Role of the carbohydrate recognition domains of mouse galectin-4 in oligosaccharide binding and epitope recognition and expression of galectin-4 and galectin-6 in mouse cells and tissues

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Abstract. Galectin-4 and its homologue galectin-6 are members of the tandem-repeat subfamily of monomer divalent galectins. Expression of mouse galectin-4 and galectin-6 by RT-PCR using primers designed to distinguish both galectin transcripts indicates that both are expressed in the small intestine, colon, liver, kidney, spleen and heart and P19X1 cells while only galectin-4 is expressed in BW-5147 and 3T3 cell lines. *In situ* hybridization confirmed the presence of galectin-4/-6 transcripts in the liver and small intestine. Galectin-4 is expressed in spermatozoons and oocytes and its expression during early mouse emryogenesis appears in 8-cell embryos and remains in later stages, as tested by RT-PCR. To study the role of carbohydrate recognition domains (CRDs) in oligosaccharide binding and epitope recognition, we cloned mouse full-length galectin-4 and galectin-6 cDNA and constructed bacterial expression vectors producing histidin-tagged recombinant galectin-4 and its truncated CRD1 and CRD2 forms. Oligosaccharide binding profile for all recombinant forms was assessed using Glycan Array available through the Consortium for Functional Glycomics. Acquired data indicate that mGalectin-4 binds to α-GalNAc and α-Gal A and B type structures with or without fucose. While the CRD2 domain has a high specificity and affinity for A type-2 α-GalNAc structures, the CRD1 domain has a broader specificity in correlation to the total binding profile. These data suggest that CRD2 might be the dominant binding domain of mouse galectin-4. Mapping of epitopes reactive for biotinylated his-tagged CRD1, CRD2 and mGalectin-4 performed on mouse cryosections showed that all three forms bind to alveolar macrophages, macrophages of red pulp of the spleen and proximal tubuli of the kidney and this binding was inhibited by 5 mM lactose. Interestingly, mGalectin-4, but not CRD forms, binds to the suprabasal layer of squamous epithelium of the tongue, suggesting that the link region also plays an important role in ligand recognition.

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Introduction

Galectins, unusual soluble-type (S-type) lectins, represent a structurally defined family of highly evolutionarily conserved ß-galactoside binding proteins which are characterized by the presence of a carbohydrate recognition domain (CRD) with a sugar-binding consensus of WG-E-R/K or WG-E-I, the lack of a leader sequence and the presence of a conservative consensus, DIAFHFNPRF (1). These features confer galectins special roles in intracellular functions and, after externalization across the membrane by non-classical secretory pathways, in cell surface interactions. Recent studies demonstrated that galectins participate in many biological processes, such as cell adhesion, cell cycle control, apoptosis, signal transduction, immune response and malignity (2).

An analysis of mouse, rat and human genome databases supports the hypothesis that up to 20 galectin and galectin-like genes can be identified in mammalian species. Based on a basic domain structure, the galectin family can be divided into a prototype monovalent monomer or dimer subfamily (galectin-1, -2, -5, -7, -10, -11, -13 and -14), chimera type members (galectin-3 and -15) and a tandem-repeat subfamily (galectin-4, -6, -8, -9 and -12) (2).

Galectin-4 is a prototype molecule of monomer divalent galectin members which shares a high structural similarity to mouse galectin-6 (3,4). Galectin-4 was originally described as a 17-kDa lectin found in rat intestinal extract (5) but later studies confirmed that the whole molecule represents 36-kDa protein (6). Several studies demonstrated its expression in the epithelium lining of the all alimentary tract from the tongue to the large intestine (7-9). An important function of galectin-4 in the alimentary canal was recently demonstrated by Hokama *et al* (10) who confirmed that this molecule contributes to exacerbation of chronic colitis. However, galectin-4 expression was detected in the lungs, testes, breast, liver and placenta by highly sensitive RT-PCR (11).

A possible role for galectin-4 in human carcinogenesis and tumor progression has been postulated. Down-regulation of human galectin-4 in colorectal cancer and its expression in HT29 and LS174T cell lines (12) and colon adenocarcinoma T84 cells (13) was described. Up-regulation of galectin-4 in human hepatocellular carcinogenesis and gastric cancer was described by Kondoh *et al* (14) and Hippo *et al* (15), respectively.

The galectin-4 gene is localized on mouse chromosome 7 and sequencing data revealed that the gene has 10 exons. In contrast, the mouse galectin-6 gene lacked a 72-bp long exon sequences in the link region of the molecule, resulting in a 24 amino acid deletion in the link spacer. In addition, some sequence differences were found scattered along the coding region, supporting the hypothesis that gene duplication in the galectin-4 locus occurred during evolution in the mouse genome. Gitt *et al* (3,4) detected galectin-6 sequences on mouse 7 chromosome using galectin-6 specific hybridization probe. However, current genome data do not confirm exactly the presence of both genes in the mouse galectin-4/-6 locus. Instead, both genes are presented as splicing variants of a single gene. In addition, human, rat or pig counterparts of galectin-6 were not identified. Therefore, whether the galectin-6 transcripts in the mouse small intestine and colon described by Gitt *et al* (3,4) represent a transcript derived from a gene other than galectin-4 remains questionable.

The presence of two CRD non-identical core domains separated by a link region suggest the possible role of tandem-repeat galectin members in crosslinking function (16,17). Rather than crosslinking one molecule it is believed that these members can crosslink two distict types of ligand. Therefore, a binding specificity for each domain was recently studied in rat and human galectin-4 (18-21). These studies confirm that both domains differ in saccharide recognition, selectivity and affinity to particular types of oligosaccharides, supporting the hypothesis for a clustering or crosslinking function of galectin-4. This was also supported by Braccia *et al* (22), demonstrating that galectin-4 can function as a core raft stabilizer in the microvillar membrane vesicles of intestinal brush borders. Ideo *et al* (23) demonstrated recently that galectin-4 binds to *O*-linked sulfoglycans (24) and to sulfated glycosphingolipids and carcinoembryonic antigen in patches on the cell surface of human colon adenocarcinoma cells.

Tissue and cell type-specific splicing variants of galectin-8, -9 and -12, were recently identified (25-30) and two isoforms of galectin-4 in porcine small intestine were identified (31). In all these cases, variants differ only in the length on the linker peptide suggesting a possible role of the peptide link region in ligand recognition. This is also supported by a cross-species conservation of particular variants in mammalin species. Although the physiological and functional relevance of the isoforms needs to be precisely elucidated, it seems likely that link region variations significantly affect the topology of the galectin molecule required for specific ligand recognition.

The major focus of this study was to investigate binding specificity for oligosaccharide chains in both galectin-4 domains and to verify the hypothesis that the link region has an important role in ligand recognition. We cloned mouse galectin-4 full-length cDNA (GenBank accession number AY044870) and mouse galectin-6 cDNA, and used the sequences for bacterial recombinant vector construction. Expression of recombinant his-tagged galectin-4 and its CRD1 (N-terminal domain) and CRD2 (C-terminal domain) allowed us to identify a pattern of specificity and high affinity oligosaccharides bound by each of the domains. Biotinylated versions of the recombinant proteins were then used for epitope screening in frozen mouse tissue. The cloning of both galectin cDNAs allowed us to design a specific set of primers selectively amplifying only one of the highly homologous galectin transcripts and to perform more detailed analysis of galectin-4/-6 expression in mouse tissue, cell lines and early mouse embryogenesis.

