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Interaction of syncollin with GP-2, the major membrane protein of pancreatic zymogen granules, and association with lipid microdomains

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Syncollin, a novel pancreatic zymogen granule protein, is present on the luminal side of the granule membrane. To address the function of syncollin, we searched for putative binding partners. Cross-linking experiments with purified syncollin, and granule content and membrane proteins revealed a direct interaction between syncollin and GP-2, a major glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein. An interaction was also observed when cross-linking was performed with recombinant GP-2. In addition, syncollin could be cross-linked to itself, supporting the suggestion that it exists as a homo-oligomer. Cleavage of the GPI anchor of GP-2 by treatment of granule membranes with phosphatidylinositol-specific phospholipase C had no effect on the membrane attachment of syncollin, indicating that it is not mediated exclusively via an interaction with GP-2. Syncollin was found to be associated with detergent-insoluble cholesterol/glycolipid-enriched complexes. These complexes floated to the lighter fractions of sucrose-density gradients and also contained GP-2, the lectin ZG16p, sulphated matrix proteoglycans and the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) syntaxin 3 and synaptobrevin 2. Our results indicate that membrane-associated syncollin is a component of lipid rafts, where it interacts both with GP-2 and membrane lipids. We suggest that the syncollin–GP-2 complex might play a role in signal transduction across the granule membrane.

Key words: apical sorting, cholesterol raft, GPI anchor.

INTRODUCTION

In endocrine and exocrine cells, secretory proteins are packaged and stored in secretory granules, and are subsequently released in response to external stimuli. Granule formation and apical sorting of digestive enzymes in pancreatic acinar cells involves the selective aggregation of these secretory proteins, and the association of the aggregates with specific membrane domains of the *trans*-Golgi network. These domains then pinch off as condensing vacuoles (reviewed in [1,2]). The selective aggregation of pancreatic secretory proteins has been well documented [3–5], but little information is available about the specific interaction of the aggregates with the *trans*-Golgi-network membrane and their sorting into condensing vacuoles. The zymogen granule (ZG) glycoprotein GP-2, which is linked to the luminal surface via a glycosylphosphatidylinositol (GPI) anchor [6–8], was the first candidate postulated to mediate this sorting event [9,10]. However, it has been demonstrated that during embryonic development, and in partially differentiated acinar carcinoma cell lines, granule formation can occur in the complete absence of GP-2 [11–13]. These findings call into question its role as a specific sortase, but do not exclude a possible function in granule formation and the packaging of secretory proteins, at least in the adult exocrine pancreas. Further observations [10] led to the proposal that GP-2, together with attached proteoglycans, forms a network at the luminal side of the ZG membrane (ZGM). We have recently identified and characterized proteoglycan and glycoprotein components of this submembranous matrix [14], and have evidence that it functions in the binding of aggregated enzyme proteins to membranes, a process that has been termed condensation sorting [15]. Furthermore, we have demonstrated that GP-2, the secretory lectin ZG16p and sulphated proteoglycans are associated with cholesterol-glycosphingolipid-enriched lipid microdomains, so-called rafts, isolated from membranes of ZGs [16]. These lipid microdomains are required for granule formation and regulated secretion of proteins.

There is now considerable interest in identifying components of the molecular machinery required to form secretory granules and in characterizing their interactions. Recently, a new ZG protein, syncollin, was identified, which in acinar cells is found tightly attached to the luminal surface of the ZGM [17,18]. It is resistant to salt-washing of the granule membranes, but is removed by sodium carbonate. Furthermore, the levels of syncollin are modulated in response to changes in feeding behaviour [19], suggesting that it is involved in the secretion of digestive enzymes, although its precise role is still unclear.

In this study, we present morphological data that confirm our previous finding that besides its membrane-associated form, syncollin is also present free in the granule content. Using a specific, trifunctional cross-linker, we demonstrate that syncollin interacts both with itself and with GP-2. Experiments with phosphatidylinositol-specific phospholipase C (PI-PLC) indicate that the membrane association of syncollin is not mediated exclusively by GP-2. Furthermore, we show that syncollin is present in lipid microdomains of the granule membrane, together with GP-2 and other membrane-associated components. We

Abbreviations used: GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; TX-100, Triton X-100; ZG, zymogen granule; ZGC, ZG-content protein; ZGM, ZG membrane; Ni-NTA, Ni^{2+}-nitrilotriacetate; sulpho-SBED, sulphosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)hexanoamido]ethyl-1,3′-dithiopropionate.

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suggest that the syncollin–GP-2 complex might play a role in signal transduction across the granule membrane.

MATERIALS AND METHODS

Antibodies

Antibodies used were as follows: 87.1, a mouse monoclonal antibody against recombinant syncollin [18]; anti-syncollin B, a rabbit polyclonal antibody raised against recombinant syncollin [20]; 69.1, a mouse monoclonal antibody against synaptobrevin 2 [21]; anti-His tag, a mouse monoclonal antibody obtained from Qiagen (Hilden, Germany); and rabbit polyclonal antibodies against rat GP-2, pig amylase [12], carboxypeptidase A (Rockland Immunochemicals, Gilbertsville, PA, U.S.A.), recombinant ZG16p [22] and syntaxin 3 [23]. Species-specific anti-IgG antibodies conjugated to horseradish peroxidase or alkaline phosphatase were obtained from Bio-Rad (Richmond, CA, U.S.A.) or Sigma Immunochemicals (Munich, Germany).

Gel electrophoresis and immunoblotting

Protein samples were separated by SDS/PAGE according to Laemmli [24], transferred to nitrocellulose (Schleicher and Schüll, Dassel, Germany) using a semi-dry apparatus and analysed by immunoblotting. Immunoblots were processed using either horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Bioscience, Arlington Heights, IL, U.S.A.) or alkaline phosphatase-conjugated secondary antibodies and BCIP (X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate)/NBT (4-Nitro Blue Tetrazolium chloride; Boehringer Mannheim, Mannheim, Germany). Silver staining of gels was performed according to Hempelmann and Kaminsky [25]. For quantification, immunoblots were scanned and processed using Pcbas software.

Isolation of ZGs

ZGs were isolated as described previously [5] from the pancreas of male Wistar rats (200–230 g; Charles River, Sulzfeld, Germany) that had been fasted overnight. The following buffer was used for homogenization: 0.25 M sucrose, 5 mM 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) and 0.1 mM PMSF. Granules were resuspended in 50 mM Hepes, pH 8.0, and lysed by freezing and thawing. The ZGM was separated from the soluble ZG-content proteins (ZGCs) by ultracentrifugation (100000 ***g*** for 30 min), resuspended in 50 mM Hepes, pH 8.0, and stored at −20 °C. For carbonate treatment, ZGM were incubated with the same volume of 300 mM Na_2CO_3, pH 11.5, on ice for 2 h and re-isolated by centrifugation at 100000 ***g*** for 30 min. For PI-PLC treatment of ZGM, 500 m-units of PI-PLC (Boehringer Mannheim) were added to ZGM (corresponding to 200–500 μg of protein), and the samples were incubated at 37 °C for 1 h in a final volume of 500 μl. Membranes and supernatant were separated by centrifugation at 21000 ***g*** for 15 min. Proteins were precipitated by methanol/chloroform and equal amounts analysed by SDS/PAGE and immunoblotting. For quantification, immunoblots were scanned and processed using Pcbas software.

