Proteoglycans support proper granule formation in pancreatic acinar cells

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Abbreviations: CEL, carboxyl ester lipase; TGN, trans-Golgi network; ZG, zymogen granule; ZGC, zymogen granule content; ZGM, zymogen granule membrane.

Abstract

Zymogen granules (ZG) are specialized organelles in the exocrine pancreas which allow digestive enzyme storage and regulated secretion. The molecular mechanisms of their biogenesis and the sorting of zymogens are still incompletely understood. Here, we investigated the role of proteoglycans in granule formation and secretion of zymogens in pancreatic AR42J cells, an acinar model system. Cupromeronic Blue cytochemistry and biochemical studies revealed an association of proteoglycans primarily with the granule membrane. Removal of proteoglycans by carbonate treatment led to a loss of membrane curvature indicating a supportive role in the maintenance of membrane shape and stability. Chemical inhibition of proteoglycan synthesis impaired the formation of normal electron-dense granules in AR42J cells and resulted in the formation of unusually small granule structures. These structures still contained the zymogen carboxypeptidase, a cargo molecule of secretory granules, but migrated to lighter fractions after density gradient centrifugation. Furthermore, the basal secretion of amylase was increased in AR42J cells after inhibitor treatment. In addition, irregular-shaped granules appeared in pancreatic lobules. We conclude that the assembly of a proteoglycan scaffold at the ZG membrane is supporting efficient packaging of zymogens and the proper formation of stimulus-competent storage granules in acinar cells of the pancreas.

Introduction

Pancreatic acinar cells are specialised in the synthesis, sorting, storage and regulated secretion of a complex mixture of digestive enzymes, which are packaged in a condensed and mainly inactive form into zymogen granules (ZG). These large, highly abundant secretory organelles are stored at the apical pole of the acinar cells and release their cargo via exocytosis in a regulated, calcium-dependent manner triggered by neuronal or hormonal stimulation. The secreted digestive enzymes reach the small intestine via the pancreatic duct system, where they are activated by enterokinase through proteolytic cleavage of trypsinogen.

The formation of ZG in the acinar cells is initiated at the *trans*-Golgi network (TGN) where the regulated secretory ZG proteins co-aggregate at the mildly acidic pH and high calcium levels and immature secretory granules, so called condensing vacuoles, are formed (Colomer et al. 1996, Dartsch et al. 1998, Freedman & Scheele 1993, Leblond et al. 1993). They mature by further concentration of the cargo proteins with selective removal of components not destined for regulated secretion. However, the molecular mechanisms of ZG formation and sorting of zymogens are still incompletely understood (Borgonovo et al. 2006, Dikeakos & Reudelhuber 2007, Gómez-Lázaro et al. 2010, Schmidt et al. 2001, Schrader 2004). This also applies to the regulation and maintenance of the size and the shape of the relatively large ZG (up to 1 µm in diameter). Using pancreatic AR42J cells, an acinar model system, we recently demonstrated that expression of ZG cargo proteins is not sufficient to trigger granule formation (Rinn et al. 2012). This is in line with previous reports indicating that specific accessory molecules are required for proper ZG formation. These components include helper proteins for cargo aggregation (de Lisle et al. 2005, Kleene et al. 1999) and/or components of the granule membrane (Colomer et al. 1996, Leblond et al. 1993, Schmidt et al. 2001, Schrader 2004). The latter enclose sulfated proteoglycans, which have been identified in ZG and in the fluid of the pancreatic ductal system (Berg & Young 1971, Reggio

& Palade 1978, Tartakoff et al. 1975). The negatively charged proteoglycans in secretory granules of hematopoietic cells and mast cells are involved in the binding of small positively charged molecules, such as amines and proteases, and have therefore been considered to promote the efficient storage of secretory products (Rönnberg et al. 2012, Wernersson & Pejler 2014). Similarly, proteoglycans within ZGs have been proposed to support ZG biogenesis and to concentrate at the ZG membrane (Kleene et al. 1999, Scheele et al. 1994, Schmidt et al. 2000, Schrader 2004), but their role in ZG formation is unclear.

In recent proteomics studies, new ZG components have been identified, which are known to interact with proteoglycans in other systems (Borta et al. 2010, Chen et al. 2008).These include chymase, a major, highly basic chymotrypsin-like serine protease initially identified in mast cells, and PpiB (Cyclophilin B), a highly basic cyclosporin A-binding protein with peptidyl-prolyl cis-trans isomerase activity (Allain et al. 2002, Borta et al. 2010). In addition, well-known constituents of ZG such as RNAse A and carboxyl ester lipase (CEL) have the potential to interact with proteoglycans, e.g. to influence enzyme function (Dvorak & Morgan 1998, Rebaï et al. 2005). Moreover, an interaction of the secretory lectin ZG16p (Cronshagen et al. 1994, Kleene et al. 1999) with heparan sulfate containing proteoglycans from ZG has recently been reported (Kumazawa-Inoue et al. 2012).

To elucidate the role of proteoglycans in exocrine granule formation, we have exploited pancreatic AR42J cells, an exocrine model system. Our data show that proteoglycans are mainly present attached to the granule membrane and indicate an important function in the maintenance of membrane shape and stability. After inhibition of proteoglycan synthesis by β-D xyloside normal granule formation was impaired. Furthermore, the basal secretion of amylase was increased. We conclude from these findings that the assembly of a proteoglycan scaffold at the ZG membrane is supporting efficient packaging of zymogens and the proper formation of stimulus-competent storage granules in acinar cells of the pancreas.

Materials and methods

Antibodies

Antibodies were used as follows: rabbit polyclonal antibodies against amylase (Sigma-Aldrich, St. Louis, MO), carboxypeptidase A (Rockland Immunochemicals, Gilbertsville, PA), ZG16p (Cronshagen et al. 1994), mouse monoclonal antibodies directed to GP2 (kindly provided by A. Lowe, Stanford University School of Medicine, Palo Alto, CA), tubulin (Sigma) and p115 (BD Transduction Lab.). A polyclonal antibody raised against components of a carbonate extract from isolated ZGM (anti-membrane-matrix) is described elsewhere (Schmidt et al. 2000). Species-specific anti-IgG antibodies conjugated to the fluorophores TRITC and Alexa 488 were obtained from Jackson ImmunoResearch (West Grove, PA), and Invitrogen (Carlsbad, CA). Species-specific IgG antibodies conjugated to HRP were obtained from BioRad (Richmond, CA).