Materials and methods

Molecular cloning of mouse galectin-4 cDNA. Mouse liver Marathon-Ready cDNA library (ClonTech Inc., USA) was screened using the panel of primers listed in Table I. Gene-specific primers were designed from a partial known mouse cDNA sequence (primers 2, 4, 5, 6, 7, 10), a rat galectin-4 cDNA sequence (primer 1) or putative galectin-4 EST clone, AA265412 (primer 13). The 3'-end untranslated region was isolated using 3'-RACE with primer 5 and an adaptor primer. Generated fragments were subcloned into pCR3.1 vector using a eucaryotic TA cloning kit (Invitrogen, USA) and sequenced. A complete full-length cDNA sequence was isolated using amplification with primers 14 and 12. For polymerase chain reaction, we used the PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA) and the following conditions for PCR amplifications: 3 U Taq DNA polymerase (Top-Bio, Czech Republic), 0.2 mM deoxynucleotides, 0.6 μM of each primer, and buffer provided by the enzyme manufacturer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100]. We amplified for 40 cycles, with initial denaturation for 3 min at 94˚C; each cycle consisted of 1 min denaturation at 94˚C, 1 min and 30 sec annealing at 60˚C, and 2 min and 30 sec extension at 72˚C; followed by a final extension of 7 min at 72˚C. Amplified fragments were visualized on ethidium bromide-stained 1% agarose gels.

Molecular cloning of mouse galectin-6 cDNA. Total RNA was isolated from mouse colon using TRIzol (Gibco, USA) as recommended by the manufacturer. RNA was transcribed into cDNA using hexanucleotides and the cDNA was used as a template for PCR with forward primer Gale4-Kzf (primer 14) and reverse primer G6TGAr (5'-gatctgagctcctcctggggaagg-3'). The generated fragment was subcloned into pCR3.1 vector

Table I. Primers designed for the cloning of mouse galectin-4 cDNA.

No.	Name	Position	Orientation	Sequence
1	mG6F-F	(-17)-1	+	5'-acgctgccctacaagag-3'
2	Gale4F1	179-203	+	5'-cgcgggatccagcggacaatggggcaaggaagaga-3'
3	Gale4F2	274-296	+	5'-cgcgggatcccaaggttgtggtgaacggaaatt-3'
4	Gale4F3	463-486	+	5'-caaccctccacagatgaacacctt-3'
5	Gale4F4u	581-604	+	5'-aagggatatgtacttcctacagcc-3'
6	rG4C-R	240-258	-	5'-accatgaacaccagctcga-3'
7	Gale4R1	430-454	-	5'-cgcgtctagaactccccgcagggtaagctggaata-3'
8	Gale4R3	606-629	-	5'-ccttgaagttgatgacaaagttcc-3'
9	Gale4R4u	614-637	-	5'-cgatcccaccttgaagttgatgac-3'
10	Gale4R2	856-879	-	5'-tccagcgtgtctaccatttggaat-3'
11	Gale4TGAr	896-919	-	5'-tcagatctggacgtaggacaaggt-3'
12	Gale4-3UTr	967-991	-	5'-ggtccttcaacattttattaggagc-3'
13	Gale4-5UTf	(-60)-(-36)	+	5'-cagccaccacttcccacacatctct-3'
14	Gale4-Kzf	(-25)-(1-)	+	5'-caagctatcagcccagactctcaag-3'

Table II. Primers designed for the generation of bacterial expression constructs carrying mouse galectin-4, CRD1 and CRD2 sequences.

No.	Name	Sequence position	Strain	Sequence
1	G4cDNAfB	3-27	+	5'-cgcgggatccggcctatgttccagcaccgggctac-3'
2	G4cDNArH	958-981	-	5'-acataagctttcagatctggacgtaggacaaggt-3'
3	G4CRD1rH	433-456	-	5'-acataagctttcagcctccgaggaagttgatggactg-3'
4	G4CRD2fB	578-606	+	5'-cgcgggatccgcgtgcgccatatgtgggggctctg-3'

using the eucaryotic TA cloning kit (Invitrogen) and sequenced. A complete full-length cDNA sequence carries a 25-bp 5'-end UTR and 906 bps of a coding region. For polymerase chain reaction, we used the PTC-200 Peltier thermal cycler (MJ Research Inc.) and the following conditions for PCR amplifications: 3 U Taq DNA polymerase (Top-Bio), 0.2 mM deoxynucleotides, 0.6 μM of each primer, and buffer provided by the enzyme manufacturer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100]. We amplified for 40 cycles, with initial denaturation for 3 min at 94˚C; each cycle consisted of 1-min denaturation at 94˚C, 1 min and 30 sec of annealing at 60˚C, and 2 min and 30 sec of extension at 72˚C; followed by a final extension of 7 min at 72˚C. Amplified fragments were visualized on ethidium bromide-stained 1% agarose gels.

Construction of bacterial expression vectors for galectin-4 and its CRD forms To obtain DNA fragments encoding full-length cDNA, CRD1 and CRD2 of mouse galectin-4, two sets of primers were synthesized which are listed in Table II. BamHI and HindIII restriction sites were incorporated into the primers for the purpose of subcloning. For expression of the galectin-4 cDNA fragment, we used primers 1 and 2, to generate CRD1 coding fragment primers 1 and 3 and for CRD2 primers 4 and 2. By PCR using pCR3.1/gale-4 cDNA as a template, cDNA encoding amino acid sequences 1-326+1 stop codon, 1-152 and 194-326+1 stop codon were separately amplified. The resulting PCR products were gel-purified (Gen-Elute kit, Sigma-Aldrich) and ligated into the pQE-31 expression vector containing a His-tag (Qiagen). Positively transformed *E.coli* M15 colonies were selected on agar plates containing ampicilin and kanamycin. Recombinant plasmids were isolated using a QIAprep spin miniprep kit (Qiagen). For DNA sequencing, the recombinant plasmids were re-transformed into *E.coli* JM109 cells. For PCR, we used the PTC-200 Peltier thermal cycler (MJ Research Inc.) and the following conditions for PCR amplifications: 3 U Taq DNA polymerase (Top-Bio), 0.2 mM deoxynucleotides, 0.6 μM of each primer, and buffer provided by the enzyme manufacturer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100]. We amplified for 40 cycles, with initial denaturation for 3 min at 94˚C; each cycle consisting of 1-min denaturation at 94˚C, 1 min and 30 sec of annealing at 60˚C, and 2 min and 30 sec of extension at 72˚C; and a final extension for 7 min at 72˚C. Amplified fragments were visualized on ethidium bromide-stained 1% agarose gels. The generated plasmids were called pQE-31/HIS-GALECTIN-4, pQE-31/HIS-CRD1 and pQE-31/HIS-CRD2.

Production and purification of recombinant mouse galectin-4 and CRD forms. To express mouse galectin-4 or its CRD1 or CRD2 domains, the *E. coli* M15 cells containing pQE-31 vector carrying the coding sequence of galectin-4 (CRD1, CRD2) were grown in LB medium at 37˚C until reaching A_{550} value 1, then the culture was shifted to 30˚C and expression of galectin-4 (CRD1, CRD2) was induced with 1 mM ETG at the A_{550} 1.2.

For isolation of the recombinant product, cells were centrifuged, resuspended in phosphate buffer (PBS, 5 mM EDTA, 5 mM ß-mercaptoethanol, 50 μM PMSF, pH 7.4) and disintegrated by sonication. Triton X-100 (0.05%) was included in the buffer for sonication of cells carrying CRD2. Cell supernatant was passed through a lactosyl-agarose column (4˚C); in the case of CRD2, the supernatant was incubated batch-wise with lactosyl-agarose beads overnight (4˚C). Bound proteins were subsequently eluted with buffer A (PBS, 5 mM EDTA, 5 mM ß-mercaptoethanol, pH 7.4) containing 200 mM lactose. Final purification of recombinant galectin-4 (CRD1, CRD2) was achieved by gel filtration on Superdex 200 HR.

