Structure, properties and enhanced expression of galactose-binding C-type lectins in mucous cells of gills from freshwater Japanese eels (*Anguilla japonica*)

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Using a Japanese-eel (*Anguilla japonica*) gill cDNA subtraction library, two novel β -D-galactose-binding lectins were identified that belong to group VII of the animal C-type lectin family. The eel C-type lectins, termed eCL-1 and eCL-2, are simple lectins composed of 163 amino acid residues, including a 22-residue signal peptide for secretion and a single carbohydrate-recognition domain (CRD) of ~ 130 residues typical of C-type lectins. The galactose specificity of the CRD was suggested by the presence of a QPD motif and confirmed by a competitive binding assay. Using Ruthenium Red staining, the lectins were shown to bind Ca^{2+} ions. SDS/PAGE showed that native eCL-1 and eCL-2 have an SDS-resistant octameric structure (a tetramer

of disulphide-linked dimers). Northern and Western blot analyses demonstrated high-level expression of eCL-1 and eCL-2 mRNAs and their protein products in gills from freshwater eels, which decreased markedly when the eels were transferred from freshwater to seawater. Immunohistochemistry showed that the eel lectins are localized in the exocrine mucous cells of the gill.

Key words: asialofetuin, carbohydrate-recognition domain, immunohistochemistry, octamer.

INTRODUCTION

The animal lectins are a group of carbohydrate-binding proteins that are involved in a variety of processes, including the innate immune response critical for the detection and elimination of infectious micro-organisms [1,2]. They can be classified into two categories: calcium-dependent animal lectins (C-type lectins) and calcium-independent animal lectins. Most members of the latter group require reducing conditions for activity and are called S-type lectins (S for sulphhydryl-dependent), or alternatively galectins, based on their galactose-binding specificities. At the molecular level, the members of the former group, C-type lectins, are defined as proteins that have at least one carbohydrate-recognition domain (CRD) of ~ 130 amino acid residues, including 14 invariable and approx. 18 highly conserved residues [3,4]. Members of the C-type lectin family can be divided further into the following seven subgroups based on their structural features other than the conserved CRD [2,5]: (I) proteoglycans, (II) endocytic type II receptors, (III) collectins, (IV) selectins, (V) leucocyte receptors, (VI) the macrophage mannose receptor, and (VII) simple or primitive lectins. In contrast with the simple structures of group VII lectins, members of groups I–VI have between one and several CRDs in their multi-domain structures.

While attempting to identify genes expressed specifically in either freshwater or seawater Japanese eels (*Anguilla japonica*), we isolated two similar but distinct cDNA clones that encode C-type lectins from a subtracted cDNA library. Northern blot analysis showed that their expression levels are markedly elevated in the gills of freshwater eels. A database search indicated that the eel lectins are members of the galactose-binding C-type lectin family, whose members have been characterized at the molecular level in a number of invertebrates [6,7], but in only a

limited number of vertebrates [8], and their homologues have not been described in fish. We therefore decided to characterize the lectins in detail, and have determined their complete amino acid sequences, the promoter regions of the corresponding genes, tissue distributions, sugar specificities, presence of Ca^{2+} -binding sites, subunit structures and cellular locations. The eel lectins, termed eCL-1 and eCL-2, have signal sequences at their N-termini and are thought to be secreted as defence molecules from the mucous cells of the gill when eels migrate from seawater to freshwater environments, where a large number of micro-organisms are present compared with the seawater environment. eCL-1 and eCL-2, identified here and shown to have simple lectin structures composed solely of a single CRD, may represent intriguing examples of the biological role of the group VII subfamily of the C-type lectin family.

MATERIALS AND METHODS

RNA isolation and construction of a subtracted cDNA library

Eels were adapted to either seawater or freshwater for 2 weeks separately. Total RNA was isolated from the seawater- and freshwater-adapted eel gills by the guanidinium thiocyanate/CsCl centrifugation method [9], and poly(A)⁺ RNA was affinity purified using an oligo(dT)-cellulose mRNA purification kit (Amersham Pharmacia Biotech). Two subtracted cDNA libraries, from seawater and freshwater eel gills, were constructed using the PCR-Select cDNA Subtraction kit (Clontech) according to the user manual (<http://www.clontech.com>, PT1117-1, PR85431). In brief, tester and driver double-stranded cDNAs were synthesized from 2 μg of poly(A)⁺ RNA preparations of

Abbreviations used: CRD, carbohydrate-recognition domain; eCL, eel C-type lectin; IPTG, isopropyl β -D-thiogalactopyranoside; PAS, periodic acid/Schiff; RACE, rapid amplification of cDNA ends; TCS, 10 mM Tris/HCl, 10 mM CaCl_2 and 150 mM NaCl, pH 7.4.

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The nucleotide sequences reported in this paper will appear in the EMBL, GenBank, GSDB and DDBJ Nucleotide Sequence Databases under the accession numbers AB060538 and AB060539.

seawater and freshwater eel gills. After hybridization, differential transcripts were amplified by PCR with *Ex Taq* (Takara, Kyoto, Japan) and inserted into a *NotI*-cleaved pBluescript II SK⁻ vector (Stratagene). Subtracted cDNA libraries (seawater and freshwater) were generated by selection using ampicillin-containing LB (Luria–Bertani) plates. Approx. 300 individual clones were sequenced from each library, and the levels of expression of the corresponding mRNAs were determined by Northern blot analysis using RNA preparations from freshwater and seawater eel gills to confirm differential expression.

