A submembranous matrix of proteoglycans on zymogen granule membranes is involved in granule formation in rat pancreatic acinar cells

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SUMMARY

The secretory lectin ZG16p mediated the binding of aggregated zymogens to the granule membrane in pancreatic acinar cells. Using a recently established in vitro condensation-sorting assay, we now show that pretreatment of zymogen granule membranes (ZGM) with either sodium bicarbonate at pH 10 or with phosphatidyl inositol-specific phospholipase C (PI-PLC) reduced the binding efficiency of zymogens to the same extent, as distinct components were liberated from ZGM. Analysis of the composition of the bicarbonate extract revealed the presence of the secretory lectin ZG16p, the serpin ZG46p and the GPI-linked glycoprotein GP-2, together with several unknown proteins, and small amounts of lipase and carboxylester lipase. The unknown proteins detected in 2-D gels represented a group of acidic and basic protein spots, which were positive in a glycan staining reaction and were soluble in methanol. One protein spot of the acidic group and several of the basic group reacted with a monoclonal antibody directed against chondroitin sulfate, indicating that the proteins represented proteoglycans. A staining pattern similar to the glycan reaction was observed in immunoblots using a polyclonal antibody directed against the whole bicarbonate extract. Immunogold electron microscopy revealed that this antibody reacted with components in the periphery of zymogen granules and strongly stained ZGM in the pellet fraction of a standard in vitro condensation-sorting assay. The amino acid composition of isolated components of both the acidic and basic group showed similarities to aggregan, a cartilage-specific proteoglycan, and to glycine-rich glycoproteins, respectively. We therefore conclude that a submembranous matrix on the ZGM composed of proteoglycans and glycoproteins is involved in granule formation in pancreatic acinar cells.

Key words: Rat, Pancreas, Pancreatic zymogen, Submembranous network, Condensation-sorting, Proteoglycan, Glycoprotein

INTRODUCTION

Granule formation in both exocrine and endocrine secretory cells involves two major steps through which the regulated secretory proteins are separated from constitutively released products and are packaged in concentrated form within secretion granules. The initial step is a pH-dependent, selective aggregation of regulated secretory proteins within the acidic environment of the trans-Golgi-network (TGN) leading to the dense granule core (for review, see Tooze, 1998). In some cells it also involves millimolar concentrations of calcium ions and represents a sorting event, since constitutively released products are excluded from ZGM. Analysis of the composition of the bicarbonate extract revealed the presence of the secretory lectin ZG16p, the serpin ZG46p and the GPI-linked glycoprotein GP-2, together with several unknown proteins, and small amounts of lipase and carboxylester lipase. The unknown proteins detected in 2-D gels represented a group of acidic and basic protein spots, which were positive in a glycan staining reaction and were soluble in methanol. One protein spot of the acidic group and several of the basic group reacted with a monoclonal antibody directed against chondroitin sulfate, indicating that the proteins represented proteoglycans. A staining pattern similar to the glycan reaction was observed in immunoblots using a polyclonal antibody directed against the whole bicarbonate extract. Immunogold electron microscopy revealed that this antibody reacted with components in the periphery of zymogen granules and strongly stained ZGM in the pellet fraction of a standard in vitro condensation-sorting assay. The amino acid composition of isolated components of both the acidic and basic group showed similarities to aggregan, a cartilage-specific proteoglycan, and to glycine-rich glycoproteins, respectively. We therefore conclude that a submembranous matrix on the ZGM composed of proteoglycans and glycoproteins is involved in granule formation in pancreatic acinar cells.

Key words: Rat, Pancreas, Pancreatic zymogen, Submembranous network, Condensation-sorting, Proteoglycan, Glycoprotein
proteins (ZGC) were separated from the zymogen granule membranes of the pellet, zymogen granules were lysed in 50 mM Hepes, pH 8.0, granules. After removal of the brownish layer of mitochondria on top centrifuged at 2000
10
500
-230 g body mass) and was homogenized in the following buffer: 0.25 M sucrose, 5 mM MES, pH 6.25, 0.1 mM MgSO4 , 1 mM DTT, (200-230 g body mass) and incubated on ice for 2 hours. To release GPI-anchored
Isolation of ZGM matrix components
Isolation and subfractionation of zymogen granules
Zymogen granules were prepared as described recently (Dartsch et al., 1994) with both the dense granule core and the ZGM at acidic pH, and an impairment of in vitro condensation-sorting after removal of both ZG16p and the proteoglycans from ZGM (Kleene et al., 1999). Since the composition of the submembranous matrix of ZGM is largely unknown, we have attempted to further characterize it and to analyze its involvement in granule formation.

