Biochemical and Biophysical Research Communications 404 (2011) 201–205

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structures of human secretory proteins ZG16p and ZG16b reveal a Jacalin-related β-prism fold

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ARTICLE INFO

Article history:
Received 13 November 2010
Available online 24 November 2010

Keywords:
ZG16p
β-Prism fold
Crystal structure
Lectin
ZG16b

ABSTRACT

ZG16p is a secretory protein that mediates condensation-sorting of pancreatic enzymes to the zymogen granule membrane in pancreatic acinar cells. ZG16p interacts with glycosaminoglycans and the binding is considered to be important for condensation-sorting of pancreatic enzymes. ZG16b/PAUF, a paralog of ZG16p, has recently been found to play a role in gene regulation and cancer metastasis. However, the detailed functions of ZG16p and ZG16b remain to be clarified. Here, in order to obtain insights into structure–function relationships, we conducted crystallographic studies of human ZG16p lectin as well as its paralog, ZG16b, and determined their crystal structures at 1.65 and 2.75 Å resolution, respectively. ZG16p has a Jacalin-related β-prism fold, the first to be reported among mammalian lectins. The putative sugar-binding site of ZG16p is occupied by a glycerol molecule, mimicking the mannose bound to Jacalin-related mannose-binding-type plant lectins such as Banlec. ZG16b also has a β-prism fold, but some amino acid residues of the putative sugar-binding site differ from those of the mannose-type binding site suggesting altered preference. A positively charged patch, which may bind sulfated groups of the glycosaminoglycans, is located around the putative sugar-binding site of ZG16p and ZG16b. Taken together, we suggest that the sugar-binding site and the adjacent basic patch of ZG16p and ZG16b cooperatively form a functional glycosaminoglycan-binding site.

1. Introduction

The pancreatic acinar cell is a secretory cell that synthesizes 15–20 different enzymes for the digestion of nutrients in the duodenum. The digestive enzymes are stored in large vesicles known as zymogen granules, which are found predominantly in the apical pole of the cell. Stimulation of the cell triggers fusion of the zymogen granule membrane specifically with the apical plasma membrane, leading to the secretion of the digestive enzymes from the cell. Over the years, significant efforts have been made to understand the condensation and sorting of secretory enzymes [1]. While selective aggregation of the enzymes and thus the formation of the granule core is well documented [2,3], their binding to the zymogen granule membrane is poorly understood.

ZG16p was originally identified by immunoscreening of a cDNA expression library of rat pancreas with an antibody against rat zymogen granule membranes [4]. Subsequently, it was revealed that ZG16p is associated with the submembranous matrix and is also found in cholesterol–glycosphingolipid-enriched microdomains in the zymogen granule membrane [5,6]. The functional importance of ZG16p in the process of condensation-sorting has been revealed by an *in vitro* aggregation assay, in which pretreatment of zymogen granule membranes with anti-ZG16p antibody inhibits the binding of content proteins to the membrane by about 50% [7].

ZG16p displays significant sequence homology to the plant lectin Jacalin, which specifically binds to Galβ1–3GalNAc [4,7]. ZG16p is therefore considered to be a secretory lectin. Competition experiments with mono- and di-saccharides show that the addition of 10 mM galactose has a weak inhibitory effect on condensation-sorting [7]. Pretreatment of membranes with chondroitinase ABC

Abbreviations: ZG16, zymogen granule membrane protein 16; PAUF, pancreatic adenocarcinoma upregulated factor; MBP, maltose binding protein; GST, glutathione S-transferase.

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doi:10.1016/j.bbrc.2010.11.093

leads to an inhibition of condensation-sorting by 40–50% [7]. Because of its locale and carbohydrate-binding properties, ZG16p could function as a linker between the submembranous matrix and lipid microdomains [1]. ZG16p does not have a transmembrane domain and interaction with the membrane is likely to be mediated by its lectin domain.