Cell culture, drug treatment and secretion experiments

AR42J cells (ATCC no. CRL-1492) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (PAA Laboratories GmbH, Linz, Austria) (Faust et al. 2008) at 37°C in a humidified atmosphere containing 5% CO_2. To improve cell adherence of AR42J cells, culture dishes and coverslips were coated with an extract of Engelbreth-Holm-Swarm tumor, prepared according to (Kleinman et al. 1979). To induce differentiation and zymogen granule formation, AR42J cells were incubated with 10 nM dexamethasone for 2-3 days (Swarovsky et al. 1988). Cell numbers and viability were determined by Trypan Blue staining in a Fuchs-Rosenthal chamber. Proteoglycan synthesis was inhibited by 4-methylumbilliferyl-β-D-xyloside (β-D-xyloside) (Sigma), which was dissolved in dimethyl sulfoxide (DMSO) (Sigma). Cells were pre-treated with the inhibitor (0-2.5 mM) or with DMSO for 4 hours

before granule formation was induced by the addition of 10 nM dexamethasone. After 48 hours cells were processed for indirect immuofluorescence, electron microscopy or for biochemical studies. Cell viability after 48 hours of treatment with β-D-xyloside was comparable to untreated controls (not shown).

Amylase secretion assays were adapted from (Limi et al. 2012). AR42J cells were seeded in 6 cm culture dishes in complete DMEM culture medium (see above) and treated with 10 nM dexamethasone. After 2 days the cells were washed twice with PBS and incubated with Krebs-HEPES buffer (KHB) (140 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 15 mM HEPES, 10 mM glucose, 2.5 mM $CaCl_2$, 0.1 mM PMSF, 2.5 mM Aprotinin, pH 7.4) without or with 1.5 nM cholecystokinin octapeptide 26-33 (CCK-8) (Sigma) for 30 min. After incubation the buffer was removed and centrifuged for 2 minutes at 16,000*g* to remove cellular debris. The cells were collected in fresh KHB and lysed by forcing them through a 26G - gauge needle followed by brief sonication. Amylase activity in buffers and cells was measured with the Liquick Cor-AMYLASE diagnostic kit (PZ Cormay, Poland). Amylase release was determined as the percentage of amylase activity secreted into KHB (with or without CCK-8) over the 30 min incubation in relation to total amylase.

Subcellular fractionation

AR42J cells were treated with β-D-xyloside and DMSO as described above. After 48 hours cells were homogenized with a Potter S homogenizer (1000 rpm, 2 strokes) in homogenization buffer (see below). The post nuclear supernatant (500*g*, 5 min, 4ºC) was applied on top of a continuous OptiPrep® gradient (0-30%) (Axis-Shield, Oslo, Norway). After centrifugation at 20,000*g* for 3.5 hours at 4°C in a swing-out rotor (SW41, Beckman Instruments, Munich, Germany), fractions (500 μl) were collected from the top of the gradient. Proteins were precipitated by 10% TCA and analyzed by SDS-PAGE.

Isolation of zymogen granules from rat pancreas

Zymogen granules (ZG) were isolated as described previously (Borta et al. 2010) from the pancreas of male Wistar rats (200-230 g) (Charles River, Sulzfeld, Germany). Animals were handled according to the German law for the protection of animals, with the permit of the local authorities. The following buffer was used for homogenization: 0.25 M sucrose, 5 mM 2-N-morpholino-ethanesulfonic acid (MES), pH 6.25, 0.1 mM $MgSO_4$, 1 mM dithiothreitol, 10 µM Foy-305 (Sanol Schwarz, Monheim, Germany), 2.5 mM Trasylol (Bayer, Leverkusen, Germany), 0.1 mM phenylmethylsulfonyl fluoride. ZGs were resuspended in 50 mM N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES), pH 8.0, and lysed by freezing and thawing. The zymogen granule membrane (ZGM) was separated from the soluble zymogen granule content proteins (ZGC) by ultracentrifugation (100,000*g* for 30 minutes), resuspended in 50 mM HEPES, pH 8.0 and stored at -20°C. For some experiments, ZGM were pre-incubated with enterokinase. For carbonate treatment ZGM were incubated with the same volume of 300 mM Na_2CO_3, pH 11.5 on ice for 2 hours and recovered by high-speed centrifugation at 100,000*g* for 30 minutes.

Gel electrophoresis and immunoblotting

Protein concentrations were determined using the Bradford assay or the BCA reagent kit (Interchim, Montlucon, France). Proteins were precipitated with 10% (w/v) trichloracidic acid (TCA) and boiled in SDS-PAGE sample buffer. Protein samples were separated by SDS-PAGE on 12.5% polyacrylamide mini gels, transferred to nitrocellulose (Schleicher and Schüll, Dassel, Germany) via a semi-dry apparatus (Trans-Blot SD, Biorad) and analysed by immunoblotting using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Bioscience, Arlington Heights, IL, USA).

Biochemical assays

Sulfated proteoglycans and glycosaminoglycans were determined using the Blyscan assay according to the manufacter's protocol (Biocolor, Belfast, Ireland). Briefly, equal amounts of TCA-precipitated protein were resuspended in 50 mM HEPES, pH 8.0 and incubated with the Blyscan dye. A precipitable complex was formed by binding of the dye to sulfated proteoglycans and glycosaminoglycans. After dissociation of the precipitated complex the samples were photometrically quantified. Agarose gel analysis of proteoglycans was performed according to (Metkar et al. 2002). Zymogen granule subfractions were run on a 1% agarose gel prepared and run in Tris-Borate-EDTA buffer, pH 8.3, followed by staining with 0.02% toluidine blue in 3% acetic acid and destaining in 3% acetic acid. Biglycan from bovine articular cartilage (Sigma) and chondroitin sulfate (Sigma) were used as standards.

