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ZG16

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Gene symbols: ZG16

1. General Information

Discovery of ZG16p & expression studies

Zymogen Granule protein 16 (ZG16p) is a 16 kDa protein which was first identified by immunoscreening of a rat pancreatic cDNA expression library with a polyspecific antiserum raised against purified zymogen granule membranes (ZGM) (7). According to its sequence homology with the plant lectin Jacalin (which specifically binds to Galβ1-3GalNAc), ZG16p was considered a secretory lectin (7, 18). It is predominantly associated with the luminal surface of ZGM, but can be removed (in conjunction with sulfated glycosaminoglycans) by carbonate- or chondroitinase-treatment (18) (Fig. 1).

Northern blot analysis of several rat organs revealed the presence of ZG16 mRNA in the duodenum and colon, where ZG16p was found to localize to mucus-producing goblet cells (7). Further experiments demonstrated that the expression of ZG16 mRNA in the rat pancreas was only moderately affected by a hormonal treatment with cholecystokinin (CCK) or cerulein (7). Both peptide hormones are known to evoke a complete release of zymogen granules (ZG) and to upregulate expression and transport of zymogens (35). On the other hand, a repeated treatment of mice with supraphysiological doses of CCK over 2 weeks, causing pancreatitis, led to a short term down regulation of ZG16 mRNA in mouse pancreas (24). In dexamethasone-treated, cultured AR42J cells, a pancreatic model system, a strong upregulation of the ZG16 mRNA was observed already after 24 h (7). Treatment with the glucocorticoid dexamethasone has been shown to induce both the differentiation of AR42J cells into acinar-like cells and the *de novo* formation of electron-opaque secretory granules, which contain the major pancreatic zymogens (22).

In a study involving biomaterial patches sutured onto rat stomach, ZG16p mRNA in gastric tissue was found to be slightly upregulated (together with amylase and lipase mRNA) already under control conditions (surgery without implant) and to a higher extent with implant (21). In a study about differentially expressed genes in the rat ileum used for bladder augmentation, ZG16 was found in a cDNA microarray to be transiently increased compared to normal ileum after 1 and 3 months post-surgery (23). By RT-PCR and

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immunoblotting, ZG16p was shown to be expressed in human liver and to be down regulated in hepatocellular carcinoma. Down-regulation appears to be a consequence of the hepatic cancer rather than the cause (37). Overexpression of ZG16p in some hepatoma cell lines inhibited cell proliferation or cell cycle progression (37). In a study on hepatotoxicity of pharmaceutical xenobiotics, rat ZG16 mRNA level was found to be upregulated two fold under the influence of the hepatotoxic substance ANIT (α-napthylisothiocyanate) (14). Furthermore, a recently identified ZG16p paralog called PAUF (pancreatic adenocarcinoma up-regulated factor) or ZG16b (17) was found to be up-regulated in human pancreatic cancer cells and in mouse pancreatic cancer tissue on mRNA and protein level. PAUF/ZG16b induction caused an increased cell proliferation, migration and invasion ability in Chinese Hamster Ovary cells (CHO) (6, 17, 20).