Recently, ZG16p has been implicated in the regulation of gene expression. Mouse ZG16p is highly expressed in the pancreas and sharply down-regulated early in the course of acinar cell injury according to DNA array analysis [8]. ZG16b/PAUF, a paralog of ZG16p, is highly expressed in human pancreatic cancer [9] and plays a role in metastasis [10].

In this study, we determine the three-dimensional structures of human ZG16p and ZG16b by X-ray crystallography in order to better understand their structure–activity relationships.

2. Materials and methods

2.1. Protein expression and purification

Full-length ZG16p and ZG16b have 167 and 208 amino acid residues, respectively. DNA fragments encoding human ZG16p (amino acid residues 21–159, comprising the core lectin domain) and ZG16b (53–208, core lectin domain) were subcloned into pCold-MBP vector [11,12] for production of recombinant proteins. The plasmid constructs were transformed into *Escherichia coli* BL21(DE3) codon plus (Stratagene) and the cells were grown in LB medium at 310 K. After induction with 0.1 mM isopropyl β-D-thiogalactoside (Wako), the cells were cultured at 288 K for 24 h. The harvested cells were resuspended in a buffer containing 50 mM Tris–HCl (pH 8.0), 50 mM NaCl and Bugbuster (Novagen) and sonicated. After centrifugation, the supernatants were collected and applied to a Ni Sepharose column (GE Healthcare) equilibrated with PBS (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl and 3 mM KCl, pH 7.4). After washing the column with PBS, the proteins were eluted with PBS containing 500 mM imidazole. The $(His)_6$-MBP-fused proteins were then digested by TEV protease at 277 K for 12 h. The digested proteins were passed through a Ni Sepharose column, and applied to HiLoad 16/60 Superdex 75 pg (GE Healthcare) equilibrated with PBS. The purified proteins were replaced with a 10 mM Tris–HCl (pH 8.0) buffer and used for the crystallization.

2.2. Crystallization, X-ray data collection and structure determination

ZG16p and ZG16b crystals were obtained by sitting drop vapor diffusion methods, in which 0.5 μl protein solution was mixed with an equal volume reservoir solution. Crystals were obtained using the following reservoir solution: ZG16p, 0.09 M MES (pH 6.5), 0.09 M NaH_2PO_4, 0.09 M KH_2PO_4 and 1.8 M NaCl; ZG16b, 0.4 M $(NH_4)H_2PO_4$ and 5% (v/v) 1,4-dioxane. Data sets were collected from synchrotron radiation (1.0000 Å wavelength) at NE3A and NW12A beamlines of Photon Factory, High Energy Accelerator Research Organization (KEK) (Tsukuba, Japan). The crystals were cryo-protected with a reservoir solution containing 25% glycerol. The diffraction data were processed using HKL2000 [13]. The structures of ZG16p and ZG16b were solved by the molecular replacement method using the program Molrep [14] with *Helianthus tuberosus* lectin (PDB ID: 1C3K) [15] and the solved ZG16p as search models, respectively. Further model building was manually performed using the programs XtalView/Xfit [16] and COOT [17]. Refinement was carried out using the programs CNS1.1 [18] and REFMAC5 [19]. The stereochemical quality of the final models was assessed by RAMPAGE [20]. Crystallographic parameters and refinement statistics are summarized in Table 1. Coordinates and structure factors for human ZG16p and ZG16b have been deposited at the Protein Data Bank with Accession Nos. 3APA and 3AQG, respectively. Figures of the structures were prepared using PyMOL (http://www.pymol.org/).

Table 1
Data collection and refinement statistics of human ZG16p and ZG16b.