Immunofluorescence and microscopy

Cells grown on glass coverslips were fixed with 4% *para*-formaldehyde in PBS, pH 7.4 for 20 minutes at RT. The samples were permeabilised with 0.2% Triton X-100 (10 minutes, RT), blocked with 1% BSA (15 minutes, RT) and incubated with primary and secondary antibodies (1 hour each, RT) as described (Borta et al. 2010, Faust et al. 2008). Samples were examined using an Olympus IX-81F microscope (Olympus Optical Co. LTD Tokyo, Japan) equipped with the appropriate filter combinations and a 100x objective (Olympus PlanApo; numerical aperture, 1.4). Fluorescence images were acquired with an F-view II CCD camera (Soft Imaging System GmbH, Münster, Germany) driven by Soft imaging software. Digital images were optimized for contrast and brightness using Adobe Photoshop (Adobe Systems, USA).

Preparation of pancreatic lobules

Pancreatic lobules were prepared as described (Scheele & Palade 1975) from animals fed with a single dose of 50 mg/kg of FOY-305 (Sanol-Schwarz, Mohnheim, Germany) dissolved in 1 ml of tap water (Schmidt et al. 2001). FOY treatment for up to 6 hours results in partial

degranulation (40-60%) of the pancreas (Rausch et al. 1987). The lobules were incubated at 37°C under agitation and were supplied with 100% O_2 every 15 minutes. For inhibition of proteoglycan synthesis lobules were incubated for 3 hours in 5 ml MEM (Sigma, Munich, Germany) containing 2.5 mM β-D-xyloside (Sigma) or DMSO (controls). Treated lobules and controls were processed for electron microscopy.

Electron microscopy

Proteoglycan staining was performed according to the Cupromeronic Blue® (CmB, Seikagaku Corp., Tokyo, Japan) procedure at critical electrolyte concentration developed by (Scott 1980, 1985). Pancreatic tissue was fixed with 1% glutaraldehyde for 30 minutes and stained overnight in 25 mM sodium acetate, pH 5.7, containing 0.5% glutaraldehyde, 0.05% CmB and 0.3 M $MgCl_2$. AR42J cells were stained for 15-60 minutes. At this electrolyte concentration highly sulfated acidic glycosaminoglycan side chains of proteoglycans are preferentially stained by CmB (Scott 1985). After three washes in acetate-$MgCl_2$ specimens were contrasted with 0.5% sodium tungstate (Scott 1985) in water and in 50% ethanol, dehydrated in an ascending series of ethanol and embedded in Epon. For routine electron microscopy, pancreatic lobules were fixed with Ito-fixative for 30 minutes at room temperature (Faust et al. 2008). AR42J cells were fixed in 1% glutaraldehyde in 0.2M PIPES buffer (pH 7.2) for 15 minutes before scraping the cells from the culture dish in a small volume of the fixative. The cells were then pelleted (15 minutes at 13.300 x g), washed three times in buffer followed by post-fixation for 1 hour at RT in 1% osmium tetroxide containing 1.5% w/v potassium ferrocyanide to improve membrane contrast. The cells were subsequently washed with distilled water and dehydrated through a graded ethanol series followed by embedding in Durcupan resin (Sigma Aldrich, Poole, UK). Ultrathin sections (70 nm) were collected on pioloform-coated EM copper grids (Agar Scientific, Stansted, UK) and contrasted using lead citrate with or without prior staining with 2% w/v aqueous uranyl

acetate. Sections were analysed using a JEOL JEM 1400 transmission electron microscope operated at 80 kV. Images were obtained with a side mounted Gatan ES1000W digital camera (Gatan, Abingdon, Oxon, UK) at a nominal magnification of 50 or 100k X. Isolated granule membrane fractions were fixed in 0.1% cacodylate buffer, pH 7.3 containing 1% glutaraldehyde (Serva, Heidelberg, Germany). After postfixation with 1% $K_4Fe(CN)_6$-reduced osmium tetroxide (1 hour at RT), samples were stained with 0.3% uranyl acetate (1 hour at 4°C). The samples were dehydrated in a graded series of alcohol and embedded in Epon 812 (Polysciences Ltd., Eppenheim, Germany) according to standard procedures. Thin sections (70 nm) were stained with lead citrate and examined using a Zeiss EM 109 electron microscope.

Quantitation and statistical analysis of data

For quantitative analysis of granule morphology in AR42J cells, 100-200 cells per coverslip were examined and categorized as ‘granular’ when showing a typical punctate staining pattern (**see Fig. 4B**). Analysis was done blind. Four coverslips per preparation were analyzed, and 4-6 independent experiments were performed. Quantitative analysis of ultrastructural alterations of pancreatic granules after different experimental treatments was performed on EM micrographs. Sections were randomly photographed at a magnification of 3000-12000×. The results of the quantitative analysis presented were from 3 independent experiments. Significant differences between experimental groups were detected by analysis of variance for unpaired variables using Microsoft Excel. Data are presented as means ± S.D., with an unpaired t-test used to determine statistical differences. P-values <0.05 are considered as significant, and p-values <0.01 are considered as highly significant. For estimating granule size, AR42J cells were scanned systematic uniform random using the digital camera and a systematic graticule with regular spacing which was positioned orthogonal to the scanning direction. One edge of the graticule markings was assigned for recording intersections

between the graticule scale and the granule profiles and the number of markings crossed by each granule during the scan was counted. For selecting the granules for sizing an unbiased counting band was used during the scan with one edge of the band defined as an acceptance line, the other as the forbidden line (Gundersen 1977). Granules that translocated within the band or crossing the acceptance line were selected for sizing. Granule sizes were estimated by multiplying the number of intersections and the spacing between the graticule markings.