MATERIALS AND METHODS
Isolation and subfractionation of zymogen granules
Zymogen granules were prepared as described recently (Dartsch et al., 1998). Briefly, the pancreas was removed from fasted male Wistar rats (200-230 g body mass) and was homogenized in the following buffer: 0.25 M sucrose, 5 mM MES, pH 6.25, 0.1 mM MgSO4, 1 mM DTT, 10 µM FOY-305 (Sanol Schwarz, Monheim, Germany), 2.5 mM Trasylol (Bayer, Leverkusen, Germany), 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 500 g and 4°C for 10 minutes, and the resulting postnuclear supernatant was centrifuged at 2000 g and 4°C for 10 minutes to sediment zymogen granules. After removal of the brownish layer of mitochondria on top of the pellet, zymogen granules were lysed in 50 mM Hepes, pH 8.0, and stored at −20°C. After thawing the soluble zymogen granule content proteins (ZGC) were separated from the zymogen granule membranes (ZGM) by centrifugation at 100000 g and 4°C for 30 minutes.

Isolation of ZGM matrix components
500 µl of ZGM corresponding to 200 µg of protein were resuspended in 50 mM Hepes, pH 8.0, mixed with 500 µl of 0.3 M NaHCO3, pH 11.5, and incubated on ice for 2 hours. To release GPI-anchored proteins (e.g. GP-2), ZGM corresponding to 800 µg of protein were incubated in a final volume of 500 µl of 50 mM Hepes, pH 8.0 containing 500 µU of PI-PLC (EC 3.1.4.10, Boehringer Mannheim, Germany) at 37°C for 1 hour. After each treatment membranes were recovered by centrifugation at 100000 g for 30 minutes.

Sulfate labeling and detection of glycans
Pancreatic lobules were prepared as described by Scheele and Palade (1975) and were preincubated in 5 ml sulfate-free medium per one pancreas. The medium was prepared using the SelectAmin Kit (Life Technologies, Eggenstein, Germany) with the following modifications: l/10 of methionine and cysteine was applied, and MgSO4 in the Eagle’s salt solution was replaced by MgCl2. The medium was supplemented with 4.5 g glucose/l and was buffered with 10 mM Hepes, pH 7.2. 2 mM carrier-free Na135I (ICN, Eschwege, Germany) were added and incubation continued at 37°C for 120 minutes, with agitation. 100% O2 was supplied every 15 minutes. Subsequently, the lobules were washed in PBS and zymogen granules were prepared as described above.

For the labeling of glycans the DIG Glycan Labeling Kit (Boehringer Mannheim, Germany) was used. Labeling with digoxigenin was performed using method A according to the manufacturer’s instructions.

Determination of sulfated glycosaminoglycans and of protein-bound radioactivity
To determine the amount of sulfated proteoglycans in fractions of zymogen granules the Blysan Proteoglycan and Glycosaminoglycan Assay was performed according to the manufacturer’s protocol (Biocolor, Belfast, Northern Ireland). Briefly, a precipitable complex was formed by binding of the Blysan dye to free or proteoglycan-bound glycosaminoglycans (GAGs). After dissociation of the precipitated complex the samples were spectrometrically quantified. For determination of protein-bound radioactivity, samples were applied to 3MM filters (Whatman, Maidstone, UK). The filters were swirled in 10% TCA on ice for 30 minutes, washed twice with ice-cold 5% TCA for 5 minutes, and once with ethanol. After drying the protein-bound radioactivity precipitated on the filters was determined by liquid scintillation counting using a scintillation counter (Raytest, Straubenhardt, Germany).

Methanol precipitation
Since it is known that certain proteoglycans and glycoproteins are methanol-soluble, we subjected the bicarbonate extract of ZGM to methanol precipitation in order to specifically isolate glycosylated components. Methanol precipitation was performed according to Wessel (1984). Briefly, 100 µl of bicarbonate extract corresponding to 100 µg of protein were mixed in consecutive steps with 400 µl of methanol, 100 µl of chloroform and 300 µl of dH2O. After centrifugation at 13000 g for 3 minutes the upper phase was collected, concentrated and subjected to 2-D gel electrophoresis. The interphase and lower phase were mixed with 300 µl of methanol and centrifuged at 13000 g for 3 minutes to precipitate methanol-insoluble proteins, which were also subjected to 2-D gel electrophoresis.