	ZG16p (3APA)	ZG16b (3AQG)
Crystallographic data		
Space group	$P2_12_12$	$P2_12_12_1$
Cell dimensions		
a, b, c (Å)	58.9, 73.2, 30.1	61.4, 66.6, 67.6
Resolution (Å)	50–1.65 (1.68–1.65)	50–2.75 (2.80–2.75)
Total/unique reflections	228,953/16,473	78,108/7629
R_{merge}	7.8 (50.0)	16.4 (50.1)
I/σ (I)	30.3 (6.2)	14.0 (3.5)
Completeness (%)	99.3 (99.9)	99.9 (99.7)
Redundancy	14.0 (13.8)	10.3 (8.8)
Refinement statistics		
Resolution (Å)	20.00–1.65 (1.69–1.65)	40.00–2.75 (2.82–2.75)
R_{work}/R_{free}	20.4/22.8	22.0/28.3
R.m.s deviations		
Bond lengths (Å)	0.015	0.013
Bond angles (°)	1.47	1.50
Ramachandran plot (%)		
Favored	97.8	93.2
Allowed	2.2	6.8
No. atoms		
Protein	1084	2132
Ligand/ion	7	10
Water	124	50
B-factors (Å^2)		
Protein	16.5	27.0
Ligand/ion	21.4	33.1
Water	28.2	17.6

3. Results and discussion

3.1. Preparation of recombinant ZG16p and ZG16b

Initial attempts to express a large amount of ZG16p with a hexahistidine tag or a GST fusion tag were unsuccessful. After an extensive trial of bacterial expression vectors, we succeeded in obtaining a large quantity of recombinant ZG16p as well as ZG16b using pCold-MBP vector [11,12]. The recombinant MBP-fused proteins were treated with TEV protease and the MBP tag was removed by chromatography through a Ni Sepharose column. The ZG16p and ZG16b proteins were further purified by size exclusion chromatography and subject to crystallization.

3.2. Quality of crystallographic data and overall view of the structures

The structure of human ZG16p was solved by the method of molecular replacement using the coordinates of Heltuba (*H. tuberosus* lectin) as a search model (PDB ID: 1C3K) [15]. The refined structure of ZG16p has a crystallographic *R*-factor value of 20.4% (*R*-free = 22.8%) in the 20.00–1.65 Å resolution range (Table 1). The structure of ZG16b, a human ZG16p paralog, was then solved by molecular replacement using the coordinates of human ZG16p. The final structure of ZG16b has a crystallographic *R*-factor value of 22.0% (*R*-free = 28.3%) in the 40.00–2.75 Å resolution range (Table 1). One ZG16p molecule is included in an asymmetric unit while two ZG16b molecules are in an asymmetric unit. All Jacalin-related mannose-binding-type lectins for which the 3D structure has been solved are dimers or tetramers [15,21–25]. However, in the case of ZG16p and ZG16b, the evidence points to their being monomeric. The maximum contact areas of the ZG16p and ZG16b molecules in the crystals are relatively small

(535 and 644 Å^2, respectively), and both proteins eluted at positions corresponding to a monomer in solution following size exclusion chromatography (data not shown). Full-length ZG16p has two cysteine residues Cys164 and Cys167 in the C-terminal region following the lectin core domain (residues 21–159), and they are more likely to form an intramolecular disulfide bridge than the less common intermolecular link [7], while ZG16b has no cysteine residues. Thus the crystallographic contacts of ZG16p and ZG16b are most probably nonphysiological, and the proteins likely function as monomers in solution.

ZG16p assumes a β-prism fold consisting of three β-sheets (Fig. 1A), and the overall structure is similar to Jacalin-related mannose-binding-type lectins such as Banlec [21] (Fig. 1C). ZG16p is the first example of mammalian lectin having β-prism I fold; all previous solved β-prism I fold lectins have come from plants. In ZG16p, each β-sheet is made up of three to four β-strands (sheet I: β1, β2, β11 and β12; sheet II: β3, β4 and β6; sheet III: β7–β10), forming three Greek key motifs (Fig. 1D). Numbering of the β-strands is according to the scheme of Heltuba [15]. In the Jacalin-related mannose-binding-type lectins there is a β5 strand that is absent in the corresponding region of ZG16p. A short α-helix occurs between the β2 and β3 strands of ZG16p – a unique feature not previously seen in β-prism I fold lectins. The overall structure of ZG16b is similar to that of ZG16p except for the absence of the short α-helix and the β1 strand (Fig. 1B and D).