Isolation of RNA and RT-PCR. Total RNA was isolated from various mouse tissues and cell lines using TRIzol (Gibco) as recommended by the manufacturer. The amount of RNA in each sample was estimated by absorbance at 260 nm. RT-PCR was performed using an Access RT-PCR system (Promega, USA), which allowed us to perform the reverse transcription and DNA polymerization in one tube. Samples of total RNA were heated at 95˚C for 2 min and cooled to 4˚C. The amount of 0.5 μg of RNA was used for RT-PCR reaction. The temperature conditions used for RT and PCR cycling were as described by the manufacturer.

RT-PCR analysis of galectin-4 expression during early mouse embryogenesis was performed using a Superscript III One-Step RT-PCR system with Platinum TaqDNA polymerase (Invitrogen). RT-PCR was performed with 50 μl reaction mixture containing 2X Reaction Mix, 90 U/50 μl RNaseOUT™ recombinant ribonuclease inhibitor, a forward and reverse primer at 0.2 μM, 2 μl SuperScript™ III RT/Platinum Taq Mix and embryos which were washed in PBS and, in a volume of 3 μl, added to the RT-PCR reaction mix. Samples were denaturated for 30 min at 55˚C and reverse-transcribed for 2 min at 94˚C. Then, 40 cycles of PCR amplification were performed with denaturation at 94˚C for 15 sec, annealing at 56˚C for 30 sec, and extension at 68˚C for 1 min/kb followed by a final extension at 68˚C for 5 min. An amount of 3 μl from this RT-PCR mixture was used as a template for 30 μl nested PCR under the following conditions: 3 U TaqDNA polymerase (Top-Bio), 0.2 mM deoxynucleotides, 0.6 μM of each primer, and buffer provided by the enzyme manufacturer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100]. Amplification was performed after 30 cycles, with initial denaturation for 3 min at 94˚C. Each cycle consisted of 30-sec denaturation at 94˚C, 30-sec annealing at 56˚C, and 1-min extension at 72˚C. The final extension ran for 7 min at 72˚C. Amplified fragments were visualized on ethidium bromide-stained 1% agarose gels.

For specific detection of galectin-4 mRNA expression, we used forward primer Gale4F3 (5'-CAACCCTCCACAGA TGAACACCTT-3') and reverse primers Gale4R2 (5'-TCCA GCGTGTCTACCATTTGGAAT-3'), where the former one corresponds to galectin-4 specific exon sequence. For specific detection of galectin-6 mRNA expression, we used forward mG6Rf (5'-GTCCAACCTGTTGAAACCAA-3') and reverse primer mG4Kr (5'- CCTATGTCCAGATCTGAGC-3'). As controls, we used the primers corresponding to mouse ß-actin (GenBank™ accession no. M32599; ß-actin forward primer, 5'-CCCAGAGCAAGAGAGCTATCCTG-3', ß-actin reverse primer, 5'-ATGCCACAGCATTCCATACCCAAG-3').

In situ hybridization on mouse tissue sections

Probes. A 275-bp galectin-4 cDNA fragment, generated by Gale4F1 and Gale4R1 primers (Table I), was cloned in sense and antisense orientation into pCR3.1 vector. ^{35}S-cDNA probes were then prepared by T7 polymerase transcription from both recombinant pCR3.1 vectors. DNA of these plasmids was linearized with XhoI. After agarose electrophoresis and gel purification using a GenElute purification kit (Sigma-Aldrich), sense and antisense transcripts were generated with T7 RNA polymerase, using the Riboprobe combination system - SP6/T7 (Promega) with 10 mM each of ATP, GTP, CTP, 0.5 mM UTP and 50 μCi of 5'[α-^{35}S] UTP (10 μM) per transcription. The transcripts were purified by incubation with RQ1 DNase and ethanol precipitation. Activity of the probes was evaluated on a Wallac 1410 liquid scintilation counter and $2.5x10^4$ cpm/μl was used for hybridization.

Tissue samples. Mouse organs were cut and fixed with 10 volumes of 4% paraformaldehyde and embedded in paraffin according to standard procedure. These specimens were cut into 5-μm sections and stored at -20˚C. The paraffin embedded sections were prepared for *in situ* hybridization as follows: incubation, 2 h at 40˚C; in xylen, 10 min; in 100% ethanol, 5 min; 95, 90, 80, 70, 50, 30% ethanol, 1 min; 1X PBS, 1 min; 4% paraformaldehyde, 20 min; 1X PBS, 5 min; proteinase K (20 μg/ml), 10 min at 37˚C; 1X PBS, 5 min; 4% paraformaldehyde, 10 min; 1X PBS, 5 min; 1.86% triethanolamin/0.25% acetanhydrid, 10 min; 1X PBS, 5 min; 0.85% NaCl, 5 min; 30, 50, 70, 85, 95% ethanol, 1 min; and 100% ethanol, 2 min. The antisense and sense transcripts were hybridized with the mouse tissue sections in a wet chamber overnight at 55˚C. The hybridization solution consisted of 50% formamide, 10% dextran sulfate, 1X Denhardt's solution, 20 mM Tris (pH 7.5), 5 mM EDTA, 500 μg/ml tRNA 0.3 M NaCl, and 10 mM DTT. Samples were washed in 5X SSC/0.1% ß-mercaptoethanol for 10 min at 55˚C, 2X SSC/0.1% ß-mercaptoethanol/50% formamide for 20 min at 65˚C, and three times in 0.5 M NaCl/10 mM Tris (pH 7.5)/5 mM EDTA (=NTE) for 10 min at 37˚C, incubated with RNase A 20 μg/ml in NTE for 10 min at 37˚C, NTE for 15 min at 37˚C, 2X SSC for 15 min at 37˚C and 0.5X SSC for 15 min at 37˚C. After washing, the tissue samples were incubated in 0.3 M NH4Ac/30, 60, 80, 95% ethanol, respectively, and twice in 100% ethanol and dried. Then the samples were covered with photography emulsion in a dark room. After 5 days, exposition and development was performed and the samples were stained with Mayer's hematoxylin. Dark field and light microscopy photos demonstrate the results.

Immunohistochemical analysis on frozen mouse tissue sections. Tissue of C57Bl/6J mouse male (Table IV) was collected into

Tissue-Tek (Sakure-Finetek, Zoeterwoude, The Netherlands) as cryoprotectivum and frozen with liquid nitrogen. Frozen sections were made using Cryocut-E (Reichert-Jung, Vienna, Austria). Both carbohydrate recognition domains, CRD1 and CRD2, as well as the whole molecule, CRD1+2, of galectin-4 were biotinylated (Exbio, Prague, Czech Republic) and employed as probes as described after short-term paraformaldehyde fixation (32). Probes were diluted to the final concentration of 20 μg of lectin/1 ml of incubation medium. Specificity of reaction was tested by preincubation of diluted lectin with 5 mM of lactose as competitive inhibitor for 60 min at room temperature. ExtrAvidin-TRITC (Sigma-Aldrich, Prague, Czech Republic) was used as a second step reagent. The nuclei were counterstained with DAPI (Sigma-Aldrich) and specimens were mounted to Vectashield (Vector Laboratories, Burlingame, CA, USA). Specimens were analyzed by fluorescence microscope Nikon 90i (Optoteam, Prague, Czech Republic) equipped with a cooled high-resolution CCD camera, Vosskhüler, and computer assisted image analysis system, LUCIA 5 (both from Laboratory Imaging, Prague, Czech Republic).