Protein purification
To isolate the methanol-soluble, glycosylated acidic and basic components of the bicarbonate extract, samples were subjected to preparative isoelectric focusing using the Rotofor System (Bio-Rad Laboratories, Munich, Germany). The samples were desalted using PD-10 desalting columns (Amersham Pharmacia Biotech, Uppsala, Sweden) and mixed with 3M urea, 2% ampholytes (Servalyte pH 3.0-10.0, Serva, Heidelberg, Germany), 10 µM FOY-305 (Sanol Schwarz, Monheim, Germany), 2.5 mM Trasylol (Bayer, Leverkusen, Germany) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in a volume of 20 ml. Isoelectric focusing was performed according to the manufacturer’s protocol (Bio-Rad Laboratories, Munich, Germany). After the initial purification step, samples were separated by SDS-PAGE and recovered by electro-elution using the Whole Gel Eluter system (Bio-Rad Laboratories, Munich, Germany).

Antibody production
To generate antibodies directed against the bicarbonate extract, immunization of rabbits was performed at Eurogentec (Seraing, Belgium) using a high-speed supernatant of bicarbonate-treated membranes (bicarbonate extract) as antigen in a standard immunization protocol (four cycles of injection of 100 µg protein). The antibodies directed against the recombinant ZG16p (Crønshagen et al., 1994) and the recombinant GP-2 (Dittie and Kern, 1992) were raised in rabbits.
Gel electrophoresis and immunoblotting

Protein samples were separated under denaturing conditions by SDS-PAGE (Laemmli, 1970) or by 2-D gel electrophoresis using 8 M urea in the isoelectric focusing (Scheele, 1975). Silver staining was performed according to Hempeleman and Kaminsky (1986). The identification of the individual enzyme spots in 2-D gels was performed according to Schick et al. (1984). For immunoblotting proteins were transferred to nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany) by semi-dry blotting. Membranes were blocked with 1% BSA in TBS for at least 1 hour, subsequently incubated for 2 hours with diluted anti-membrane matrix antibody (1:500) or monoclonal anti-chondroitin sulfate antibody (1:50) (Clone CS-56, Sigma, Deisenhofen, Germany), washed in TBS and finally incubated in diluted peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (BioRad, Richmond, CA, USA) for 1 hour. Immunoblots were processed using diaminobenzidine (DAB) reagent (0.05% DAB, 0.04% CoCl2, 0.05% H2O2 in 10 mM Tris-HCl, pH 7.5).

In vitro condensation-sorting assay

To test the functional significance of the submembranous matrix in condensation-sorting a previously established in vitro assay was used (Dartsch et al., 1998). Briefly, 100 μg of protein extract of bicarbonate- or PI-PLC-treated membranes were adjusted to pH 8.0 with 0.5 M MES, pH 4.5, and were added to 1 mg of ZGC. The mixture was adjusted to pH 7.5 or pH 5.9 by adding the same volume (200 μl) of 100 mM MES, pH 4.5 or pH 6.25, respectively. After incubation under rotation at room temperature for 120 minutes the reaction mixture was centrifuged at 13000 g for 30 minutes. The resulting pellets were resuspended in 50 mM Hepes, pH 8.0 and further analyzed. For quantitation of membrane-bound aggregates [35 S]methionine/cysteine-labeled ZGC was added to the samples as a tracer (Dartsch et al., 1998).

Electron microscopy

Biopsies from the pancreas of fasted rats, isolated ZGM samples and supernatant and pellet fractions of a standard condensation-sorting assay were fixed in 2% paraformaldehyde/0.1% glutaraldehyde. In the case of tissue biopsies the fixatives were dissolved in 0.1% cacodylate buffer, pH 7.3, while the samples from isolated fractions were fixed in their original buffer by adding concentrated fixatives to a final concentration of 1%. The samples were dehydrated in a graded series of alcohol and either embedded in Epon according to standard procedures or in water-soluble resin Lowicryl K4M (Polysciences Ltd., Eppenheim, Germany) by polymerisation at −20°C and UV light at −7°C. The matrix components were localized in both pancreatic tissue and in isolated fractions using thin sections of K4M-embedded probes, which were incubated with a polyclonal antiserum directed against the whole submembranous network of zymogen granules (Kleene et al., 1999). To further characterized these components the supernatant and the precipitate was embedded in Epon and subjected to electron microscopy of thin sections.