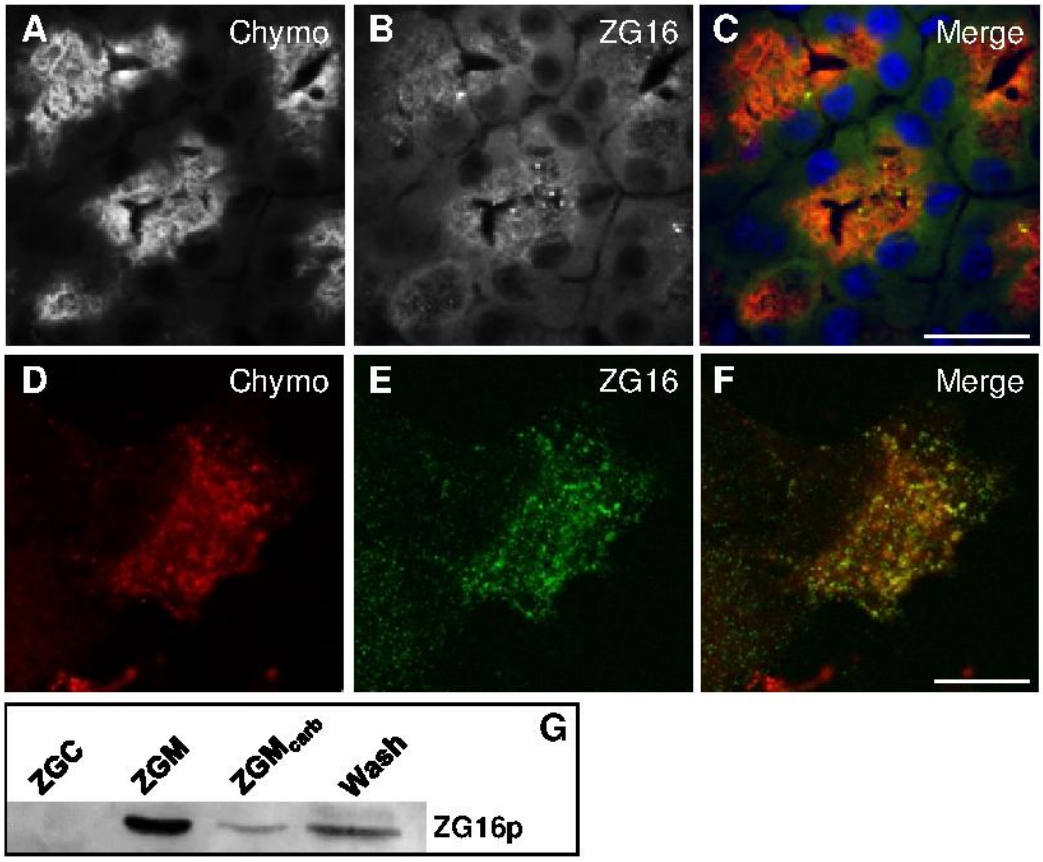

Figure 1. Immunofluorescence microscopy of pancreatic sections from rat pancreas and pancreatic AR42J cells. Cryosections of rat pancreas (A-C) and AR42J cells (D-F) were immunostained with antibodies directed to chymotrypsin (Chymo; A, D) and ZG16p (B, E). (C, F) Overlay of (A, B) and (D, E). Nuclei in (C) were stained with Hoechst 33258. Scale bars: 10 µm (C, F). (G) Immunoblot of zymogen granule subfractions from rat pancreas. Note that ZG16p is mainly associated with the zymogen granule (ZG) membrane (ZGM), but behaves like a peripheral membrane protein and can be released by carbonate-treatment (Wash). Equal amounts of protein (20 µg) were loaded onto a 12.5% SDS-polyacrylamide gel, blotted onto nitrocellulose membranes and incubated with an antibody to ZG16p. ZGC, zymogen granule (ZG) content proteins; ZGM, ZG membrane proteins; ZGM_{carb}, carbonate-treated ZG membranes; Wash, peripheral ZG membrane proteins released by carbonate treatment.

Immunolocalization & Proteomics

Immunoblotting of ZG subfractions revealed that ZG16p is mainly associated with the ZGM fraction, but can be removed by carbonate treatment indicating that it is a peripheral, membrane-associated protein (7) (Fig. 1). ZG16p has no transmembrane domain, and membrane interaction may be mediated by its lectin domain (18). Two dimensional gel electrophoresis demonstrated that ZG16p (*pI* 9.2) belongs to a group of basic, membrane-associated proteins (2, 28). In addition, ZG16p was successfully identified in recent proteomics studies on ZG from rat pancreas (2, 5, 27). It was identified by mass spectrometry in the ZGM fraction. Furthermore, a quantitative approach confirmed that ZG16p behaves like a peripheral ZGM protein (2). Immunohistochemistry of rat pancreas showed staining of the apical, granule-rich area of acinar cells (Fig. 1) (7). Immuno-electronmicroscopy revealed staining of isolated ZGM using ZG16p-specific antibodies (18). Furthermore, in an immunohistochemical staining of rat duodenum and colon, the luminal surface as well as goblet cells were labeled with ZG16p-specific antibodies after immunoperoxidase/DAB cytochemistry (7).

