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THE INTEGRAL AND PERIPHERAL PROTEINS OF THE ZYMOGEN GRANULE MEMBRANE *

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This study reports the lipid and protein composition of purified pig zymogen granule membranes. Lipids made up more than 70% of the membrane dry weight and phosphatidylserine constituted 17% of the total phospholipids. The membrane was shown to contain nine major proteins. A protein of M_r 92000 was the major constituent accounting for more than 25% of the Coomassie blue staining on gels. Six glycoproteins, including the latter, were revealed by periodic acid-Schiff staining and concanavalin A binding. A phase separation technique using partition of intrinsic and extrinsic membrane proteins in Triton X-114 solutions has shown that most of the proteins were integral membrane proteins. The ATP diphosphohydrolase, which is distinctive of the zymogen granule membrane, segregated with integral proteins. These data constitute a detailed description of a purified membrane fraction from pig pancreatic zymogen granules.

Introduction

The zymogen granule membrane is a central component of the secretory system in the exocrine pancreas. It is involved in zymogen packaging and storage in the acinar cell. In response to appropriate stimuli, this membrane is also responsible for the fast release of the granule content through its specific fusion with the apical membrane of the cell (exocytosis). The aim of our research is to resolve the different functions of this membrane. Proteins of the zymogen granule membrane are the first targets of these studies. Indeed each protein of the membrane should be involved at different steps of these multiple functions of the membrane. The proteins expected to play a major role in a membrane are the integral membrane proteins. They are characterized by a hydrophobic intramembrane domain. Such integral proteins as the Ca^{2+}-ATPase of the sarcoplasmic reticulum, the $(Na^+ + K^+)$-ATPase of the renal tubule, the band III of the erythrocyte, and the rhodopsin of the rod cell outer segment, constitute the major protein distinctive for each one of these membranes. A major protein has already been identified for zymogen granule membranes. This protein is a glycoprotein having a molecular mass between 80 and 92 kDa [1–4]. The zymogen granule membrane has also been characterized by the presence of an ATP diphosphohydrolase activity [5–7]. In this paper, we report the protein composition of this specialized membrane from pig pancreas. Integral as well as peripheral membrane proteins have been identified. The major protein of the membrane, a 92 kDa glycoprotein, and the ATP

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Abbreviations: Endo D and Endo H, endo-β-*N*-acetylglucosaminidases; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; lysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; Pipes, 1,4-piperazinediethanesulphonic acid; PMSF, phenylmethylsulphonyl fluoride; PS, phosphatidylserine.

diphosphohydrolase were shown to be both integral proteins of the zymogen granule membrane.

Materials and Methods

Experimental Procedures

Materials

Pig pancreases were obtained from local abattoirs, Abattoir Provigo and Bienvenue (St-Valérien, Québec, Canada). They were immediately placed in ice-cold Krebs Ringer Phosphate buffer, pH 7.4, and processed within less than 90 min after slaughter.

Triton X-114, ATP, ADP and PMSF were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Electrophoresis reagents were from Bio-Rad Laboratories, (Mississauga, Ontario, Canada). Concanavalin A, Endo H and Endo D were purchased from Miles Laboratories (Toronto, Ontario, Canada). All other chemicals used were at least of reagent grade quality.

Preparative methods

Tissue was processed at 4°C by modifications of a previously described method [4]. Pancreases were first trimmed of excess fat and of blood vessels. For a typical batch preparation, 200 g were homogenized for 8 s in a Waring blender. One liter of 8 mM cacodylate buffer pH 6.0, 2 mM $CaCl_2$, 0.65 M sucrose, 0.1 mM PMSF was then added. This suspension was homogenized by two strokes of a Teflon-glass homogenizer and filtered through four layers of cheese-cloth. The filtrate was centrifuged 15 min at $700 \times g$ (Sorvall GSA rotor). The supernatant was recentrifuged in the same conditions to remove all gross cellular debris. This second $700 \times g$ supernatant was centrifuged for 15 min at $4000 \times g$. The brownish overlayer of mitochondria was removed by vortexing and scraping with a cotton swab. The white granule pellet was then suspended in the washing buffer (10 mM Pipes, pH 6.0/0.56 M sucrose/0.1 mM PMSF) and incubated at 0°C for 1 h in order to produce swelling of the mitochondria. This suspension was then overlaid on a 0.65 M sucrose solution in a conical tube and centrifuged at 4500 $\times g$ for 10 min. The pellet obtained was gently vortexed to get rid of the very loose layer of mitochondria. This pellet was resuspended in an appropriate buffer depending if the granules were to be kept intact (Pipes, pH 6.0/0.6 M sucrose) or further processed to obtain the membranes (25 mM Hepes, pH 8.0/0.2 M KCl (the lysis buffer)). The granules were kept on ice for 1 h in order to optimize their lysis. The membranes are then obtained by centrifuging (38000 rpm, 45 min, SW41 rotor) the lysed granule preparation over a discontinuous sucrose gradient made of 3.5 ml of 0.3 M sucrose and 2.5 ml of 1 M sucrose. Membranes were collected on the 1 M sucrose cushion, resuspended in 0.2 M KCl, and pelleted at 30000 rpm for 30 min in SW41 rotor. This pellet constituted the purified zymogen granule membranes. The pellet obtained under the 1 M sucrose (1 M sucrose pellet) was also collected and kept for analysis.

