Purification and characterization of the N-terminal domain of galectin-4 from rat small intestine

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Abstract Using affinity chromatography on lactose-agarose, five β-galactoside binding lectins of 14 to 20 kDa were detected in the rat small intestinal mucosa. The prominant proteins of 17 and 19 kDa were purified to homogeneity by 2D-electrophoresis. Direct N-terminal sequencing of the 17 kDa protein and intra-chain sequencing of the 19 kDa protein produced sequences which are part of the N-terminal domain of the L-36/galectin-4. A rabbit polyclonal antibody was raised against the 19 kDa lectin, which specifically recognized the 17 and 19 kDa lectins and detected a related 36 kDa protein in human undifferentiated HT29 cells.

Key words: β-Galactoside binding lectin; Galectin; Intestinal mucosa; HT29

1. Introduction

Galectins represent a growing family of abundant β-galactoside binding lectins which share characteristic amino acid sequences [1]. Though their function is not yet known with certainty, they could play important roles in many cellular or cell–matrix interactions through specific recognition of carbohydrate structures [2]. Many β-galactoside binding lectins have been described in the rat intestine [3], but few of them were fully characterized. Particularly, a 17 kDa lectin, named RIH, has been purified and led to the isolation of a cDNA sequence encoding a 36 kDa lectin [3,4], now recognized as galectin 4, consisting of two carbohydrate binding domains bound by a link sequence and the C-terminal domain II being the RIH sequence. During the purification of an rat intestinal endogenous proteins inhibitor of fucosyltransferase activity (fuctinin (fuctinin [5]), preliminary experiments were performed to rule out the possibility that fuctinin could be an endogenous lectin inhibiting fucosyltransferase activity by interaction with galactoside-substrates. A lectin fraction, different from fuctinin, was obtained which also inhibited in vitro fucosyltransferase activity in a non-competitive way [6]. To get more information about the inhibition of this glycosylation process [7], a more refined study of this lectin fraction was undertaken, which led to the purification of the N-terminal domanin I of galectin 4 and to the generation of specific antibodies against this domain.

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Abbreviations: EDTA, ethylene diamine tetraacetic acid; 2D-electrophoresis, two-dimensional electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TST, Tris-buffered saline containing 0.1% Tween 20 and 10% fetal calf serum.

2. Materials and methods

2.1. Purification of β-galactoside binding lectins from rat intestine

Scraped small intestinal mucosa of adult male Sprague–Dawley rats was homogeneized at 4°C in a buffer consisting of 50 mM Tris-HCl, 150 mM Lactose, 4 mM 2-mercaptoethanol, pH 7.5, containing 2 mM EDTA and 0.25 mM PMSF to inhibit protease activities. Purification of the lectin fraction from mucosa was essentially performed as described by Leffler et al. [3], with the addition of an ammonium sulfate fractionation step (80% saturation) for lactose elimination before affinity chromatography. After dialysis of the pellet and removal of insoluble proteins by centrifugation, the soluble fraction was loaded onto a column of Lactose-Agarose (Sigma, USA) equilibrated in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl (buffer A), washed with four column volumes of buffer A and one column volume of buffer A containing 100 mM sucrose. Lectins were eluted with buffer A containing 100 mM lactose. Protein concentration in the fractions was determined by the method of Schaffner and Weissmann [8] and analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE).

2.2. Gel electrophoreses

For proteins, SDS-PAGE was performed under reducing conditions by the method of Laemmli [9], using a 15% separating gel at a constant power of 6 W for 2 h. Peptide electrophoresis was carried out according to the method of Schagger and von Jagow [10] using a 10% stacking gel, a 5% spacer gel and a 15% separating gel. Two-dimensional electrophoreses (2D-electrophoreses) were carried out according to O'Farrel for equilibrium (IEF) [11] and for non-equilibrium (NEPHGE) [12] pH gradient electrophoreses.

2.3. Amino acid sequence determinations and analysis

Lectins isolated by 2D-electrophoresis in non-equilibrium conditions were blotted onto PVDF membranes (Immobilon P^{SQ}, Millipore, USA) according to Matsudaira [13] for direct sequencing. For the 19 kDa lectin, PVDF spots were incubated overnight with 10 mg/ml of CNBr in 70% HCOOH. Generated peptides were eluted, separated by electrophoresis and blotted onto PVDF membranes. Edman analysis of protein or peptide spots was performed at the Service Central d'Analyses of the Centre National de la Recherche Scientifique (CNRS, Solaize, France).