Preparation of specific antisera against mouse galectin-4. The peptide named PM02 (H-GAMTIPAYPAGSPGTNPPQMNT LPG-OH), representing a part of the link between protein domains, was synthesised by PolyPeptide Laboratories (Prague, Czech Republic). Two immunogens were prepared by coupling the peptide to OVA-Ovalbumin A5503 (Sigma, USA) and T-Thyroglobulin T1126 (Sigma) with EDCI [1-ethyl-3-(dimethylaminopropyl)-carbodiimide] HCl E1769 (Sigma-Aldrich). The 30 mg of PM02 for OVA and 3 mg of PM02 for T were dissolved in 1 ml of phosphate-buffered saline (PBS) pH 7.4. The 5.5 mg of OVA was dissolved in 3 ml PBS and 8 mg of T was dissolved in 2 ml PBS. Appropriate solutions of peptides and protein carriers were mixed. The solution of 5 mg of EDCI in 300 μl of PBS (OVA) and 1 mg of EDCI in 100 μl of PBS (T) was dropped into the reaction mixture. The mixtures were allowed to react for 12 h at 25˚C under gentle agitation. After this time, 4 ml of 1 M hydroxylamine (OVA) and 3 ml of 1 M hydroxylamine (T) were added and the reaction continued for 4 h. Reaction mixtures were centrifuged at 5,000 x g for 10 min at 4˚C and supernatant was chromatographed on Sephadex G-25 fine (Pharmacia, Sweden)/PBS in a 1.6x36-cm (70 ml) column. The flow rate was 2 ml/min and volume of fraction was 3 ml. Conjugates were eluted in an interval of 4th-9th fraction. These fractions were concentrated via ultrafiltration through a partition wall with cut-off at 5 kDa. The final concentration of the conjugate solution was 3 mg/ml and these solutions were frozen (-70˚C) in 1-ml aliquots.

Two New Zealand rabbits, three to four months old, were used for immunization. The first rabbit (020) was immunized with OVA-conjugate and the second rabbit (019) was immunized with T-conjugate. The immunization dose was 150 μg of conjugate for one animal and individual doses were applied in three-week intervals. For the first immunization, solutions of conjugates were emulsified with complete Freund adjuvant (Sigma-Aldrich) and injected subcutaneously at two sites. For the second, fourth and sixth immunizations, solutions of conjugates were emulsified with incomplete Freund adjuvant and injected subcutaneously at two sites. In the third and fifth doses, solutions of conjugates were emulsified with AL-SPAN-OIL adjuvant (Sevapharma, Prague, Czech Republic) and injected intramuscularly at two sites. Before the immunization and 7-10 days after the last dose, rabbits were bled from the central ear artery and blood was centrifuged at 4˚C. The obtained serum was tested and stored at -70˚C.

The obtained antisera were tested using ELISA titration. As the antigen, immunogenic conjugates (OVA, T) and recombinant mouse galectin-4 were used. The 96-well polystyrene microtitre plates, High Binding, 3590 (COSTAR, USA), were coated with 4.0 μg/ml immunoconjugate solution and 5.0 μg/ ml galectin-4 solution in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated for 12 h at 4˚C. The plates were washed three times with PBS supplemented with 0.5% Tween-20 (PBS-Tw). The remaining binding sites were blocked with 200 μl of 1% skim-milk powder, F 70166 (Fluka, Germany), in PBS-Tw buffer for 1 h at room temperature. The plates were washed three times with PBS-Tw buffer. Antisera were diluted 1:100 and further 2^n series, maximum dilution was 1:102,400 in 0.2% skim-milk powder in PBS-Tw buffer. These samples (100 μl) were applied to wells of a microtitre plate and incubated for 2 h at 37˚C. The plates were washed three times with PBS-Tw buffer. Peroxidase-labelled goat anti-rabbit IgG A-6154 (Sigma-Aldrich) diluted 1:2,000 in PBS with 1% BSA were added in 100-μl volumes to each well and incubated for 1 h at room temperature. The plates were washed four times with PBS-Tw buffer. Citric acid buffer (0.04 M, pH 5.0) in phosphate buffer containing orthophenylenediamine P-6912 (Sigma-Aldrich) 0.05% and hydrogen peroxide 0.1% was added in 100-μl volumes to each well. The reaction was stopped after 10 min by adding 100 μl of 2 M sulphuric acid. Absorbances were read at 492 nm on the microELISA reader, Hidex Chameleon, Driver Version 4.20 (Hidex, Finland).

Glycan array screening of the binding specificity of mouse galectin-4 and CRD1 and CRD2 forms. Prior to screening, recombinant mouse galectin-4 and CRD forms were labeled using an Alexa Fluor® 488 protein labeling kit (Molecular Probes, A-10235) according to the manufacturer's protocol. The fluorescently labeled recombinant proteins were screened on the Consortium plate based glycan array in binding buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween-20 and 1% BSA. The lectin was screened as previously described (33). Briefly, the plate consists of array biotinylated glycosides (34) bound to streptavidin-coated microtiter plates in replicates of n=4. Pre-coated plates were washed three times with 100 μl of wash buffer (binding buffer minus BSA) prior to incubation. A stock solution of fluorescently labeled recombinant galectin-4, or the CRD forms, (mGal-4, 2.8 μg/ml; CRD1, 17.7 μg/ml; and CRD2, 12.3 μg/ml) was added to each well and incubated at room temperature for 1 h. The plates were washed and read in 25 μl of wash buffer on a Victor-2TM 1420 Multi-label Counter (Perkin-Elmer Life Sciences) at excitation 485/ emission 535.

To identify primary binding affinity, the glycans were ranked according to their signal to noise (S/N) by dividing their mean relative fluorescence units by the mean background generated in control wells lacking glycosides. This value was

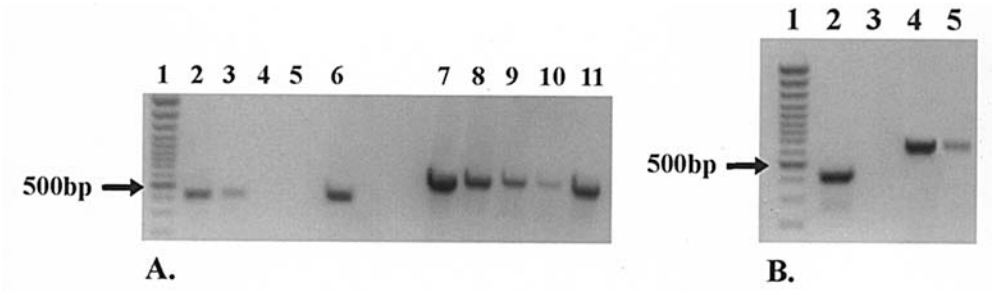

Figure 1. Analysis of galectin-4 expression during early mouse embryogenesis. (A) Results indicate galectin-4 expression in spermatozoons (lane 2), oocytes (lane 3) and 8-cell embryos (lane 6). Galectin-4 expression was not detectable in 2-cell embryos (lane 4) and 4-cell embryos (lane 5). Lanes 7-11 show the corresponding ß-actin expression in the same order. The molecular weight marker is in lane 1. (B) Lane 2 shows galectin-4 expression in 16-cell embryos, galectin-4 expression was not detectable in 4-cell embryos (lane 3). Lanes 4 and 5 show the corresponding ß-actin expression. The molecular weight marker is in lane 1.

compared to the average S/N for all wells in the array and the results were then ranked as high affinity (>3*avg. S/N) and medium affinity (>2*avg. S/N).

The streptavidin/biotin glycan array is listed as Plate array v3 on the Consortium website at: http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh5.shtml.

Results

Mouse galectin-4 and galectin-6 cDNA cloning. To isolate mouse galectin-4 cDNA, we screened mouse liver Marathon-Ready cDNA library using PCR primers designed from a partial known mouse cDNA sequence, a rat galectin-4 cDNA sequence and a putative galectin-4 EST clone, AA265412, as described above. In combination with 3'RACE technique, a full-length cDNA sequence carrying a 60-bp 5'-end UTR, 981 bps of a coding region and 71 bps of a 3'-end UTR including an AATAAA box was obtained, subcloned and sequenced. Three independent clones shared 100% sequence similarity. The found sequence was compared to genomic sequences presented in the genome databases and then submitted to the GenBank under accession number AY044870 as the first purified mouse galectin-4 full-length sequence. The sequence was then used for the construction of bacterial expression vectors required for recombinant galectin-4 preparation. For galectin-6 cDNA isolation, we designed a gene-specific reverse primer containing the point mutations found between galectin-6 and galectin-4 known genome sequences. Using RT-PCR and RNA isolated from the colon, we amplified a cDNA sequence which shares 100% sequence similarity to the mouse galectin-6 cDNA formerly published (3,4).