Enzyme assays

Amylase was measured by the method of Bernfeld [8]. ATP diphosphohydrolase assays were performed as previously described [7] by monitoring the P_i released from ADP or ATP [9]. Succinate-cytochrome *c* reductase was monitored spectrophotometrically at 550 nm according to a modification of the method of Hodges and Leonard [10] in a final volume of 3 ml containing 50 mM sodium phosphate, pH 7.0, 0.83 mM KCN, 0.5 mg/ml cytochrome *c* and 83 μg/ml of succinate.

Triton X-114 separation of zymogen granule membrane proteins

The experimental conditions for the separation of integral and peripheral proteins in Triton X-114 solutions were those used by Bordier [11]. Usually 100 to 200 μg of membrane proteins were extracted in 200 μl of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1.0% Triton X-114.

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose and detection of concanavalin A binding proteins

The SDS gel electrophoresis was performed as previously described [12]. The proteins were transferred according to Burnette [13] using a current of 60 V for 2.5 h in a Trans-Blot cell (Bio-Rad Laboratories). Staining of the nitrocellulose filter for protein was done according to Schaffner et al. [14] using Amido black (Bio-Rad Laboratories).

The binding of concanavalin A was performed according to Clegg [15] using the divalent property of the lectin and its affinity for the enzyme glycoprotein horseradish peroxidase. Tween 20, instead of bovine serum albumin, was used as blocking agent according to Batteiger et al. [16]. The control included 0.1 M α-methyl-D-mannoside as specific inhibitor at the step of incubation with concanavalin A.

Endo-β-N-acetylglucosaminidase H treatment of the membranes

To 100 μg of zymogen granule membranes, 200 μg of SDS were added and incubated at 100°C for 2 min. The % SDS was brought down to 0.2% using 50 mM, pH 5.5 citrate buffer. PMSF was added to 1 mM and Endo H to 50 munits/ml. Incubation was done at 37°C for 8 h.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis were carried out according to the procedure of Laemmli [12] for slab gels and Weber and Osborn [17] for disc gels. Coomassie blue staining was done according to Fairbanks [18]. periodic acid-Schiff staining, according to Glossman and Neville [19].

Analytical procedure

Protein was determined with the procedure of Lowry et al. [20], phospholipid phosphorus according to Bartlett [21] and cholesterol according to Ott et al. [22]. Phospholipids were separated by two-dimensional thin-layer chromatography on Silica gel G (Merck). The first dimension solvent system was chloroform/ methanol/ ammonium hydroxide (65 : 25 : 5, v/v), and the second was chloroform/ acetone/ methanol/ acetic acid/ water (30 : 40 : 10 : 10 : 5, v/v).

Results

Morphological and enzymatic characterization of the zymogen granules and their membranes

Fig. 1 shows the purified zymogen granule preparation and their membranes. Few mitochondria, the expected contaminants, were observed. The

TABLE I

LEVELS OF MARKER ENZYMES IN THE DIFFERENT FRACTIONS LEADING TO THE PURIFICATION OF ZYMOGEN GRANULE MEMBRANE

Marker enzymes	Homogenate	Zymogen granules	Zymogen granule membranes
Amylase			
Specific activity [a]	185.3 ± 13.0	450.4 ± 52.0	10.7 ± 8.0
Enrichment	–	2.4	0.06
Recovery (%)	100	2	0.0002
Succinate-cytochrome *c* reductase			
Specific activity [b]	17.6 ± 2.9	5.0 ± 1.0	12.9 ± 5.2
Enrichment	–	0.3	0.7
Recovery (%)	100	2	0.003
ATP diphosphohydrolase			
ATP substrate			
Specific activity [c]	54.5 ± 3.5	165.1 ± 13.6	3910 ± 440
Enrichment	–	3.0	71.7
Recovery (%)	100	2.6	0.3
ADP substrate			
Specific activity	48.6 ± 3.8	123.5 ± 3.11	3170 ± 440
Enrichment	–	2.5	65.2
Recovery (%)	100	2.2	0.3

[a] μmol of maltose released per min per mg protein at 37°C.
[b] nmol of cytochrome *c* reduced per min per mg protein at 25°C.
[c] nmol of P_i released per min per mg protein at 37°C.

