We have characterized pufflectin, a novel mannose-specific lectin, from the skin mucus of the pufferfish, *Fugu rubripes*. Molecular mass estimations by gel filtration and matrix-assisted laser desorption ionization time-of-flight mass spectrometry and the SDS-PAGE pattern suggest that pufflectin is a homodimer composed of non-covalently associated subunits of 13 kDa. The full-length pufflectin cDNA consists of 527 bp, with 116 amino acid residues deduced from the open reading frame. The amino acid sequence of pufflectin shows no homology with any known animal lectin. Surprisingly, pufflectin shares sequence homology with mannose-binding lectins of monocotyledonous plants and has conserved two of three carbohydrate recognition domains of these plant lectins. The pufflectin gene is expressed in gills, oral cavity wall, esophagus, and skin. In addition, an isoform occurs exclusively in the intestine. Pufflectin differs from mannose-binding lectins purified from the blood plasma of *Fugu*. Whereas pufflectin did not agglutinate five bacterial species tested, it was demonstrated to bind to the parasitic trematode, *Heterobothrium okamotoi*. This finding suggests that pufflectin contributes to the parasite-defense system in *Fugu*.

The integument of fishes is covered with mucus (1), just as the inner surface of the mammalian gut. In mammals, the peculiar mucosal immune system serves as an extra-epithelial first line of defense against pathogens that originate from the contents of the intestine (2, 3). Because water is a perfect medium for bacteria and parasitic microbes, the fish integument is constantly exposed to pathogen attack. It is generally believed that the barriers against pathogen invasion exist in the surface of the fish body and that cutaneous mucus serves as a mechanical as well as a biochemical barrier (1). However, the biochemical details are poorly understood. Various humoral defense factors such as immunoglobulin, complement, C-reactive protein, lysozyme, and hemolysin have been detected in the skin mucus of fish (1, 4). Lectins, carbohydrate-binding proteins that are neither antibodies nor enzymes (5, 6), are also present in the mucus (1, 4) and might function in pathogen defense.

**EXPERIMENTAL PROCEDURES**

**Preparation of Skin Mucus Extract—*Fugu* were supplied by Fukui Prefecture Fisheries Experimental Station (Japan). The skin mucus was scraped off with a spatula and was homogenized with an equal volume of phosphate-buffered saline (pH 7.4) containing 0.9 mM CaCl$_2$ and 0.33 mM MgCl$_2$ (**PBS**($^+$) buffer). The homogenate was centrifuged at 15,000 × *g* for 30 min at 4 °C, and the supernatant (crude skin mucus extract) was further analyzed.

**Hemagglutination Tests—**Serial 2-fold dilutions of the samples (25 °C) were assayed for agglutination on microtiter plates coated with five bacterial species. Agglutination was considered to occur when visible clumping was observed in the final dilution.

Fish species with lectins in the integumental mucus include the windowpane flounder *Lophopsetta maculata* (7), the ling *Genypterus blacodes* (8), the Arabian Gulf catfish *Arius thalasinus* (9), the Oriental catfish *Plotosus lineatus* (10), the conger eel *Conger myrista* (11, 12), the dragonet *Repomucenus richardsonii* (13), the loach *Misgurnus anguillicaudatus* (14), the kingklip *Genypterus capensis* (15), and the Japanese eel *Anguilla japonica* (16). Lectins are probably present in the skin mucus of many additional species, because the mucus of many fishes induces hemagglutination, a property typical of lectins (17). Despite this apparent ubiquity of mucus lectins, experimental data that elucidate their assumed function in pathogen defense are scarce.