Results

Sulfated proteoglycans are concentrated at the inner surface of the zymogen granule membrane

To localise proteoglycans within zymogen granules at the ultrastructural level**,** pancreatic tissue and AR42J cells were stained using Cupromeronic Blue (CmB). CmB preferentially binds to highly sulfated acidic glycosaminoglycan side chains of proteoglycans (see Materials and Methods) (**Fig. 1**) and has been used successfully to stain proteoglycans in secretory granules of leucocytes (Unger et al. 1997). In ultrathin pancreatic sections CmB staining mainly appeared as elongated small electron-dense filaments or 'prisms' underneath the granule membrane with some variation between individual granules (**Fig. 1A, B**). CmB-proteoglycan complexes were also observed in the intermembrane space (**Fig. 1A**), presumably due to a staining of proteoglycans of the extracellular matrix. Similar results were obtained with the exocrine cell line AR42J after treatment with the synthetic glucocorticoid dexamethasone, which is known to induce differentiation into acinar-like cells and the formation of secretory granules, which contain the major pancreatic zymogens (Logsdon et al. 1985, Rinn et al. 2012). Electron-dense CmB-proteoglycan complexes were found underneath the granule membrane and in the intermembrane space, but were absent in controls treated with buffer (compare **Fig. 1C and D**). Those complexes were less prominent than in pancreatic granules, which may be explained by a lower concentration of proteoglycans in AR42J granules (which are much smaller than pancreatic zymogen granules; see **Fig. 5**) or by the presence of different proteoglycans (e.g. less sulphated ones).

Next, we analyzed the distribution of granule marker proteins and proteoglycans in suborganellar fractions of zymogen granules. Isolated ZG from rat pancreas were separated in a content (ZGC) and membrane fraction (ZGM). Isolated ZGM were further treated with carbonate at pH 11.5 to liberate membrane-associated components (Borta et al. 2010, Schmidt et al. 2000) (**Fig. 2A**). Major soluble granule markers such as amylase or carboxypeptidase A

were predominantly found in the content fraction (ZGC, ~ 80%) and do not contribute much to the peripheral ZGM components (Borta et al. 2010). In contrast, the secretory lectin ZG16p, a peripheral ZGM protein (Borta et al. 2010, Cronshagen et al. 1994, Kleene et al. 1999) which has recently been demonstrated to interact with heparan sulfate proteoglycans in ZG (Kumazawa-Inoue et al. 2012), was concentrated on isolated ZGM (~ 77%). However, most of the membrane-bound ZG16p (~ 68%) was removed by carbonate treatment (**Fig. 2A**), whereas GP2, a major GPI-anchored glycoprotein of ZG, was retained. In addition to ZG16p, ~ 70% of the membrane-associated proteoglycans were removed by carbonate (**Fig. 2B**). Interestingly, we also observed an alteration of the migration of ZG16p in immunoblots of isolated ZGM. ZG16p is usually detected in a distinct protein band of about 16kD when the protein samples are heated at 95ºC prior to electrophoresis (**Fig. 2C)**. However, incubation at RT results in a shift of the ZG16p band to the stacking gel indicating that the lectin is interacting with a large high molecular mass complex, which prevents it from entering the gel. In line with this, ZG16p and proteoglycans have been described as components of a submembranous protein matrix (Borta et al. 2010, Gómez-Lázaro et al. 2010, Schmidt et al. 2000), which is supposed to aid in granule formation and protein sorting.

Interestingly, electron microscopy of isolated ZGM and carbonate-treated ZGM fractions revealed that the untreated membranes appeared overwhelmingly curved or curled (**Fig. 2D**). In contrast, most of the carbonate-treated membranes had a strikingly linear appearance (**Fig. 2E**). When immunogold electron microscopy with an anti-membrane matrix antibody was performed, gold labelling of peripheral ZGM components was predominantly found on the untreated, curved membranes (**Fig. 2F**). However, only few gold particles were visible on the linear membranes obtained after carbonate treatment (**Fig. 2G**).

These observations suggest that peripheral ZGM components (as part of a membrane scaffold) may support the membrane shape and curvature of the relatively large ZG granules (1 µm in diameter). We assume based on the above observations that proteoglycans and

associated granule components have additional mechanical functions during granule formation at the TGN and for the maintenance of granule shape and stability.

The carbonate extract was also subjected to agarose gel analysis (**Fig. 3**) and stained with toluidine blue, a dye which forms complexes with anionic glycoconjugates such as proteoglycans and glycosaminoglycans. A diffuse band and a high molecular smear indicative of proteoglycans/GAGs was detected in the ZGM extract. A minor portion of proteoglycans/anionic glycoconjugates was also found in the untreated ZGC fraction containing soluble content proteins. Carbonate treatment of this fraction revealed a larger amount of glycoconjugates which may become more accessible to agarose separation or dye staining. The inaccessibility might be due to a tight interaction with content proteins, which have to be removed before separation. It is possible that additional glycoconjugates are present within ZGs, which resist separation and staining according to the method described above. Despite several attempts (Kumazawa-Inoue et al. 2012) the nature of the proteoglycan core proteins within ZG has not yet been identified.

Inhibition of proteoglycan synthesis perturbs granule formation in AR42J cells and pancreatic lobules

To analyse the role of proteoglycans in granule formation in more detail, pancreatic AR42J cells were treated with 4-methylumbilliferyl-β-D-xyloside (β-D-xyloside) to inhibit proteoglycan synthesis (**Suppl. Fig. S1**). This drug blocks the formation of glycosaminoglycan chains on proteoglycans (Schwartz 1977). AR42J cells were pretreated with 2 mM β-D xyloside for 4 hours before granule formation was induced by the addition of 10 nM dexamethasone (**Fig. 4A-C**). Normally, dexamethasone induces 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 (Logsdon et al. 1985) (**Fig. 1C, D and Fig. 4B**). Interestingly, treatment with β-D-xyloside perturbed the formation of granules

in AR42J cells (**Fig. 4C**). Instead of the typical punctate staining pattern with numerous secretory granules labelled with carboxypeptidase A antibody (as shown in **Fig. 4B**; 60-70% of the cells show this staining), an unusual fine punctate staining pattern for carboxypeptidase A was observed in the majority of the cells (70-80%) (**Fig. 4C**). It has to be noted that the small punctate staining pattern was very characteristic for β-D xyloside treatment. Incubation of AR42J cells with mannosamine, an inhibitor of GPI anchor synthesis and N-glycosylation, for example, resulted in an accumulation of zymogens in the Golgi apparatus, and the formation of granular or fine punctate structures was completely inhibited (not shown). In unstimulated controls (without dexamethasone), 4-8% of the cells exhibited a strong labelling of the Golgi apparatus as well as a fine punctate staining pattern, which might represent constitutive cargo containers (**Fig. 4A, Suppl. Fig. S2**).