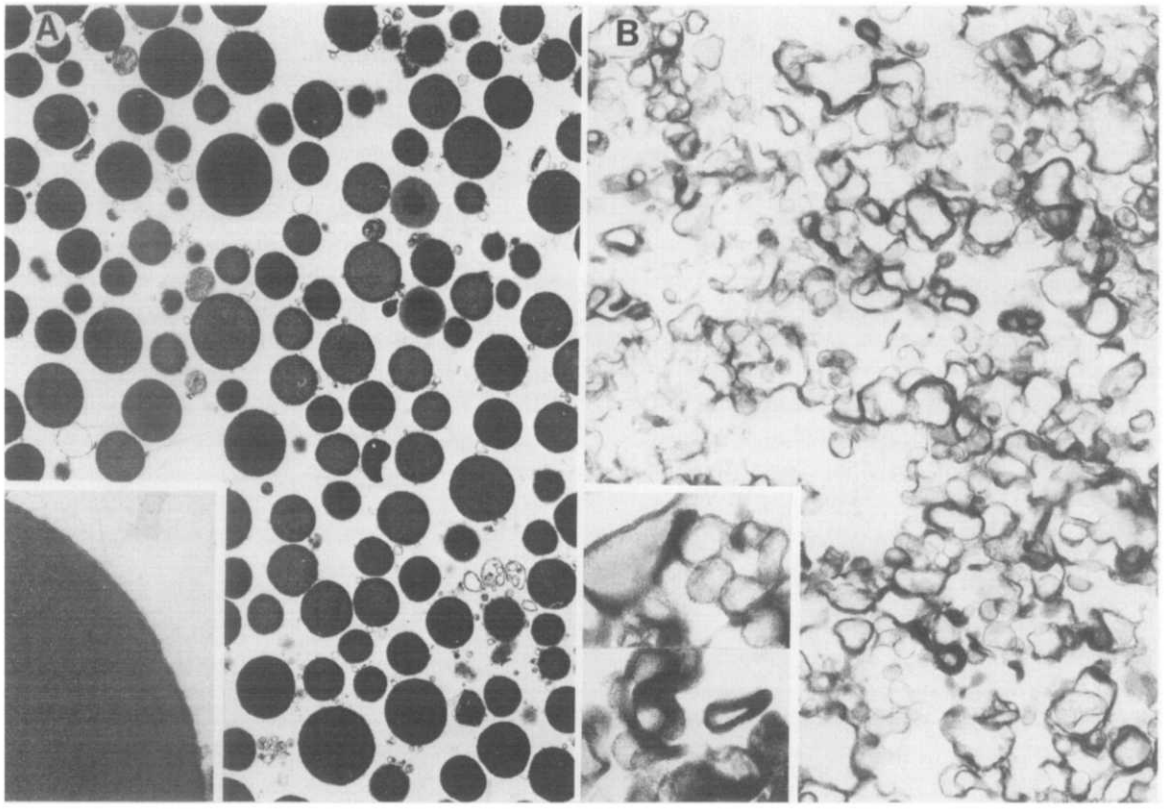

Fig. 1. Transmission electron microscopy of a typical granule and granule membrane preparations. (A) Zymogen granules; magnification 6720×. The integrity of the granule membrane can be observed in the higher magnification (inset, 43380×). (B) Zymogen granule membranes; magnifications, 21000× and insets 43680×. Only small smooth vesicles are seen. Aggregation to form multilayered figures can also be observed (insets).