Expression of galectin-4 and galectin-6 in mouse tissues and cell lines. For analysis of galectin-4 expression during early mouse embryogenesis, one-step RT-PCR was used. Results indicate the expression of galectin-4 in spermatozoons, oocytes, 8-cell embryos, and 16-cell embryos. In contrast, the expression was not detectable in 2- and 4-cell embryos (Fig. 1).

To examine the tissue distribution of galectin-4 and galectin-6, we used RT-PCR assay with mRNA isolated from selected mouse tissues and cell lines. The analysis was performed using a Gale4F3 primer specific for galectin-4 amplification, and a Gale4R2 reverse primer. Similarly,

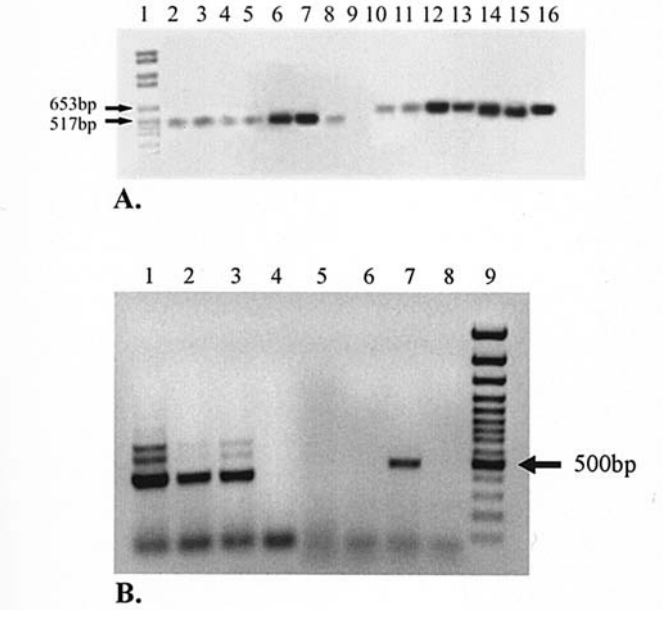

Figure 2. Expression of the galectin-4 and galectin-6 genes in mouse tissues and cell lines. (A) Results indicate galectin-4 (lanes 2-8) and galectin-6 (lanes 10-16) expression in the small intestine (7/15), colon (6/14), liver (8/16), kidney (4/12), spleen (5/13) and the heart (3/11) and also in cultured cells of the mouse T cell lymphome BW-5147 (2/10) line. Lane 1 shows molecular weight marker and lane 9 negative control with no reverse transcription. (B) Galectin-4 is expressed in the in the BW-5147 (1), 3T3 (2) and P19 (3) cell lines. Galectin-6 is expressed in the P19 (7) cells but no expression was found in the BW-5147 (5) and 3T3 (6) cell lines. The molecular weight marker is in lane 9 and negative controls are in lanes 4 and 8.

galectin-6 specific expression was investigated using RT-PCR with primers mG6Rf and mG4Kr, which were used previously (3). We found that galectin-4 as well as galectin-6 specific transcripts are presented in the small intestine, colon, liver, kidney, spleen and heart (Fig. 2A). While galectin-4 was found to be expressed in cultured BW-5147, 3T3 and P19X1 cells, galectin-6 was expressed only in P19X1 cells (Fig. 2).

To confirm the galectin-4 expression in mouse tissues, we also performed *in situ* hybridization on the sections of mouse liver and small intestine. Strong expression was detected in the small intestine and a weaker signal was obtained for the liver tissue. As the control experiments with the sense cRNA probe do not give a positive output from any tissue, we assumed that the detection is specific (Fig. 3).

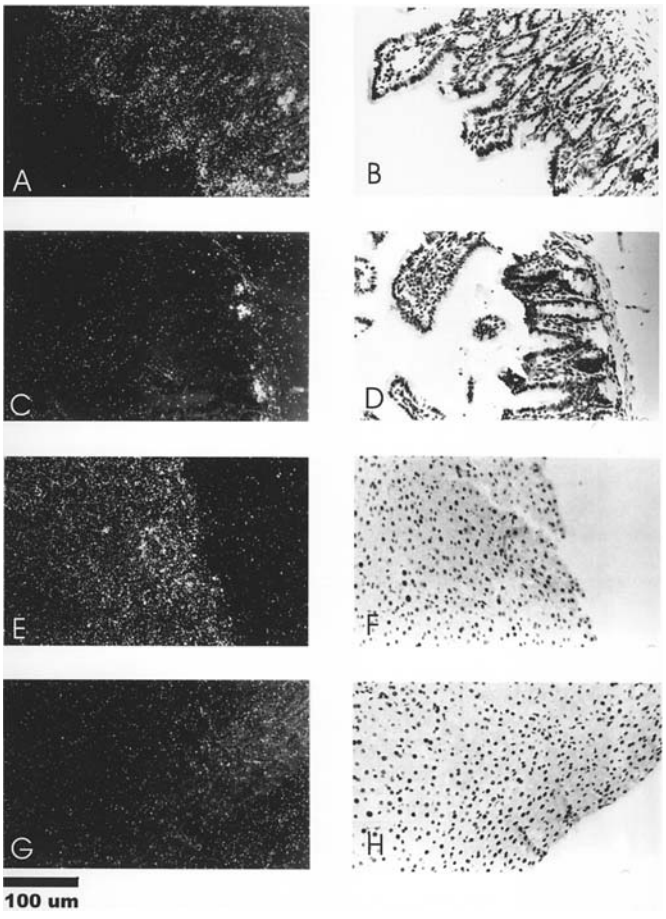

Figure 3. *In situ* hybridization. Staining of the mouse small intestine (A) and liver (E) with the 275-bp antisense galectin-4 cRNA probe and control negative signal with the sense cRNA probe (C and G, respectively). Brighfield view of the corresponding sections after hematoxylin staining. (B, D, F, H).

Preparation of recombinant galectin-4, CRD1 and CRD2 as His-tag fusion proteins. After PCR reaction using galectin-4 cDNA as a template and the primers listed in Table II, we obtained products with restriction sites of approximately 1000, 480 and 420 bp. The DNA products were ligated into the expression vectors containing a His-tag (consisting of 6 histidine residues), and recombinant His-tag proteins were expressed in *E. coli* M15 cells after induction with IPTG. SDS-PAGE analysis of a crude extract from non-induced and induced bacteria revealed IPTG-dependent induction of His-tag/galectin-4, His-tag/CRD1 and His-tag/CRD2 proteins at a molecular weight of approximately 36, 20 and 18 kDa, respectively (Fig. 4). Successful production of fusion proteins was further confirmed by Western blotting and specific detection of the His-tag by Ni-NTA-HRP conjugate. Recombinant proteins were water-soluble and partially secreted into cultivation media.

Anti-galectin-4 antisera were raised in rabbits using PM02 coupled to ovalbumin and thyroglobulin via the EDCI conjugation method presented in Materials and methods. Its immunoreactivity was examined by ELISA system. The antisera react with both immunogenic conjugates as well as with recombinant mouse whole-complex galectin-4 while control pre-immune antisera do not react to these chemicals.