On the basis of the structure of the carbohydrate recognition domain, animal lectins are divided into four main groups: C-type (20), galectins (21, 22), P-type (23), and I-type (24). So far, sequences of mucosal lectins have been determined in only two species of the Anguilliformes, namely the conger (congerin I and II) and the Japanese eel (AJL-2). Congerin I and II were classified as galectins (18, 19); this class probably dominates among the skin mucus lectins because many of them interact specifically with galactose or lactose. On the other hand, AJL-2 has a sequence typical of C-type lectins (16). Lectins of ling and kingklip might belong to the same class because of the Ca$^{2+}$-dependence of their activity and their typical SDS-PAGE pattern (8, 15). However, it is questionable whether all skin mucus lectins belong to the galectins or the C-type. Unfortunately, the sound knowledge of lectin structure that is necessary to establish the biological role and the functional mechanisms of lectins in the fish integumental mucus is mostly lacking to date.

The pufferfish, *Fugu rubripes*, is a model species in which the genome sequencing project has reached an advanced stage (25). We have studied the mucus of the skin and intestine of this species and found interesting lectins that resemble those of monocotyledonous plants. Here we report the primary structure, tissue-specific expression, and biochemical characteristics of these lectins. In addition, we describe binding of the lectin to *Heterobothrium okamotoi*, a serious parasite of the *Fugu* (26).
with PBS(+) buffer were made on microtiter plates with 96 wells. Twenty five µl of a 5% suspension of rabbit red blood cells purchased from commercial dealers were added to each well. After 1 h of incubation at room temperature, samples were checked for hemagglutination. The lectin titer was expressed as the greatest sample dilution in which agglutination could be observed.

Partial Purification by Ion-exchange Column Chromatography—The crude skin mucus extract was analyzed by fast protein liquid chromatography (FPLC, Amersham Biosciences) on a Poros HQ/M (4.8 × 100 cm, PerSeptive Biosystems) ion-exchange column with a linear gradient of 0–1 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 1 ml/min. The eluted proteins were monitored by absorbance at 280 nm, and hemagglutination activity was tested in each 1 ml fraction. Active fractions were pooled, concentrated and concentrated for determination of carbohydrate specificity.

Determination of Specific Sugars—Sugar-dependent inhibition of lectin-induced hemagglutination was determined to establish specific sugar–lectin interactions. Twenty five µl of the active fraction identified by FPLC was serially 2-fold diluted with PBS(+) buffer containing 200 mM of either α-glucose, α-galactose, α-mannose, α-fucose, N-acetyl-α-glucosamine, N-acetyl-α-galactosamine, maltose, lactose, saccharose, or melibiose in microtiter plates. After incubation at 4 °C for 1 h, 25 µl of a 5% rabbit erythrocyte suspension was added to each well, and the lectin titer was determined after 1 h as detailed above. Lectin Isolation by Affinity Chromatography—α-Mannose, which specifically interacted with the mucus lectin, was coupled to epoxy-activated Sepharose 6B (Amersham Biosystems) as a ligand according to the manufacturer’s instructions. In brief, the epoxy-activated Sepharose 6B matrix was incubated with 3 mM α-mannose in NaOH solution containing 0.5 M NaCl (pH 11.5) at 40 °C for 16 h; then the remaining active sites on the gel were blocked with 1 mM ethanolamine (pH 8.0). The crude skin mucus extract was pumped at 10 ml/h through a column (1 × 10 cm) packed with the gel that had been pre-equilibrated with PBS(+) buffer. Afterward, the column was washed with PBS(+) buffer to remove unabsorbed material. Then proteins were eluted from the column with PBS(+) buffer containing 50 mM α-mannose and were collected manually by monitoring the absorbance of the eluate at 280 nm.

Enzymatic Digestion and Amino Acid Sequencing—Enzymatic digestion was performed using 4% polyacrylamide isoelectric focusing gel containing 8 M urea and 2% ampholine (pH 3.5–10) for 16 h with 300 mM of rabbit skin mucus extract (0.5 ml) in the presence of 0.5 ml of a 5% solution of lysyl endopeptidase (Wako) at an enzyme to substrate ratio of 1:20 (w/w) at 37 °C for 16 h. The digest was fractionated by high performance liquid chromatography (HPLC, Hitachi) on a TSKgel ODS-120T reversed-phase column (4.6 × 250 mm) ( Tosoh) using a linear gradient of acetonitrile (0–80%) in 0.05% trifluoroacetic acid. The flow rate was 1 ml/min. Amino acid sequences of digested fragments were determined using a model 470A protein sequencer (PerkinElmer Life Sciences).