Northern blot analysis

Total RNA was isolated from various tissues from seawater and freshwater eels by the acid guanidinium thiocyanate/phenol/chloroform method. For Northern analysis, 20 µg of total RNA/lane was electrophoresed on formaldehyde/agarose (1%, w/v) denaturing gels in 1 × Mops running buffer (20 mM Mops, pH 7.0, 8 mM acetate and 1 mM EDTA) and then transferred on to Hybond-N⁺ nylon membranes (Amersham Pharmacia Biotech) by the vacuum blotting method using 10 × SSC (1 × SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0) as the transfer buffer. After transfer, membranes were baked for 2 h at 80 °C and prehybridized for 2.5 h at 68 °C in PerfectHyb hybridization solution (Toyobo, Tokyo, Japan). The cDNA clones isolated from the above-described subtraction libraries were used as probes. The probes were labelled with [α -³²P]dCTP (3000 Ci/mmol) using a Ready-To-Go DNA labelling kit, and the unincorporated nucleotides were removed by passage through a Sephadex G-50 column (Amersham Pharmacia Biotech). The membranes were hybridized separately with each ³²P-labelled probe in the same buffer at 68 °C for 16 h. The blots were subsequently washed in increasingly stringent conditions (final wash: 0.1 × SSC/0.1% SDS for 30 min at 60 °C). Membranes were exposed to imaging plates (Fuji Film, Tokyo, Japan) in a cassette overnight. The results were analysed using a Fujix BAS2000 Bio-image analyser (Fuji Film).

cDNA library screening

An eel gill cDNA library in λ ZAP II (Stratagene) was prepared as described [10]. Plaque lifts were prehybridized for 2 h at 42 °C in a solution containing 20% formamide, 5 × Denhardt's solution (0.1% each of Ficoll, polyvinylpyrrolidone and BSA), 6 × SSPE (1 × SSPE is 0.15 M NaCl, 1 mM EDTA and 10 mM NaH₂PO₄, pH 7.4) and 0.1% SDS. The probe was labelled with [α -³²P]dCTP (Amersham Pharmacia Biotech) as described above for Northern blotting. Hybridization was performed for 16 h at 42 °C in prehybridization solution by adding the radiolabelled cDNA probe at approx. 10⁶ c.p.m./ml. To identify positive clones, the filters were rinsed twice with 2 × SSC/0.1% SDS for 15 min at room temperature, and washed subsequently with increasingly stringent buffers (final wash: 0.1 × SSC/0.1% SDS for 30 min at 55 °C). Finally, the filters were exposed to Kodak X-Omat AR film for 48 h at -80 °C with an intensifying screen. Positive plaques were isolated by three rounds of screening under identical conditions as for the primary screening, and transformed into pBluescript II SK⁻ by *in vivo* excision using ExAssist helper phage and *Escherichia coli* XL1-Blue MRF'.

DNA sequencing and analysis

The nucleotide sequences of both strands were determined by the dideoxy chain termination method with an automated sequencer (LI-COR model 4000L) using a SequiTherm Long-read Cycle Sequencing kit-LC (Epicentre Technologies). The DNA

sequences were compiled and analysed using the Genetyx MAC computer program (Software Development, Tokyo, Japan), and were also compared with the GenBank/EMBL/GSDB/DBJ database using the advanced BLAST program of the National Center for Biotechnology Information (NCBI). Multiple protein sequence alignments were carried out using the program ClustalW, and final adjustments were made manually. The hydropathy plot was constructed by the Kyte–Doolittle method.

Determination of the 5' terminal sequence of cDNA by 5' RACE (rapid amplification of cDNA ends)

To obtain full-length cDNAs, the 5' ends of the eel gill lectin cDNAs were amplified using a 5'/3' RACE kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 µg of total RNA from freshwater eel gill using AMV (avian myeloblastosis virus) reverse transcriptase and a gene-specific primer SP1 (5'-CT-TCCCCGCTGCGTTTCGAAC-3'). To attach a known sequence to the 3' end of the first-strand cDNA, a homopolymeric tail was appended using terminal transferase and dATP. The tailed cDNA was directly amplified by PCR using the oligo(dT)-anchor primer and the gene-specific nested primer SP2 (5'-TGTCAGGCTGCTTAGAATCC-3'). To reduce the background, the original PCR products were amplified again using the PCR anchor primer and a second nested gene-specific primer SP3 (5'-ATTCCACATTGCTGTGCACC-3'). Next, the PCR products were purified by 1.5% (w/v) agarose gel electrophoresis, and an approx. 300-bp band was isolated and subcloned into the pZero-2 vector using a Zero Background/Kan cloning kit (Invitrogen) and sequenced.