RESULTS

A putative submembranous matrix on ZGM is involved in condensation-sorting

We recently obtained evidence that one of the possible mechanisms by which the granule core is bound to ZGM might be by linkage through the secretory lectin ZG 16p, which was found to be associated with proteoglycans and seems to be part of a matrix on the luminal surface of ZGM (Kleene et al., 1999). To investigate this in more detail, we attempted to remove the submembranous matrix from the ZGM to characterize its composition and to analyze the ‘stripped’ ZGM in a standard condensation-sorting assay (Dartsch et al., 1998). Incubation of ZGM in Hepes buffer either at 30°C for 1 hour or at 4°C for 2 hours removed about 30-40% each of the GPI-anchored glycoprotein GP-2, the lectin ZG16p and proteoglycans from the granule membrane (Fig. 1, left). However, incubation in bicarbonate buffer liberated about 50% of GP-2, 60% of ZG16p and 70% of the proteoglycans (Fig. 1, middle). Treatment of ZGM with PI-PLC in Hepes buffer removed only an additional 10-15% of the matrix components on top of that removed by Hepes buffer alone (Fig. 1, right). Even after a sequential treatment (bicarbonate followed by PI-PLC), about 20-30% of the matrix components remained tightly associated with ZGM (not shown). The differently treated ZGM and the resulting extracts were incubated with ZGC proteins at pH 5.9 in a standard in vitro condensation-sorting assay (Fig. 2) (Dartsch et al., 1998).

As expected, the binding efficiency of zymogen aggregates to bicarbonate-treated ZGM was reduced to a lower percentage than to PI-PLC-treated membranes (Fig. 2A), indicating that bicarbonate treatment removed the majority of the matrix components required for the binding process. In contrast, addition of the liberated matrix components to the assay, which also contained remnants of ZGM (Dartsch et al., 1998), enhanced the binding capacity (Fig. 2B). This effect was more pronounced with the bicarbonate extract (Fig. 2B). These findings support the conclusion that ZGM contain peripheral membrane components which are involved in condensation-sorting.

Identification of ZGM-associated components

To further characterize these components the supernatant...
fractions of PI-PLC- and bicarbonate-treated membranes were separated by 2-D gel electrophoresis and after silver staining the protein patterns were compared to those of separated ZGC proteins (Fig. 3A). The PI-PLC extract contained the GPI-anchored glycoprotein GP2, the membrane-associated serpin ZG46p (Chen et al., 1997), the secretory lectin ZG16p, lipase and, in addition, 6-7 spots in the acidic range and 4 in the neutral or basic range (Fig. 3B). These protein spots did not cross-react with antibodies directed to chymotrypsinogen, trypsinogen, procarboxypeptidase or amylase (data not shown), and were not identical with major zymogen proteins. The bicarbonate extract showed a similar pattern, except that it contained carboxylester lipase (CEL) and additional protein spots in both the acidic and basic range (Fig. 3C). We then used the whole complement of matrix components released from ZGM by bicarbonate treatment to raise a polyclonal antiserum in rabbits. This antibody was used in immunoblots on ZGC (Fig. 4A), the PI-PLC extract (Fig. 4B) and the bicarbonate extract (Fig. 4C). In ZGC the antisem recognized carboxylester lipase, lipase, the serpin ZG46p and in trace amounts the secretory lectin ZG16p, which were known to be associated with the membrane matrix (Fig. 4A). In addition, several proteins in the acidic and basic range of the 2-D gel showed a reaction with the antibody, but the exact nature of these protein spots is still unknown. The PI-PLC extract showed a strong reaction of the antibody with GP-2, ZG46p and weak reactions with both ZG16p and a major spot.
The submembranous network of zymogen granules in the acidic range of 30-31 kDa (rectangle, Fig. 4B). However, several protein spots of PI-PLC extracts visible after silver staining did not react with the antibody (compare Figs 3B and 4B). In the bicarbonate extract of ZGM carboxylester lipase, 8-10 spots in the acidic (arrows) and 6-8 spots in the basic range (arrowhead) were detectable in addition to the proteins shown in Fig. 4B. These spots were not visible in silver-stained gels (compare Figs 3C and 4C).