Mucosal Lectins of the Pufferfish—Total RNA was extracted from the skin of three individuals using RNA extraction solution (Isogen, Nippon gene). First strand cDNA was synthesized from the total RNA with the SMART® RACE cDNA amplification kit (Clontech) for 5′- and 3′-rapid amplification of cDNA ends (RACE). Based on the amino acid sequences of the fragments LP-5 and LP-13 (described under “Results”), two degenerate primers were designed: LP-13F, 5′-GA/AGA/ATATA/CT/GC-IC/AT/T/GTTT/C/A-3′, and LP-5R, 5′-CAT/C/CA/GTG/AG/TIC/GA/T-A/G/TIC/CG/AT/GTTT/C-3′. The first strand cDNA was amplified by PCR (i-Cycler, Bio-Rad) in 20 µl of a reaction mixture containing 5 units of Taq DNA polymerase (Takara), 5 µM of LP-5R (Clontech), and 500 nM of LP-13F primer for 3′-RACE or LP-5R primer for 5′-RACE, respectively. Amplification was performed in 40 cycles consisting of 94 °C for 5 s, 60 °C for 10 s, and 72 °C for 2 min; the final elongation reaction was conducted at 72 °C for 10 min. To confirm independently the sequence of the 5′-untranslated region, which had been found to lack a signal peptide, first strand cDNA was synthesized from 1 µg of skin total RNA of another individual using the GeneRacetm kit (Invitrogen) for 5′-RACE. Reverse transcription was performed after 60 s at 90 °C with Thermostar (Invitrogen) to remove any RNA secondary structure. GeneRacetm 5′ primer and the specific primer FML-5′-RACE, 5′-CT/CTTGAGCCTAGCAGCC/TC/TCTC-3′, which was synthesized on the basis of the determined sequence, were used for PCR amplification.

The nucleotide sequence obtained by 3′- and 5′-RACE over the coding region was confirmed by PCR amplification with the two specific primers FML-SP-2 (5′-ATC/TCC/CTGC/TGCT/GAG/GTA/TCA-3′) and FML-AP4 (5′-GT/ATGAGAAGTTGGC/TTC-3′). Denaturation was performed at 94 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. The resulting PCR product was also used as a probe for Northern blot analysis, and the above-mentioned primers were employed for the RT-PCR analysis described below.

Each PCR product generated was ligated into the pCR®-2.1-TOPO vector (Invitrogen). The plasmid DNA was purified and sequenced using the Applied Biosystems DNA sequencing kit (Applied Biosystems). Sequences were analyzed using the SeqEd™ version 1.0.3 software (Applied Biosystems). Analyses were repeated with at least five independent PCRs to avoid PCR errors.

Homologs of the deduced amino acid sequence were searched with the BLAST 2.0 program accessed via GenomNet (blast.genome.ad.jp/). Multiple alignment of sequences was conducted using the ClustalW Multiple Sequence Alignment Program version 1.8 (clustalw.genome.ad.jp/).