Identification of 5' flanking regions upstream of the lectin genes by PCR amplification of genomic DNA sequences

The PCR amplification of genomic DNA was performed using two sets of primers complementary to the cDNA sequence. Eel genomic DNA (2 µg) was digested with *ScaI* and *StuI* at 37 °C for 3 h and self-ligated using a DNA ligation kit (Takara) at 16 °C overnight to produce circular DNA. In this approach, one of the two restriction enzymes should have a cleavage site in the cDNA sequence, and the primers should be designed using the cDNA sequence upstream of the restriction site. The first PCR amplification was performed using antisense primer N1 (5'-TGGTGGATCTGTAGATACCA-3') and sense primer C1 (5'-GGTCTCCTTCCCAATGTCC-3'). The PCR reaction was carried out in an MJ Research thermal cycler for 30 cycles of denaturation (94 °C, 20 s), annealing (60 °C, 30 s) and extension (72 °C, 45 s), with an additional 7 min primer extension after the final cycle using *Ex Taq* polymerase. To reduce background and increase the yield of target fragments, a second PCR amplification was performed using another set of nested primers, N2 (5'-GAAGAAAACAGACAGGTG-3') and C2 (5'-GTGGCTGT-TCTTTCTGGCC-3'), under identical PCR conditions. The PCR reactions produced a band of approx. 700 bp, which was purified, subcloned and sequenced as described above.

Antibody production

A 430 bp fragment encoding the mature form of an eel gill lectin (amino acid residues 22–163 of eCL-2) was subcloned into bacterial expression vector pRSET B (Invitrogen), and the construct was transformed into *E. coli* XL1-Blue. When the turbidity of the bacterial culture measured at 600 nm reached 0.6, isopropyl β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued

for an additional 5 h at 37 °C. The cells were harvested from a 1-litre culture by centrifugation, resuspended in 20 ml of lysis buffer (50 mM Na₂PO₄ and 300 mM NaCl), disrupted by sonication and centrifuged at 10000 *g* for 20 min. The pellet was solubilized by resuspension and mixing in buffer containing 8 M urea, 50 mM Na₂PO₄ and 300 mM NaCl, pH 7.6, for 2 h at 4 °C. The mixture was then centrifuged at 10000 *g* for 40 min to remove any insoluble material. Urea-solubilized His₆-tagged recombinant fusion protein (His₆-eCL-2) was purified in the denatured state using a Ni²⁺-nitrilotriacetate agarose column (Qiagen) and dialysed against 0.9 % NaCl at 4 °C. Polyclonal antibodies to His₆-eCL-2 were prepared in Japanese White rabbits by injecting 400–450 µg of the purified recombinant protein emulsified in complete Freund's adjuvant (1:1, w/v) subcutaneously at multiple sites. The rabbits were injected five times at 2-week intervals, and were bled 7 days after the fifth immunization. The antiserum cross-reacted with eCL-1 as well as with eCL-2, and was named 'anti-eCL'.

Affinity purification of anti-eCL antibody

The antibody was purified on an *N*-hydroxysuccinimide-activated HiTrap affinity column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. His₆-eCL-2 fusion protein (500 µg in 500 µl of saline) was coupled to 1 ml of *N*-hydroxysuccinimide-activated Sepharose, and then 1 ml of anti-eCL antiserum (diluted 1:1 in PBS) was applied to the column and incubated overnight at 4 °C. The bound antibody was eluted with 4 M MgCl₂ and dialysed against 1 litre of PBS overnight at 4 °C.

Preparation of asialofetuin–agarose for affinity chromatography

The asialofetuin–agarose gel was prepared from fetuin–agarose as described by Den and Malinzak [11]. Briefly, for removal of terminal sialic acid, the fetuin–agarose gel (Sigma) was suspended in an equal volume (10 ml) of 100 mM sodium acetate, pH 5.6, and incubated at 37 °C for 12 h in the presence of 1 unit of neuraminidase (type III from *Vibrio cholerae*; Sigma) and 1 mg of CaCl₂ per ml. The desialized gel was washed with 8 M urea before equilibration with homogenization buffer TCS (10 mM Tris/HCl, 10 mM CaCl₂ and 150 mM NaCl, pH 7.4).

Isolation and determination of sugar specificity of eel C-type lectins

Eel gills (2 g) were homogenized in 5 vol. of ice-cold TCS containing 2 mM PMSF, 20 µg/ml leupeptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin. The homogenates were centrifuged at 100000 *g* for 1 h at 4 °C and the supernatant was carefully collected in fresh tubes. Portions (7 ml) of the supernatant were incubated with 5 ml of asialofetuin–agarose beads under gyration overnight at 4 °C. The mixture was poured into a column and allowed to settle. After extensive washing of the column with TCS, bound proteins were eluted with TCS containing 200 mM lactose, 300 mM sucrose, 300 mM maltose, 300 mM glucose, 200 mM D-mannose, 200 mM L-fucose, 200 mM D-galactose, 200 mM *N*-acetyl-D-galactosamine, 150 mM IPTG or 150 mM methyl-β-D-galactose. Ca²⁺ dependency was tested by elution with 10 mM EDTA in TBS (10 mM Tris/HCl and 150 mM NaCl, pH 7.4). The eluates were subjected to SDS/PAGE (15 % gel) and lectin bands were detected by means of Western blot analysis using specific antiserum to eel gill C-type lectin (anti-eCL).

Ruthenium Red staining

Lectin (4 µg) was electrophoresed under reducing conditions at 4 °C and electroblotted on to a PVDF membrane by applying low voltage (12–15 V) overnight at the same temperature. The membranes were incubated in 20 mM Hepes, pH 7.8, containing 1 mg/ml Ruthenium Red for 30 min at room temperature and then rinsed for 2–3 min in 20 mM Hepes, pH 7.8. Identical blots used as controls were incubated in the same solutions containing 50 mM CaCl₂.