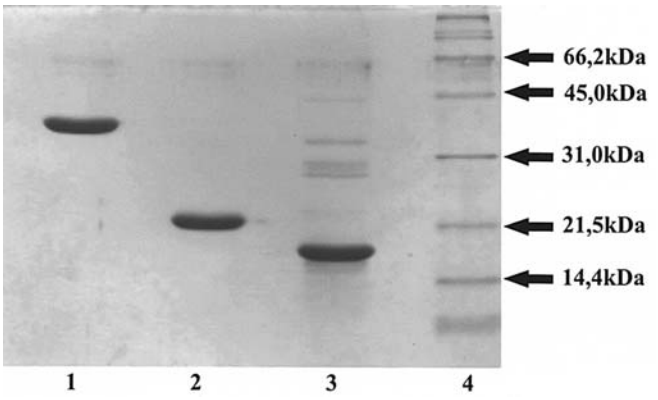

Figure 4. SDS-PAGE of the recombinant galectin-4 and CRD1 and CRD2 protein forms. Results indicate the expression of the complete galectin-4 protein in lane 1, CRD1 in lane 2, CRD2 in lane 3 and protein weight marker in lane 4.

Glycan array analysis of the glycan binding profile of mouse galectin-4 and both CRD domains. Table III summarizes the glycan array binding specificity data for mouse galectin-4 and the CRD1 and 2 domains. Fig. 5 shows the binding profile of each recombinant protein and the differential binding pattern displayed by each. The holoprotein bound with highest affinity to blood group A and B structures in the array. It also bound to lactose sulfated on the 3 and 3' positions, as well as LNT and LNnT and, very specifically, to GalNAcα1-3GalNAcα-. The two CRD domains exhibited binding that distinctly differentiated the blood group A and B specificity. CRD1 binding was specific for type 2 blood group B structures. CRD2 was specific for type 2 blood group A structures. CRD1 had a broader specificity for other ligands approaching that of the holoprotein.

Detection of epitopes reactive for CRD1, CRD2 and the complete galectin-4. Table IV and Fig. 6 summarize results of the monitoring of tissue distribution of epitopes reactive for CRD1, CRD2 and the whole-complex of galectin-4 (CRD1+2) in selected mouse tissues. No remarkable differences in the binding reactivity of all three probes to tissue were detected except for the observation that CRD1+2 recognizes the suprabasal layer of squamous epithelium covering the tongue that was negative for the binding of both CRD1 and CRD2 separately. This epithelium reactivity seems to be restricted to mucosa only because epidermis representing the same tip of epithelium was areactive. Large and small intestine as well as myocardium, striated musculature, liver and splenic lymphocytes were negative for the expression of epitopes reactive for all three used probes. Macrophages of red pulp of the spleen and alveolar macrophages were recognized by both CRD1 and CRD2 as well as by CRD1+2.

To study whether anti-galectin-4 rabbit antiserum can inhibit the binding of galectin with reactive epitopes, we used double color immunochemistry on the section of the tongue where the complete CRD1+2 form of galectin-4 was bound but no binding of the CRD1 or CRD2 domain was observed (Fig. 7). The result confirmed that galectin-4 is expressed in the epithelium of the tongue as documented by binding of green labeled antibody but reactive epitopes for this molecule

Table III. For each of the proteins analyzed, the glycans listed are ranked as high or medim affinity based on S/N values within each assay. For comparative analysis of their relative binding affinity, the values are normalized to the S/N of B-tetra Lac (glycan no. 200).

Glycan no.	Glycan	Trivial name	S/N	Rank	Normalized
Mouse galectin-4					
200	Galα1-3(Fucα1-2)Galß1-4Glc-SP1	B-tetra Lac	20.13	High	1.00
202	GalNAcα1-3(Fucα1-2)Galß1-4Glc-SP1	A-tetra Lac	16.97	High	0.80
201	Galα1-3(Fucα1-2)Galß1-4GlcNAc-SP1	B-tetra type 2	16.01	High	0.75
168	Galα1-3(Fucα1-2)Galß1-4GlcNAcß-Sp2	B (type 2)	13.31	High	0.62
73	GalNAcα1-3GalNAcα-Sp2	Fs-2	10.85	High	0.51
172	GalNAcα1-3(Fucα1-2)Galß1-4GlcNAcß-Sp2	A (type 2)	9.30	High	0.44
203	GalNAcα1-3(Fucα1-2)Galß1-4GlcNAc-SP1	A-tetra type 2	8.27	High	0.39
169	Galß1-3GlcNAcß1-3Galß1-4Glcß-Sp2	LNT	6.72	High	0.32
199	[3OSO_3]Galß1-4Glcß-SP1	3'SuLac	5.65	High	0.27
193	Galα1-3Galß1-4Glcß-Sp1	Galili-tri	5.57	High	0.26
104	Fucα1-2Galß1-4Glcß-Sp1	2'FL	5.53	High	0.26
191	Galß1-4[6OSO_3]Glcß-Sp1	6SuLac	5.47	High	0.26
161	3'-O-Su-Galß1-3GalNAcα-Sp2	3'-O-su-TF	4.74	Med	0.22
32	Galß1-4GlcNAcß1-3Galß1-4Glcß-Sp1	LNnT	4.54	Med	0.21
17	Galß1-3Galß-Sp2	Galß1-3Gal	4.10	Med	0.19
CRD1					
200	Galα1-3(Fucα1-2)Galß1-4Glc-SP1	B-tetra Lac	21.31	High	1.00
73	GalNAcα1-3GalNAcα-Sp2	Fs-2	11.05	High	0.52
199	[3OSO_3]Galß1-4Glcß-SP1	3'SuLac	9.47	High	0.44
201	Galα1-3(Fucα1-2)Galß1-4GlcNAc#SP1	B-tetra type 2	9.05	High	0.42
191	Galß1-4[6OSO_3]Glcß-Sp1	6SuLac	7.21	High	0.34
33	GlcNAcß1-3Galß1-4Glcß-Sp1	LNT-2	5.39	High	0.25
32	Galß1-4GlcNAcß1-3Galß1-4Glcß-Sp1	LNnT	5.03	High	0.24
17	Galß1-3Galß-Sp2	Galß1-3Gal	4.46	High	0.21
193	Galα1-3Galß1-4Glcß-Sp1	Galili-tri	4.39	High	0.21
161	3'-O-Su-Galß1-3GalNAcα-Sp2	3'-O-su-TF	4.01	Med	0.19
104	Fucα1-2Galß1-4Glcß-Sp1	2'FL	3.86	Med	0.18
169	Galß1-3GlcNAcß1-3Galß1-4Glcß-Sp2	LNT	3.35	Med	0.16
168	Galα1-3(Fucα1-2)Galß1-4GlcNAcß-Sp2	B (type 2)	3.22	Med	0.15
21	Galß1-4GlcNAcß-Sp1	LacNAc	2.92	Med	0.14
CRD2					
203	GalNAcα1-3(Fucα1-2)Galß1-4GlcNAc-SP1	A-tetra type 2	27.31	High	13.58
202	GalNAcα1-3(Fucα1-2)Galß1-4Glc-SP1	A-tetra Lac	22.68	High	11.28
172	GalNAcα1-3(Fucα1-2)Galß1-4GlcNAcß-Sp2	A (type 2)	22.39	High	11.13
201	Galα1-3(Fucα1-2)Galß1-4GlcNAc-SP1	B-tetra type 2	2.85	Low	1.42
200	Galα1-3(Fucα1-2)Galß1-4Glc-SP1	B-tetra Lac	2.01	Low	1.00
168	Galα1-3(Fucα1-2)Galß1-4GlcNAcß-Sp2	B (type 2)	1.94	Low	0.96
184	9-OS-Sp3	9-OS	1.75	Low	0.87

are expressed only in highly restricted areas in the suprabasal layer of the epithelium as indicated by the red color of recombinant galectin-4. Polyclonal antibody did not suppress the galectin-epitope binding.