Reverse Transcription-PCR Analysis—Total RNA from muscle, liver, heart, kidney, head kidney, spleen, brain, gonad, Gill, oral cavity wall, esophagus, intestine, and skin was isolated from three individuals as described above. Each total RNA sample was treated with the SMART® RACE cdNA amplification kit. PCR amplification was performed in a total volume of 20 µl with Taq DNA polymerase and specific primers, FML-SP2 and FML-AP4 (500 nM each), for 25 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. Alternatively, the above oligonucleotide primers were synthesized based on the sequence of the intestine-type isoform: FIL-SP1, 5′-CAGTCTTGGAGAAT-3′; and FIL-AP2, 5′-I(C/T)TITTTCA-3′. The PCR products were also described with the SMART® RACE cdNA amplification kit. PCR amplification was performed in a total volume of 20 µl with Taq DNA polymerase and specific primers, FML-SP-2 and FML-AP4 (500 nM each), for 25 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. The resulting oligonucleotide primers were synthesized based on the sequence of the intestine-type isoform: FIL-SP1, 5′-CAGTCTTGGAGAAT-3′; and FIL-AP2, 5′-ATCGAG/CTGAGCAGGGCATTGTTGC/TTC-3′. These were used to detect the expression of the intestine-type isoform. The protocol of PCR amplification was the same as described above. The PCR products were analyzed by 2.0% agarose gel electrophoresis and staining with ethidium bromide.

Multiple Sequence Alignment Program version 1.8 (clustalw.genome.ad.jp/).
Characterization of Hemagglutination Activity of the Lectin—To examine divalent cation requirements of the purified lectin, serial 2-fold dilutions of the lectin having hemagglutination titer values of $2^6$ (25 μl) was made with PBS(−) buffer containing either 50 mM EDTA or 50 mM CaCl$_2$. After 1 h incubation with 25 μl of 5% rabbit erythrocyte suspension, the lectin titer was measured as detailed above.

The inhibitory effects of saccharides on the hemagglutination activity of the lectin were assayed as follows. Twenty five μl of the saccharide solutions tested in this study were diluted 2-fold in series on microtiter plates with PBS(−) buffer and incubated with the same volume of lectin solution diluted to a titer of $2^6$ (25 μl) for 1 h at room temperature. Then 50 μl of a 5% rabbit erythrocyte suspension were added to each well and incubated for 2 h at room temperature, and the concentration of each saccharide leading to complete inhibition was determined.

Agglutination Test with Bacteria—Five strains of fish pathogenic bacteria (provided from the National Research Institute of Aquaculture, Fisheries Research Agency, Japan) were tested for agglutination with affinity-purified lectin. The Gram-negative bacterium, *Aeromonas hydrophila* FPC 866, *Edwardsiella tarda* FPC 499, and *Pseudomonas plecoglossicida* FPC 337 were cultured in tryptic soy broth (TSB), and *Vibrio anguillarum* FPC 675 was grown in TSB containing 2% NaCl. The Gram-positive *Streptococcus difficile* FPC 576 was cultured in brain heart infusion with 0.5% glucose. The number of cells was estimated by measuring colony-forming units/ml. The cells were collected and suspended at ~$10^9$ colony-forming units/ml in PBS(+) buffer. Aliquots of these cells were mixed with equal volumes of affinity-purified lectin at a concentration equivalent to a titer of $1 \times 10^6$ as determined in hemagglutination tests with rabbit erythrocytes. After incubation at room temperature for 1 h, agglutination was observed under a light microscope BX60 (Olympus).

Binding Test with Parasite—Fluorescence-labeled lectin was prepared using the Alexa Fluor® 488 protein labeling kit (Molecular Probes) according to the manufacturer’s directions. By using tweezers, the adult *H. okamotoi* (about 1 cm long), which is a monogenean trematode that parasitizes *Fugu* (26), was excised from the gill chamber of infected fish. Parasites were fixed with 0.5% formalin in PBS(−) buffer. Aliquots of these cells were mixed with equal volumes of affinity-purified lectin at a concentration equivalent to a titer of $1 \times 10^6$ as determined in hemagglutination tests with rabbit erythrocytes. After incubation at room temperature for 1 h, agglutination was observed under a light microscope BX60 (Olympus).
lectin and were placed on glass slides for observation under a fluorescence microscope BX60 (Olympus).