Western blot analysis

Total protein concentrations in the samples were measured using a BCA protein assay kit (Pierce) and adjusted to approx. 1 µg/µl with TCS. The soluble proteins (20 µg/lane) were separated by SDS/PAGE using a 15 % (w/v) polyacrylamide gel and electroblotted on to a PVDF membrane. After blocking in 10 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 0.05 % Tween 20 and 5 % (w/v) non-fat milk for 1 h at room temperature, the membrane was incubated with anti-eCL antiserum, preimmune serum or preabsorbed antiserum at 1:1000 dilution overnight at 4 °C. Preabsorption was carried out by incubating 10 µl of the antiserum with 500 µg of affinity-purified recombinant protein (His₆-eCL-2) or 100 µg of purified native lectin in 500 µl of PBS overnight at 4 °C. The immune complexes on the membrane were then reacted with alkaline phosphatase-conjugated goat anti-(rabbit IgG) at 1:3000 dilution for 1 h at room temperature. The bound secondary antibody was visualized using 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue tetrazolium chloride as chromogenic substrates.

Immunohistochemistry

Gills from freshwater eels were fixed in 0.1 M PBS, pH 7.4, containing 4 % (w/v) paraformaldehyde, incubated successively in 1 × HBSS (130 mM NaCl, 5 mM KCl, 0.5 mM Na₂PO₄ and 100 mM Hepes, pH 7.4) containing 10 % (w/v) sucrose overnight and 1 × HBSS containing 20 % (w/v) sucrose for 30 min at 4 °C, and finally frozen in Tissue-Tek O. C. T. compound. Frozen sections (6 µm) were cut in a cryostat at –20 °C, and mounted on Vectabond-coated glass slides and dried in air for 45 min. The sections were washed twice with PBS, treated with 2.5 % (v/v) H₂O₂ in PBS for 1 h at room temperature, and washed again with PBS. They were subsequently incubated for 2 h at room temperature with 2.5 % (v/v) normal goat serum, and then for 48 h at 4 °C with affinity-purified anti-eCL antibody, preabsorbed antiserum or preimmune serum at 1:1000 dilution unless stated otherwise. After washing with PBS, the sections were incubated with peroxidase- and goat anti-(rabbit IgG)-conjugated dextran polymer (EnVision+; Dako) for 2 h at room temperature. The bound antibody was visualized using 0.55 mM 3,3'-diaminobenzidine tetrahydrochloride containing 1.5 mM nickel chloride and 0.02 % H₂O₂ in 50 mM Tris/HCl, pH 7.4. The mucous cell was visualized by the periodic acid/Schiff (PAS) staining method as described previously [12].

RESULTS

Identification of differentially expressed genes from subtracted cDNA libraries

In an attempt to isolate genes that are expressed specifically in freshwater- or seawater-adapted euryhaline fishes, we have generated two subtracted cDNA libraries from poly(A)⁺ RNA

Figure 1 Differential expression of eCL mRNA in the gills of freshwater- and seawater-adapted eels

Total RNA preparations (20 µg/lane) isolated from gills from freshwater (FW)- and seawater (SW)-adapted eels were analysed by Northern blot analysis using a ³²P-labelled eCL-2 probe under high-stringency conditions. A single transcript was detected at 0.8 kb. Hybridization to an eel β-actin probe (corresponding to nucleotides 206–343 of the rat sequence) demonstrated equal loading of each lane.

preparations of gills from freshwater and seawater eels, as described in the Materials and methods section. The subtracted cDNA libraries were expected to be enriched in sequences of genes expressed specifically either in freshwater or in seawater. We obtained approx. 300 individual clones from each library, sequenced their inserts, and classified them by a database search into several categories, including channels, transporters, receptors, transcription factors, structural proteins, housekeeping enzymes and proteins with no match to previously studied ones. The subtraction is usually not complete and therefore false positives should be eliminated. Furthermore, for the analysis of the functional role of the freshwater- or seawater-specific clones, identification of the cells that express the corresponding proteins is necessary, which means that the levels of expression should be relatively high. To fulfil these criteria, we performed Northern analysis using total RNA preparations from gills from freshwater and seawater eels, and obtained seven candidate clones that exhibited both differential expression between freshwater and seawater and relatively dense bands on Northern analysis. Among these there was a 136 bp clone encoding a part of a protein similar to a galactose-binding C-type lectin whose expression is confined to the gill and is markedly elevated in freshwater (Figure 1).

Cloning and sequence analysis of full-length cDNAs encoding eel gill C-type lectins

On Northern blot analysis, the 136 bp lectin probe hybridized to a 0.8 kb transcript (Figure 1). To isolate the lectin cDNA clones of this size, we constructed a freshwater eel gill cDNA library in the λZAP II vector, and obtained eight positive clones by screening 3×10^5 recombinant plaques. The longest clone was of 689 bp and contained a complete open reading frame and the apparent 3' end of the sequence, including two polyadenylation signals (AATAAA) and a poly(A) tail. The 5' end was determined by 5' RACE, which yielded 25 bp of additional sequence, extending the total length of the sequence from 689 to 714 bp (accession no. AB060538).