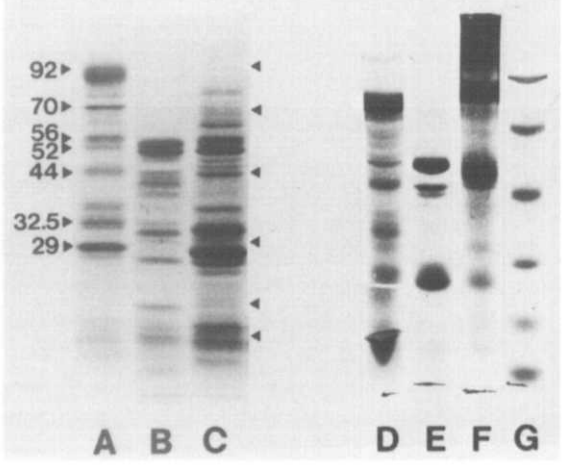

Fig. 2. SDS-polyacrylamide gel electrophoresis of the zymogen granule membrane proteins. Zymogen granules, lysed at pH 8.0, were centrifuged in a step gradient of 1 M and 0.3 M sucrose. The membranes were collected on the 1 M sucrose and washed with 0.2 M KCl (A). (B) The 0.3 M sucrose overlay showing the granule content. (C) The pellet under the 1 M sucrose. A, B, C are on 6–15% gradient Laemmli gel. D, E, F, G are 7% Weber and Osborn gels. (D) Same as A; (E) same as (B); (F) pig erythrocyte membranes. Arrowheads and (G) show the molecular weight standards: 94, 67, 43, 30, 20.1 and 14.4 ($\cdot 10^3$).

granules have kept their limiting membranes (Fig. 1A, inset). The purified membranes formed small vesicles and had a tendency to clump, forming myelin figures with many layers of membranes (Fig. 1B). The purity of the zymogen granule membranes was confirmed by a 70-fold enrichment in its specific enzymatic marker the ATP diphosphohydrolase [5,6,23]. The recovery of the latter activity in the membrane preparations was

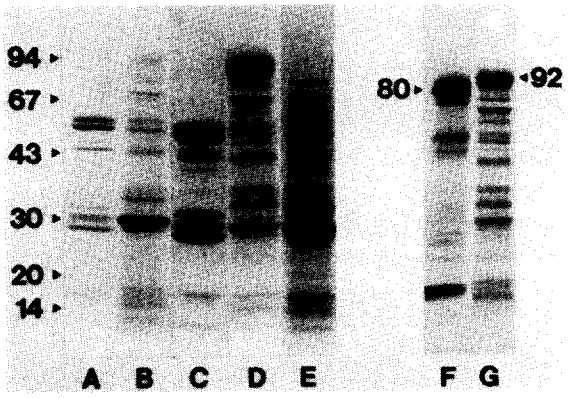

Fig. 3. KCl-extractable proteins of the zymogen granule membrane and comparison between rat and pig membranes. Zymogen granule membranes collected on the 1 M sucrose cushion were washed with 0.2 M KCl (D). Supernatant was dialysed, lyophilysed and dissolved. Soluble (A) and insoluble (B) fractions were obtained. (C) The granule content and (E) the 1 M sucrose pellet fraction. Right panel shows zymogen granule membrane from rat (F) and pig (G). Laemmli 6–15% gradient gels.

100- and 1500-times higher than the recovery of the mitochondrial and the granule content markers, respectively (Table I). The specific activity of amylase of the membranes was 17 times lower than in the homogenate and no important amylase bands could be detected in the electrophoretic patterns of the membranes (Fig. 2 and 3). Mitochondria are the most likely contaminant in zymogen granule membrane preparations. In order to have an estimation of this contamination, succinate-cytochrome *c* reductase has been measured. Since no values for a good porcine mitochondria preparation could be found, bovine heart mitochondria were used as a reference [24]. Comparing a specific activity of 12.9 nmol/min per mg in our preparation to 960–1100 nmol/min per mg for pure mitochondria, a maximum mitochondrial content of 1.3% is obtained. This fits well with the relative recovery of mitochondria in our membrane fraction.

Lipid composition of the zymogen granule membranes

Zymogen granule membranes have a ratio of phospholipid to protein of 2.49 (w/w). This ratio is very high if compared with sarcoplasmic reticulum, 0.43 [25], with erythrocyte membranes, 0.75 [26] or with chromaffin granule membranes, 1.65 [27]. Such a ratio implies that more than 70% of the membrane dry weight is lipid. This proportion is close to myelin's with 79% lipid [26]. The membrane is mainly composed of PC (37.2%), PE (18.1%), sphingomyelin (17.1%) and PS (17.0%) (Table II). The level of PS is relatively important, twice as much as in the chromaffin granule membrane [27]. The lysoPC concentration is not very high (8.9%), half of the amount in the chromaffin granule. The absence of cardiolipin and the high concentration of sphingomyelin confirmed the low level of mitochondrial contamination of the membrane preparations. Indeed mitochondrial membranes are known to have a high proportion of cardiolipin and a low amount of sphingomyelin in their phospholipids [28,29].