RESULTS

Detection of Sugar Specificity—First, we used crude skin mucus extract in hemagglutination inhibition experiments to test for specific lectin-sugar interaction. However, none of the sugars tested inhibited lectin hemagglutination activity. Therefore, we produced partially purified lectin by ion-exchange chromatography. Hemagglutination activity against rabbit erythrocytes was detected in the fraction eluted by 0.25–0.265 M NaCl in Tris-HCl buffer (Fig. 1); it was inhibited exclusively by D-mannose (Table I).

Purification of Skin Mucus Lectin—A single protein peak was obtained from the crude skin mucus extract of Fugu by D-mannose affinity chromatography when the medium was changed to PBS(+/H11001/100) buffer containing 50 mM D-mannose (Fig. 2). The peak fraction showed hemagglutination activity (data not shown).

In both unreduced and reduced SDS-PAGE, the affinity-purified fraction yielded a protein band of 13–14 kDa, which was one of the major soluble proteins in the skin mucus (Fig. 3). This protein, termed pufflectin, was negative to periodic acid-Schiff staining (data not shown), suggesting that it is not glycosylated. The isoelectric point of the lectin was 6.42 (data not shown).

The molecular mass of pufflectin was determined to be ∼26 kDa by gel filtration, whereas MALDI-TOF mass spectrometry suggested a mass of about 13.05 kDa (Fig. 4). Together with the results from SDS-PAGE, these findings suggest that native pufflectin is a dimer of identical 13–14-kDa subunits, which are non-covalently linked.

Amino Acid Sequence Analysis—Affinity-purified pufflectin was subjected to Edman degradation, but no sequence could be
determined, indicating that the N terminus was blocked. Therefore, we analyzed internal fragments obtained by enzymatic cleavage. Four fragments separated by reversed-phase HPLC (LP-2, -5, -10, and -13; Fig. 5) were scrutinized, and two internal amino acid sequences could be established (LP-5: NH2-Asp-Gly-His-Gln-Leu-Trp-Asn-Ser-Asp-Gly-His-Gly-Met-COOH; LP-12: NH2-Trp-Ile-Ala-Leu-Phen-Gln-COOH).

Molecular Cloning—The partial cDNAs encoding pufflectin including the 5′- and 3′-untranslated regions were isolated by 5′- and 3′-RACE methods, respectively. The overlapping sequence was confirmed by PCR amplification with two specific primers, FML-SP2 and FML-AP4. The resulting pufflectin cDNA sequence consisted of 527 bp, including 59 bp of 5′-untranslated region, 348 bp of open reading frame, a stop codon (TGA), and 120 bp of 3′-untranslated region containing a polyadenylation signal (Fig. 6). The open reading frame encoded 116 amino acids with a calculated molecular mass of 13,133 Da, which was in good agreement with the conclusions drawn from the biochemical analyses discussed above. There were no ATG translation start sites upstream of the open reading frame, indicating that this lectin did not possess a signal peptide. This feature is the common characteristic of the galectins, a super-family of animal lectins that bind to β-galactosides (21, 28).

Homology Analysis—A data base search indicated that the amino acid sequence of pufflectin was not similar to other animal lectins but to mannos-binding lectins of monocotyledoneous plants such as common snowdrop, Galanthus nivalis (29). Narcissus hybrid cultivar (GenBank™ accession M88120), Clivia miniata (30), leek, Allium prorrum (31), and garlic, Allium sativum (32) (Fig. 7). The highest degree of homology was found in the common snowdrop lectin, G. nivalis agglutinin (29.4% identity).

RT-PCR Analysis—To establish the organ specificity of pufflectin expression, total RNA from different tissues was reverse-transcribed into cDNA. The PCR amplification of the cDNA provided bands of the predicted size (432 bp) in gill, oral cavity wall, esophagus, and skin but not in muscle, liver, heart, kidney, head kidney, spleen, brain, gonads, and intestine (Fig. 8A). There were no differences in the expression patterns between the three individuals tested. The PCR products amplified from positive tissue samples were subcloned and sequenced and were found to be the pufflectin cDNA partial sequences.