The open reading frame encodes a protein of 163 amino acid residues (Figure 2) with a calculated molecular mass of 18490 Da. Hydropathy analysis indicated that the first 22 amino acid

residues from the N-terminus are uncharged, mostly hydrophobic and likely to form a signal peptide (Figure 2), as expected for a secreted protein. There is a potential amidation site (Gly-162) at the end of the C-terminus. In addition, a putative N-glycosylation site (Asn-Xaa-Thr/Ser) is present at position 37. Comparison of the sequence with those of lectins from other species indicated that the eel sequence shares 30–40% similarity with the known sequences of the C-type lectins (Figure 2). There are several signature sequences of the C-type lectin family [3,5,13]: the C-type lectin domain signature (residues 130–154, including three conserved Cys residues), the QPD (Gln-Pro-Asp) motif (residues 120–122) that is a determinant of sugar specificity, and the WND (Trp-Asn-Asp) motif (residues 141–143) that is important in binding carbohydrate and Ca²⁺. The protein was therefore named eel C-type lectin-2, or eCL-2 for short.

Sequencing of all eight positive cDNA clones revealed the presence of another clone, eCL-1, which shares more than 88% sequence similarity with eCL-2 (Figure 2; accession no. AB060539). The relative proportions of the two forms were approx. 1:1. Our nomenclature of eCL-1 and eCL-2 is not in the order of their discovery, but reflects sequence similarities with the previously described group II members of C-type lectins: eCL-1 with rat and human hepatic lectin-1, and eCL-2 with rat and human hepatic lectin-2 (Figure 2).

Sequences of the promoter regions of the eCL genes

To establish the sequences of the promoter regions, we next isolated and sequenced genomic DNA clones containing the corresponding regions. Figure 3 shows a partial 551 bp sequence of the eCL-1 gene and a 646 bp sequence of the eCL-2 gene covering the possible transcription and translation initiation sites. The nucleotide sequence around the initiator methionine codon ATG (TCCACCATGG) conforms well with the consensus sequence of the common Kozak box for translation initiation in higher eukaryotes [GCC(A/G)CCATGG] [14]. The genes contain a consensus TATA box, several CAAT boxes and a number of putative transcription factor-binding sites, including those for NF-IL6, p53, GAL-4 and GATA-1. The potential promoter regions of the two genes are surprisingly similar; their sequence identity is 98% except for deletions of the following regions: nucleotides –13 to 21 in the eCL-1 gene, and nucleotides –217 to 239 and –310 to 391 in the eCL-2 gene (Figure 3). This similarity explains the observation mentioned above that the eCL-1 and eCL-2 gene transcripts are present in approximately equal abundance.

Tissue distribution and time course of alterations in expression

To determine the multiple tissue distribution of eCL mRNAs and to compare levels of expression in seawater and freshwater eels, we performed Northern blot analysis using total RNA preparations from various tissues from seawater and freshwater eels, including the gill, kidney, head kidney, heart, liver, posterior intestine, anterior intestine, stomach and muscle. As a probe, we used ³²P-labelled eCL-1 cDNA, which was expected to hybridize to both eCL-1 and eCL-2 mRNAs because of their high sequence similarity. A strong signal of ~0.8 kb was detected in the gill, and a faint band in the stomach (Figure 4A) when studying tissues from freshwater eels. The other tissues examined were negative. Comparison between the seawater and freshwater eel gill samples indicated that there was a marked difference in eCL mRNA expression between freshwater and seawater eels (Figure 4). Figure 5 shows the time course of alterations in eCL mRNA expression and protein levels following the transfer of eels from

Figure 2 Amino acid sequence alignment of eCLs and other members of the galactose-binding C-type lectin family (groups II and VII)

Abbreviations, accession numbers and references are as follows: rat hepatic lectin-1, -2/3 (RHL-1, -2/3) [32,33], K02817, J02762; human hepatic lectin-1, -2 (HHL-1, -2) [34,35], M10058, M11025; human macrophage lectin-2 (hML-2) [36], D50532; human scavenger receptor with C-type lectin (hSRCL) [37], AB038518; *Trimeresurus stejnegeri* lectin (TSL) [8], AF119097; rat Kupffer cell receptor (rKCR) [38], J03734. The putative signal sequences for the two novel proteins identified here, eCL-1 and eCL-2, are boxed. Black shading denotes amino acid residues identical in all members; grey shading denotes residues identical with those in eCL-1; white areas denote non-identity. In addition, four Cys residues (marked with asterisks) that form two conserved disulphide bonds (S-S), the putative optional disulphide bond, and the position of the C-type lectin signature sequence (dotted underline) are indicated [3,39]. The conserved QPD motif for galactose binding is overlined with a bold bar, and the WND motif, which is important in binding carbohydrate and Ca^{2+} , is double-overlined. Gaps (—) have been introduced to optimize identity. The percentage identity of the CRD of eCL-2 (Cys-29 to Cys-154) with the CRDs of other galactose-specific C-type lectins are as follows: RHL-1, 36.1%; RHL-2/3, 37.4%; HHL-1, 33.1%; HHL-2, 36.8%; hML-2, 34.6%; hSRCL, 33.3%; TSL, 36.4%; rKCR, 40.7%.

freshwater to seawater. The adaptive alterations occurred relatively slowly.

Production of antiserum and immunochemical characterization of eCLs

A rabbit polyclonal antiserum was generated against a bacterially expressed His-tagged protein containing amino acid residues 22–163 corresponding to the mature form of eCL-2. The antiserum recognized both eCL-1 and eCL-2 produced recombinantly in *E. coli* (Figure 6A). The specificity of the antiserum was characterized by Western blot analysis. A single band of 18 kDa was observed when crude extracts of the eel gill containing soluble proteins were subjected to Western blotting (Figure 6B, lane 1). The size of the band corresponds well with the calculated molecular masses of eCL-1 and eCL-2 if one assumes that native lectin is glycosylated at Asn-37. Samples from freshwater eels gave a much denser band than those from seawater eels, indicating higher levels of expression of eCL-1 and eCL-2 in fresh-

water eels. This confirms the Northern analysis result at the protein level. The staining was almost abolished by preabsorption of the antiserum with the antigen (Figure 6B, lane 2).