Sequence homologies & Crystal structure

The predicted amino acid sequence of ZG16p indicates that it represents a novel secretory lectin (7). The N-terminal part of ZG16p contains an ER-targeting signal, which is presumably cleaved in the mature form of the protein. Because of sequence similarities to the carbohydrate recognition domain of the plant lectin Jacalin from jack fruit, ZG16p belongs to the Jacalin lectin family (7, 18) (Fig. 2). Lectins are carbohydrate binding proteins other than enzymes or antibodies. Their specificity is defined by the mono- or oligosaccharides with which they interact best (1, 12). In animals, lectins play important roles in cell adhesion, maintenance of membrane polarisation, the recognition of pathogens (immune system), glycoprotein synthesis and in various protein trafficking/sorting processes (4, 10, 11, 26, 31, 32).

Sequence analyses uncovered that ZG16p is highly conserved amongst mammals (16) but also appears in many other species, e.g. salmon (*S. salar*) and its copepod parasite, the sea louse *Caligus rogercresseyi*. In rat, it shares sequence homologies, specifically at the C-terminus, with two secretory proteins from other exocrine glands, prostatic spermine binding protein and common salivary protein 1, possibly belonging to a group of evolutionary related proteins (7). In humans the paralog ZG16b/PAUF was recently found to play a role in gene regulation and cancer metastasis (17, 20) (Fig. 2).

For both human proteins the crystal structure was recently solved (16) revealing a β-prism fold with 3 β-sheets, each consisting of 3-4 β-strands forming three Greek motifs in both proteins, similar to jacalin related mannose-binding type lectins (16). (ZG16p: http://www.rcsb.org/pdb/explore/explore.do?structureId=3APA). In both cases the structural informations were obtained from the core lectin domain of the proteins by cloning and expressing the sequences without their signal peptides (for ZG16p aa 20-160 and for ZG16b aa 53-208). Additionally, in ZG16p, seven amino acids from the C-terminus (aa 161-167) containing two cystein residues (Cys164 and Cys167), which do not exist in the sequence of ZG16b (16), were removed. The structure of ZG16p contains a short α-helix between the β2 and β3 strand, which does not exist in any other β-prism fold lectin and also not in ZG16b, which in addition lacks the β1 strand (16) (ZG16b: http://www.rcsb.org/pdb/explore/explore.do?structureId=3AQG). In contrast, ZG16p lacks the β5 strand which usually exists in Jacalin-related mannose-binding type lectins (16). Kanagawa et al. (16) suggest that the sugar-binding capacity of ZG16p originates from a well conserved functional motif of three different loops (GG-loop, recognition loop, and binding loop) all situated on top of the β-

prism fold which act together as one motif (Fig. 2). This motif is shared by all mannose-binding-type Jacalin-related lectins (16). The putative sugar-binding site of ZG16p was occupied by a glycerol molecule (used as cryoprotectant), which may mimic the mannose bound to Jacalin-related mannose-binding-type plant lectins. These lectins bind mannose/glucose-type glycans and glycosaminoglycans (GAGs). In addition, a positively charged basic patch of lysine and arginine residues, which may bind sulfated groups of GAGs, is located around the putative sugar-binding site of ZG16p and ZG16b (Fig. 2).

