rXCL-3a proteins were examined by western blotting using the 5G7 or 4G12 mAbs. (C) N-glycosylation of Intl-3. The purified Intl-3s, rIntl-3, and rXCL-2 were incubated with (+) or without (−) protein N-glycosidase (PNGase) and changes in the size were examined by western blotting.

Intl family proteins. Sequence analyses of the isolated cDNA clones revealed those encoding the known Intl family proteins, XCL-1, XCL-2, and XCL-3; a novel splicing isoform of XCL-3, designated XCL-3a (Supplementary Fig. S1); and two novel Intls, now designated Intl-3 and Intl-4 (Supplementary Figs. S2 and S3). Fig. 2A illustrates a comparison of the amino acid sequences of deduced Intl-3 and Intl-4 translates with that of the human Intl-1, the molecular structure of which has been best correlated with its carbohydrate recognition properties (Wesener et al., 2015). The Intl-3 and Intl-4 translated consisted of 340 and 335 amino acids that showed 67% and 63% sequence identity with that of human Intl-1, respectively. Both had a possible signal peptide, N-glycosylation residue, and fibrinogen-like domain. In the amino acid residues forming the carbohydrate recognition structure in human Intl-1 (Asn-243, Glu-244, Asn-260, Glu-262, His-263, Glu-274, Thr-288, and Tyr-297), both Intl-3 and Intl-4 had minor amino acid substitutions that possibly did not alter the basic carbohydrate recognition structure (Wesener et al., 2015). The Intl-3 and Intl-4 translates showed 61–79% and 59–68% amino acid sequence identities to known Xenopus Intl family proteins, respectively (Supplementary Fig. S4). Notably, the Intl-3 translate contained an amino acid sequence, REDGAVSVE, following the signal peptide that showed a perfect match with the N-terminal amino acid sequences of the 39 and 40.5 kDa peptides identified in intestines with 5G7 mAb. This fact indicates that Intl-3 was the major Intl in the intestines of X. laevis.

To estimate the evolutionary relationships among Intl-3, Intl-4, and the Intl family proteins in Xenopus, amphioxus, lamprey, zebrafish, mice, and humans, a joining-tree was constructed (Fig. 2B). Xenopus, mouse, and human Intls were split into two main branches: one branch contained Xenopus Intl-3, Intl-4, XCL-2, and mouse and human Intls; the other branch contained the remaining four Xenopus Intls. This suggests that the expansion of the Intl family occurred before divergence of these species and mammalian Intls evolved from one of these branches. Search for the Intl-3 and Intl-4 genes in the Xenbase J-strain X. laevis genome database 9.1, identified single genome sequences at chr7S:24354410-24366743 and chr8L:99454479-99444250, respectively.

To characterize the protein encoded by the isolated Intl-3 cDNA, the expression vector containing the entire coding region was transfected to human epithelial kidney cell line, 293T cells and the rIntl-3 protein was affinity-purified from the culture supernatants using a Gal-Sepharose column. When the purified rIntl-3 protein was analyzed by SDS-PAGE under non-reducing conditions, the rIntl-3 protein was found as a major band at about 420 kDa and a minor band at 70 kDa that were both reactive to the 5G7 mAb (Fig. 3A, left lane). In contrast, under reducing conditions (right lane), it was identified as a predominant 39 kDa protein. Under partially reducing conditions (middle lane), two major rIntl-3 bands appeared at 35–39 kDa and 78 kDa. These results indicate that the rIntl-3 protein consisted of a major 12-mer and a minor dimer of subunit peptides encoded by the cloned cDNA, suggesting that the Xenopus Intl-3 protein could be produced from a single gene.

Similarly, the recombinant Intl family proteins were produced by 293T cells following transfection of the recombinant expression vectors and compared with Intls purified from the frog intestinal extracts by western blotting. The results showed that among the recombinant Intl family proteins recognized by the 4G12 mAb, the rXEL, rXCL-2, and Intl-3 and Intl-4 proteins were recognized by the 5G7 mAb (Fig. 3B) and that Intl-3 is predominant among the intestinal Intls. When the purified intestinal Intl-3 was treated with N-glycosidase, the sizes were reduced from 39 kDa to 40.5 kDa–36 kDa and 37.5 kDa, respectively (Fig. 3C), indicating that the subunit peptides were N-glycosylated. The same treatment reduced the size of rIntl-3 from 39 kDa to 36 kDa, whereas it did not affect the size of the rXCL-2 protein at 33/34 kDa as estimated from its amino acid sequence, which was deduced from cDNA (Supplementary Fig. S4). The 40.5 kDa subunit that was absent in the rIntl-3 protein might be a splicing isoform or a product of alternative post-translational modification of the 39 kDa peptide.