2.4. Production of a specific antibody against the purified 19 kDa lectin

Pieces of wet polyacrylamide gel containing the 19 kDa lectin were used for rabbit immunization [14]. Before injection, 10 ml of blood was collected as preimmune control serum. For the initial injection, the suspension of polyacrylamide gel containing 150 μg of the 19 kDa lectin was emulsified in an equal volume of complete Freund's adjuvant [15]. Booster injections with 75 μg lectin without Freund's adjuvant were performed one and two months later. Diluted immune serum was used as a source of antibodies without further purification.

2.5. Immunoblotting

SDS–PAGE separated proteins were transferred onto nitrocellulose sheets using a semi-dry electrophoretic blotting apparatus (Sartorius, France) at 0.9 mA/cm^2 for 90 min [16]. The sheets were incubated in

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50 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS) containing 0.1% Tween 20 and 10% newborn calf serum (TST), for 30 min to block non-specific antibody binding. After a 2 h incubation in the presence of rabbit immune serum diluted to 1/2,000 in TST followed by several washings with TST, the bound rabbit antibodies were detected by incubation with alkaline phosphatase-labeled goat anti-rabbit IgG (Dako, Denmark). Control blots with preimmune serum performed in each case.

2.6. Protein staining

Proteins were stained with Coomassie brillant blue R-250 and molecular weight markers blotted on membranes with Protogold (Biocell Research Laboratories, UK).

2.7. HT29 cells

Undifferentiated HT29 cells (from ATCC, USA) were cultured in DMEM medium (Sigma) supplemented with 10% calf serum. At confluency, cells were collected on PBS buffer, pH 7.4 (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) by scraping and washed three times with the same buffer. Cells were made more fragile by 10 min treatment at 4°C with 0.25 M sucrose, 10 mM triethanolamine buffer, pH 7.0, then cells were broken by 20 strokes of a Potter-Elvejhem homogeneizer.

3. Results

Preliminary experiments were conducted in order to optimize the preparation of mucosal intestinal lectins by affinity chromatography on Lactose Agarose. The best yield (3% of soluble proteins of intestinal mucosa) was obtained with a ratio of 4 mg of intestinal soluble proteins for 1 ml of affinity phase. The yield sharply decreased using higher ratio, falling down to 1% with a load of 12 mg proteins/ml affinity phase. Analysis of the lectin fraction by SDS-PAGE indicated the presence of five protein bands in the 14–20 kDa range (Fig. 1, left), with two prominent bands at 17 and 19 kDa (named thereafter 17 and 19 kDa lectins). Two rows of spots at 17 and 19 kDa were present on the IEF-2D electrophoresis gel (Fig. 1, right top panel). Under non-equilibrium conditions the 2D electrophoresis pattern shows less numerous spots (Fig. 1, right bottom panel): the 17 kDa lectin consisted of a major spot at pH 7.6 (lower arrowhead) and several more basic proteins around pH 8.2–8.3; for the 19 kDa lectin, a major spot appeared at pH 7.3 (upper arrowhead) and two minor spots at pH 7.1 and 7.8. Just above the 19 kDa major spot, two small spots were also present. The reproducibility of this pattern allowed us to use NEPHGE for preparative purpose: the major spot of both 17 and 19 kDa lectins were either used for antibody generation or blotted onto PVDF membranes for microsequencing.

Direct microsequencing of the 17 kDa lectin provided an unambiguous NH_2-terminal sequence (YNPTLPYKRPIPGG-LSVGMSIYIQG) which was found to correspond to amino acids 13–37 of the domain-I of L36, a previously characterized 36 kDa lectin. Direct sequencing of the 19 kDa lectin was unsuccessful, despite treatment of a larger amount of protein than for the 17 kDa lectin. Peptides of the 19 kDa lectin were generated by CNBr cleavage of the blotted spot. After peptide separation and blotting, a sequence of 23 amino acids was obtained for one peptide (RRFHVNFEVGQDEGADIAFHF-NP), which completely matched the amino acids 44–66 of the same L36 lectin. A possible structural relationship of both the 17 and 19 kDa lectin with the L-36 lectin and the RIH lectin are shown in Fig. 2.

Production of polyclonal antibodies from gel pieces was undertaken in the rabbit for the 19 kDa lectin (used spot is indicated by arrowhead in the right bottom panel of Fig. 1), which produced a powerful and highly specific antiserum. Intense staining of both 17 and 19 kDa lectins was obtained by Western blotting with a 1/2,000 dilution of the antiserum. On blots from soluble intestinal mucosa proteins as well as from the lectin fraction, only the 17 and 19 kDa lectins were detected (Fig. 3, left and middle panel). No binding of the antiserum to a 36 kDa component was ever observed despite repeated experiments under various conditions. In undifferentiated HT29 cells, the antiserum produced a quite different: a major protein at 36 kDa and one minor protein at 22 kDa were specifically detected in the soluble extract of the HT-29 cells (Fig. 3, right panel).