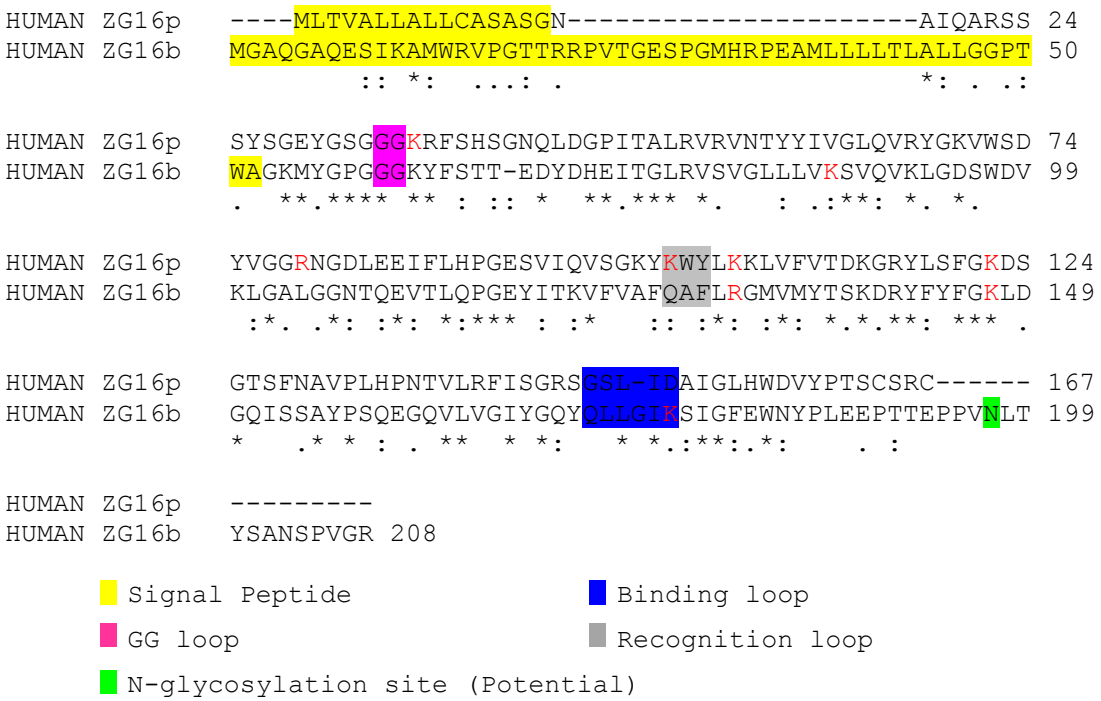

Figure 2. Alignment of the human ZG16p and ZG16B protein sequences. The online version of ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to generate the alignment. The information of the Binding, Recognition and GG loops was taken from (16). Red letters indicate putative glycosaminoglycan-binding residues (16). Signal Peptides and N-glycosylation sites were extracted from the Protein Knowledgebase (UniprotKB) (http://www.uniprot.org/uniprot/O60844 for ZG16p and http://www.uniprot.org/uniprot/Q96DA0 for ZG16B). (*) identical residues; (:) conserved substitutions; (.) semi-conserved substitutions.

2. Pancreatic Function/s

ZG16p is predominantly associated with the luminal surface of ZGM, and can be removed in conjunction with proteoglycans/GAGs by carbonate- or chondroitinase-treatment (18, 28, 30). Thus, it was suggested to represents a component of the *submembranous granule matrix* (a putative proteoglycan/glycoprotein scaffold at the luminal side of the ZGM contributing to cargo sorting, packaging and granule stability), where it interacts with sulfated proteoglycans (18, 28, 30). Interestingly, ZG16p has also been found to associate with cholesterol-glycosphingolipid-enriched microdomains in the ZGM together with GP-2 and sulfated proteoglycans (29). In addition, a tightly membrane-associated form of about 32 kDa, presumably a dimer, has been identified (15,

18). However, the ZGM association of ZG16p was not influenced in GP-2 knock-out mice (36). In line with this, a function as a linker/helper protein in the binding of aggregated zymogens to the granule membrane has been proposed from *in vitro* studies (18). Pretreatment of ZGM with anti-ZG16p antibody in an *in vitro* condensation-sorting assay (8) inhibited the binding of aggregated content proteins to the membrane by about 50% whereas pretreatment with anti-amylase antibody had no significant effect (18). Pretreatment of membranes with chondroitinase ABC (which also removes ZG16p from the ZGM indicating that it may interact with proteoglycans/GAGs) resulted in an inhibition of condensation-sorting by 40-50% (18). Competition experiments with mono- and disaccharides showed that the addition of 10 mM galactose had only a weak inhibitory effect on condensation-sorting (18). The recent structural data on ZG16p (16) suggest that the putative sugar-binding site and the adjacent basic patch cooperatively form a functional GAG-binding site. Thus, ZG16p could function as a linker between the submembranous matrix and lipid microdomains and contribute to the sorting and packaging of zymogens during ZG biogenesis (15, 18, 19, 28, 30).