Northern Blot Analysis—Pufflectin mRNA was present in gill, oral cavity wall, esophagus, intestine, and skin but not in muscle, liver, heart, kidney, head kidney, spleen, brain, and gonads as detected by Northern blot analysis (Fig. 9). The relative gene expression in the skin was higher than in the other positive tissues. The expression pattern was identical in the three individuals tested.

Isolation of Isoforms—The organ specificity of expression patterns determined by two alternative methods was reversed except for the intestine, which was positive in the Northern blot analysis but negative in the RT-PCR-based approach. This result could be explained by the existence of an intestine-specific isoform of pufflectin. We attempted to isolate this isoform by 3′- and 5′-RACE using degenerate primers (LP-13F and LP-5R, respectively), and we were able to isolate the cDNA encoding the intestine-type pufflectin (Fig. 10). In its nucleotide sequence, adenine 9, guanine 31, and thymine 432 residues were found in pufflectin (Fig. 10). Therefore, neither of the primers used in RT-PCR analysis (FML-SP2 or FML-AP4) could recognize this isoform. The deduced amino acid sequence of the isoform showed 91.4% identity with pufflectin.

Expression of the intestine-type isoform gene in muscle,
liver, heart, kidney, head kidney, spleen, brain, gonad, gill, oral cavity wall, esophagus, intestine, and skin was examined by RT-PCR analysis. However, the isoform was detected exclusively in the intestine (Fig. 8B).

Effects of Divalent Cation and Sugars on Pufflectin—The hemagglutination activity of pufflectin was not altered by the addition of either EDTA or CaCl₂, indicating that pufflectin-induced agglutination does not require calcium.

Inhibition of hemagglutination activity via the addition of sugars occurred with D-mannose at a concentration of 125 mM (Table II). Other saccharides showed no inhibition at concentrations of 500 mM.

Bacterial Agglutination—At a concentration equivalent to the titer of 2⁵ that was fully effective in inducing rabbit erythrocyte hemagglutination, pufflectin showed no agglutination activity against any of the five bacterial species tested (see under “Experimental Procedures” for list of species).

Binding to Parasitic Trematode—H. okamotoi treated with Alexa-labeled pufflectin in blocking solution showed pronounced fluorescence on the surface of its body, which was reduced substantially in the presence of 1 mM D-mannose (Fig. 11).

DISCUSSION

In the present study, we have characterized pufflectin, a novel lectin from the integumental mucus of Fugu. Pufflectin interacts specifically with D-mannose, whereas other fish skin mucus lectins characterized so far mostly are specific for either D-galactose or lactose (33). Intriguingly, the amino acid sequence of pufflectin does not resemble that of any known animal protein but shows relatively high homology to mannose-binding lectins of monocotyledonous plants. The common snowdrop (G. nivalis) lectin, G. nivalis agglutinin, possesses three mannose-binding motifs (Gln-X-Asp-X-Asn-X-X-X-Tyr; boxed in Fig. 7), which are conserved among mannose-specific

![Fig. 8. RT-PCR analysis of organ specificity of pufflectin (A) and its intestine-type isoform (B). Total RNA from muscle, liver, heart, kidney, head kidney, spleen, brain, gonad, gill, oral cavity wall, esophagus, intestine, and skin was isolated and reverse-transcribed to cDNA. PCR amplifications were performed with specific primers, FML-SP2 and FML-AP4 (A) or FIL-SP1 and FIL-AP2 (B), respectively. Amplification products and DNA marker were electrophoresed in 2% agarose gel and stained with ethidium bromide.](image)

![Fig. 9. Northern blot analysis of tissue specificity of pufflectin gene expression. Total RNA from each tissue indicated (10 µg) was electrophoresed on a 1.5% formamide-agarose gel, blotted onto a nylon membrane, and probed with alkali phosphatase-labeled pufflectin cDNA.](image)

