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Galectin-related protein: An integral member of the network of chicken galectins
2. From expression profiling to its immunocyto- and histochemical localization and application as tool for ligand detection

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ABSTRACT

Background: Galectin-related protein (GRP), present in vertebrates, is special within this family of adhesion/growth-regulatory proteins due to its strong positive selection and loss of canonical lectin activity.
Methods: RT-PCR and Western blotting together with flow cytofluorimetry and immunocyto- and histochemistry monitor expression and localization of chicken GRP. The promoter sequence of the GRP gene is processed computationally to detect putative sites for binding transcription factors. The labeled protein is applied as probe to detect binding sites on cells and in sections, along with glycocompounds to test inhibition of the association.
Results: Expression of GRP in chicken is limited to bursa of Fabricius, immunohistochemically found in B cells, also in bursal epithelium and vessels. Presence in B cells is shared with only one canonical galectin, i.e. CG-8. Binding to a chicken lymphoma line was specific and saturable, not affected by lactose but completely blocked by heparin, as also seen in sections.
Conclusions: Expression monitoring initiated for GRP reveals a distinct site of localization in chicken, much more restricted than for any of its canonical galectins.

1. Introduction

Their increasingly emerging physiological significance justifies efforts to detect, to structurally characterize and correspondingly classify animal and human lectins [1,2]. The resulting formation of families gives direction to aim first at accomplishing a complete listing of the members of each family on the genomic level, next at defining biochemical properties of each protein. Measuring their expression and localization profiles and then performing a detailed network analysis are the following steps. This work will relate the evolutionary course of gene/sequence diversifications to characteristics of the individual homologous proteins from their structure to their cell/tissue presence and localization. When applying this concept to the adhesion/growth-regulatory galectins, to bring the status of knowledge for the galectin-related protein (GRP), an integral part of the phylogeny of this class of lectins [3,4], to the same level as already attained for canonical proteins was an obvious necessity. In this case, the indications for an exceptionally high level of sequence conservation among vertebrates [3], the loss of binding to the common ligand lactose for human GRP [5,6] and the general status of the five canonical chicken galectins (CGs) as model suited for comprehensive network analysis of general relevance [7] had prompted detailed biochemical characterization of chicken GRP (C-GRP) [8]. Except for the occurrence of C-GRP-specific mRNA in bursal lymphocytes [3], no information on expression of this gene was available for this sixth member of the galectin family in chicken or for any other vertebrate.

In this report, monitoring of C-GRP expression in 4-week-old chickens is documented on the level of mRNA (RT-PCR) and of protein (Western blotting) as well as of its localization in tissue sections and isolated/cultured cells. Using the full set of non-cross-reactive anti-CG antibodies, C-GRP positivity is set into relation to the corresponding properties for each canonical CG. A computational inspection of the promoter region flanked the experimental work to track down putative regulatory sites for a tissue-specific expression. In addition to the immunocyto- and histochemical monitoring, labeled C-GRP, along with the labeled forms of the other family members, is introduced as tool to detect sites in the C-GRP-positive tissue/cells, which are reactive with this protein. The combined results of the biochemical [8] and cyto-

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and immunohistochemical [this study] investigations shape the notion for C-GRP to be an acquisition to the galectin family with strong positive selection, a unique binding capacity and its own, very particular profile of expression. The documented data initiate GRP localization and detection of cellular binding sites as a step toward functional characterization. Equally important, the addition of C-GRP to the network of canonical CGs is assumed to have relevance beyond this model organism, prompting detailed comparative analysis for GRP among vertebrates.

2. Materials and methods

2.1. Materials

The monoclonal antibody AV20 against the chicken B cell marker chB6 [9,10] was kindly provided by S. Härtle (Chair of Animal Physiology, Faculty of Medicine, Ludwig-Maximilians-University, Munich, Germany), FITC-labeled anti-mouse IgG1 was a generous gift from H. Ammer (Institute of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany). CGs and C-GRP, which were purified after recombinant production using affinity chromatography on lactosylated Sepharose 4B as crucial step (CGs) or as fusion protein (C-GRP) as described [8,11,12], were labeled under activity-preserving conditions with the *N*-hydroxysuccinimidyl derivative of biotin, fluorescein isothiocyanate or the succinimidyl ester derivative of the Alexa Fluor® 488 dye (Invitrogen, Darmstadt, Germany), as described previously [13]. The fungal (*Polyporus squamosus*) lectin (PSL) was purified, controlled for purity and labeled as described [14–16]. Extent of labeling was routinely determined by mass spectrometry [17], labeled lectins were rigorously checked for activity and maintained specificity by haemagglutination (CGs) and by binding assays using asialofetuin as matrix or mammalian cells, especially Chinese hamster ovary (CHO) parental cells and mutant lines altered in distinct aspects of glycosylation (for details, please see [8]). The solid-phase assay for assessment of binding of surface-presented C-GRP (using 0.1 μg/mL, 0.25 μg/mL and 0.5 μg/mL for coating) to biotinylated CGs (titrations with 5–40 μg/mL) was performed under identical conditions. Biotinylated plant lectins (MAA-I, *Maackia amurensis* agglutinin-I; *Phaseolus vulgaris* erythroagglutinin, PHA-E; *Phaseolus vulgaris* leukoagglutinin, PHA-L; Jacalin, JAC; peanut agglutinin, PNA) and Texas-Red® Avidin D for visualizing their binding were purchased from Vector Laboratories (distributed by Enzo Life Sciences, Lörrach, Germany). DT40 B lymphoma cells (avian leukosis-induced bursal lymphoma line; ATCC® CRL-2111™) [18,19] were kindly provided by S. Härtle and B. Kaspers (Chair of Animal Physiology, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, Munich, Germany).