Thévenod and co-workers proposed an additional function of ZG16p in the regulation of a K^+ conductance in zymogen granules (3). In an approach to identify a ZGM protein involved in the regulation of an ATP-sensitive K^+ and Cl^- conductance they used a dihydropyridine derivative in photoaffinity labeling experiments. To their surprise, they labeled and purified ZG16p as a high-affinity dihydropyridine binding protein of rat ZGM. They suggest a regulatory role for ZG16p in the direct coupling between granule fusion to the plasma membrane and the activation of channels in the ZGM (33). It had already been proposed that anion-cation channels might promote “flushing out” of the granule content (i.e., enzymes or mucins) during exocytosis in pancreatic acinar cells and in intestinal goblet cells (9, 13). It is speculated that a similar mechanism may take place after an initial, fusion pore-mediated granule swelling by the granule matrix, including ZG16p (33, 34). This intriguing possibility remains to be established.

Recently, ZG16p and PAUF/ZG16b have been implicated in the regulation of gene expression (17, 20, 24). For the ZG16p paralog, ZG16b/PAUF (Fig. 2), it is assumed that it plays a role in gene regulation enhancing the expression of β-catenin and TLR/CXCR4. It induces the extracellular signal-regulated kinase (ERK) phosphorylation and activates the IKK-β-mediated TPL2/MEK/ERK signaling pathway through TLR2 leading to a rapid proliferation of pancreatic cells and to an increased expression of the protumorigenic cytokines RANTES and MIF in THP-1 cells (Human acute monocytic leukemia cells) (6, 20, 25). Thus, it plays an important role in the progression of pancreatic cancer. PAUF/ZG16b was as well found to be highly expressed in human and mouse pancreatic cancer tissue (6, 17, 20, 25). In Chinese Hamster Ovary (CHO) cells the induction of ZG16b/PAUF increased cell proliferation, migration and invasion ability (17).

3. Tools to study ZG16

a. cDNA clones

Various plasmids encoding for rat or human ZG16 have been generated. Cronshagen *et al.* (7) cloned the complete coding sequence of rat ZG16 into a pQE32 expression vector in frame with the encoding 6xHIS-tag for protein expression and purification. Kanagawa *et al.* (16) cloned the core sequence of human ZG16 from aa20-160 into a top Cold-MBP vector for the production of recombinant human ZG16p for crystal structure analysis. Zhou *et al.* (37) generated plasmid pcDNA3.1A-hZG16, encoding a human ZG16p fusion protein with a Myc-His-tag at the C-

terminus as well as pGEX-hZG16 (19–167aa) for the expression of GST-tagged human ZG16p.

b. Antibodies

Specific rabbit anti-ZG16p antibodies generated against the rat (7, 16) and human ZG16p (36, 37) have been described and used for immunoblotting, immunofluorescence, immunohistochemistry and binding assays by different laboratories.

Commercially available antibodies are offered. An affinity purified rabbit IgG anti human ZG16p antibody is offered by "the Protein Tag Group" (Cat. Nr. 17397-1-AP; reactive in human, rat and mouse; tested in ELISA, WB, IHC and raised against a His-tagged version of the full length ZG16p). The corresponding antigen is as well available ("the Protein Tag Group"; Cat. Nr. ag11332 for the His-tagged protein; Cat. Nr. ag11300 for a GST-tagged ZG16p fusion protein). An affinity purified polyclonal rabbit anti human ZG16p antibody raised against a synthetic peptide is available from "Life Span Biosciences" (Cat. Nr. LS-C111443).

c. ZG16 silencing

siRNA for rat ZG16 is commercially available (e.g. ambion.com).

d. Mouse lines

Currently none available.

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