3.3. Structural insights into the sugar-binding site

The sugar-binding site of mannose-binding type Jacalin-related lectins consists of three exposed loops (GG loop, Recognition loop, and Binding loop) at the top of the β-prism fold [26]. We found that the putative sugar-binding site of ZG16p is occupied by a glycerol molecule (Figs. 1A and 2A). Soaking experiments using various sugars failed to bring a sugar molecule into the binding site, probably because of competition with glycerol present in high concentration (20–25% (v/v)), which is used as cryoprotectant during the

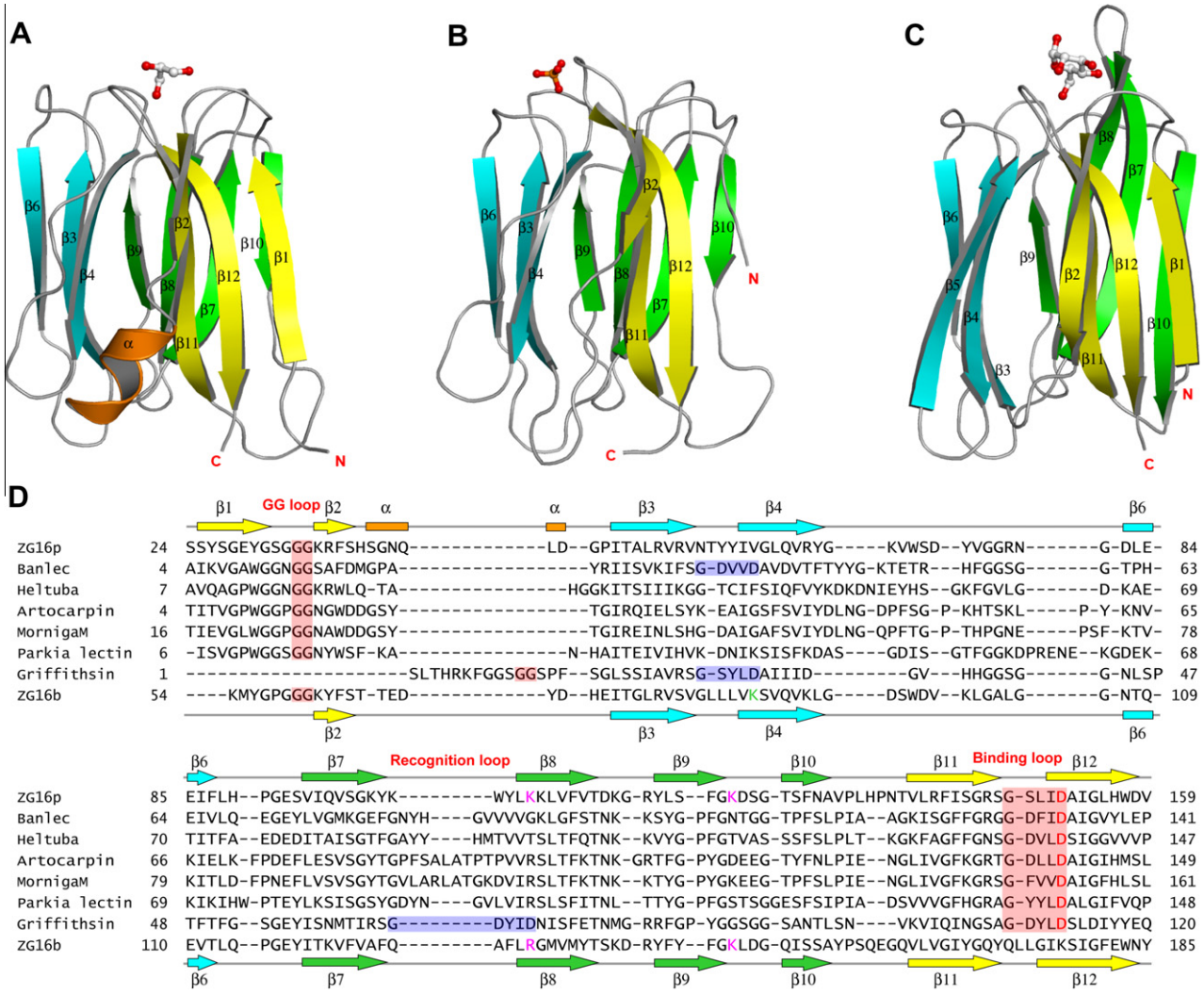

Fig. 1. Human ZG16p and ZG16b have a β-prism fold. Overall structures of ZG16p (A), ZG16b (B) and Banlec (C) from *Musa acuminata* (PDB entry 3MIT [21]) are shown with ribbon models. The secondary structures are highlighted (β-strands belonging to the first β-sheet (β1–2 and β11–12), yellow; second β-sheet (β3–6), cyan; third β-sheet (β7–10), green; α helix, orange) and the loops are colored gray. Bound glycerol (A), phosphate ion (B) and mannose (C) are shown as ball-and-stick models. (D) Structure-based alignment of ZG16p and ZG16b with mannose-binding-type Jacalin-related lectins (Banlec, 3MIT [21]; Heltuba, 1C3M [15]; Artocarpin, 1VBO [25]; MornigaM, 1XXQ [22]; *Parkia* lectin, 1ZGS [23]; Griffithsin, 2GUD [28]). Red boxes indicate residues involved in mannose binding at the first sugar-binding site. Blue box in Banlec (GDVVD) indicates residues involved in mannose binding at the second binding site [26,29] and blue boxes in Griffithsin (GSYLD and GDYID) indicate residues involved in mannose binding at the second and third sites, respectively [28]. Secondary structures of ZG16p and ZG16b are shown above and below the amino acid sequences, respectively, and are colored as in (A) and (B). Magenta and letters indicate the basic residues conserved among ZG16p homologs and the corresponding residues in ZG16b are also in magenta. Phosphate-binding residue of ZG16b is shown in green. Sequences were aligned with MATRAS [30].