Quantitative analysis of the morphological observations revealed that after β-D-xyloside treatment only 25% of the dexamethasone-stimulated cells exhibited a typical granular staining pattern, in contrast to ~ 65% in stimulated controls (**Fig. 4D**). Interestingly, the fine punctate staining pattern in β-D-xyloside-treated cells was more intense than in unstimulated controls (without dexamethasone) indicating that the expression of zymogens was induced (see also **Fig. 8A**) but normal granule formation was influenced.

These observations were confirmed by ultrastructural studies (**Fig. 5**). In dexamethasone-stimulated control cells electron-opaque secretory granules were observed in 72 ± 6% of the cells, whereas in unstimulated controls electron-dense granular structures were almost absent (4 ± 2% of the cells) (**Fig. 5A, B**). In β-D xyloside-treated cells electron-opaque granules were mainly absent as shown in **Fig. 4D**. However, electron-opaque granules could be identified in 12 ± 6% of the cells which appeared to be smaller than the secretory granules localized in the stimulated control cells (**Fig. 5C)**. Therefore, the granule size was quantified for both, stimulated as well as β-D xyloside-treated cells, using unbiased sampling and sizing

methods (see Materials and Methods). The mean granule profile size was estimated to be 125.8 nm in the stimulated control and 72.6 nm in β-D xyloside-treated cells (**Fig. 5D**). Those ultrastructural studies also confirmed the intactness of the cells after β-D xyloside treatment. The morphology of other intracellular organelles was unchanged when compared to untreated cells.

Immunoblot analysis of fractions collected after density gradient centrifugation of cell homogenates revealed differences between secretory granules from controls and β-D xyloside-treated AR42J cells. We prepared continuous OptiPrep gradients (0-30%) from post nuclear supernatants of AR42J cells. We analysed equal amounts of protein of the fractions obtained after density gradient centrifugation by immunoblotting and quantified by densitometry (**Fig. 6**). Secretory granules from controls, identified by an antibody to amylase, were found mainly in gradient fractions 17-21 with some immunoreactivity in fractions 14-16. Some soluble amylase was also detected in the uppermost gradient fractions reflecting the fragmentation of granules during homogenization and centrifugation. However, amylase-positive structures from β-D xyloside-treated cells exhibited a much broader distribution and were recovered in fractions 12-20 (**Fig. 6**). These data indicate that β-D xyloside treatment of dexamethasone-stimulated AR42J cells results in the formation of secretory organelles with different buoyant densities. This is in agreement with the ultrastructural studies indicating a shift towards smaller granule size in β-D xyloside treated cells (**Fig. 5D**).

We also examined the effect of β-D-xyloside on granule morphology in rat pancreas (**Fig. 7**). To investigate granule formation in the presence of inhibitor only, pancreas was first partially degranulated by feeding rats with a single dose of FOY-305 leading to a hormonally stimulated release of preexisting/stored zymogen granules (Rausch et al. 1987, Schmidt et al. 2001). After degranulation pancreatic lobules were isolated, treated with β-D xyloside and processed for electron microscopy.

Ultrastructural studies of control and treated lobules provided evidence for the intactness and the polarity of the isolated acinar cells. Based on morphological criteria, the junctional complexes at the apical pole, the plasma membrane and membranes of intracellular organelles (Golgi, ER, mitochondria, granules) of the majority of the cells appeared intact after β-D xyloside treatment (compare **Fig. 7A and B**). Interestingly, granules exhibiting a strikingly irregular shape were observed in β-D-xyloside-treated cells, whereas the granules in controls had an overwhelmingly spherical appearance (**Fig. 7**). It has to be noted that the irregular-shaped granules were also found distant from the Golgi complex in different locations of the apical region. It is likely that their formation is caused by β-D-xyloside treatment, and that they do not represent typical immature granules. Furthermore, some of the irregular-shaped granules were increased in diameter about 2-3-fold when compared to normal granules. This increase in size may be due to homotypic granule fusion. In addition, granular structures with an unusually small diameter (0.21 - 0.27 µm) were observed in β-D-xyloside-treated cells (**Fig. 7D, insert, F**). These findings are consistent with the results obtained in AR42J cells indicating that also in pancreatic lobules abnormal granules are formed in the presence of β-D-xyloside.

Treatment with β-D xyloside influences secretion of zymogens in AR42J cells

We next examined whether treatment of AR42J cells with β-D xyloside had an impact on the induction and expression of zymogens induced by dexamethasone-treatment (**Fig. 8A, B**). Quantification of the cellular amount of amylase in controls (+/– dexamethasone) and inhibitor-treated cells verified that the observed effect of β-D xyloside on secretory granules was not due to differences in protein expression. **Fig. 8A, B** shows that addition of dexamethasone induces the expression of amylase in AR42J cells. The increase in the cellular

amount of amylase by dexamethasone-treatment is comparable in controls and β-D xyloside-treated cells.

Dexamethasone not only induces secretory granule biogenesis, the expression and storage of amylase but it also sensitizes AR42J cells for stimulated exocytosis by up-regulation of CCK receptors (Logsdon 1986, Logsdon et al. 1985). To study whether the proteoglycan inhibitor β-D-xyloside had an impact on basal and stimulated secretion in AR42J cells, we determined the release of amylase under different conditions. Controls and cells treated with β-D-xyloside were stimulated for granule fomation with dexamethasone. After 48 hours cells were washed with Krebs-Hepes buffer and incubated with or without CCK-8. Supernatants and cell lysates were collected after 30 min and assayed for amylase activity (**Fig. 8C, D**). Interestingly, we observed a significant increase in the basal (unstimulated) secretion of amylase after treatment with β-D-xyloside (**Fig. 8A**). Stimulation with CCK induced amylase secretion in controls and β-D-xyloside-treated cells, but the ratio of stimulated vs. basal secretion was lower in β-D-xyloside-treated cells (**Fig. 8B**). These results further suggest that after β-D xyloside treatment part of the amylase is not located in typical, CCK-sensitive storage granules of AR42J cells and is constitutively secreted.