**Fig. 4.** Expression of Intl-3 and Intl-4 in adult tissues and embryos. RT-PCR assays were performed using total RNA fractions isolated from the tissues indicated and embryos (stage 35 embryos on the left panel). The primers used and the expected sizes of the cDNA fragments amplified are shown in Supplementary Table S1. The assays without RT reaction resulted in no DNA amplification. ODC was a loading control.

3.3. Expression of Intl-3 and Intl-4 in adult tissues and embryos

To examine Intl-3 and Intl-4 expression in adult tissues and developing embryos, RT-PCR assays were performed using specific primers. Intl-3 expression was strong in the intestine; at
binding of Intl-3 to bacterial cells, LPS, and PGN is dependent on the presence of Ca\(^{2+}\). WhenIntl-3 was incubated with fixed E. coli cells in TBS-Ca containing various competitors, the binding was blocked most effectively with xylose (Xyl), and partially with LPS and PGN (Fig. 5B). However, the binding was not affected by glucose (Glc), LTA, PrtA, N-acetyl-N-galactosamine (GalNAc), and N-acetyl-N-glucosamine (GlcNAc). Intl-3 was incubated with Gal-Sepharose in TBS-Ca, and after washing, the beads were incubated in TBS-EDTA or TBS containing 100 mM of various saccharides or amino sugars. The competitively eluted Intl-3 was examined by western blotting and the intensities of the 39–40.5 KDa protein bands on the blot were quantified by densitometry. The bound Intl-3 was most efficiently released with EDTA and the pentoses Xyl and ribose (Rib), whereas arabinose (Ara) and the hexose Gal were less effective (Fig. 5C). The other hexoses such as fructose (Frc), Glc, and mannose (Man), disaccharides such as lactose (Lac), maltose (Mal), and melibiose (Mel), and amino sugars (GalNAc and GlcNAc) were even less competitive for elution.

To examine whether the Intl-3 binding could cause bacterial agglutination, fixed GFP-E. coli cells were incubated with the purified Intl-3 protein or Intl-3 (both 10 \(\mu\)g/ml) and observed under a fluorescence microscope. The results showed that as compared with the control (Fig. 6A), both the purified Intl-3 and Intl-3 preparations agglutinated the bacteria in TBS-Ca (Fig. 6B and E). This agglutination was abolished by addition of EDTA (Fig. 6C and F) or Xyl (Fig. 6D and G).

3.5. Injection of LPS increases intestinal Intl-3 content

As intraperitoneal injection of LPS has shown to increase XCL-1 contents in the serum and spleen (Nagata et al., 2013), whether or not it would also change Intl-3 content in the digestive tract was investigated. Three days after the LPS injection, digestive tracts were isolated, divided into 5 sections (Fig. 7A), and the extract of each section was examined by western blotting. In control APBS-injected frogs, Intl-3 content was highest in the intestinal sections (Fig. 7B, histogram). Although Intl-3 increased Intl-3 contents throughout the intestines and rectum, Quantitative analyses of the blots prepared from four APBS-injected and four LPS-injected frogs revealed 2.5–4 fold increases of Intl-3 contents in the intestinal and rectal sections (Fig. 7B, histogram). Although XCL-1 was barely detectable in control frogs, following LPS stimulation, it was identified in all the sections of the digestive tract. To examine the expression levels of Intl transcripts, total RNA fractions were isolated from the intestines of frogs and RT-PCR assays were performed. The expression of XCL-1, XCL-3, and Intl-3 exhibited definite increases following LPS stimulation, whereas the expression of XEEL, XCL-2, and Intl-4 appeared to remain unchanged (Fig. 7C).

Intl-3 contents in the sera and spleens of five APBS-injected and five LPS-injected frogs were compared. The results showed that LPS-stimulation increased XCL-1 contents in the serum and spleen (Fig. 8A), confirming a previous report (Nagata et al., 2013). However, Intl-3 was undetectable in both serum and spleen, regardless of LPS injection. When similar analyses were expanded to include other tissues, LPS stimulation resulted in increases in XCL-1 contents of the liver, lung, heart, muscle, in addition to the spleen and serum (Fig. 8B). In contrast, the Intl-3 protein was detected only in the intestine regardless of LPS stimulation, although mRNA was found in the brain, testis, stomach, and liver, as well as the intestine (Fig. 4), suggesting that the tissue Intl-3 contents might be regulated by not only transcription, but also by translation or post-translational modification steps.