Purification of syncollin

Syncollin was purified as described in [18]. Briefly, freshly isolated ZGMs from three to five rats were washed with 0.6 M KI for 30 min at 4 °C with gentle agitation. The membranes were then recovered by centrifugation at 21000 ***g*** for 20 min and washed with 0.1 M Na_2CO_3, again for 30 min at 4 °C. The membranes were pelleted by centrifugation at 21000 ***g*** for 30 min. The supernatant was recovered and dialysed overnight against 50 mM Hepes, pH 7.6. The resulting precipitate was collected by centrifugation at 21000 ***g*** for 5 min. The pellet was either dissolved in 50–100 μl of 0.5 % taurodeoxycholate in 50 mM Hepes, pH 7.6, or resuspended in 50 mM Hepes, pH 8.0.

Detergent extraction and sucrose gradients

Membranes (500 μg) were first carbonate-treated at pH 10.0 to reduce the amount of membrane-associated matrix components, which were found to interfere with flotation. Washed membranes were incubated on ice for 30 min in 50 mM Hepes, pH 8.0, containing Lubrol 17A17 (0.5 %, v/v; Serva, Heidelberg, Germany), Triton X-100 (TX-100; 0.5 %, v/v; Serva) or octylglucoside (60 mM; Boehringer Mannheim), and then centrifuged at 120000 ***g*** for 30 min. The detergent-insoluble pellet fraction was resuspended in 50 mM Hepes, pH 8.0, and adjusted to 1.2 M sucrose, containing the appropriate concentration of detergent. The pellet fraction (500 μl) was overlayered with 1 ml of 1.1 M sucrose and 1 ml of 0.15 M sucrose, again containing detergent. After centrifugation for 1 h at 120000 ***g*** in a swing-out rotor (TLA 120.1; Beckman Instruments, Munich, Germany), fractions (220 μl) were collected from the top of the gradient. Similar results were obtained with 1 % concentrations of the detergents. For saponin treatment, the Lubrol-insoluble membrane pellet was resuspended in 0.25 % Lubrol and 0.25 % saponin. Proteins were precipitated by methanol [26] and analysed by SDS/PAGE. Lipids were extracted by Bligh–Dyer two-phase extraction [27] and analysed by TLC.

For continuous sucrose gradients the detergent-insoluble pellet fraction was resuspended in 50 mM Hepes, pH 8.0, and adjusted to 40 % sucrose, containing the appropriate detergent at 0.5 %. The pellet fraction (500 μl) was then overlayered with 4 ml of a continuous sucrose gradient (30–5 %), centrifuged for 1 h at 120000 ***g*** in a swing-out rotor (SW 50.1) and processed as described above.

Production of recombinant GP-2

The following primer sequences were used to amplify the coding sequence of rat GP-2 (amino acids 20–515) from a rat pancreas cDNA library (Clontech, Heidelberg, Germany) by PCR: 5′-ATTGGATCCTCCATCTACAACACATCAAGGTTATG-3′ (forward primer GP-2.up) and 5′-TATGAGCTCCTACAGG-AACCCTGTATTGCGG-3′ (reverse primer GP-2.down). Using the restriction sites for *Bam*HI and *Sac*I at the ends of the PCR products (underlined; *Bam*HI in GP-2.up and *Sac*I in GP-2.down), the cDNA was cloned in frame with a 6 × His tag into the pQE vector (Qiagen). Induction of expression in *Escherichia coli* M15 (Stratagene, La Jolla, CA, U.S.A.) and purification of recombinant proteins under denaturing conditions was according to the manufacturer's instructions using Ni^{2+}-nitrilotriacetate (Ni-NTA) affinity chromatography. Recombinant GP-2 was renatured in 50 mM Hepes, pH 7.6, concentrated in an ultrafiltration cell (Millipore, Eschborn, Germany) and used for cross-linking experiments.

Cross-linking experiments

All protein solutions were desalted before use. Protein (1 mg) was incubated with 0.6 mg of the trifunctional cross-linker sulpho-SBED {sulphosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)hexanoamido]ethyl-1,3′-dithiopropionate; Pierce, Rockford, IL, U.S.A.} at room temperature for 30 min in the

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dark, in order to couple the cross-linker to the protein via the *N*-hydroxysuccinimide ester group. To remove unbound cross-linker, the reaction mixture was applied to a desalting column (PD-10; Amersham Bioscience, Freiburg, Germany) or dialysed. Recombinant GP-2 or isolated syncollin (100 μg) carrying the cross-linker was then added to ZGC (1 mg of protein), to ZGM (100 μg), to ZGM/ZGC or to the supernatant fraction (200 μg) of carbonate-treated ZGM, in a volume of 400 μl of 50 mM Hepes, pH 8.0. After incubation for 30 min in the dark, the samples were split and the pH was shifted to either pH 7.5 or 5.9 by addition of an equal volume of 0.1 M Mes, pH 6.75 or 4.7 respectively. After incubation at room temperature for 2 h the samples were exposed to UV light (265 nm) for 15 min to cross-link interacting proteins with the photoactivated aryl acid group of the cross-linker. Samples were centrifuged at 13000 ***g*** for 30 min and supernatant and pellet fractions were analysed separately. In some cases enrichment of biotinylated proteins was achieved by the use of streptavidin-coated magnetic beads (Dynal, Hamburg, Germany).

For analysis, samples were subjected to SDS/PAGE under reducing conditions, which causes cleavage of the disulphide bridge within the cross-linker, leaving the biotin moiety of the sulpho-SBED coupled to the binding partner. For detection of the biotin the proteins were transferred to nitrocellulose or PVDF membranes, incubated with NeutrAvidin (Pierce) according to the manufacturer's instructions and processed using SuperSignal Ultra (Pierce).

Electron microscopy

Pancreatic tissue, isolated ZGs and ZGM fractions were fixed in 0.1 % cacodylate buffer, pH 7.3, containing 1 % glutaraldehyde or 4 % paraformaldehyde (Serva). The samples were dehydrated in a graded series of alcohol, embedded in Lowicryl K4M (Polysciences, Eppenheim, Germany) and polymerized at −20 °C under UV light (360 nm) for 48 h. Thin sections (70 nm) were incubated with the polyclonal anti-syncollin B antibody at a dilution of 1:200–500, and visualized using 10 nm Protein A–gold solution (provided kindly by Dr J. Slot, University of Utrecht, Utrecht, The Netherlands) at a dilution of 1:60 or 1:70, both in PBS containing 0.5 % (w/v) BSA. Sections were stained with uranyl acetate/lead citrate and analysed using a Zeiss EM 9S electron microscope.

Statistical analysis of data

Significant differences between experimental groups were detected by ANOVA for unpaired variables, using Microsoft Excel. Data are presented as means ± S.D., with an unpaired Student's *t* test used to determine statistical differences. P values of < 0.05 were considered significant, and P values of < 0.01 were considered highly significant.