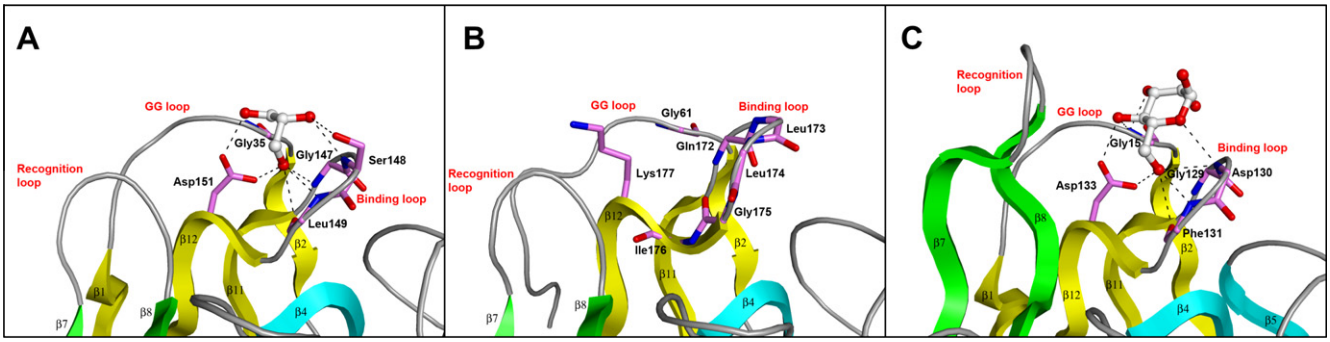

Fig. 2. Putative sugar-binding site of ZG16p is similar to mannose-binding site of Jacalin-related mannose-binding-type lectins. Close-up view of the putative sugar-binding site of ZG16p (A), ZG16b (B) and mannose-binding site of Banlec (C). Bound glycerol (A) and mannose (C) are shown as ball-and-stick models. Residues of Banlec binding to mannose and the corresponding residues in ZG16p and ZG16b are shown as stick models. Potential hydrogen bonds are indicated as dotted lines. Ligand-binding loops of Banlec, the so called "Binding loop", "Recognition loop" and "GG loop" [26] and the corresponding loops of ZG16p and ZG16b, are indicated with red letters.

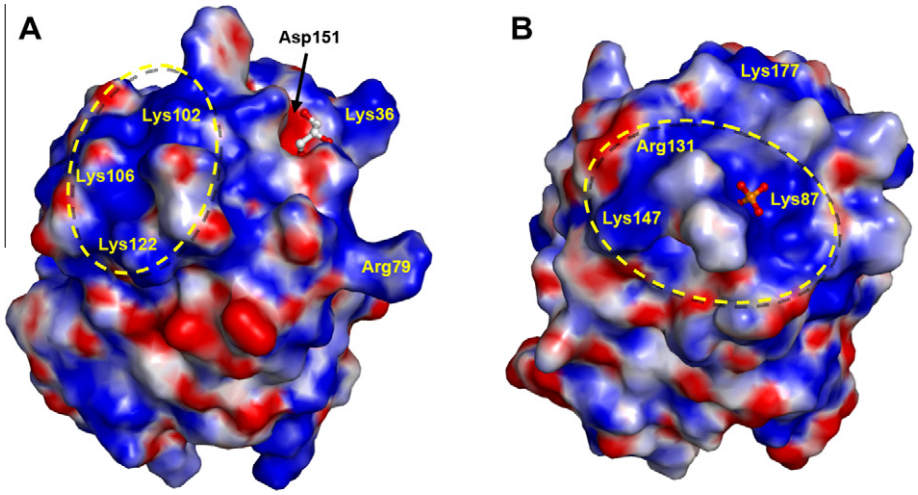

Fig. 3. Positively charged patches of ZG16p and ZG16b. The surface models of ZG16p (A) and ZG16b (B) are colored according to the electrostatic surface potential (blue, positive; red, negative; scale from −10 to +10 kT/e). Bound glycerol (A) and phosphate ion (B) are shown as ball-and-stick models.

X-ray data collection. Other cryoprotectants such as 20% (w/v) mannose or 20% (v/v) ethyleneglycol dramatically worsened the quality of the diffraction data (4–8 Å resolution). We are currently screening for better cryoprotectants. A structural comparison of ZG16p with Banlec bound to mannose revealed that the glycerol bound to ZG16p mimics the C4, O4, C5, O5, C6 and O6 atoms of the mannose (Fig. 2A and C). Gly147, Ser148, and Leu149 in the Binding loop and the Asp151 on the β12 strand create a network of hydrogen bonds with the glycerol oxygen atoms (Fig. 2A), which is also observed in the Banlec/mannose complex (Fig. 1C) [21], Heltuba/mannose [15], Artocarpin/methyl-mannose [27], MornigaM/mannose [22], *Parkia platycephala* seed lectin/5-bromo-4-chloro-3-indolyl-mannose [23] and Griffithsin/mannose [28] (Supplementary Fig. 1). Since Jacalin-related mannose-binding lectins do not recognize the 2-OH group of mannose, the lectins also interact with glucose, glucosamine and *N*-acetylglucosamine residues. These striking similarities strongly suggest that ZG16p and the mannose-binding-type Jacalin-related lectins have the same mannose-binding mode. The putative sugar-binding residues in ZG16p are well conserved among mammalian ZG16p-type lectins, suggesting functional conservation as well (Supplementary Fig. 2). In contrast, most of the corresponding sugar-binding residues are replaced in ZG16b and there is a residue insertion in the corresponding Binding loop (Figs. 1D and 2B). Aspartic acid residue in GxxxD motif of ZG16p is replaced with Lys177 in the corresponding site of ZG16b (Fig. 2), suggesting that ZG16b does not bind mannose-type sugars, but possibly binds another type of carbohydrate ligand, such as sulfated glycosaminoglycans through Lys177.