TABLE II

LIPID COMPOSITION OF PIG ZYMOGEN GRANULE MEMBRANES

The number of determinations is given in parenthesis.

Phospholipids	% [a] (2)
Phosphatidylcholine	37.2
Phosphatidylethanolamine	18.1
Sphingomyelin	17.1
Phosphatidylserine	17.0
Lysophosphatidylcholine	8.9
Phosphatidylinositol	1.8
Cholesterol	325.6 ± 89 μg/mg protein (3) (130.8 μg/mg phospholipid)
Phospholipid : protein ratio (w/w)	2.49 ± 0.63(5)

[a] Relative percentage by weight of each phospholipid.

Zymogen granule membrane proteins

Analytical polyacrylamide gel electrophoresis of the zymogen granule membrane showed a dozen proteins using the Laemmli as well as the Weber and Osborn gel systems (Fig. 2). Apparent molecular weights of these proteins have been calculated and are summarized in Table III. Low concentration gels have been run to determine more accurately the molecular weights of bands with low mobility. The mobility of the major bands has also been verified on acidic gel using the technique of Dam et al. [30] and showed no difference in mobility.

TABLE III

PIG ZYMOGEN GRANULE MEMBRANE PROTEINS: PROPERTIES OF THE MAJOR COMPONENTS

M_r	Glycoprotein [a]	Intrinsic [b]	Extrinsic [b]
140000	GP	I	
92000	GP	I	
70000			E
65000	GP	I	
56000	GP	I	
52000	GP	I	
44000	GP	I	
32500			E
29000		I	

[a] Periodic acid-Schiff-positive and concanavalin A binding proteins.

[b] Based on their partition in Triton X-114 solutions (Fig. 5).

The major protein of the zymogen granule membrane has a molecular weight of about 92000. This protein accounted for 25% of the Coomassie blue staining as extracted from the gel with 0.2% SDS, 0.1 N NaOH [31], or by integration of the peak of the scanning pattern. This band always had such a broad appearance even when much less material was applied on the gel. That led us to compare this band with band III of the erythrocyte which has a very similar behavior upon electrophoretic separation [32]. Fig. 2, lane F, shows the Coomassie blue staining pattern of pig erythrocyte membranes. Pig band III has a significantly higher M_r of around 94000. It must be pointed out that human band III has a slightly lower molecular weight than the porcine protein. This could be misleading in a comparison with pig zymogen granule membrane proteins. Rat and pig zymogen granule membrane proteins have been run on the same gel (Fig. 3). The protein pattern was very different. The rat major band ran significantly lower than pig major glycoprotein as already mentioned [4].

Zymogen granule membrane preparation led to the production of a pellet under the 1 M sucrose cushion used for membrane isolation. This pellet has been tested for the presence of mitochondrial enzymes and ATP diphosphohydrolase. It was found devoid of such enzymatic activities. A ratio of 0.20 mg of phospholipids per mg protein was found for the material of this pellet. Protein composition of this fraction (Fig. 2, lane C) showed some similarities with the content and the membrane of the zymogen granule. However, the 92 kDa protein characteristic of the membrane was absent. Bands at M_r 61000, 35500 and 31500 were more specific to this fraction. In addition, bands of 29000 and three in the range of M_r 14000 were major constituents of this pellet. These latter bands were shared with the membrane. Some other bands could be identified as coming from the granule content.

Contamination of the membrane by secretory proteins was not detectable on gels as Fig. 2 shows. Only a doublet close to amylase's could be misleading but these bands did not align with one another on the gel. In addition, the membrane bands were found to be glycoproteins (Fig. 4), which is not the case for amylase [33].

Characterization of the zymogen granule membrane glycoproteins

Fig. 4 shows the periodic acid-Schiff staining of the zymogen granule membrane electrophoretogram. Periodic acid-Schiff-positive bands corre-