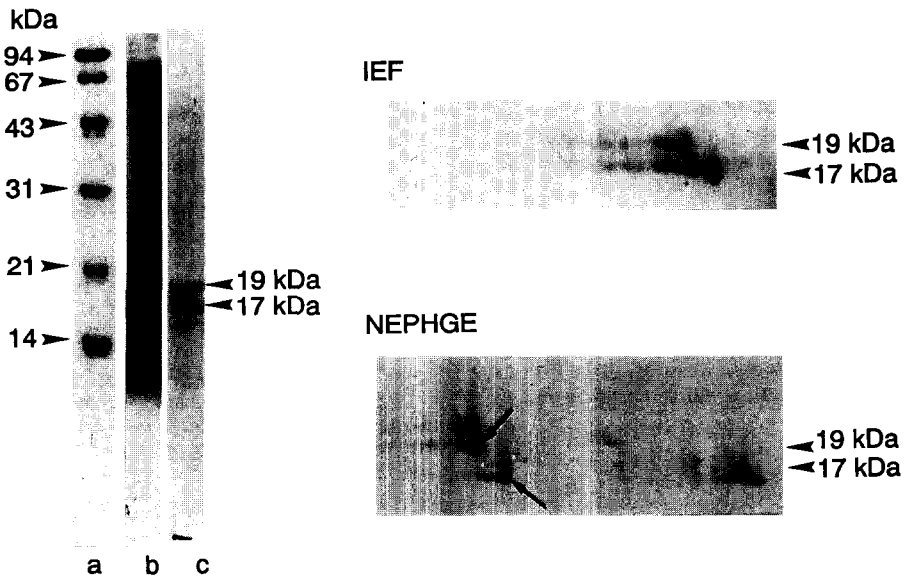

Fig. 1. Characterization of the lectin fraction from rat small intestinal mucosa. Left panel: SDS-PAGE analysis of the lectin fraction obtained by lactose-agarose affinity chromatography. (a) Calibration kit; (b) total soluble proteins; (c) lectin fraction. Right top panel: 2D-electrophoresis in equilibrium condition (IEF) of the lectin fraction. Right bottom panel: 2D-electrophoresis in non-equilibrium condition (NEPHGE); black arrows show the 17 and 19 kDa spots used for microsequencing and antibody production. Alkaline pH of the 2D-gels are located at the right side.

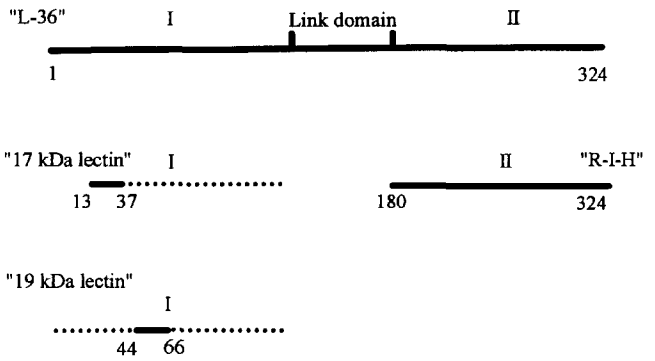

Fig. 2. Structural relationship between lectin 19 kDa, lectin 17 kDa, RIH lectin and the 36 kDa lectin (Galectin 4). Amino acid numbers are referred to the numerotation of amino acids of the 36 kDa lectin [4].

4. Discussion

This work on intestinal lectins was initiated [6] before the publication of the extensive work by Oda et al. [4] concerning molecular cloning of L36 (galectin 4) of the rat intestine and was pursued thereafter since the protein pattern of the lectin fraction that we prepared from rat intestine was different from that previously reported in the same organ by Leffler et al. [3]. The two major lectins we purified originated from the N-terminal domain I of galectin 4, whereas the major lectin purified by Leffler et al. [3], called RIH, corresponds to the C-terminal domain II of this galectin. Only minor differences existed between the two purification procedures: in our case, we use ammonium sulphate precipitation for lactose elimination, a commercially available lactose–agarose and perhaps a different ratio (protein load/phase volume) in affinity chromatography. As reported by Leffler et al. [3], this ratio is critical in lectin purification and we used the ratio giving the best yield for the preparation used. The yield of 3% of soluble protein for the lectin fraction was higher than previously reported (1% [3]). Use of scraped mucosa instead of whole intestine could be reponsible for the higher yield and the different pattern in our work as compared to the study of Leffler et al. [3]. Oda et al. [4] raised the question about the fate of the N-terminal domain of galectin 4 which they were unable to detect. Obviously, our work demonstrates that a large amount of the N-terminal fragment of galectin 4 is present in intestinal mucosa. Conversely, the 17 kDa C-terminal domain of galectin 4 (RIH) could not be detected in our preparation, though, since its pHi is above 9, it might correspond to the minor basic protein observed on NEPHGE gels.