Discussion

Galectin-4 and galectin-6 found in the mouse are closely related tandem-repeat members of the galectin family. Both galectins were described to be expressed in the small intestine and colon in recent studies (3,9). Therefore, the biological function of both galectins is generally expected to play an important role in tissue of the alimentary tract. This was recently supported by Hokama *et al* (10) who demonstrated that endogenous galectin-4 contributes to the exacerbation of chronic colitis and that this molecule functions to prevent recovery from acute intestinal injury. The functional role of galectin-6 has not been defined nor has detailed expression

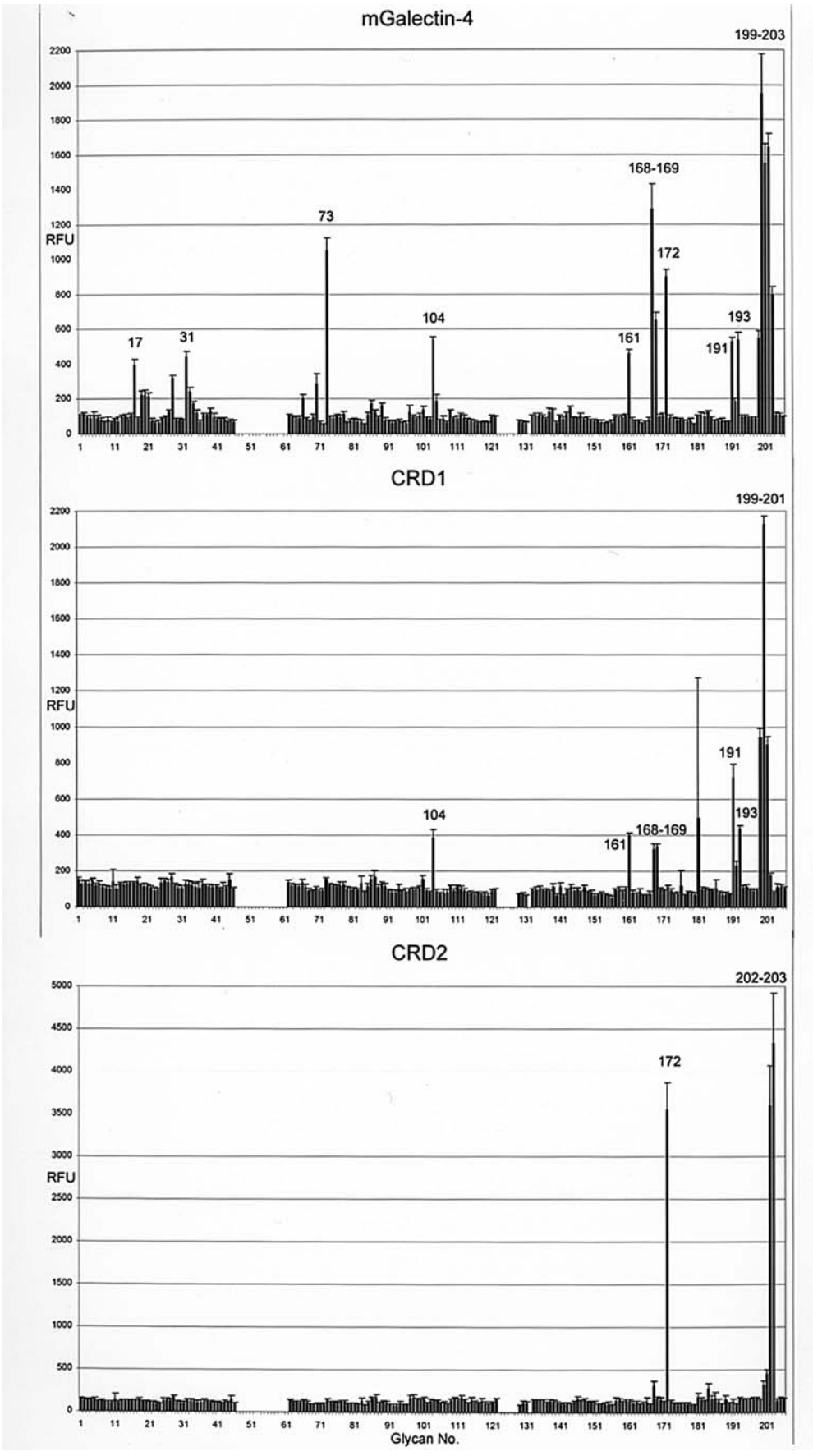

Figure 5. Glycan array binding specificity profiles for mouse galectin-4, CRD1 and CRD2 using Glycan Array v3. Binding specificity profiles are presented for each recombinant protein screened against an array of 185 unique glycans. The holoprotein mouse galectin-4 bound to type 2 A and B tetra lactose and LacNAc with and without fucose (glycans no. 200-203, 168, 172). It also bound uniquely to several other sulfated glycans (199, 161) and to LNT and LNnT structures (169, 32) and Fs-2 (73). The CRD1 displayed a very similar profile to the holoprotein; it recognized type 2 B-tetra Lac as the highest affinity ligand (200). The major difference was that it did not recognize type 2 blood group A structures. CRD2 bound with high affinity to type 2 blood group A structures (172, 202, 203) and with only low affinity to blood group B structures (168, 200, 201) and none of the other structures were recognized by the holoprotein.

Table IV. Tissue distribution of epitopes reactive for CRD1, CRD2 and the whole-complex galectin-4 molecule (CRD1+2).

Probe	Liver	Spleen		Kidney		Myocardium	Lung			Tongue		Skin	
		WP	RP-MPh	Gl	T		B	A-E	A-MPh	M-E	M	E	D
CRD1	-	-	+	-	++	-	-	-	+	-	-	-	-
CRD1 (+ lactose)	-	-	-	-	-	-	-	-	-	-	-	-	-
CRD2	-	-	+	-	++	-	-	+	-	-	-	-	
CRD2 (+ lactose)	-	-	-	-	-	-	-	-	-	-	-	-	-
CRD1+2	-	-	+	-	++	-	-	-	+	++	-	-	-
CRD1+2 (+ lactose)	-	-	-	-	-	-	-	-	-	-	-	-	-

WP, white pulp; RP-MPh, red pulp macrophages; Gl, glomeruli; T, tubuli; B, bronchioli; A-E, alveolar epithelium; A-MPh, alveolar macrophages; M-E, mucosa epithelium; M, musculature; E, epidermis; D, dermis; E, epidermis. Scale: -, negative signal; +, positive signal; ++, strong positive signal.

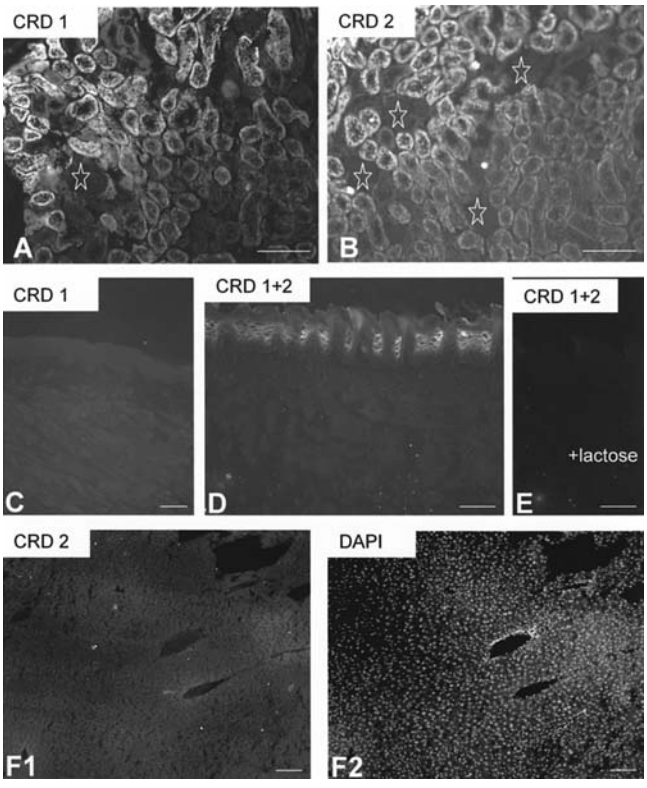

Figure 6. Detection of epitopes reactive for CRD1, CRD2 and the complete galectin-4 on frozen mouse tissue. Detection of epitopes reactive to CRD1 (A and C), to CRD2 (B and F1) and whole galectin-4 (CRD1+2; D and E) to mouse kidney (A and B), tongue (C-E) and liver (F1) without (A-D, F1) and after lactose (E) administration as competitive inhibitor. The same field of liver after detection of DNA in cell nuclei by DAPI is in panel F2. Asterisks indicate the position of glomeruli, bar is 100 μm.