3.4. Positively charged patches and implications for glycosaminoglycan binding

ZG16p is known to interact with glycosaminoglycan, and positively charged residues in the protein are potentially involved in the binding of the negatively charged sulfate groups on the glycosaminoglycan. Hence electrostatic potentials were mapped on the molecular surface of ZG16p and ZG16b (Fig. 3). ZG16p has a basic patch composed of Lys102, Lys106 and Lys122, located close to the putative-mannose/*N*-acetylglucosamine binding site including Asp151 (Fig. 3A). Lys106 and Lys122 are especially well conserved among mammalian ZG16p homologs (Supplementary Fig. 2). Intriguingly, Lys106 is placed in a region corresponding to the third mannose-binding site of Griffithsin [28] (Fig. 1D). Lys36 is also adjacent to the putative-mannose/*N*-acetylglucosamine binding site and may be involved in the binding as well (Fig. 3A). These findings indicate the functional importance of these basic residues for glycosaminoglycan binding in concert with the residues in mannose/*N*-acetylglucosamine-binding site (Supplementary

Fig. 3). Considering the potential of mannose/glucose-type glycan and glycosaminoglycan binding, ZG16p may interact with a variety of partner molecules in the submembrane space of pancreatic acinar cells (Supplementary Fig. 4). ZG16b also exhibits a basic patch comprising Lys87, Arg131 and Lys147 adjacent to the putative sugar-binding site including positively-charged Lys177. Arg131 and Lys147 of ZG16b correspond to the conserved basic residues, Lys106 and Lys122 of ZG16p. Interestingly, a phosphate ion interacts with Lys87 of ZG16b which structurally corresponds to Asp38 in the second sugar-binding site (GDVVD) of Banlec (Figs. 1B and D & 3B). The phosphate ion probably mimics a sulfate group. Thus, the basic patch of residues of ZG16b may be involved in binding sulfated glycosaminoglycans as in the case of ZG16p.

We have solved the first three-dimensional structure of a mammalian β-prism fold lectin, ZG16p. ZG16b, a paralog of ZG16p, is also demonstrated to have a β-prism fold. The overall structure of ZG16p is quite similar to mannose-binding type Jacalin-related lectins such as Banlec. A glycerol molecule with a characteristic hydrogen bonding pattern located in a putative mannose binding site of ZG16p suggests a common sugar-binding mode between ZG16p and other mannose-binding-type Jacalin-related lectins. In ZG16p and ZG16b, positively-charged lysine and arginine residues cluster close to the putative sugar-binding site and potentially interact with sulfated glycosaminoglycans. ZG16p and its ability to bind mannose/glucose-type glycans and glycosaminoglycans could work as a platform for localizing proteoglycans and aggregated secretory proteins in the submembranous environment of the zymogen granule.

Acknowledgments

We sincerely thank Mr. Masaki Kato (RIKEN) for the bioinformatic analysis, Dr. Shinya Hanashima (RIKEN) for critical suggestions and Ms. Kana Matsumoto (RIKEN) and Ms. Kiyomi Senda (Nagoya City University) for initial attempts to produce ZG16p proteins. We thank the beamline staff of PF/KEK (Japan) and SPring-8 (Japan) for providing data collection facilities and support. This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.093.

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