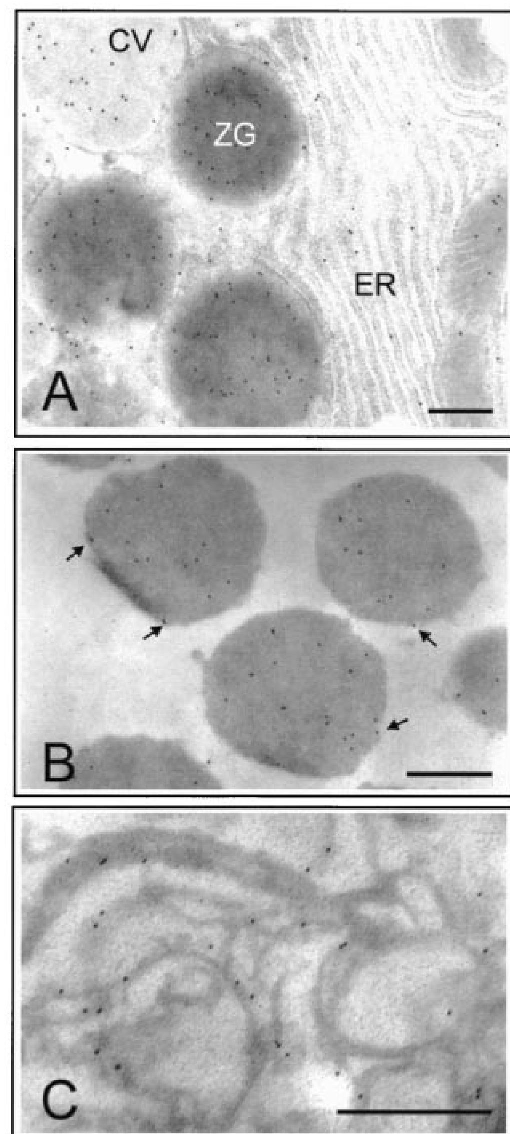

Figure 1 Syncollin is present both free in the ZG lumen and attached to the granule membrane

Pancreatic tissue (**A**), isolated ZGs (**B**) or a membrane fraction of ZGs (**C**) were processed for immunogold electron microscopy, incubated with the polyclonal anti-syncollin B antibody and visualized using 10 nm Protein A–gold. Arrows indicate gold particles at or close to the membrane. CV, condensing vacuole; ER, endoplasmic reticulum. Scale bars, 0.5 μm.

RESULTS

Syncollin is present both free in the ZG content and attached to the granule membrane

In previous biochemical studies [18,28] it has been demonstrated that syncollin is a peripheral protein of ZGMs that resists high-salt washing, but which is removed by treatment of the membranes with Na_2CO_3 at pH 11.0. Furthermore, by immunoblotting, a population of syncollin was found in the granule content [28]. We now present morphological data supporting these previous findings (Figure 1). When pancreatic tissue or isolated ZGs were embedded for immunoelectron microscopy and incubated with an antibody against syncollin, a clear labelling over the whole of the ZGs and a sparser labelling of the membranes was visible (Figures 1A and 1B). To confirm the membrane association of syncollin, immunoelectron microscopy was performed with a membrane fraction isolated from lysed ZGs (Figure 1C). In these preparations, gold labelling was found predominantly on the membranes. When both isolated ZGMs and ZGCs were incubated at pH 5.9 under conditions that allow aggregation of zymogens (condensation) and binding to the membranes (sorting) [5], gold labelling was also found on the surface of the dense-core aggregates, which form at pH 5.9 (results not shown). However, only a small number of gold particles were found inside the aggregates, indicating that free syncollin is not likely to be involved in the aggregation of zymogens.

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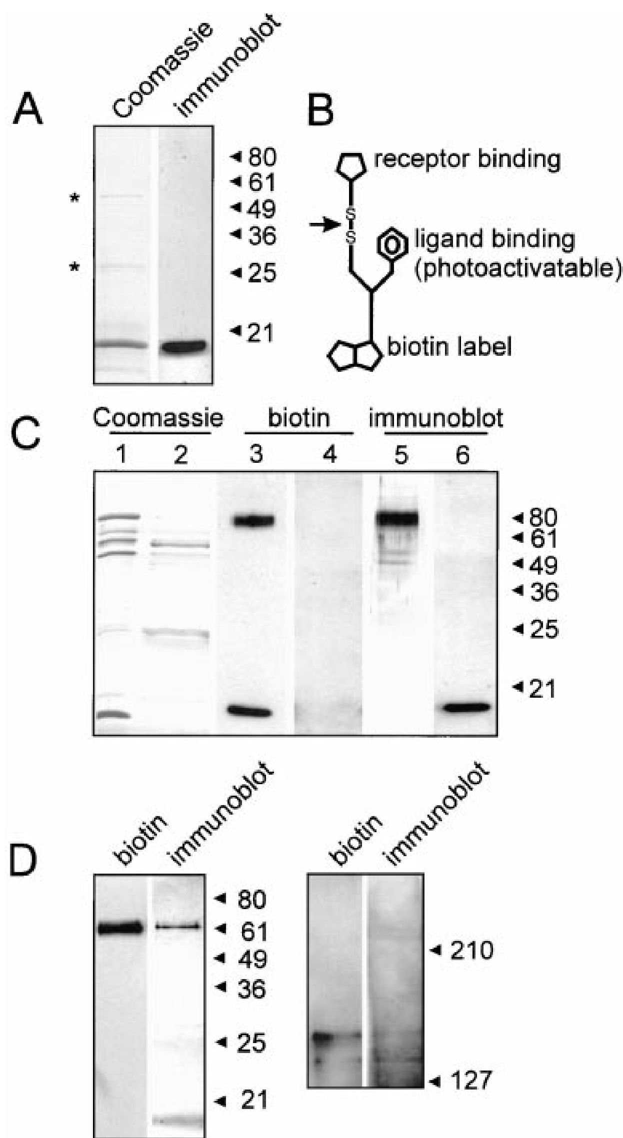

Figure 2 Cross-linking of syncollin to proteins in the ZGM

Syncollin was isolated from the carbonate wash of ZGMs (**A**). The purity of the sample was analysed by Coomassie Brilliant Blue staining and by immunoblotting using the monoclonal anti-syncollin antibody 87.1. Minor protein contaminants are marked with asterisks. (**B**) Schematic of the trifunctional cross-linker sulpho-SBED. The disulphide bond that is cleaved under reducing conditions is marked with an arrow. (**C**) The cross-linker shown in (**B**) was coupled to isolated syncollin and incubated with isolated ZGM (see also Table 1) at a pH of 5.9. The mixture was separated by centrifugation into pellet (lanes 1, 3, 5 and 6) and supernatant fractions (lanes 2 and 4). Both fractions were run on 12.5% acrylamide gels and either stained with Coomassie Brilliant Blue (lanes 1 and 2) or blotted on to nitrocellulose membranes (lanes 3–6). Biotinylated proteins (biotin) were detected by ECL using streptavidin-peroxidase. Afterwards the blots were incubated with antibodies against GP-2 (lane 5) or syncollin (87.1; lane 6) and alkaline phosphatase-conjugated secondary antibodies. The same membrane was used for lanes 3 and 5. (**D**) Pellet fractions were separated on 12.5% (left-hand panel) and 5% (right-hand panel) acrylamide gels under non-reducing conditions and blotted on to nitrocellulose. Biotinylated proteins (biotin) and syncollin (left-hand panel) as well as GP-2 (right-hand panel) were detected as described in (**C**). The positions of molecular-mass markers (in kDa) are indicated on the right.