Discussion

In this study we combined biochemical and morphological experiments using both AR42J cells and pancreatic tissue, which support the involvement of proteoglycans in the formation of normal-sized and stimuli-sensitive storage granules in acinar cells of the exocrine pancreas. Secretory granule biogenesis is a complex process involving the pH-dependent selective aggregation of regulated secretory proteins within the acidic environment of the TGN (for review see: Tooze et al. 2001; Dikeakos & Reudelhuber 2007; Bonnemaison et al. 2013) and the interaction of the aggregated zymogens with specific membrane domains. These sorting steps allow the separation of regulated secretory proteins from constitutively released cargo and their packaging in a concentrated form within secretory granules. In contrast to the well documented selective aggregation of the complex mixture of zymogens in pancreatic acinar cells (Dartsch et al. 1998, Freedman & Scheele 1993, Leblond et al. 1993), relatively little information is available on the specific interaction of zymogens with the TGN/granule membrane and their ultimate sorting (Dikeakos & Reudelhuber 2007, Gómez-Lázaro et al. 2010, Schrader 2004). However, there is evidence that specific accessory or 'helper' molecules including proteoglycans, sulfated glycoproteins and lectins support exocrine granule formation (de Lisle 2002, Forsberg et al. 1999, Kleene et al. 1999, Leblond et al. 1993). Expression of the sulfated mucin-type glycoprotein pro-Muclin, for example, has been shown to induce granule size in AR42J cells (de Lisle et al. 2005).

A role for proteoglycans in the homeostasis and storage of proteases and amines in secretory granules has been well studied in mast cells (Rönnberg et al. 2012, Wernersson & Pejler 2014). For the exocrine pancreas, however, studies showing the presence of proteoglycans in ZGs have so far been descriptive only. *In vitro* studies suggested that sulfated proteoglycans within ZGs might mediate the sorting and aggregation of cationic zymogens via ionic interactions of their negatively charged sulfate groups (Reggio & Dagorn 1978, Reggio & Palade 1978, Tartakoff et al. 1975). Using the CmB protocol as well as gel-

based analyses, we provided direct morphological and biochemical evidence for the presence of proteoglycans within the ZGs of rat pancreas. Interestingly, the majority of the proteoglycans appears to be associated with the ZG membrane, since CmB-proteoglycan complexes were detected predominantly underneath the granule membrane. This is consistent with biochemical findings, where the majority of sulfate-labeled proteoglycans in pancreatic acinar cells of the rat were found to be associated with ZG membranes and could be released by carbonate treatment (Scheele et al. 1994, Schmidt et al. 2000) (see **Fig. 2**). Furthermore, the lectin ZG16p, which has been shown to interact with proteoglycans (Kumazawa-Inoue et al. 2012), is mainly localised at the ZG membrane and interacts with a large high molecular mass complex liberated from ZG membranes. In contrast to ZG, CmB-proteoglycan complexes in secretory granules of leucocytes were found to be distributed over the whole granule (Unger et al. 1997).

We and other groups have put forward the hypothesis that specific proteoglycans (and associated components) form a submembranous protein scaffold (*submembranous matrix*) at the luminal side of the granule membrane, which functions in membrane targeting of zymogens as well as in granule formation and stability (Borta et al. 2010, Freedman et al. 1998, Kleene et al. 1999, Scheele et al. 1994, Schmidt et al. 2000, 2001; Schrader 2004). Our ultrastructural data further support the existence of such a membrane-associated protein matrix. The striking loss of membrane shape and curvature after removal of proteoglycans and other matrix components by carbonate treatment of isolated ZG membranes implies that the submembranous protein scaffold has an influence on granule shape, structure and stability (**Fig. 9**). This notion is further supported by the appearance of irregular-shaped granules after the inhibition of proteoglycan synthesis by treatment of pancreatic lobules with β-D-xyloside.

Interestingly, proteoglycans were also required for the dexamethasone-induced formation of normal ZGs in AR42J cells. It is likely that proteoglycans contribute to the proper packaging, concentration and sorting of zymogens into secretory granules (**Fig. 9**), since

treatment of AR42J cells with β-D-xyloside reduced the appearance of electron-dense secretory granules and resulted in the formation of granule structures with a smaller size. After density centrifugation those structures exhibited a much broader distribution and thus, different buoyant densities when compared to control cells. However, amylase expression was not reduced in β-D-xyloside-treated cells when compared to controls. These findings are indicative for the formation of smaller secretory organelles and/or a reduction in the amount of cargo in the presence of β-D-xyloside.

In support of this, it has been reported, that mast cells from mice with a defect in heparin synthesis contained smaller granules and large empty vacuoles and failed to store several proteins that are normally bound to heparin in secretory granules (Forsberg et al. 1999, Humphries et al. 1999). A reduction in cargo can be explained by inefficient aggregation, packaging and sorting/membrane targeting of zymogens. However, proteoglycans may display multiple functions during granule formation (**Fig. 9**). The membrane-associated proteoglycans can function not only as a submembranous scaffold for the attachment of zymogen complexes, but also as a mechanical device for membrane deformation at the TGN (Huttner & Zimmerberg 2001), and maintenance of granule size. The impairment of such a mechanical function could also contribute to the formation of smaller secretory organelles instead of normal-sized granules.

Furthermore, the basal secretion of amylase was increased in AR42J cells after β-D-xyloside treatment. This might be indicative for misrouting of secretory proteins to a constitutive pathway and is in line with the morphological observations, e.g. the appearance of abnormal granules and/or non-granular, presumably constitutive transport containers.