Characterization of sugar-binding properties of eCLs

The eel lectins eCL-1 and eCL-2, identified by subtraction cloning and their presence being confirmed by Western blot analysis, were purified from freshwater eel gill extracts by affinity chromatography on asialofetuin-agarose and used for determination of sugar specificity. eCL was readily eluted from the affinity gel with EDTA, establishing its bivalent metal ion dependency (Figure 7, lane 3). For determining the sugar-binding specificity of eCLs, competitive elution of eCLs from the asialofetuin affinity gel with various sugars was employed. Lactose, a galactose-containing disaccharide, was effective, achieving complete elution of eCLs (Figure 7, lanes 4 and 5). Among the monosaccharides tested, only galactose and its derivatives IPTG and methyl- β -D-galactose were effective (Figure

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eCL-1  GTGAGTGTGTTTACCTCAGACGGTTAGCTATACACAGGTTATGGTGCATTAATTGAAGGTTGTCGTTTGTGCGTCTCATTATGACAGTTG -461
eCL-2  GTGAGTGTGTTTACCTCAGAGTGTAGCTATACACAGGTTATGGTGCATTAATTGAGGTTGTCGTTTGTGTCGTCATTATGACAGTTG -556
          CAAT-box
eCL-1  ACTGTGCCAATGAGAGAAAAAGCATTGGTTAAATAACATCTGGTTAAAGGAGGAGGGGTTGGGAATATAACTATACCTGTCTGCTTTC -370
eCL-2  ACTGTGCCAATGAGAGAAAAAGCATTGGTTAAATAACATCTGGTTAAAGGAGGAGGGGTTGGGAATATAACTATACCTGTCTGCTTTC -465
          CAAT-box
eCL-1  CAACGTATGTCTGATTGTGACGTACAAAGCATTAGTATTTGAAGGTAATTCAGAAAGCCTTCCAAACATC----- -297
eCL-2  CAACGTATGTCTGATTGTGACGTACAAAGCATTGGTATTTGAAGGTAATTCAGAAAGCCTTCCAAACATCGCAACACTGTGAGCACAC -374
          NF-IL6
eCL-1  -----AGACATGGCTCTCAAGCCCGTGGAAAAAG -269
eCL-2  AACTAAATCATTTAAATGAGGTAATGATGTTGCTGGGAAGCGGAGACGTATACAGACGTAAACGACATGGCTCTCAAGCCCGTGGAAAAAG -283
          NF-IL6
eCL-1  CAAACCGGTTCTTAGAAGGTTGCAAGTTGAACCAACTGCGAA-----CCAGAACTAGGTTACGTTGGTGA -201
eCL-2  CAAACCGGTTCTTAGAAGGTTGCAAGTTGAACCAACTGCGAACCGGCACTAGCACCAGCCAGAACCAAGCACTAGGTTACGTTGGTGA -192
          p53
eCL-1  AAAGAGGTATTAGTGAACGTGAGTTAATTCCTCTATCAATCATGTAGCAAAATTTCTAAGGAATCCTTTGTGAAACTTGTTCACATTA -110
eCL-2  AAAGAGGTATTAGTGAACGTGAGTTCAATTCCTCTATCAATCATGTAGCAAAATTTCTAAGGAATCCTTTGTGAAACTTGTTCACATTA -101
          GAL-4 GATA-1 p53
eCL-1  CTTATCTAATCATCTGCGAAATCCAGTAAATCCCTTCGTGAAACTTGTTCACATTAATGCTATCTGATCGCTTTATAAAGGCTGAGAAA -19
eCL-2  CTTATCTAATCATCTGCGAAATCCAGTAAATCCCTTCGTGAAACTTGTTCACATTAATGCTATCTGATCGCTTTATAAAGGCTGAG--- -13
          GAL-4 GATA-1 CAAT-box TATA-box
eCL-1  TAGAAGAATATGTTTCATTGACAGCAGAACAGAACCATAGATCCACCGTCTGTTTCTTCCAGCTGGTATCTACAGATCCACCATGG -70
eCL-2  -----AATATGTTTCATTGACAGCAGAACAGAACCATAGATCCACCGTCTGTTTCTTCCAGCTGGTATCTACAGATCCACCATGG -70
          -1 +1 Met

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Figure 3 Nucleotide sequences of upstream regulatory regions of the eCL-1 and eCL-2 genes

Sequences of 0.55 kb of the eCL-1 gene and 0.65 kb of the eCL-2 gene upstream of the transcription start sites are shown. Negative numbers to the left refer to the nucleotide positions upstream of the 5' end of eCL cDNAs. TATA box and CAAT box sequences are boxed. The regulatory elements NF-IL6, GAL-4, GATA-1 and p53 are indicated by solid underlines or overline. The dashed regions indicate the nucleotides that are absent from the corresponding eCL gene. The dots represent the nucleotides that differ between the two eCL genes. The 5' ends of the eCL cDNAs are indicated by a bent arrow.