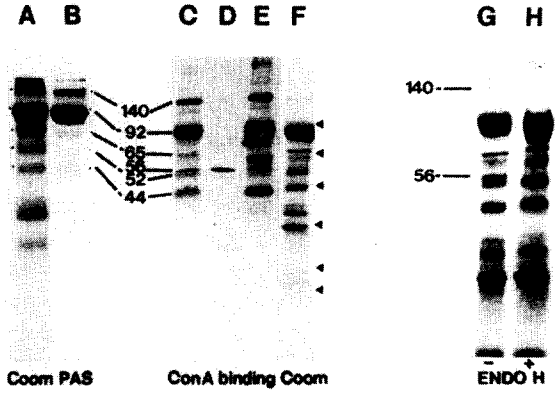

Fig. 4. Zymogen granule membrane glycoproteins. Zymogen granule membrane (150 μg protein) were run on 10% Laemmli gel, stained for glycoproteins with Schiff's reagent (B) and overstained with Coomassie blue (A). Zymogen granule membrane proteins were run on Laemmli 6–15% gradient gel (F) transferred to nitrocellulose sheet by Western blotting, reacted with concanavalin A and revealed by horseradish peroxidase according to Clegg [15]. Lane C, Triton X-114 phase and E, total granule membrane proteins: lane D, 1 M sucrose pellet. Arrowheads show M_r standards (see Fig. 2). Right panel shows the indubation of granule membrane with (H) or without (G) Endo H in presence of 0.2% SDS.

sponded to M_r 140 000, 92 000, 65 000, 56 000 and 44 000. The latter three were stained quite faintly.

Concanavalin A binding to proteins transferred on nitrocellulose membranes was a much more sensitive and accurate approach to look at these glycoproteins. Western blotting of the proteins separated on SDS gel was quantitative. Indeed no remaining band could be significantly detected on the gels after transfer (results not shown). Six bands were specifically binding concanavalin A corresponding to M_r 140 000, 92 000, 65 000, 56 000, 52 000 and 44 000 (Fig. 4). The 140 kDa protein had the highest capacity to bind concanavalin A. Two of the glycoproteins were sensitive to Endo H digestion. A reduction of 4 to 5 kDa in the apparent molecular mass for the 140 and 56 kDa bands was observed (Fig. 4, lane H). These differences in their molecular masses is believed to account for the glycosidic moiety of the two proteins. Endo D digestion was also performed and did not lead to any changes in the electrophoretic pattern of the membrane proteins. No glycoprotein in the different preparations could be stained blue using Stains-all (Eastman). This coloration would have been indicative of acidic proteins [34]. The 1 M sucrose pellet was shown to have only one glycoprotein of M_r 56 000 (Fig. 4, lane D). No major membrane glycoprotein was detected in this fraction.

Characterization of the 0.2 M KCl extraction of the zymogen granule membranes

KCl washes of the membranes have been dialysed, lyophilysed and run on gels in order to determine their protein composition. A soluble and an insoluble fraction were obtained. Fig. 3 shows that the proteins of the soluble fractions were of secretory origin. The insoluble fraction was composed of bands common to three fractions: the zymogens, the membrane and the 1 M sucrose pellet. A similar set of proteins could be extracted from zymogen granule membranes with 3 mM EDTA. These proteins extractable with KCl are bound to the membrane via ionic interactions and can be considered as peripheral proteins.

Separation of integral and peripheral membrane proteins

The phase separation of integral membrane proteins in Triton X-114 solutions has been performed. This technique takes advantage of the property of integral membrane proteins, and only these, to form mixed micelles with nonionic detergents [11]. Fig. 5 shows the two fractions obtained with zymogen granule and the 1 M sucrose pellet. Triton X-114 interfered in the electrophoresis making the bands look fuzzy. The detergent phase contained most of the membrane proteins and all the glycoproteins (Fig. 4 lane C). The other fraction, containing water soluble (hydrophilic, peripheral) proteins, gave four major bands. Table III summarizes the behavior of the membrane proteins in the Triton X-114 phase separation system. One protein was found in both fractions. Indeed the 92 kDa protein, the membrane's major protein, was observed in the two phases. Partition of this band was not always in the same proportion depending on the batch of membranes used.