Cross-linking reveals an interaction of syncollin with GP-2, a major membrane protein of ZGs, and also a homotypic interaction

To identify the binding partner(s) of syncollin, cross-linking experiments were performed with isolated syncollin carrying the trifunctional cross-linker sulpho-SBED. Native syncollin was isolated from the supernatant of sodium carbonate-treated ZGM [18]. The preparation was at least 95% pure, as judged by Coomassie Brilliant Blue staining (Figure 2A). Solubilized syncollin isolated by this procedure appears to exist as a homo-oligomer [18]. Purified syncollin was coupled to sulpho-SBED (Figure 2B) and incubated with ZGCs or ZGM (Figure 2C), or a mixture of both (Table 1) at pH values of 7.5 or 5.9, to mimic either the conditions found in the pre-Golgi compartments or the slightly acidic milieu of the *trans*-Golgi network. As mentioned above, exposure to a pH of 5.9 triggers the selective aggregation of zymogens *in vitro*, leading to the formation of dense cores [5]. Under reducing conditions, the disulphide bridge of the cross-linker is cleaved (Figure 2B), and the biotin moiety remains attached to interacting proteins. The biotinylated proteins were then detected by ECL, using streptavidin-conjugated peroxidase (NeutrAvidin).

Table 1 Summary of results of cross-linking experiments

Isolated syncollin (a) or recombinant GP-2 (b) was coupled to the trifunctional cross-linker sulpho-SBED and incubated with ZGCs, ZGM, a mixture of both (ZGM/ZGC) or the supernatant fraction of carbonate-treated ZGM. The pH was either 7.5 or 5.9. After cross-linking, samples were centrifuged at 13000 ***g*** for 30 min, and supernatant (Sup) and pellet (P) fractions were subjected to SDS/PAGE under reducing conditions. This leads to cleavage of the disulphide bridge within the cross-linker, leaving the biotin moiety of the sulpho-SBED coupled to the binding partner. Proteins were transferred to nitrocellulose membranes and biotinylated proteins were detected by ECL, using streptavidin-conjugated peroxidase. The presence (+) or absence (−) of specific biotinylated proteins under the different experimental conditions is indicated. Unless indicated, similar results were obtained at pH 5.9 and 7.5: the results shown are for pH 5.9, and + in parentheses indicates that the protein was present at pH 7.5.

(a)

	Biotinylated protein					
	ZGM		ZGC		ZGM/ZGC	
	P	Sup	P	Sup	P	Sup
Syncollin	+	−(+)	+	−(+)	+	−
GP-2	+	−	−	−	+	−

(b)

	Biotinylated protein							
	ZGM		ZGC		ZGM/ZGC		Carbonate-wash ZGM	
	P	Sup	P	Sup	P	Sup	P	Sup
Syncollin	+	−	−	−	+	−	+	−
23 kDa	−	+	−	+	−	+	−	+
12 kDa	−	+	−	+	−	+	−	+

Cross-linking in the presence of ZGM proteins revealed predominantly two biotinylated protein bands with molecular masses of 16 and 75–80 kDa in the pellet fraction (Figure 2C, lanes 3 and 4). After immunoblotting, the biotinylated 16 kDa protein could be labelled with an antibody to syncollin, indicating that the isolated protein was self-interacting (Figure 2C, lane 6). The biotinylated 75–80 kDa protein, which was only found when ZGM were used in the assay (Table 1), could be labelled with an antibody to GP-2 (Figure 2C, lane 5). In all cases, the cross-linking reaction appeared to be pH-independent (Table 1). Biotinylation of GP-2, but not of syncollin, could be reduced or inhibited by the addition of syncollin lacking the cross-linker (results not shown), supporting the specificity of the interaction between syncollin and GP-2. When the cross-linking experiments were performed with BSA, which is known to bind to lipids, no

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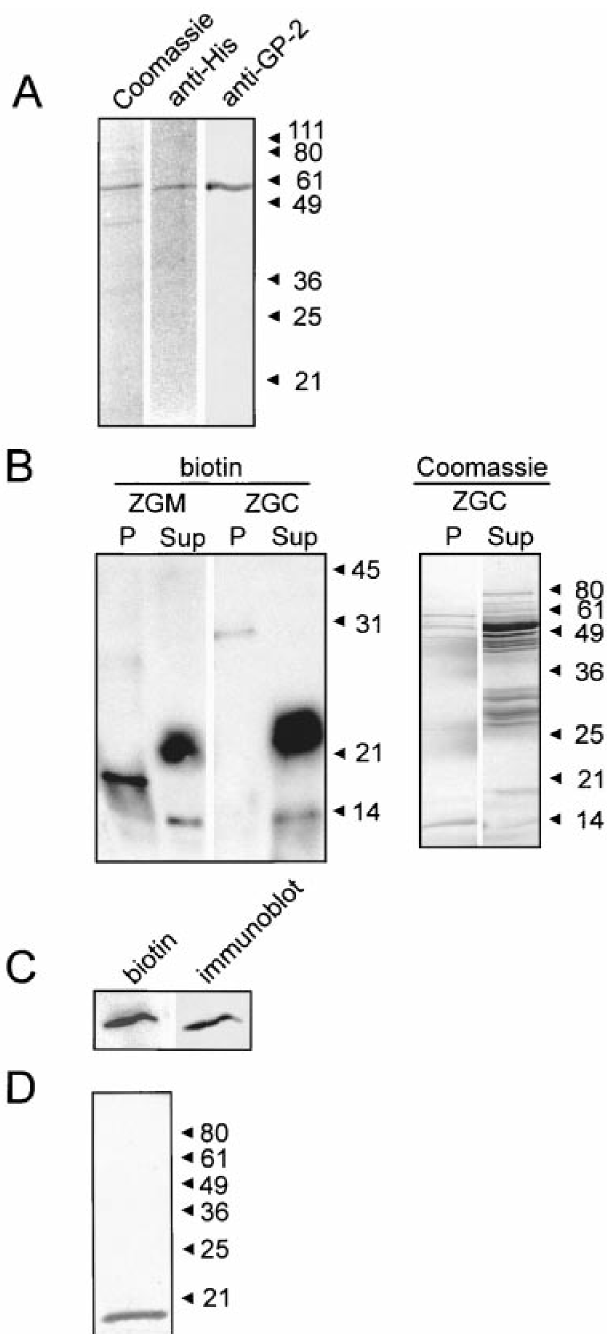