It should be noted that regardless of the absence or presence of serglycin proteoglycans, the major constituents in mast cells, granules are of about equal size and are present in approximately equal numbers (Åbrink et al. 2004, Braga et al. 2007, Wernersson & Pejler 2014). Although serglycin is crucial for dense core formation and supports storage of granule

compounds it does apparently not contribute to the biogenesis of mast cell granules in the systems studied. This appears to be in contrast to ZG granules, and might be explained by the predominant membrane-association of proteoglycans in ZGs. The glycosaminoglycan side chains of heparin sulphate proteoglycans from rat pancreatic ZG were recently characterized by Kumazawa-Inoue and colleagues (Kumazawa-Inoue et al. 2012). Furthermore, the digestion of those proteoglycans with heparin lyase II exposed the core proteins having a molecular weight of 66 and 35-40 KDa, but the identification of the core protein was unsuccessful. Nevertheless, serglycin is not supposed to be present in ZG or to contribute to ZG biogenesis in mice (Niemann et al. 2009). On the other hand, several ZG components have been identified which can interact with proteoglycans including the lectin ZG16p, the chymase mast cell protease 1, PpiB (peptidyl-prolyl cis-trans isomerase B), CEL and RNase A. Interestingly, those proteins have been found to be associated with the ZG membrane (Borta et al. 2010, Chen et al. 2008). Thus, proteoglycans might be involved in their sorting/packaging to ZG (**Fig. 9**). In addition, the interaction with proteoglycans is supposed to influence enzyme function, which might be important after secretion (Pejler & Sadler 1999) Proteoglycans have also been found associated with detergent-insoluble cholesterol/glycolipid-enriched complexes of the ZG membrane (Kalus et al. 2002, Schmidt et al. 2001). If they contribute to the clustering of lipid raft components and to the assembly of functional protein complexes (Ikonen 2001), and thus, influence granule fusion, has to be elucidated (**Fig. 9**). In line with this, proteoglycans may have a role in facilitating ZG maturation by regulating the removal of membranes via clathrin-mediated processes. This would be consistent with an increase in granule size observed for some ZG in the exocrine pancreas (**Fig. 7**).

We propose that proteoglycans within ZG are important accessory components in granule biogenesis which can support multiple steps in granule formation (**Fig. 9**): ZG proteoglycans are able to interact electrostatically and through specific protein-protein and carbohydrate-

protein binding domains with the secretory proteins of the granule content (Scheele et al. 1994, Schmidt et al. 2000, Schrader 2004). In addition to a potential mechanical function in granule formation and in the maintenance of granule shape and stability, they contribute to the proper packaging and sorting/membrane binding of zymogens (**Fig. 9**). Additional functions may be the modulation of enzyme function before or after secretion and granule fusion/exocytosis. It will be a challenge for future studies to identify and characterize these proteoglycans, and to elucidate their complex functions and interactions in health and disease.

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Figure legends

Figure 1. Pancreatic zymogen granules possess a submembranous proteoglycan scaffold. Pancreatic tissue (**A-C**) and AR42J cells (**D**, **E**) were stained according to the Cupromeronic Blue (Cmb) protocol and embedded for electron microscopy (see Materials and Methods). (**A**) Control treated with buffer without Cmb. (**B**, **C**) Electron dense proteoglycan complexes were found to form filaments or 'prisms' underneath the granule membrane in acinar cells of rat pancreas (**B**; asterisks) and in AR42J cells (**D**; white arrows). In addition, Cmb staining was detected within the intracellular space (**B**, **D**; black arrows). (**C**) Higher magnification of zymogen granules in acinar cells of rat pancreas after Cmb staining. Arrows point to proteoglycan complexes. (**E**) AR42J control treated with buffer without Cmb. Note that in controls no staining is detectable underneath the granule membrane. Bars, 1 µm.

Figure 2. Membrane-associated proteoglycans and granule matrix components influence membrane curvature. (**A**) Zymogen granules (ZG) were isolated from rat pancreas, and granule subfractions were prepared as described in Materials and Methods. Lysed granules were separated into a content (ZGC) and membrane fraction (ZGM). In addition, isolated membranes were treated with carbonate and separated into a pellet (P, **treated membranes**) and supernatant (Sup, **carbonate-extract**) fraction. Equivalent amounts were analysed by SDS-PAGE and immunoblotting, using antibodies against carboxypeptidase A (carb), amylase (amyl), ZG16p and GP2. (**B**) The amount of proteoglycans (PG) in the supernatant (Sup) and pellet (P) fractions obtained after carbonate treatment of ZGM was determined with the Blyscan assay. The results are expressed as percent of total proteoglycans (supernatant + pellet fraction). (**C**) Immunoblotting of ZG membranes (ZGM) using anti-ZG16p antibody. Protein samples were heated at 95ºC or incubated at room temperature (RT). Note the accumulation of ZG16p in the stacking gel (dotted line). (**D-G**) Ultrastructure of isolated ZG membranes. Isolated membranes (ZGM) were either left untreated (**D, F**) or incubated with

carbonate (**E, G**) to remove granule matrix components, and afterwards processed for electron microscopy. (**F, G**) Immunogold electron microscopy of isolated membranes incubated with a polyclonal anti-matrix antibody and visualized using 10-nm protein A-gold. Note the overwhelmingly curved (arrowheads) or curled membranes (arrows) in (**D, F**), in contrast to their linear appearance in (**E, G**), and the reduced number of gold particles at the membranes in (**G**). Con, control. Bars, 1 µm.

Figure 3. Detection of glycoconjugates in zymogen granule subfractions. Zymogen granules were isolated from rat pancreas, and membrane (ZGM) and content fractions (ZGC) were prepared. Isolated ZGM and ZGC were treated with carbonate and the supernatants (carb) collected after centrifugation. Samples were afterwards separated on 1% agarose gels and stained with toluidine blue. 1 mg (ZGC; ZGCcarb) and 500 µg (ZGMcarb) of protein were applied per lane. Chondroitin sulfate and biglycan were used as standards.