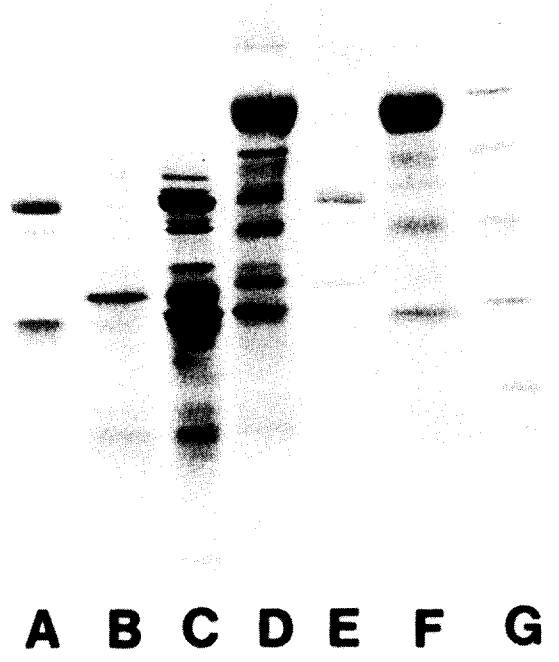

Fig. 5. Phase separation of zymogen granule membrane proteins in Triton X-114 solutions. Granule membranes (150 μg protein) (D) and 1 M sucrose pellet (C) (150 μg protein) were separated with Triton X-114 according to Ref. 11 in a detergent phase (F, granule membrane; B, 1 M sucrose pellet) and an aqueous phase (E, membranes; A, pellet). Lane G shows the molecular weight standards (see Fig. 2).

TABLE IV

PHASE SEPARATION WITH TRITON X-114 SOLUTIONS OF ATP DIPHOSPHOHYDROLASE ACTIVITY

Fractions	ATP substrate		ADP substrate	
	Specific activity (μmol/min per mg protein)	Total activity (μmol/min)	Specific activity (μmol/min per mg protein)	Total activity (μmol/min)
Zymogen granule membranes	2.87	0.247	3.10	0.192
Zymogen granule membranes + Triton X-114 (0.2–3.0%)	1.79	0.111	1.40	0.087
Triton X-114 phase	3.10	0.074 (67%)	2.8	0.067 (77%)
Aqueous phase	Undetectable	–	Undetectable	–

The equipartition of some membrane proteins has been reported for band III, Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase [35]. However, no soluble proteins have been shown to partition in the detergent phase. This ambivalent behavior of some proteins has been related to a property of pore-former proteins, or to the state of monomer or polymer of these proteins [35]. The detergent phase of the 1 M sucrose pellet gave a protein pattern very different from the one obtained with the membranes. The 31 kDa protein, one of the major specific constituents of this fraction was found in the Triton phase. No major bands were shared, thus stressing a very clear difference between the two fractions. However, the aqueous components of both fractions had in common, proteins from the content of the granule, amylase (M_r 53000) for example.

ATP diphosphohydrolase activity has been assayed in both fractions obtained with zymogen granule membranes. Table IV shows that 67% of the residual ATP hydrolytic activity and 77% of ADP's were recovered in the detergent phase. No activity could be detected in the aqueous phase. These results are in accordance with the maximal recovery possible in the detergent fraction for an intrinsic membrane protein if compared with bacteriorhodopsin (80% recovery), a true intrinsic membrane protein [11].

Discussion

In the present study we have determined that the purified pig zymogen granule membranes produced more than 15 bands on standard one-dimensional SDS gel electrophoresis. Six of them are glycoproteins. Table III summarizes the properties of these proteins. One of them, a 92 kDa glycoprotein accounts for more than 25% of the zymogen granule membrane proteins, thus providing a unique and very convenient marker for this membrane. Our studies agree on this point with the work of Ronzio and collaborators [1,2,3] that the zymogen granule membrane was characterized by a major glycoprotein named GP2. However, the M_r attributed to this major protein in the literature varies considerably; from 74000 [3], 80000 [4] to 92000. This variation can be explained for two reasons: (1) the broadness of this band on gels and, (2) differences in the amino acid composition and in the oligosaccharide moiety of the protein between the different species studied. The overall complexity of the protein composition that we observed for this membrane is very different from the simplicity of previous studies by Ronzio et al. [3] with four observable bands, or by Meldolesi and Cova [36] who could not use high resolution gels in 1971.

Ronzio detected four glycoproteins; we detected six using two different methods. Two of these glycoproteins are sensitive to Endo H digestion which splits the chitobiosyl core of *N*-linked glycoproteins rich in mannose [37]. M_r 140000 and 56000 proteins are typical examples of such glycoproteins [38] with sugar chains accounting for 4 to 5 kDa of their molecular mass. This sensitivity to Endo H fits well for the 140 kDa protein with its high binding capacity for concanavalin A. Indeed both proteins, Endo H and concanavalin A, require a high mannose content for their biological

activities. Since its insensitivity to both Endo H and Endo D, (Endo D being specific for complex chains) the 92 kDa protein oligosaccharide chains seemed to be *O*-glycosidically linked such as for glycophorin of the erythrocyte membrane [39].