Figure 3 Cross-linking of GP-2 to proteins in the ZGM and ZG contents

(**A**) Recombinant His-tagged GP-2 was purified by Ni-NTA affinity chromatography and the eluate separated by SDS/PAGE on 12.5% acrylamide gels. The sample was analysed by Coomassie Brilliant Blue staining or by immunoblotting using anti-His or anti-GP-2 antibodies. (**B**) Recombinant GP-2 was coupled to the trifunctional cross-linker sulpho-SBED and incubated with isolated ZGM or ZGCs at pH 5.9 (see also Table 1). The samples were separated by centrifugation into pellet (P) and supernatant (Sup) fractions, subjected to SDS/PAGE (15% acrylamide) and transferred to nitrocellulose membranes. Biotinylated proteins (biotin) were detected by ECL using streptavidin-peroxidase. Coomassie Brilliant Blue staining of the pellet and supernatant fractions of ZGC is shown on the right. (**C**) After cross-linking, the pellet fraction of ZGM was separated by SDS/PAGE, and biotinylated proteins were detected as described above. The same blot was afterwards incubated with anti-syncollin antibody 87.1 and an alkaline phosphatase-conjugated secondary antibody. (**D**) Recombinant GP-2 carrying the cross-linker sulpho-SBED was incubated with isolated syncollin at pH 5.9. After centrifugation, the pellet fraction was separated by SDS/PAGE, transferred to nitrocellulose and biotinylated proteins were detected as described above. The positions of molecular-mass markers (in kDa) are indicated on the right.

labelling of GP-2 or other membrane and content proteins was observed (results not shown). Furthermore, we have shown previously that similar cross-linking experiments with the granule content proteins amylase, trypsinogen or ZG29p result in the labelling of a distinct set of proteins [29].

When the protein separation is performed under non-reducing conditions, the disulphide bridge of the cross-linker is not cleaved (Figure 2B) and the interacting proteins remain attached to each other. Under these conditions the biotinylated protein bands were shifted to higher molecular masses (Figure 2D). On 12.5% acrylamide gels a biotinylated protein band of about 60 kDa was visible, which after immunoblotting was recognized by an antibody to syncollin (Figure 2D, left-hand panel). On 5% acrylamide gels two additional biotinylated protein bands with molecular masses of 135–165 kDa were detected, which were recognized by antibodies to GP-2 (Figure 2D) and syncollin (results not shown). The 135 kDa band might represent a complex of GP-2 and a syncollin tetramer. It should be stressed that the detection of the high-molecular-mass complexes under non-reducing conditions was extremely difficult, presumably because of inefficient transfer to the membrane and reduced antibody binding. However, these observations further support the suggestion that syncollin is self-interacting and forms oligomeric structures [28].

In order to verify the putative interaction of syncollin with GP-2, we produced His-tagged recombinant GP-2 and purified it by Ni-NTA affinity chromatography (Figure 3A). After immunoblotting, the purified protein, which was neither glycosylated nor GPI-anchored, was recognized by both anti-His and anti-GP-2 antibodies. Purified GP-2 was coupled to the trifunctional cross-linker sulpho-SBED and incubated with either ZGCs, ZGM, a mixture of both ZGCs and ZGM, or with the carbonate wash of ZGM (Figure 3B and Table 1) at pH values of 7.5 or 5.9. After centrifugation at 13000 ***g***, the biotinylated proteins of the supernatant and pellet fractions were separated by SDS/PAGE, transferred to nitrocellulose membranes and incubated with streptavidin-conjugated peroxidase (NeutrAvidin). Cross-linking with recombinant GP-2 coupled to sulpho-SBED revealed three putative binding partners of GP-2 in the low-molecular-mass range: a biotinylated protein of about 12–13 kDa, which was present in all supernatant fractions (biotinylation seemed to be more pronounced at pH 7.5 than at pH 5.9), a prominent biotinylated protein of about 22–24 kDa, which was present in all supernatant fractions independently of pH, and a biotinylated protein of about 16 kDa that was only found in the pellet of ZGM-containing fractions, again independently of pH. This protein seemed to be membrane-associated, and after immunoblotting it could be labelled with an antibody to syncollin (Figure 3C). The soluble biotinylated proteins were not identified. They might be components of the submembranous granule matrix [14], or content proteins that are loosely attached to the matrix or the membrane. When recombinant GP-2 coupled to sulpho-SBED was incubated with purified syncollin in the cross-linking assay, syncollin was again found to be biotinylated (Figure 3D). Taken together, these data indicate that GP-2 interacts directly with syncollin.

The membrane attachment of syncollin is not exclusively mediated by GP-2

To investigate whether the membrane attachment of syncollin was mediated via its interaction with the GPI-anchored GP-2, isolated ZGMs were treated with PI-PLC, which has been shown to cleave the GPI anchor [8]. After PI-PLC treatment, membranes were re-isolated by centrifugation and the pellet and supernatant

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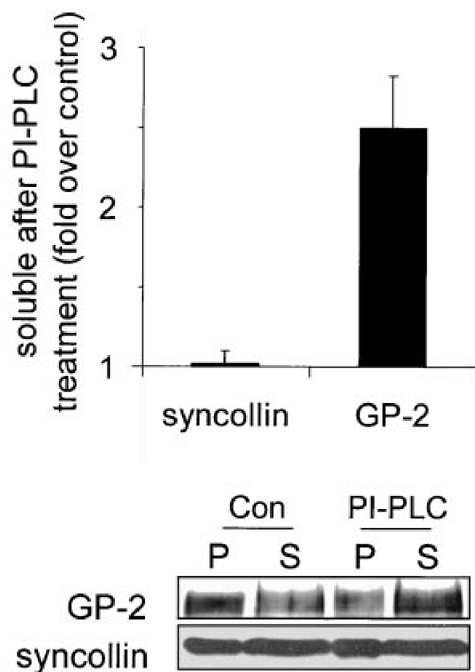

Figure 4 Effect of PI-PLC treatment on the association of syncollin with the ZGM

Isolated ZGM were treated with PI-PLC and separated by centrifugation into supernatant (S) and pellet (P) fractions. Equal amounts of the samples were further analysed by SDS/PAGE (12.5% acrylamide) and immunoblotting, using antibodies against syncollin (87.1) or GP-2. The resulting immunoblots were quantified by densitometry. The graph shows the amount of soluble syncollin or GP-2 after PI-PLC treatment (fold over control). The data are from three or four experiments and are expressed as means ± S.D. Con, untreated control.