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3.6. Immunohistochemical localization of Intl-3

Immunohistochemical and immunofluorescent examination using the 5G7 mAb were performed to localize the Intl-3 protein in frozen transverse sections of the digestive tract. The Intl-3 protein was not observed in the stomach (Fig. 9A); however, in comparison to the control section (Fig. 9I and J), it was clearly visible in the epithelium of intestinal and rectal villi (Fig. 9B–F). Immunoreactivity was stronger in the anterior intestine (Fig. 9B) and weaker toward the distal portion of the rectum (Fig. 9C), which appeared to be consistent with the results of western blot analyses (Fig. 7B).

Detailed observation showed more intense Intl-3 staining at the base of villi (Fig. 9D) in comparison to the apical portions (Fig. 9E). Higher magnification of the labeled cells revealed an accumulation of immunoreactivity in apical granules of the goblet cells and weak labeling in the cytoplasm (Fig. 9F). In the intestines of LPS-stimulated frogs, stronger Intl-3 immunoreactivity was observed in granules of the goblet cells (Fig. 9F). Preabsorption of the 5G7 mAb with rIntl-3 abolished the staining of granules and cytoplasm of the goblet cells (Fig. 9G), whereas the preabsorption control gave the staining similar to the non-preabsorbed mAb (Fig. 9H), supporting that the 5G7 mAb visualized Intl-3 on the intestinal sections. Staining with the anti-XCL-1 mAb 4C8 or the control mAb XT-1 yielded no noticeable labeling in the intestine of a LPS non-stimulated frog (Fig. 9I and J). Similar to the immunohistochemical assay, the 5G7 mAb specifically visualized the Intl-3 immunoreactivity in immunofluorescent staining (Fig. 9K and L). In this staining, multiple fluorescent granules were clearly visible in apical cytoplasm of the goblet cells (Fig. 9K).

4. Discussion

The present findings of Intl-3 and Intl-4 transcripts add novel members to the list of X. laevis Intl family lectins and present previously unidentified counterparts of Intls expressed in the intestines of mammals (Komiya et al., 1998; Tsuji et al., 2001; French et al., 2009), fish (Russell et al., 2008), and amphioxus (Yan et al., 2012). Intl-3 was shown to be the major Intl in Xenopus digestive tracts, by virtue of its cross-reactivity with the anti-XEEL mAb 5G7. Although Intl-4 transcripts were distributed among various tissues, analyses of the Intl-4 protein were not performed in the present study because the specific probe was unavailable. The Intl-3 protein is highly homologous to other chordate Intl family proteins in terms of overall amino acid sequences and basic structural characteristics, i.e., an oligomeric (probably 12-meric) structure consisting of Nglycosylated subunit peptides containing a conserved fibrinogen-like domain (Lee et al., 2004). Human Intl-1 is a trimer of subunit proteins that are linked by disulfide bonds with Cys-31 and Cys-46 in the N-terminal region (Tsuji et al., 2009; Wesener et al., 2015). Xenopus Intl-3 probably uses Cys-21, Cys-39, or Cys-68 for oligomerization within a similar region.

Consistent with a previous report on XCL-1 (Nagata et al., 2013), Intl-3 binds to the surface of bacterial cells depending on the presence of Ca2+. This binding is blocked by saccharides such as Xyl. In addition, similar to XEEL (Wangkanont et al., 2016), the Intl-3 binding caused agglutination of the bacterial cells. These results suggest that Intl-3 crosslinks bacterial surface glycans through its multivalent carbohydrate binding. Immunohistochemical analysis demonstrated an accumulation of Intl-3 protein in secretory mucous granules of intestinal and rectal goblet cells and its levels increased shortly after intraperitoneal injection of LPS. Thus, as has been suggested with mammalian Intls, Intl-3 is most likely involved in the innate immune function of mucus secretions that protect the walls of the digestive tract against pathogenic bacteria and parasites (Komiya et al., 1998; Pemberton et al., 2004).

Mammalian Intl expression has been shown to increase in...
intestinal and airway epithelia following parasitic infection (Pemberton et al., 2004; Datta et al., 2005) and allergen-induced inflammation (Gu et al., 2010; Kerr et al., 2014), respectively. In the present study, intraperitoneal injection of LPS, a prominent inflammatory substance from the outer membrane of Gram-negative bacteria, caused increased production of Xenopus Int-3 protein in the intestinal goblet cells and XCL-1 protein in the spleen, serum, and several other tissues (Nagata et al., 2013). Although little is known about the signals regulating tissue Int-3 levels, these findings suggest that Int-3 production is stimulated by cytokines or chemokines secreted by inflammatory cells migrating from local inflammation sites. Increasing knowledge has been accumulated on detailed signaling mechanisms in intestinal epithelia that are triggered by the recognition of pathogenic microbes and lead to the induction of inflammatory and innate immune responses (Wells et al., 2011). In particular, a recent study (Courth et al., 2015) reported that monocYTE-derived cytokines are potential stimulators of defensin gene expression in intestinal Paneth cells that are known to produce Intls in mice and humans (Komiya et al., 1998; Wrackmeyer et al., 2006). Since the present data show that variation in the levels of Intl family gene transcripts does not necessarily parallel with the levels of corresponding proteins, tissue or cellular Intl contents are probably regulated at various steps, including transcription, translation, and post-translational modification.