**Immunolocalization of matrix components**

We then used the antiserum directed against the components of the bicarbonate extract to stain various tissues from the rat. At the light microscope level the strongest reaction was found in pancreatic acinar cells but not in duct cells or the islets of Langerhans. In the parotid gland the reaction was restricted to certain portions of the duct system, and a strong reaction was found in the apical cytoplasm of the cells of the prostate (data not shown). Using immunogold techniques at the electron microscope level (Fig. 5) the anti-ZGM matrix antibody reacted with the Golgi complex, with zymogen granules and with the content released into the acinar lumen (Fig. 5A). Inside zymogen granules a concentration of gold particles in the periphery of the condensed content was observed (Fig. 5B). During exocytosis only few gold particles remained associated with the ZGM, which is inserted into the apical plasma membrane (Fig. 5C, arrows). The content proteins released into the acinar lumen formed a fibrillar network with immunogold particles often arranged in line. This observation supports the existence of a network-like structure (Fig. 5D).

The matrix components are methanol-soluble proteoglycans and glycoproteins

Certain proteoglycans are known to be methanol-soluble (Faltynek et al., 1982). It was therefore likely that the acidic and basic proteins of the bicarbonate extract, which were only visible after immunoblotting but not in silver-stained gels, represented methanol-soluble proteoglycans. To test this assumption we subjected the high-speed supernatant of bicarbonate-treated ZGM to a standard methanol precipitation according to Wessel (1984). After centrifugation, both the supernatant and the pellet fractions were separated on 2-D gels and analyzed by immunoblotting using the anti-matrix antibody. The pellet representing the methanol-insoluble fraction contained predominantly carboxylester lipase, ZG46p, lipase, ZG16p and a few minor, as yet unidentified protein spots (Fig. 6A). The methanol-soluble fraction revealed a similar staining pattern to the whole bicarbonate extract shown in Fig. 4C, consisting of a prominent group of acidic and basic proteins (Fig. 6B). To further verify their proteoglycan nature we used both a monoclonal antibody directed against chondroitin sulfate A and C (clone CS-56, Sigma), and a commercial glycan labeling kit. The anti-chondroitin sulfate antibody selectively reacted with one protein spot of the acidic group (Fig. 7A, rectangle), which was prominent in the PI-PLC and in the bicarbonate extract (compare Figs 4B,C and 7A), and also with four spots of the basic group (arrowheads). We conclude that these protein spots represent chondroitin sulfate-containing proteoglycans. The glycan detection reaction revealed strong labeling of the methanol-soluble acidic group of matrix components supporting their glycoconjugate nature (arrows, Fig. 7B).
**ZGM matrix components are enriched after condensation-sorting**

In Fig. 2 it has been demonstrated that solubilized submembranous matrix components enhance the binding efficiency of aggregated zymogens to ZGM. To further identify which components of the matrix were involved in condensation-sorting, the bicarbonate extract was incubated with ZGC either at pH 7.4 or at pH 5.9. After centrifugation the pellet fractions were subjected to 2-D gel electrophoresis and immunoblotting using the anti-matrix antibody. A significant enrichment of both the acidic and the basic group of glycoproteins was observed in the pellet fractions at pH 5.9, indicating their association with either aggregated zymogens or with ZGM present in the assay (Fig. 8B). This was further examined by immunogold electron microscopy (Fig. 9). The antibody stained isolated ZGM (Fig. 9A) and also the matrix components after their detachment by bicarbonate treatment (Fig. 9C). When the matrix was fixed in the presence of Alcian Blue, a cationic dye which binds to acidic groups on mucosubstances and proteoglycans, a fine fibrillar network was observed (Fig. 9B). Addition of the matrix components to a standard condensation-sorting assay revealed a significant enrichment of immunogold particles along the membranes and in the periphery of aggregated zymogens (Fig. 9D-F).