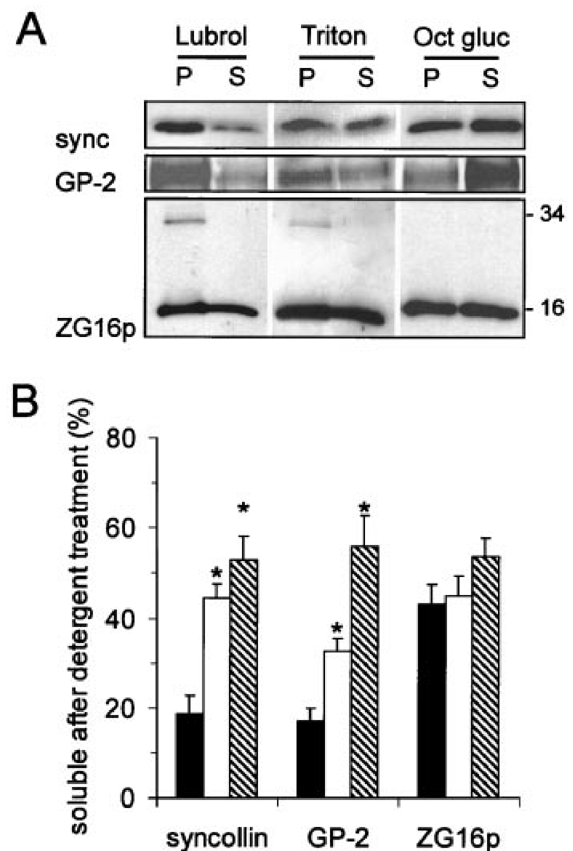

Figure 5 Treatment of ZGMs with different detergents

Isolated ZGMs were incubated with 0.5% Lubrol 17A17, 0.5% TX-100 or 60 mM octylglucoside at 4 °C for 30 min, and separated into pellet (P) and supernatant (S) fractions by high-speed centrifugation. (**A**) Equal amounts of the supernatant and pellet fractions obtained after high-speed centrifugation of Lubrol-treated (black bars), TX-100-treated (white bars) or octylglucoside-treated (hatched bars) ZGM were further analysed by SDS/PAGE and immunoblotting, using antibodies to syncollin (87.1; sync), GP-2 or ZG16p. (**B**) The resulting immunoblots were quantified by densitometry. The graph shows the amounts of soluble syncollin, GP-2 or ZG16p after detergent treatment. The data are from three or four experiments and are expressed as means ± S.D. $^{*}P < 0.01$ when compared with Lubrol treatment.

fractions were analysed by SDS/PAGE and immunoblotting. Quantification of immunoblots performed with antibodies to GP-2 and syncollin revealed that GP-2 was partially removed from the membranes by PI-PLC treatment, and was found to be enriched in the supernatant fractions when compared with untreated controls (Figure 4). In contrast, syncollin was not enriched in the supernatant fractions of PI-PLC-treated membranes, indicating that the membrane attachment of syncollin was not mediated exclusively via its interaction with GP-2. This result is in agreement with our previous demonstration that syncollin is able to interact directly with membrane lipids, and to insert into the granule membrane in a cholesterol-dependent manner [28].

Syncollin is associated with detergent-insoluble glycolipid- and cholesterol-enriched complexes

We recently reported the existence of glycosphingolipid- and cholesterol-enriched microdomains, or rafts, in the ZGM [16]. These microdomains contain GP-2, the lectin ZG16p and sulphated proteoglycans, and form TX-100-insoluble glycolipid-rich complexes at 4 °C, which float to light fractions on sucrose-density gradients [30]. In light of the discovery of an interaction between syncollin and GP-2, we decided to determine whether syncollin was also associated with lipid microdomains in the granule membrane. Membranes were extracted with the non-ionic detergents TX-100 [30], Lubrol [31] or octylglucoside [32] at 4 °C, and equal amounts of the detergent-insoluble and -soluble fractions obtained after high-speed centrifugation were analysed by immunoblotting, using antibodies against syncollin, GP-2 or ZG16p, and quantified by densitometry (Figure 5). In the absence of detergent, about 90–95 % of GP-2 and syncollin, and about 80–90 % of ZG16p, could be recovered in the pellet fraction (results not shown). Syncollin was found to be significantly more soluble in TX-100 and octylglucoside than in Lubrol. GP-2 was also more sensitive to TX-100 and octylglucoside treatments. Both TX-100 and Lubrol had a similar effect on ZG16p, whereas it was slightly more soluble in octylglucoside (Figure 5). Interestingly, on the ZG16p immunoblot, a protein band of approx. 32 kDa, presumably representing a dimer, was detected exclusively in the pellet fractions. The 32 kDa protein band could also be detected after octylglucoside treatment, but in some cases it was less prominent (Figure 5A). The behaviour of syncollin in TX-100 extracts of granule membranes has been studied previously [17,18]. When solubilized in TX-100 it behaves as a monomer, whereas in taurodeoxycholate it behaves as a protein of approx. 120 kDa [18]. We assume that the loss of the oligomeric structure might account for its higher solubility after TX-100 extraction.

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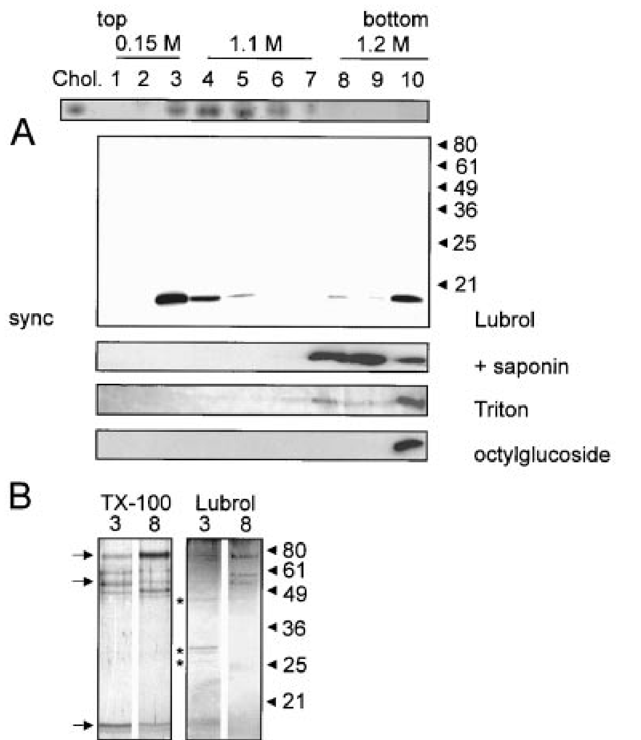

Figure 6 Syncollin is associated with cholesterol-rich microdomains isolated from ZGMs

(**A**) Equal amounts (1 mg of protein) of carbonate-treated ZGM were incubated with 0.5% Lubrol 17A17, 0.5% TX-100 or 60 mM octylglucoside at 4 °C for 30 min. The detergent-insoluble pellet fraction obtained after high-speed centrifugation was adjusted to 1.2 M sucrose (containing the appropriate detergents or a combination of Lubrol and saponin) and overlayered with 1.1 M/0.15 M sucrose. The density gradient was then centrifuged at 120000 ***g*** for 1 h. Fractions were collected from the top of the gradient and analysed by TLC (upper panel) or by SDS/PAGE and immunoblotting, using antibodies directed against syncollin (87.1; sync). Chol., cholesterol marker. (**B**) Fraction 3, containing the detergent-insoluble complexes after flotation, and fraction 8, representing the 1.1 M/1.2 M sucrose interface, from TX-100 and Lubrol gradients were analysed by SDS/PAGE and silver staining. Arrows indicate protein bands that were found in the fractions after both TX-100 and Lubrol treatment. Protein bands that were present only after Lubrol treatment are marked with asterisks. The positions of molecular-mass markers (in kDa) are indicated on the right.