From the work of Oda et al. [4], the rat intestine expresses the full-length mRNA of galectin 4 and the 36 kDa lectin can be immunodetected on blots after extraction under denaturing conditions. Therefore, the obtention of separate domains probably depends on proteolytic cleavage during homogeneization and purification, despite the use of two protease inhibitors. However, this cleavage appeared to be remarkably constant, since 2D-electrophoresis produced the same pattern in many independent preparations. Moreover, unambiguous sequence of the 17 kDa and 19 kDa lectins were obtained despite pooling of lectin fractions from independent experiments. Whether this cleavage can also occur physiologically and lead to functional lectins generated from the 36 kDa lectin can only be hypothetized.

Other post-translational modifications of galectin 4 can be suspected. The blocking of the N-terminal end of the 19 kDa lectin precluded N-terminal sequencing. Though N-terminal blocking can be artificially produced during sample processing, it could be physiologically relevant: N-terminal blocking was also reported for several lectins isolated by ion-exchange chromatography [3] and N-terminal acetylation of galectin 3 has been demonstrated [17]. Thus, the N-terminal blocked 19 kDa lectin could likely correspond to the complete domain I of galectin 4. On IEF-2D gels, both 17 and 19 kDa lectins appeared as two rows of spots of quite similar molecular mass. In non-equilibrium conditions, most of these spots became gathered in one major spot for each lectin and Edman's reaction (directly or after hydrolysis) indicated a single N-terminal sequence in this spot. This fact might be interpreted as a differental but limited proteolysis of the C-terminal part of domain I, since the link sequence between the two domains of galectin is very susceptible to proteolysis [1]; it could also suggest other post- translational modifications strongly altering protein charge such as phosphorylation and sulfatation. For example, phosphorylation has been described for CBP35 (galectin 3) in the mouse and the dog [18,19].

That the 17 and 19 kDa proteins belong to the same galectin was confirmed by the production of an anti-19 kDa protein antibody which specifically recognized both lectins. Of interest is the cross reaction of this antibody to a human protein of 36 kDa extracted from undifferentiated HT29 cells derived from a human colonic adenocarcinoma [20] and which could likely be the human conterpart of the galectin 4. By contrast, Oda et al. [4] could not detect any hybridization of rat galectin 4 cDNA with human DNA. However, at the protein level, rat and

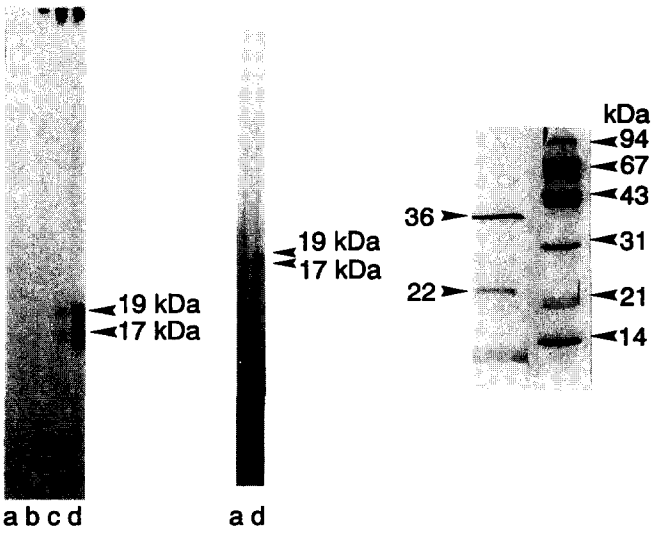

Fig. 3. Immunoblotting using the anti-19 kDa lectin antiserum. Left panel: immunoblotting after SDS-PAGE of soluble proteins of small intestinal mucosa; (a) preimmune serum; (b,c,d) immune serums after the first, the second and the third boost injection, respectively. Middle panel: immunoblotting of the lectin fraction obtained by lactose–agarose affinity chromatography (letters as above). Right panel: immunoblotting of soluble proteins from human undifferentiated HT29 cells.

human galectin 4 could share sufficient homologies at least in the N-terminal domain to give such a cross reaction. This highlight antibody provides a powerful tool for further studies of galectin 4 in rat and human tissues.

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