Figure 4 Tissue distribution of eCL mRNA determined by Northern analysis

Eels were adapted to seawater or freshwater for 2 weeks, and total RNA was isolated from the indicated tissues, as described in the Materials and methods section. (A) Autoradiogram of a Northern filter containing RNA preparations from freshwater (FW) eels; (B) Northern analysis of samples from seawater (SW) eels. The amounts of total RNA were adjusted to approx. 20 μ g by measuring their absorbance at 260 nm, except for the liver samples (60 μ g). Hybridization with an eel β -actin probe was used to estimate relative amounts of mRNA loaded in each lane; weak signals in the liver lanes are due to relatively low abundance of the actin mRNA compared with rRNA.

7, lanes 7–14), demonstrating the galactose specificity of eCL-1 and eCL-2. The observed specificity is consistent with that predicted from the sequence data (for details, see Discussion).

Figure 5 Time course of alterations in eCL expression following transfer of eels from freshwater to seawater

Freshwater eels were transferred to seawater, and total RNA and crude soluble proteins were isolated from the gills of three eels separately at the times indicated and subjected to Northern or Western blot analysis. The set of three samples gave similar results; two of them are shown to demonstrate low variability between individuals. Hybridization to an eel β -actin probe demonstrated equal loading in each lane.

Calcium-binding site on eCL demonstrated by Ruthenium Red staining

Ruthenium Red, an inorganic dye, is known to stain Ca^{2+} -binding proteins specifically [15]. We used this stain to confirm the Ca^{2+} dependency of eCL-1 and eCL-2. Affinity-purified eCLs were separated by SDS/PAGE and transferred to a PVDF membrane. Incubation of the blots with Ruthenium Red resulted in strong staining of an 18 kDa band corresponding to the eCLs

Figure 6 Western blot analysis of eCLs to determine their sizes and specificity of antiserum

(A) Reactivity of antiserum (anti-eCL) with recombinant eCL-1 and eCL-2. (B) Western blot analysis of native eCLs. The soluble protein fractions from gill homogenates were separated by SDS/PAGE under reducing conditions, transferred to PVDF membranes, and stained with anti-eCL antiserum (lane 1), absorbed antiserum (lane 2) or preimmune serum (lane 3).

Figure 7 SDS/PAGE monitoring of binding of eCLs to asialofetuin-agarose and their elution with EDTA and various saccharides

eCLs were purified by affinity chromatography on an asialofetuin-agarose column and subjected to SDS/PAGE. A 15% (w/v) polyacrylamide gel and specific anti-eCL antiserum were used. Lane 1, crude eel gill soluble protein fraction; lane 2, flow-through fractions from affinity gel; lane 3, 10 mM EDTA eluates from the affinity column; lanes 4 and 5, samples eluted successively with 200 mM lactose (lane 4) and then with 10 mM EDTA (lane 5); lane 6–14, eluates obtained with 300 mM sucrose, 300 mM maltose, 300 mM glucose, 200 mM D-mannose, 200 mM L-fucose, 200 mM D-galactose, 200 mM N-acetyl-D-galactosamine, 150 mM IPTG and 150 mM methyl-β-D-galactose (Me-Gal) respectively.

(Figure 8, lane 2). An identical incubation carried out in the presence of 50 mM CaCl_2 showed no dye binding to the protein (Figure 8, lane 1), indicating that the eCLs are indeed Ca^{2+} -binding proteins and can be classified in the C-type lectin family.

Octameric subunit structure

SDS/PAGE under mild conditions, namely without heat treatment and reducing agents and at 4 °C, revealed a higher-order structure of eCLs. On mild SDS/PAGE, native eCLs migrated as a band of ~148 kDa (Figure 9, lane 3); on heat treatment, a band of ~37 kDa was apparent (lane 2), and on reduction the size of the band was lowered further to ~18 kDa (lane 1). These

Figure 8 Ruthenium Red staining of eCLs

Affinity-purified eCLs were separated by SDS/PAGE under reducing conditions on a 15% (w/v) polyacrylamide gel, electroblotted on to a PVDF membrane and incubated with Ruthenium Red as described in the Materials and methods section. Membranes were incubated with Ruthenium Red in the presence (lane 1) or absence (lane 2) of competing Ca^{2+} ions.

results indicate that eCL-1 and eCL-2 have an octameric subunit structure consisting of four non-covalently associated dimers whose constituent monomers are linked by disulphide bonds $[(18 \text{ kDa})\text{-SS-(18 kDa)}]_4$. A unique property we found is that the octameric structure of eCLs is very stable and persists even in the presence of SDS if not heated.

Mucous cell localization of eCL

The localization of eCL in the gill was examined by immunohistochemistry using the anti-eCL antiserum. The antiserum specifically stained mucous cells located near the basal region of the secondary lamella (Figure 10A). The mucous cell localization was confirmed by staining consecutive sections with carbohydrate-specific PAS, a well established chemical stain for mucous cells (Figure 10B). No specific staining was observed

Figure 9 Determination of the subunit structure of eCLs by SDS/PAGE

Affinity-purified eCLs were mixed with SDS sample buffer and run on an SDS/polyacrylamide gel under the indicated conditions. Two types of gel were used to achieve maximum resolution of low- and high-molecular-mass species: a 15% (w/v) gel (lanes 1 and 2) and a 10% gel (lane 3). Positions of molecular mass markers are shown on the left and right. 2-ME, 2-mercaptoethanol.