Although it is very important to know the whole protein composition of a specific membrane, it is much more important to know which one of these proteins is very intimately inserted into the phospholipid bilayer of this membrane. Indeed intrinsic proteins are the most specific biochemical and functional markers of a specialized membrane [40–42]. In this study we used the phase separation technique in Triton X-114 solutions developed by Bordier [11] to separate intrinsic from extrinsic membrane proteins. Most of the zymogen granule membrane proteins were shown to be intrinsic. This characteristic could be expected as the membranes were previously extracted with 0.2 M KCl. The association of the remaining extrinsic proteins is then quite strong and therefore more specific to this membrane than the commonly adsorbed secretory proteins [43] which are merely absent from our preparations. Consequently these extrinsic proteins can be considered specifically associated with the zymogen granule membrane and can play a role at this level.

Among intrinsic membrane proteins, so called due to their affinity for the nonionic detergent, some are of particular interest for their possible role in the zymogen granule functions. The first one is the ATP diphosphohydrolase which is very tightly and specifically associated with this membrane [4,5]. This study shows that the activity is intrinsic to the membrane. It also shows that bands of M_r 56 000, 44 000 and 29 000 which correspond to ATP diphosphohydrolase subunits [5], all segregate into the detergent fraction (Table III). The 56 and 44 kDa subunits are glycoproteins, the former being sensitive to Endo H digestion.

Another intrinsic protein, the 92 kDa glycoprotein is of prime interest in this membrane. It constitutes by itself more than 25% of the protein content of this membrane. It has been shown to be a glycoprotein of ambivalent behavior in our hands. Indeed it has never been confined to only one phase of the Triton X-114 separation. This behavior has also been observed with channel-forming proteins [35]. Such a role for the 92 kDa protein would be of major importance in a membrane responsible for the granule integrity through the maintenance of optimal intragranular osmolarity and pH (LeBel, D., unpublished observations). Understanding of its transmembrane localization should help in finding a role for this major polypeptide of the zymogen granule membrane.

The phospholipid composition of the membrane has some interesting particularities in relation to its participation in the process of membrane fusion. LysoPC has been shown to facilitate membrane fusion [47,48]. LysoPC constitutes 9% of the zymogen granule membrane phospholipids (Table II). However, in the chromaffin granule for instance, where the LPC concentration is 16.7% [27], at least 60% is in the inner leaflet of the membrane [49], thus making their participation in the fusion process less likely. The location of lysoPC in the zymogen granule membrane is also most probably internal due to two reasons: (1) the presence of active phospholipase A_2 inside the granule has already been observed [50,51], and (2) the absence of detectable levels of lysoPE and lysoPS argues for the preferential localization of PE and PS on the leaflet of the membrane protected against phospholipase digestion (external) and of lysoPC on the unprotected leaflet (internal). The other interesting feature of the lipid composition is the presence of 17% PS (Table II). PS content has been shown to be important in the Ca^{2+}-mediated fusion of liposomes [44] and binding of divalent cations to PS appears to be correlated with this ability of liposomes to fuse [45]. The high content of PS (17%) in the zymogen granule membrane and its possible external localization should be more than sufficient to allow such a Ca^{2+}-mediated phenomenon since chromaffin granules which are believed to fuse in such a way [46], have a PS concentration of 10% [26].

The formation of a pellet under 1 M sucrose cushion used for the isolation of membranes from lysed granules was an unexpected phenomenon. Electrophoretic patterns showed that few proteins of the pellet are shared with the granule membrane. Five of the six membrane glycoproteins were very neatly absent from this fraction, making its origin from the zymogen granule membrane very unlikely. Moreover, enzyme markers did not allow to associate this fraction with zymogen gran-

ule membrane nor with mitochondria. The simultaneous presence of soluble zymogens and of little phospholipids in the 1 M sucrose pellet make us believe that this fraction could arise from the molecules responsible for the condensation of the granule content and its osmotic inactivation [52–54]. Upon granule lysis, this material would aggregate, trapping some zymogens in the process and get pelleted after centrifugation.

In this study, the zymogen granule membrane of pig pancreas has been purified and examined for its lipid and protein composition. The ratio of phospholipid to protein was very high at 2.49. The membrane contained more than 15 proteins, including six glycoproteins. Most of them were integral membrane proteins. ATP diphosphohydrolase activity has been observed to segregate with intrinsic membrane proteins. The three proteins of the membrane whose molecular weight corresponded to the ATP diphosphohydrolase subunits were all intrinsic membrane proteins. The finding of a role for the ATP diphosphohydrolase subunits and for the 92 kDa glycoprotein in the zymogen granule membrane should be of critical importance in resolving the mechanism of cellular secretion in the pancreas.

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