**Characterization of individual matrix components**

To isolate the methanol-soluble glycosylated acidic and basic components the bicarbonate extract was subjected to a preparative isoelectric focusing in solution. The fractions obtained after focusing were analyzed by immunoblotting using the anti-ZGM matrix antibody to identify the methanol-soluble proteins. In a second purification step the samples containing the methanol-soluble acidic and basic proteins were separated preparatively by vertical SDS-PAGE. The proteins were recovered from the gel by electro-elution. The fractions containing the methanol-soluble proteins were again identified by immunoblotting using the anti-ZGM matrix antibody. The amino acid composition of these proteins was determined. It revealed a similarity of one protein of the acidic group (fraction no. 13) with the proteoglycan aggrecan of the rat, a major constituent of the extracellular matrix. The amino acid composition of the other proteins of the acidic group (fraction numbers 12, 14, 15) were similar and revealed a high degree of glycine residues, characteristic of glycine-rich proteins found in the cell wall of plants (Table 1). The amino acid composition of the basic group of proteins showed similarities to the putative polysaccharide binding protein precursor from porphyra purpurea and to a 27 kDa primary mesenchyme-specific spicule protein precursor of the purple sea urchin (Table 2). N-terminal sequencing of the acidic glycine-rich protein no. 15 revealed that the protein starts with six consecutive glycines, while N-terminal sequencing of the
The submembranous network of zymogen granules revealed the sequence (D,S,G or T)(Q,V or L)DFP(E or R)TGA(A or V), which does not match to any known sequence. Sequencing of tryptic peptides was not possible, because all proteins of the acidic and basic group were protease-resistant, supporting their high degree of glycosylation.

Although the exact identification of individual matrix proteins is so far not possible, we conclude that proteoglycans and glycine-rich glycoproteins are components of the submembranous matrix.

**DISCUSSION**

Proteoglycans are macromolecules composed of glycosaminoglycan (GAG) chains covalently bound to a protein core. The GAGs are arranged in an alternating unbranched sequence and carry sulfate substituents in various positions (Kjellen and Lindahl, 1991). Within the organism proteoglycans occur in three major locations: as components of the extracellular matrix (ECM; for review, see Iozzo, 1998), as part of the glycocalyx on the cell surface (reviewed in Bernfield et
Proteoglycans in both the ECM and on the cell surface are involved in a variety of biological processes, such as growth and organization of cells and tissues, acting as filters or modulating growth factor activities. These complex regulatory processes rely on interactions of both the GAG chains and domains in the protein core with various partners in their surrounding. Intracellular proteoglycans have been observed in various cellular compartments, such as in the endosomal compartment, in lysosomes, in the nucleus and most predominantly in secretion granules of the various cell types of the haemopoetic system including mast cells in the gut and in connective tissues (for review, see Kolset and Gallagher, 1990).

Secretory granule proteoglycans contain heparan sulfate and chondroitin sulfate chains attached to a unique core protein, which is composed of repetitive units of serine and glycine, and therefore is referred to as serglycin (Ruoslahti, 1988). Within the secretion granules of haemopoetic cells and mast cells the proteoglycans are complexed to basic proteins, such as proteases and cytolytic agents, and therefore these proteins have been considered as matrix structures involved in the efficient concentration of secretory products (Matsumoto et al., 1995). Sulfation of the GAG chains on granule proteoglycans seems to be important for an efficient storage of secretory products, as indicated by recent studies in transgenic animals in which a key enzyme for sulfation has been knocked out (Forsberg et al., 1999; Humphries et al., 1999a,b). Using sulfate incorporation in vitro into pancreatic lobules of the guinea pig, sulfated proteoglycans have been identified within zymogen granules of acinar cells and in the secretion of the duct system (Tartakoff et al., 1975; Reggio and Palade, 1978). Scheele et al. (1994) reported that more than 90% of sulfate-labeled proteoglycans in pancreatic acinar cells of the rat were associated with zymogen granule membranes (ZGM). Furthermore, they mentioned that 70% of the $^{35}\text{SO}_4$-labeled molecules were released from ZGM by treatment with sodium carbonate at pH 11.2, while the glycoprotein GP-2 remained bound to ZGM. Since PI-PLC treatment in this study liberated about 50% of GP-2 together with 27% of radiolabeled proteoglycans, the authors postulated a submembranous matrix at the luminal surface of ZGM, which at least in part is composed of the GPI-anchored glycoprotein GP-2 with sulfated proteoglycans attached to it. The present study corroborates and 

**Fig. 9.** Immunolocalisation of membrane-matrix components on ZGM and in the in vitro condensation-sorting assay. The anti-membrane-matrix antibody shows a strong reaction with isolated ZGM (A, enlarged 180000× in inset) and also with the fixed bicarbonate extract (C). When the fixation of bicarbonate extract is performed in the presence of Alcian Blue a fibrillar network is observed (B, arrows). In a standard in vitro condensation-sorting assay to which matrix components have been added, immunogold particles are observed in the periphery of aggregated zymogens (D) and along the membranes which have aggregated zymogens attached (arrows, E,F). Bars, 0.1 µm.
The submembranous network of zymogen granules

The bicarbonate extract of ZGM was subjected to preparative isoelectric focusing in solution. The fractions obtained after focusing containing the unidentified glycoconjugates were separated preparatively by vertical SDS-PAGE and recovered from the gel by electro-elution using the Whole Gel Eluter. The methanol-soluble proteins of the acidic group were subjected to amino acid analysis.