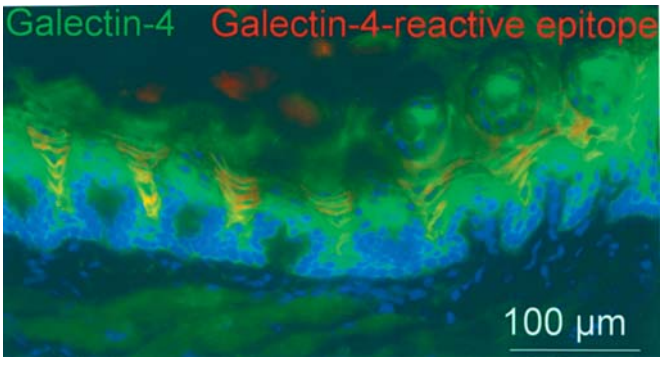

Figure 7. Double-color immunochemistry on a section of tongue using anti-galectin-4 polyclonal antibody and the complete form of recombinant galectin-4. For immunohistochemical staining, a section of mouse tongue was used and anti-mouse galectin-4 antiserum was applied. Then, the section was stained with biotinylated recombinant galectin-4 (red color). The result shows that galectin-4 is expressed in tongue tissue (antibody-green colour) but reactive epitopes for this molecule are expressed only in restricted areas of the suprabasal layer of the epithelium (red, complete recombinant galectin-4, CRD1+2).

analysis been performed in the mouse. As both galectins share more than 90% DNA sequence similarity, formerly published data on the expression of mouse galectin-4 using hybridization techniques do not distinguish well between galectin-4/-6 specific expression due to a possible cross-hybridization. Therefore, we studied the expression of both galectins in mouse tissues and cell lines using a highly sensitive RT-PCR approach in combination with a panel of galectin-4 and galectin-6 specific primers that we developed and tested on the cloned galectin-4 and -6 cDNA sequences. Our data confirmed formerly published data acquired by hybridization techniques (3) that both galectins are expressed in the small intestine and colon. However, we found that these galectins are expressed also in the kidney, spleen, liver and the heart, and also in cultured embryonal carcinoma P19X1 cells. In addition, the expression of galectin-4 in the liver is documented by *in situ* hybridization data in combination with the fact that the cDNA sequence was cloned from the mouse liver cDNA library. Thus, these data clearly indicate that mouse galectin-4

and galectin-6 are more broadly distributed and that the function of both galectins may not be limited to the gastrointesinal tract. The system of specific primers helped us to indicate the expression of galectin-4 found in mouse cultured cells of T lymphome BW-5147 and and 3T3 fibroblast cells while galectin-6 was not found to be expressed in these cell lines.

While expression of galectin-4 was largely studied in adult human, pig and rat tissues, we decided to study the expression of galectin-4 during early mouse embryogenesis using a highly sensitive RT-PCR approach. We found that galectin-4 is expressed in mouse spermatozoons and oocytes but its expression disappeared in two- and four-cell stage embryos. The reconstitution of the expression occurred in 8-cell embryos and was found to remain in the stage of morula. This finding suggests that galectin-4 might also be involved in early developmental processes.

Galectin-4 was described to be expressed by tissue of the digestive system from tongue to large intestine (9). To study the distribution of epitopes reactive to recombinant galectin-4 in a mouse model, we prepared recombinant biotinylated galectin-4, CRD1 and CDR2 protein probes. We detected epitopes reactive for galectin-4 in squamous epithelium of the tongue only, while the intestine (small and large) was negative. This observation can be explained by the occupation of reactive ligands in the intestine by endogenous galectin. This observation seems to be specific, because the epidermis was negative. The epitopes of tongue epithelium were accessible only for the complete galectin-4, when the whole molecule was employed as a probe, and no binding of CRD1 and CRD2 forms was detected. This can be explained by the necessity of the whole-complex molecule for the recognition of epitope in this biological sample, supporting the possible structural role of the link region found in tandem-repeat galectin members in a ligand recognition. Our previous finding using both separate CRD domains of human galectin-4 indicated differences in the binding pattern of galectin-4 to human epidermis (35) that were not observed in the mouse model. The selectivity of binding of galectin-4 to the suprabasal non-proliferating layer of tongue epithelium is similar to the reactivity of galectin-3, which recognizes the same tissue compartment (36). The binding of galectin-3 to postmitotic differentiated cells was even observed in squamous cell carcinomas of the head and neck, where this feature can be employed as an independent prognostic marker (37).

Preparation of recombinant forms of mouse galectin-4 allowed us to investigate the oligosaccharide binding profile using Glycan Array analysis of 185 different glycan structures. The highest binding specificity of mouse galectin-4 was primarily to α-GalNAc and α-Gal A and B type structures with or without fucose. We found that the CRD1 domain significantly differs in selectivity for oligosaccharide recognition and affinity binding compared to the CRD2 domain. CRD1 bound with highest affinity to blood group B glycans, while CRD2 bound with very high affinity to blood group A glycans and low affinity to group B glycans. This supports the hypothesis that galectin-4 may have a crosslinking function. While the CRD1 domain bound to more structures with middle or low affinity, the CDR2 domain shows clear specificity supported by a high affinity for A type-2 α-GalNAc structures, yet B type structures can be recognized with lower affinity. On the other hand, the CRD1 domain has a broader specificity than CRD2 when compared to the total binding profile of mouse galectin-4. Complete galectin-4 also bound several unique structures, suggesting that the holoprotein, CRD, has a conformation which has broader specificity than the monomeric CRD domains. These data suggest that CRD2 might be the dominant binding domain of mouse galectin-4. Nevertheless, further investigation is needed to determine a physiological functional relevance to the observed behavior as it relates to the natural ligand recognition for each galectin-4 domain.

As mouse galectin-4 and galectin-6 share high sequence similarity, antibody distinguishing between both these molecules is of special value. During our study, we developed anti-mouse galectin-4 specific rabbit polyclonal antiserum which was raised against the 24-amino acid peptide of the galectin-4 link region. As this part of the protein is missing in galectin-6, this polyclonal antibody can selectively bind to the whole-complex galectin-4 molecule. This antibody can be useful for studies of the structural role of the link region in ligand recognition and it can clearly separate the protein expression of both galectins. We used anti-galectin-4 antiserum to study whether the antibody reacting to the link region of the galectin can inhibit the binding of the galectin to its reactive epitopes. To this goal we used the section of the tongue where only the complex galectin form was found to be reactive with galectin epitopes while CRD1 or CRD2 forms were negative. The result that the antiserum bound to the galectin did not prevent epitope recognition still did not exclude the possibility that the link region of tandem repeat galectin members has an important role in ligand recognition.

Molecular cloning of galectin-6 full-length cDNA confirmed formerly published sequence data, as our cloned galectin-6 cDNA sequence was 100% indentical to that described by Gitt *et al* (4). This documents that two closely related galectin-4/-6 transcripts exist in mouse tissues. Differences found in our cloned galectin-4 and galectin-6 cDNA sequences support the conclusion of Gitt *et al* (3) that two distinct genes might exist in the mouse genome due to a possible gene duplication. Whether the expression of both galectin transcripts has biological consequence and the overlapping or distinct functions remain to be analyzed.

Acknowledgements

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