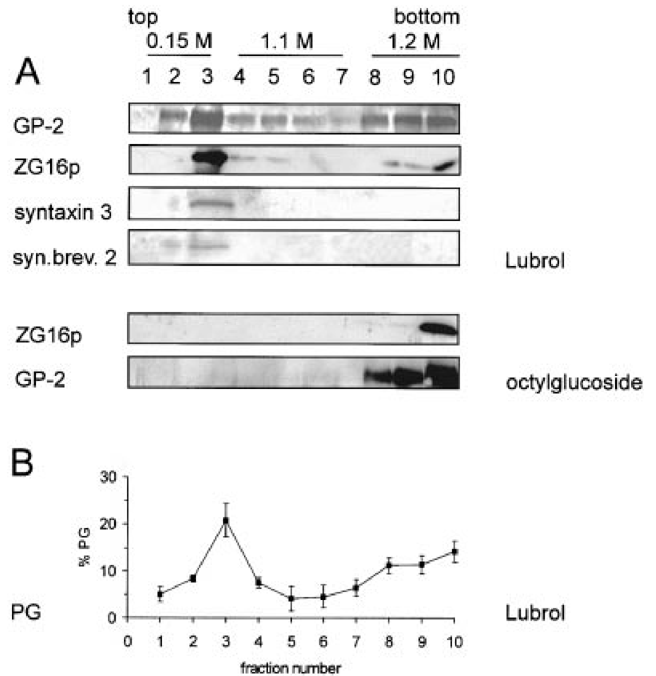

Figure 7 GP-2, ZG16p, SNARE proteins and sulphated proteoglycans are associated with cholesterol-rich microdomains after Lubrol treatment of isolated ZGMs

(**A**) Isolated ZGM were incubated with 0.5% Lubrol 17A17 or 60 mM octylglucoside and subjected to sucrose-density gradient centrifugation, as described in Figure 6. Fractions were collected from the top of the gradient and analysed by SDS/PAGE and immunoblotting, using antibodies directed against GP-2, ZG16p, syntaxin 3 or synaptobrevin 2 (syn.brev. 2). (**B**) The amount of matrix proteoglycans (PG) in the fractions was determined using the Blyscan assay, which specifically precipitates sulphated glycosaminoglycans on proteoglycans. After dissociation of the precipitated complex, the samples were quantified photometrically. The data in (**B**) are from four independent experiments (means ± S.D.).

The detergent-insoluble fractions were analysed further by sucrose-density gradient centrifugation (Figure 6). Only a very small amount of syncollin was found to float to lighter fractions after TX-100 treatment, and no flotation was observed after octylglucoside treatment (Figure 6A). In contrast, when Lubrol was used instead of TX-100 or octylglucoside, syncollin was found to float mainly to the low-density fractions 3 and 4, corresponding to the 0.15 M/1.1 M interface (Figure 6A). Lipid analysis of the gradient fractions revealed that cholesterol was present in the same fractions. Flotation was abolished when the membranes were treated with a combination of saponin (which disrupts cholesterol) and Lubrol. These data indicate that syncollin is associated with cholesterol-rich lipid rafts.

When the protein composition of TX-100 gradient fractions 3 and 8, which contained most of the floating proteins, was analysed by silver staining and compared with the corresponding fractions from a Lubrol gradient, significant differences were observed (Figure 6B). Fraction 3 of the Lubrol gradient contained protein bands in the 25–30 kDa range and a protein band of about 45 kDa, which were absent from the TX-100 fractions (Figure 6B, asterisks). However, protein bands of approx. 16, 53 and 75–80 kDa were visible after both treatments (Figure 6B, arrows).

It is known that GP-2, ZG16p and sulphated proteoglycans, which are believed to form a submembranous granule matrix [14], float to lighter fractions after TX-100 treatment [16]. As shown in Figure 7, these proteins were also recovered in the lighter fractions of the Lubrol gradients, as well as in fractions 8 and 9, and in the pellet fraction 10. In contrast, GP-2 and ZG16p, like syncollin, did not float in octylglucoside gradients. As lipid microdomains are thought to play a role in signal transduction [33], we looked for the presence of the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) syntaxin 3 and synaptobrevin 2, which are involved in granule fusion with the apical plasma membrane [34–36]. Surprisingly, both proteins were found to float to lighter fractions after Lubrol treatment (Figure 7A), but not after TX-100 extraction or octylglucoside treatment of granule membranes (results not shown).

To analyse the differences between the Lubrol- and TX-100-insoluble membrane domains in more detail, we prepared continuous sucrose gradients (5–30%) for flotation experiments (Figure 8). Interestingly, the TX-100-insoluble membranes, identified by an antibody to ZG16p, floated mainly to fraction 13, whereas the Lubrol-insoluble membranes exhibited a much broader distribution and were recovered in fractions 9–14. Similar

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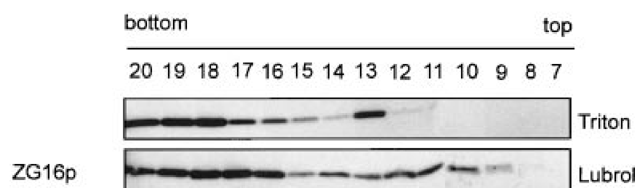

Figure 8 Detergent-insoluble complexes isolated by TX-100 or Lubrol show different buoyant densities

Carbonate-treated ZGM were incubated with 0.5% Lubrol 17A17 or 0.5% TX-100 as described for Figure 6. The detergent-insoluble pellet fraction obtained after high-speed centrifugation was adjusted to 40% sucrose (containing the appropriate detergents) and overlayered with a continuous sucrose gradient (5–30%). The density gradient was then centrifuged at 120000 ***g*** for 1 h. Twenty fractions were collected from the top of the gradient and analysed by SDS/PAGE and immunoblotting, using antibodies directed against ZG16p.

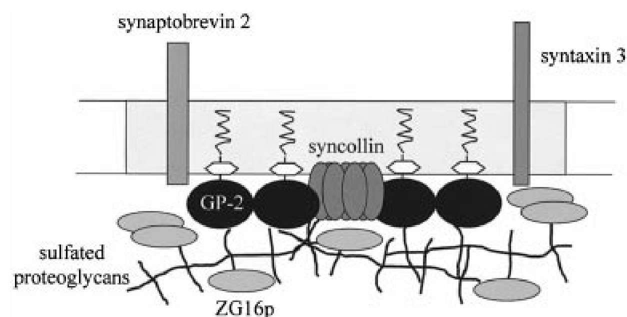

Figure 9 Schematic model of the components found to be associated with detergent-insoluble complexes isolated from ZGMs

We favour a model in which zymogen aggregates are bound to a submembranous protein matrix composed of proteoglycans, glycoproteins and the secretory lectin ZG16p at the luminal side of the ZGM [15,16]. The proteoglycan/glycoprotein matrix that is believed to be involved in the sorting/binding of zymogens is attached to specific membrane domains via GPI-anchored proteins, such as GP-2, which is thought to exist as a tetramer. Additional components of these microdomains are the SNARE proteins syntaxin 3 and synaptobrevin 2, and syncollin. Syncollin is thought to exist as a homo-oligomer and might even form a membrane pore complex [18].

results were obtained with an antibody to GP-2 (results not shown). These data indicate that Lubrol treatment results in the formation of multiple membrane domains with different buoyant densities, whereas TX-100 treatment reveals a distinct detergent-resistant complex of greater buoyant density.