The methanol-soluble proteins of the basic group were isolated as described in Table 1 and subjected to amino acid analysis.

The methanol-soluble membrane matrix components (Fig. 4C). In immunogold electron microscopy this antibody reacts with components in the periphery of zymogen granules (Fig. 5B,C), but most importantly it intensely stains ZGM in a standard condensation-sorting assay to which the bicarbonate-soluble membrane matrix components have been added (Fig. 9D-F). The equivalent to this staining reaction is the enrichment of the same components in the pellet fraction of the assay at pH 5.9 (Fig. 8B). The exact biochemical nature of these membrane-matrix components has not yet been elucidated. Microsequencing of individual components separated by isoelectric focusing in solution followed by preparative vertical SDS-gels has so far failed, because most of the components are protease-resistant. The methanol-soluble acidic protein no. 13 (Table 1) shows a similar amino acid composition to the cartilage-specific proteoglycan of the rat, although aggrecan has a high molecular weight core protein with a mass of 221 kDa. With the exception of protein no. 13, the other proteins of the acidic group seem to be glycoproteins. They could be deglycosylated using trifluoro methan sulfonic (TFMS) and their amino acid composition suggests that they represent glycine-rich glycoproteins. A number of different studies indicate that glycine-rich glycoproteins can function as adhesion molecules (Schirmer et al., 1994) and that they act as scaffold structures in plants (Wojtaszek and Bolwell, 1995). We therefore assume that the acidic glycerine-rich proteins as components of the ZGM matrix could function as a submembranous scaffold involved in binding of the granule core and in supporting granule shape and stability. The chondroitin sulfate-containing basic proteins (Fig. 7A) have a similar amino acid composition, pl and molecular mass to the 27 kDa primary mesenchyme-specific spicule protein precursor of the sea urchin (Table 2), which represents a skeletal protein containing a lectin domain (Harke et al., 1995).

Future work is required to further identify the exact nature of these membrane-matrix components. It is, however, already possible to conclude that the binding of aggregatedzymogens to the granule membrane is ensured by a macromolecular

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**Table 1. Amino acid composition of the acidic methanol-soluble proteins of the membrane matrix**

<table>
<thead>
<tr>
<th>Acidic protein no. 13</th>
<th>Acidic proteins nos 12, 14, 15</th>
<th>Glycine-rich CWS2P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala 5.9</td>
<td>6.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Arg 5.3</td>
<td>3.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Asx 5.6</td>
<td>6.8</td>
<td>2.1</td>
</tr>
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<td>Glx 11.7</td>
<td>12.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Gly 12.7</td>
<td>12.1</td>
<td>45.0</td>
</tr>
<tr>
<td>Ile 2.6</td>
<td>3.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Leu 5.4</td>
<td>6.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Lys 1.8</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Met 0.0</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Phe 1.8</td>
<td>2.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Pro 8.0</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser 12.8</td>
<td>13.2</td>
<td>7.2</td>
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<tr>
<td>Thr 6.4</td>
<td>9.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Tyr 1.2</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Val 6.1</td>
<td>6.5</td>
<td>3.3</td>
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**Table 2. Amino acid composition of the basic methanol-soluble proteins of the membrane matrix**

<table>
<thead>
<tr>
<th>Basic proteins (23-35 kDa, pl 8-9)</th>
<th>PSBP* (22 kDa)</th>
<th>PM27‡ (27 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala 3.0</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Arg 3.8</td>
<td>2.6</td>
<td>8.1</td>
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*PSBP, putative polysaccharide binding protein precursor, porphyra purpurea, 22 kDa, pl 8.5; §PM 27, 27 kDa primary mesenchyme-specific spicule protein precursor, purple sea urchin, 29 kDa, pl 8.1.

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The submembranous network of zymogen granules 2241
matrix consisting of several glycoconjugate components, which are reversible bound to ZGM during granule formation in the TGN. They are released from ZGM during exocytosis, predominantly by bicineartate ions, which in turn are secreted into the acinar lumen by the initial duct system.

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REFERENCES