Figure 4. Inhibition of proteoglycan synthesis perturbs granule formation in AR42J cells. AR42J cells were cultured in the absence (**A**) or presence of dexamethasone (**B, C**). Cells in (**C**) were also treated with 2 mM β-D-xyloside (b-D-xyl). Images show immunostaining with an antibody directed against carboxypeptidase A. Note the presence of typical punctate granules in (**B**), in contrast to their absence in (**A**) and (**C**). Arrows in (**A**) and (**C**) point to cells exhibiting an unusual fine punctate staining pattern. Higher magnification images of boxed regions are shown in (**A**) and (**C**). Bars, 10 µm. (**D**) Quantitative analysis of granular staining after the different treatments (see Materials and Methods). The data are from 4-6 independent experiments and are expressed as means ± S.D. (* $p<0.01$ when compared to dexamethasone-stimulated controls (Con)). (+) with or (-) without dexamethasone (dexa).

Figure 5. Presence of secretory granules in AR42J cells after treatment with β-D-xyloside and dexamethasone. AR42J cells were cultured under the experimental conditions described in **Fig. 4** and processed for electron microscopy (see Materials and Methods). Note the presence of electron-opaque secretory granules in (**A**, arrows, mean granule size 125.8 nm, n = 93), in contrast to their absence in (**B**). After treatment with β-D-xyloside (xyl) small granules can be seen clustered around the Golgi region (C, mean granule size 72.6 nm, n = 107). (**D**) Granule size distribution. Granules were sampled using an unbiased scanning band and sizes estimated through counting line intersections of granule profiles with a systematically spaced graticule. G, Golgi apparatus; (+) with or (-) without dexamethasone (dexa). Bars, 500 nm in overview, 200 nm in enlargement.

Figure 6. Secretory vesicles isolated from β-D-xyloside-treated AR42J cells and controls show different buoyant densities. Dexamethasone-stimulated AR42J cells were either treated with β-D-xyloside (xyl) or left untreated (control). After 48 hours cells were homogenized, and the postnuclear supernatant was subjected to density gradient centrifugation. Fractions were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting. The presence of amylase in the gradients is shown as a densitometric diagram measured from the immunoblots.

Figure 7. Fine structure of pancreatic lobules after treatment with β-D-xyloside. Pancreatic lobules were incubated in the absence (**A, C**) or presence (**B, D**) of β-D-xyloside (b-D-xyl). After 3 hours lobules were fixed, embedded in Epon and processed for electron microscopy as described in Materials and Methods. (**A, C**) Control lobules (Con). Note the presence of junctional complexes (arrows) and the apical localization of zymogen granules. (**B, D**) After β-D-xyloside treatment individual Golgi cisternae had a dilated appearance.

Irregular-shaped zymogen granules (asterisks) as well as unusually small granules (**D**, insert) were frequently observed. (**E, F**) Quantification of irregular-shaped granules (**E**) and unusually small granules (0.21 - 0.27 µm in diameter) (**F**) in controls and after β-D-xyloside treatment. Data are expressed as means ± S.D. (* $p<0.01$ when compared to controls (Con), n= 1011-1251 from 95-107 cells). G, Golgi apparatus; L, acinar lumen. Bars, 5 µm (**A, B**), 1 µm (**C, D**).

Figure 8. Effect of β-D-xyloside on amylase secretion in AR42J cells. (**A**) Expression of amylase in AR42J cells. Cells were treated with 2 mM β-D-xyloside (bDXyl) or DMSO (Con) and cultured in the absence (-) or presence (+) of dexamethasone to induce granule formation. After 48h, cells were lysed and equal amounts of protein were separated by SDS-PAGE and immunoblotted using antibodies directed to amylase and tubulin. (**B**) Densitometric quantification of immunoblots in (**C**) of total amylase normalized to tubulin. (**C, D**) Effect of β-D-xyloside on amylase release. Cells were washed with Krebs-Hepes buffer (KHB) and incubated with (CCK) or without CCK-8 (KHB) for 30 min. Supernatants and cell lysates were collected and assayed for amylase activity. Values for amylase release in (**C**) are expressed as percentage of the total cellular amylase. Note the increased basal secretion of amylase in β-D-xyloside treated cells (**C**) and the lower ratio of stimulated vs. basal secretion (**D**). Fold-stimulation was calculated as the secreted amount in the presence of CCK-8 divided by the secreted amount in the absence of CCK-8. Data are presented as means ± S.D. from 3 independent experiments.

Figure 9. Potential functions of proteoglycans during zymogen granule biogenesis and secretion. During the generation of immature ZG at the TGN, a proteoglycan scaffold at the TGN/granule membrane can support granule formation by promoting membrane curvature

and can aid in the sorting and packaging of zymogens (①). Proteoglycans are supposed to interact with membrane components, e.g. GP2, a major GPI-anchored glycoprotein of ZG and are associated with lipid microdomains of the granule membrane. Proteoglycans can contribute to dense core formation and maturation of granules (②). The lectin ZG16p can potentially bridge interactions between proteoglycans and cargo proteins. During exocytosis, the proteoglycan-cargo meshwork is released into the acinar lumen and the pancreatic duct system (③). During this process, certain cargo molecules may detach (due to changes in pH) whereas others remain associated. This can have an impact on enzyme function (④). Certain complexes may bind to the surface of duct cells or enterocytes after secretion. CV, condensing vacuole; ISG, immature secretory granule; ZG, zymogen granule; ZGM, ZG membrane; ZGC, ZG content; ZGM_{carb}, carbonate-treated ZG membranes.

Supplementary Material

Supplementary Figure S1. Inhibition of proteoglycan synthesis in AR42J cells. Dexamethasone-stimulated AR42J cells were treated with different concentrations of β-D-xyloside (b-D-xyl) (0-2.5 mM) for 48 hours, and the relative amounts of precipitable proteoglycans in cell homogenates were analyzed using the Blyscan assay. The data are from 4 independent experiments and are expressed as means ± S.D.

Supplementary Figure S2. Co-localisation of carboxypeptidase A and the Golgi marker p115 in unstimulated AR42J cells. Unstimulated AR42J cells (- dexa) were processed for immunofluorescence and stained with antibodies directed against the Golgi marker protein p115 (**A**) and the granule enzyme carboxypeptidase A (CBP A) (**B**). Note the co-localisation of both proteins at the Golgi complex (arrows) and the presence of fine punctate structures

positive for carboxypeptidase A, which may represent constitutive cargo containers (**B**). Bar, 10 µm.