A recent study employing X-ray crystallography established the fine structural basis of carbohydrate recognition by the human Intl-1 (Wesener et al., 2015). For carbohydrate binding, human Intl-1 uses the C-terminal region of each subunit peptide, but does not use the fibrinogen-like domain as had been previously assumed (Lee et al., 2004). In the C-terminal region, several critical amino acids and a Ca$^{2+}$ coordinate to form a flexible target recognition structure that interacts to a terminal acyclic 1,2-diol, which is a common feature unique to bacterial glycans. This provides remarkable selectivity of the human Intl-1 in the recognition of microbial over mammalian cell surface glycans. The C-terminal region of Xenopus Int-3, as well as those of XEEL and XCL-1, are
highly homologous to that of human Intl-1 and functionally conserve critical amino acids. These facts are consistent with the findings that the profiles of carbohydrate recognition specificity of these *Xenopus* lectins are similar to those of the human Intl-1, i.e., they bind to pentoses such as Xyl, Rib, and Ara with a higher affinity than they do to the hexoses Frc, Gal, and Glc, or to the amino sugars GalNAc and GlcNAc (Tsuji et al., 2001; Nagata, 2005; Nagata et al., 2013). Thus, *Xenopus* Intl-3, as well as XCL-1, XEEL, and human Intl-1, may function as microbial recognition molecules in the innate immune system.

Despite its putative selectivity in microbial recognition and capacity for multivalent binding, detailed functions of Intl-3 and other intestinal Intls are yet to be determined. Following secretion from the epithelia, Intl-3 may entrap and immobilize microbes on the intestinal mucosa to prevent them from making contact with the surface of epithelial cells. On the other hand, if microbial invasion occurs beneath the epithelia, Intl-3 may act as a tag that would facilitate the clearance of tagged microbes by the activation of phagocytic macrophages (Tsuji et al., 2009). Alternatively, by analogy with XCGL, which aggregates carbohydrate chains in the egg jelly coat and forms a layer that is impenetrable to sperm (Wyrick et al., 1974), Intl-3 may crosslink carbohydrate chains of...
mucus to form a dense barrier that prevents microbes from penetrating the mucosa.

The human Intl-1 (omentin) reportedly regulates vascular endothelial cell function (Maruyama et al., 2012) and steroidogenesis by ovarian granulosa cells (Cloix et al., 2014). It has also been suggested that human Intl-1 modulates the action of insulin on target cells (Yang et al., 2006), and has tumor suppressor activity (Mogal et al., 2007). In the aforementioned studies, recombinant Intl-1 was expressed in vivo, or added to the cell culture in vitro, and the biochemical or morphological changes were examined in the putative target tissues or cells. The receptors for Intl-1, however, have not been identified on its target cells, and it is unknown whether the action of Intl-1 is dependent on carbohydrate recognition. Other studies have reported that human Intl-1, a designated lactoferrin receptor and GPI-anchored membrane protein, could bind and transport lactoferrin across the cell membrane (Wrackmeyer et al., 2006; Suzuki et al., 2008; Akiyama et al., 2010). These studies have also not been able to correlate lactoferrin transportation by Intl-1 with carbohydrate recognition activity.

Further studies should focus on the molecular mechanisms involved in the diverse physiological functions of Intls, particularly the putative roles and characteristics of carbohydrate recognition.

It seems that Intl-3 is secreted from the intestinal epithelia and is possibly assimilated into the overlying mucus, whereas XCL-1 is secreted into the circulation. In contrast, human Intl-1 is present in both intestinal secretions and blood plasma (Tsujii et al., 2009), suggesting that the physiological roles of human Intl-1 may be similar to those of both Intl-3 and XCL-1 in Xenopus. In addition, as reported thus far, XCLG and XEEL are the lectins unique to Xenopus, suggesting that they might have evolved through functional divergence of the Intl family, for adaptation to the environment in which fertilization and development of the frog occurs. These findings partially illustrate that Xenopus has a relatively large Intl family now consisting of at least eight lectins, as compared to the human and mouse Intl families that consist of only two lectins each.

To elucidate the diverse physiological roles of the Intl family, deeper insights into the Xenopus Intls, including the identification and characterization of XCL-2, XCL-3, and Intl-4 proteins, would be helpful.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.devimm.2016.02.006

References