![Fig. 10. Comparison of nucleotide and deduced amino acid sequences between pufflectin and its intestine-type isoform. The cDNA of the isoform was isolated from intestine total RNA using 3'- and 5'-RACE using degenerate primers, LP-13F and LP-5R. Asterisks highlight mutated bases; altered amino acids are circled in the isoform. Arrows indicate locations and orientation of primers used in the RT-PCR.](image)
Served in the intestine-type isoform as Gln30, Asp32, Asn34, and skin. An intestine-specific isoform was also identified in equivalent to a titer of 1:26 for that room temperature. The concentration of each saccharide leading to complete inhibition was determined. The buffer and incubated with the lectin solution at a concentration PBS(+/−) examined. Saccharide solutions were diluted 2-fold in series with the first intestinal lectin shown to be homologous to a skin species. Therefore, the intestine-type isoform of pufflectin is (41), but skin mucus lectins have not been studied in this hand, a lactose-specific lectin, GJL-I, was isolated from stom-

mucus lectin. This finding suggests that the skin and the in-

expression patterns of mucosal lectins has been limited to the external surface tissues, i.e., the skin, oral cavity wall, esophagus, and skin. An intestine-specific isoform was also identified in this study. The two putative mannose-binding sites are conserved in the intestine-type isoform as Gln30, Asp32, Asn34, Tyr38, and Gln54, Asp56, Asn58, Tyr62 (Fig. 10), suggesting that the isoform also possesses lectin activity. These facts are well explained by the duplication-degeneration-complementation model, which predicts that pairs of duplicated genes share the ancestral functions but are expressed differentially in the various domains in which the ancestral gene was expressed uniformly before the duplication (38). Previously, information on expression patterns of mucosal lectins has been limited to the Anguilliformes. Congerins in the conger eel are expressed in skin, gills, and the upper alimentary canal from the oral cavity to the esophagus, but not in stomach and intestine (39). In the Japanese eel, the lactose-binding lectin, AJL-I, is produced in the skin only (16), and the two galactose-specific C-type lectins, eCL-1 and eCL-2, are expressed exclusively in the gills (40). None of these lectins is present in the intestine. On the other hand, a lactose-specific lectin, GJL-I, was isolated from stomach and intestine of the toxic moray eel, Gymnothorax javanicus (41), but skin mucus lectins have not been studied in this species. Therefore, the intestine-type isoform of pufflectin is the first intestinal lectin shown to be homologous to a skin mucus lectin. This finding suggests that the skin and the integuments of digestive organs share common defense mechanisms in Fugu.

Pufflectin did not induce agglutination in any of the five bacterial species investigated in this study. A number of fish skin mucus lectins were reported to possess agglutination activity against bacteria, but the activity was specific for certain bacterial species. For instance, skin mucus lectins of windowpane flounder agglutinated Micrococcus marina and Metschnikowia reukaufi, but did not affect six other strains (7). Therefore, effects of pufflectin on a greater number of bacterial species including virulent and avirulent strains should be explored before general conclusions regarding the role of pufflectin in the defense against bacterial invasions are drawn.

Pufflectin is the first fish mucosal lectin that is demonstrated to bind to parasitic metazoans. Future functional studies therefore should take into account the possibility that the action of mucosal lectins is directed not only against bacteria but also against eucaryotic parasites. H. okamotoi is restricted to the gill chamber and is not observed on the skin in Fugu (42). This might be explained by the finding that pufflectin is less expressed in gills as compared with skin (Fig. 9). However, H. okamotoi frequently colonizes Fugu, indicating that it has evolved mechanisms that enable it to overcome the putative pufflectin-based defense system. In order to resolve this hy-
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pothesis, the mechanism of pufflectin activity will have to be investigated in comparative studies on various parasite and fish species.

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