2.2. Tissue preparation and isolation of bursal B cells

Bursae of Fabricius were dissected from the proctadael region of the cloaca of five 4-week-old chickens, snap frozen in liquid nitrogen and then further processed for RNA isolation, extract preparation for Western blotting and cryo-sectioning. Bursal B cells were routinely obtained from freshly dissected tissue that was cut into small pieces in ice-cold phosphate-buffered saline, pH 7.2 (PBS). These pieces were placed on a stainless steel mesh screen (pore size: 75 μm) and gently pressed through the screen using the plunger of a 10 mL syringe into a petri dish, whose surface was covered with ice-cold PBS. The resulting suspension was transferred to a 50 mL tube and filled up to 50 mL with PBS. After 5–10 min on ice, the supernatant was gently poured into a new 50 mL tube and centrifuged for 10 min at 225 xg, the resulting cell pellet was resuspended in 10 mL PBS and this suspension layered on top of an equal volume of Ficoll solution (density: 1.077 mg/mL, Biocoll Separating Solution; Biochrom AG, Berlin, Germany). Following a centrifugation step at room temperature for 12 min at 400 ×*g*, the cell-containing interphase was collected and washed twice with ice-cold PBS. Thereafter, the cell pellet was suspended in RPMI 1640 medium (containing 8% fetal calf serum (obtained from Life Technologies, Darmstadt, Germany), 2% chicken serum (Sigma-Aldrich, München, Germany) and antibiotics) to give 2.5×10^7 cells/mL, 10 μL of this suspension were carefully pipetted onto microscope slides, cells were fixed in cold acetone for 10 s and used for cytochemical staining. Cells were also pelleted, snap frozen and used for RNA isolation and Western blotting, respectively.

2.3. Expression profiling by RT-PCR

Isolation of total RNA from bursa of Fabricius tissue and purified B cells as well as the ensuing preparation of cDNA were done, as described previously for tissues of 6-month-old chickens [12,20]. PCR amplification of C-GRP-specific mRNA was directed by the sense primer 5′-TCTAGAATGGCGGGGACCGTG-3′ and the antisense primer 5′- AAGCTT TCAGCCAAGTTTTGTAAG-3′ as described [8]. The mRNAs of the canonical CGs used primer sets as in previous studies [12,20]. In detail, CG-1A-specific mRNA was amplified with the sense primer 5′-GGATCCATGGAG CAAGGACTG-3′ and the antisense primer 5′-GAATTCAATTTTCTCCATGC CCAGCCG-3′, CG-1B-specific cDNA by the sense primer 5′-ATGTCTTGTC AGGGACCA-3′ and the antisense primer 5′-TTACTCCCAGCTGACAGA CCG-3′, CG-2-specific cDNA by the sense primer 5′-AGAATGTTTGAAAT GTTCAAC-3′ and the antisense primer 5′-TCACTCCACCTTGAAGGAG-3′, CG-3-specific cDNA by the sense primer 5′- CCCGGCGTACCCTGGATA-3′ and the antisense primer 5′- AAATCATGGAGGTCAAAACAC-3′, and CG-8-specific cDNA by the sense primer 5′-ATGATGTCCTTGGATGGA-3′ and the antisense primer 5′-CTACCAGCTCCTCACATC-3′. The lengths of amplified cDNAs for C-GRP, CG-1A, CG-1B, CG-2, CG-3, and CG-8S/L are 516 bp, 351 bp, 408 bp, 399 bp, 627 bp, and 891/948 bp, respectively. The reaction mixture for the PCR reactions was prepared, as recommended by the distributor of the *Taq* DNA polymerase (Qiagen). In general, the analyses were carried out in a volume of 20 μL containing 2.5 μg cDNA, 0.5 U Taq polymerase, 50 μM dNTPs, 1 × PCR-buffer (commercial mixture of Tris-HCl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, pH 8.7), at 20 °C, 1 × Q-Solution and 0.1 μM of sense and antisense primers. Amplification of the galectin-specific cDNAs was started with an initial denaturation step at 94 °C for 4 min, then 29 consecutive cycles of the following series of steps were performed: denaturation at 94 °C for 45 s, annealing at 52 °C (CG-1B, CG-2, CG-3, CG-8, actin) or 60 °C (C-GRP, CG-1A) for 45 s and extension at 72 °C for 1 min. The final extension step was carried out at 72 °C for 10 min. PCR products and reagents were separated in 2% agarose gel electrophoresis at 80 V for 40 min. The loading control with chicken β-actin-specific mRNA was established with the sense primer 5′-ATGGCTCCGGTATGTGC-3′ and the antisense primer 5′-ACCG TGTTCAATGGGGT-3′. The length of amplified cDNA was 185 bp.

2.4. Extract preparation and Western blotting

Tissue pieces from bursae of Fabricius and isolated bursal B cells were processed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 1% (w/v) NP-40). Polyclonal antibody preparations were obtained using recombinant C-GRP (full length)/CGs as antigens in rabbits as described [20,21]. In detail, immunization was started with 200 μg recombinant C-GRP in an emulsion of 0.5 mL PBS mixed with Freund's complete adjuvant (0.5 mL; Sigma-Aldrich) and pursued with booster injections in three-to-six-week intervals using Freund's incomplete adjuvant (Sigma-Aldrich). The titer was regularly monitored by ELISAs with the antigen. The immunoglobulin G (IgG) fraction obtained by affinity chromatography over protein A-Sepharose 4B was rigorously examined for cross-reactivity by Western blotting/ELISAs using each of the canonical CGs/C-GRP as bait, followed by removal of respective activity by chromatographic depletion on resin presenting the respective CG and subsequent controls to verify complete removal [22]. The content of protein after coupling to divinyl sulfone-activated Sepharose 4B

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