DISCUSSION

Recently, it has been demonstrated that syncollin has an N-terminal signal sequence that directs it into the lumen of the endoplasmic reticulum [18,20]. Consequently, the protein enters the secretory pathway of the acinar cell, and eventually becomes tightly attached to the luminal surface of the ZGM. The morphological results presented here support our previous conclusion that there are two populations of syncollin within the ZG: one free in the lumen and the other associated with the granule membrane. The membrane-bound form of syncollin is believed to exist as a complex of molecular mass of approx. 120 kDa [18]. We have shown here using cross-linking that membrane-bound syncollin interacts directly with GP-2, a major membrane protein of ZGs. Similar interactions could not be demonstrated with BSA or with other abundant granule proteins coupled to the cross-linker [29]. Furthermore, ZG16p, another component of lipid microdomains in the granule membrane, could not be cross-linked to either syncollin or GP-2, but was cross-linked to a proteoglycan component (M. Schrader and R. Kleene, unpublished work). These findings support further the specificity of the cross-linking approach. The interaction with GP-2 might stabilize the association of syncollin with the membrane, but is not exclusively responsible for membrane attachment, since removal of GP-2 via cleavage of its GPI anchor leaves the amount of membrane-bound syncollin almost unaffected. The fact that syncollin is also found in detergent-insoluble complexes that are enriched in cholesterol indicates an additional interaction with cholesterol or other lipids. Furthermore, flotation was inhibited by treatment of membranes with saponin, which disrupts cholesterol. In a previous study, we showed that syncollin was removed from the granule membrane after cholesterol depletion by methyl-β-cyclodextrin, and interacted with liposomes in a cholesterol-dependent manner. Furthermore, cholesterol could be co-immunoprecipitated with syncollin [28]. We conclude from these data that syncollin is able to interact with GP-2 as well as with membrane lipids, and to insert into the membrane via an interaction with cholesterol.

We recently reported the existence of lipid microdomains in the membranes of ZGs from pancreatic acinar cells [16]. These microdomains showed an enrichment in cholesterol and sphingomyelin, and formed TX-100-insoluble glycolipid-enriched complexes that floated to the lighter fractions of sucrose-density gradients. Furthermore, the complexes were found to contain GP-2, the lectin ZG16p and sulphated matrix proteoglycans [16]. Similar observations have now been made for syncollin: it was found to be associated with detergent-insoluble cholesterol-enriched complexes in conjunction with GP-2, ZG16p and proteoglycans. Furthermore, the association of syncollin with the membrane was more sensitive to TX-100 or octylglucoside than to Lubrol. As TX-100 treatment has been shown to interfere with the oligomeric structure of syncollin [18], one might speculate that an oligomeric form is important for its membrane interaction. Whereas the association of GP-2 with lipid microdomains is obviously mediated by its GPI anchor, the association of ZG16p is less clear. It is likely that its interaction with the membrane is mediated by its lectin domain, possibly by binding to microdomain-associated glycolipids or glycoproteins. Another mode of interaction may be the association of ZG16p with chondroitin sulphate moieties of proteoglycans, which are themselves attached to lipid microdomains [15] (Figure 9).

It is not yet clear whether single or multiple types of lipid raft, which are able to form specialized membrane domains by aggregation, exist in the membranes of secretory granules [2]. It is also unknown whether the composition of these rafts is changed after granule fusion with the apical membrane. In a previous study [31], Lubrol treatment was used to define cholesterol-based lipid rafts, which differ from those isolated by TX-100. In the ZGM, the presence of syntaxin 3, synaptobrevin 2 and syncollin in detergent-insoluble complexes produced by extraction with Lubrol, but not with TX-100, might support the existence of separate or structurally different microdomains. Interestingly, TX-100 extraction of granule membranes produced a detergent-resistant complex of greater buoyant density than that seen after Lubrol treatment. One might speculate that the rafts consist of highly ordered (and TX-100-resistant) 'core' domains, containing GP-2, ZG16p and matrix proteoglycans, surrounded by semi-ordered (and TX-100-sensitive, but Lubrol-resistant) structures containing syncollin, syntaxin 3 and synaptobrevin 2. A possible function of such a marginal zone of semi-ordered lipids could be to enhance interactions between

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GPI-anchored proteins (e.g. GP-2) and transmembrane or peripheral membrane proteins that are essential for protein function (e.g. during exocytosis) [37].

Our data indicate that syncollin is a component of lipid microdomains in ZGMs. In a recent publication [16] we provided evidence that these microdomains were required for proper granule formation as well as for regulated secretion of zymogens. Raft disruption via cholesterol depletion led to a constitutive secretion of newly synthesized proteins (e.g. amylase), indicating that the zymogens were mis-sorted. Bearing in mind models proposed for granule biogenesis in neuroendocrine cells [1,2,38], we assume that granule formation in the exocrine pancreas is initiated by the interaction of glycolipids, integral and/or GPI-anchored glycoproteins and proteoglycans via their sugar moieties, leading to the formation of lipid/glycoprotein/proteoglycan patches in the membrane (Figure 9). A submembranous matrix composed of glycoproteins and proteoglycans [14] is attached to these tightly packaged lipid/protein platforms, presumably via GP-2 [8,10]. The matrix is believed to function not only in the binding and sorting of aggregated zymogens, but also in ensuring the stability of the forming granules.

We assume that syncollin's main function does not involve the condensation of zymogens or the binding/sorting of aggregated zymogens to the membrane, despite the fact that it is a membrane-associated protein. In preliminary studies using a recently developed *in vitro* condensation-sorting assay [5], preincubation of ZGM with a variety of anti-syncollin antibodies had almost no effect on the sorting/binding of zymogen aggregates to the membrane. In contrast, pretreatment of membranes with an antibody to ZG16p, a peripheral membrane protein known to be involved in sorting/binding of zymogens [15], showed a significant concentration-dependent inhibitory effect. Furthermore, by immunoelectron microscopy, syncollin was not found to be present within the zymogen aggregates that were formed *in vitro*. Rafts are thought to be involved in signal transduction [33], and furthermore syntaxin 3 and synaptobrevin 2, two SNAREs which mediate granule fusion [36], are also found in lipid microdomains after Lubrol treatment. Recently, the cholesterol-dependent clustering of SNARE proteins has been reported in PC12 cell membranes, although there is disagreement about whether the SNAREs are present in detergent-insoluble complexes [39,40]. Consequently, syncollin might have a regulatory function in granule biogenesis and secretion in conjunction with other raft-associated proteins, for example GP-2. It has been reported that after granule fusion the cleavage of GP-2 from the apical membrane is required to activate endocytosis [41], which is regulated by tyrosine kinases [42]. Freedman and co-workers [43] suggested that GP-2 may exist in a complex with Src kinases and caveolin to regulate exocytosis at the apical plasma membrane. As GPI-anchored proteins have been shown to exist in a complex with proteins that are important in signal transduction [44–46], the interaction between GP-2 and syncollin might have a related function.

The notion that syncollin exists as a homo-oligomer is supported by our cross-linking experiments, which show that isolated syncollin is self-interacting. These observations, in conjunction with the possible lipid interaction of syncollin, have led to the suggestion that syncollin might be a pore-forming protein [28]. This intriguing possibility remains to be established. In fact, the existence of pores in ZGMs has been reported [47–49], although their function remains unclear. If syncollin is indeed a pore-forming protein, it might fulfil important functions in signal transduction, and might regulate granule biogenesis and/or exocytosis of secretory proteins via lipid microdomains.

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