Figure 10 Immunohistochemical localization of eCLs in gills from freshwater eels

Serial sections of the gill were stained with affinity-purified anti-eCL, a polyclonal antibody raised against recombinant eCL-2 but also able to recognize eCL-1 (A), PAS reagent for the identification of mucous cells in the gill (B), absorbed anti-eCL (C) and preimmune serum (D). In (B), sections were counter-stained with haematoxylin. Clear staining was obtained with anti-eCL, but not with absorbed or preimmune serum. MC, mucous cell; SL, secondary lamella. Scale bars represent 25 μm .

with preimmune serum or absorbed antiserum (Figures 10C and 10D).

DISCUSSION

While attempting to identify differentially expressed genes in gills from freshwater or seawater eels using subtracted cDNA libraries, we isolated clones encoding β -D-galactose-binding C-type lectins that are highly expressed in freshwater eel gills, which we termed eCL-1 and eCL-2. They are simple secretory lectins composed of a single CRD that is defined by two conserved disulphide bonds and 14 invariable and 18 highly conserved amino acid residues (Figure 2). The invariant residues include the WND (Trp-Asn-Asp) motif near the C-terminal region of the CRD, which is extremely important in binding carbohydrate and Ca^{2+} [3,5]. The CRDs of eCL-1 and eCL-2 contain the QPD (Gln-Pro-Asp) motif that is considered to confer galactose ligand specificity [13,16]. This motif is, however, not completely conserved in all of the galactose-binding lectins [6,7]. It is also recognized that a Trp (W) residue next to the QPD motif is important for high-affinity galactose binding with pronounced selectivity [17]. The CRD sequences of the eCLs also satisfy this criterion, consistent with the results of a competitive binding assay that demonstrated high-affinity and highly selective binding of galactosides (Figure 7). The galactose-specific, QPD-containing CRDs have so far been found only in members of group I, group II and group VII of the animal C-type lectins (Figure 2). The secretory nature of eCL-1 and eCL-2, suggested by the presence of signal sequences, is consistent with their location in the mucous cells, which secrete mucus to protect the gills by forming a boundary layer between the water and the epithelium of the gills. eCL-1/eCL-2 detected in the stomach in low but significant amounts (Figure 4A) may

also play a similar protective role by creating barriers against invasive pathogenic micro-organisms. Other examples of secretory lectins include *Pleurodeles* lectin [18], acorn barnacle lectin [19] and *Trimeresurus stejnegeri* venom lectin [8].

A large number of lectins have been identified in the skin and serum of fish, but few of them have been characterized with respect to both structure and functionality [12,20–26]. Restricting the examples to galactose-binding lectins, for example, a galactose-binding lectin with a bacteria-recognition activity has been identified in coho salmon (*Oncorhynchus kisutch*) eggs [24], but it has not yet been characterized at the molecular level. In addition, an *N*-acetyl-D-galactosamine-specific C-type lectin, that could serve as an opsonin for the phagocytosis of foreign pathogens, has recently been identified in blue gourami (*Trichogaster trichopterus*) serum, but its sequence has not been established [26]. The eel gill lectins eCL-1 and eCL-2 reported here are thus the first soluble β -D-galactose-binding fish lectins with a true C-type CRD that have been fully characterized at the molecular level. Fish lectins have been suggested to be involved in host defence, based on their pathogen-binding and opsonic activities [25–28]. It can therefore be speculated that when euryhaline eels, which lack a highly developed immune system, reach the freshwater environment on upstream migration in their life cycle, they face the problem of an abundance of invading pathogenic bacteria as compared with the seawater environment, and thus secrete galactose-binding C-type lectins from the mucous cells of the gill as defence molecules.

Another interesting feature of eCL-1 and eCL-2 is the environment-dependent regulation of their expression. They exhibited markedly elevated expression under freshwater conditions. Although the mechanism of the regulation of expression remains to be clarified, there are two possibilities: (i)

activation of the eCL-1 and eCL-2 genes by a salinity-dependent mechanism, or (ii) activation by a factor other than salinity, such as the abundance of infectious bacteria. In this context, it is noteworthy that lectin production in some fish species is subject to seasonal variation; for instance, in the winter skate (*Raja ocellata*), the serum haemagglutinin level increases during summer when there is an abundance of micro-organisms [29]. Similarly, in the acorn barnacle, a marine invertebrate, the mRNA and protein levels of a secreted galactose-binding lectin (BRA-3) increase during early summer [19]. To obtain further insight, we determined the potential promoter sequences of the eCL-1 and eCL-2 genes, which revealed the presence of canonical TATA and CAAT boxes in the appropriate locations. These elements are common to the promoters of a variety of highly regulated eukaryotic genes, and their presence is consistent with the observed tight regulation of expression of the eCL-1 and eCL-2 genes. The eCL-1 and eCL-2 promoter regions also contain several putative transcription regulatory elements, including binding sites for NF-IL6, p53, GAL-4 and GATA-1. Although the functionalities of these regulatory elements are not clear, their presence may be worthy of comment in conjunction with the other reports. Thus a C-type lectin (Mincle) has been identified in mouse macrophages that is strongly induced in response to several inflammatory stimuli under the regulation of NF-IL6, which belongs to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors [30]. In addition, a soluble galactose-binding lectin has been shown to have a strong promoter activity that is down-regulated by p53 [31].

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