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Purification and Characterization of the Apical Plasma Membrane of the Rat Pancreatic Acinar Cell

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Summary. A method is described for the rapid purification of the apical plasma membrane from the rat pancreatic acinar cell. It makes use of wheat germ agglutinin affinity chromatography to selectively bind vesicles with N-acetyl glucosamine present at their surface. Particular conditions (150 mM NaCl) had then to be used to keep membrane vesicles in the coveted orientation, i.e. as right-side-out vesicles. Due to its specific apical location in many epithelial cells, γ-glutamyltranspeptidase was chosen to monitor the purification procedure. The final fraction was enriched in γ-glutamyltranspeptidase by a factor of 75 relative to the homogenate. Na,K-ATPase, a strict basolateral membrane marker, was not detectable in the fraction. No membranes originating from other compartments, more particularly expected from zymogen granules, or from other cell types, did contaminate the preparation. As expected for an epithelial cell apical plasmalemma, lipid composition showed a very high ratio of glycolipids (37.5%). The absence of membrane-bound GP-2, and the exceptionally high specific activity of γ-glutamyltranspeptidase suggest that the apical membrane would not be made up by the exocytosis of secretory granule, but instead by the fusion of specialized secretory vesicles very likely originating from the constitutive secretory pathway. In conclusion, this report describes a method of obtaining a fraction highly enriched in the secretory apex of the pancreatic exocrine cell that would be directly involved in exocytosis with zymogen granules and also in local anion transport.

Key Words plasmalemma · secretion · membrane biogenesis · membrane retrieval · endocytosis · cystic fibrosis

Introduction

In the exocrine pancreas, secretion involves the fusion of the zymogen granule membrane with the acinar cell apical membrane. So far, most efforts have been focused on the membrane of the secretory granule [18–20]. In previous reports, labeling of intact zymogen granules have been carried out in order to identify proteins at their surface [18, 19] which might be involved in the recognition and fusion of the granule with the apical membrane. Due to their location, these proteins could thereby regulate exocytosis at the plasma membrane level [11]. Little has been done, however, on the isolation and characterization of the other partner in exocytosis, the apical plasma membrane. This fraction would be essential to identify and characterize all the proteins involved in the specific recognition with the zymogen granule, as nonspecific fusions are essentially not observed under normal conditions [39], and as high secretory response of secretory tissues is intimately correlated with the regional specialization of the luminal plasma membrane [40]. One of the reasons explaining the difficulty of acquiring such a fraction is the small proportion made up by this particular portion of the plasmalemma in the resting pancreas. It indeed constitutes in vivo less than 5% of the total plasma membrane area of the pancreatic acinar cell [9].

The present study describes a method for the purification of the apical plasmalemma of the rat pancreatic acinar cell to a high level of purity. It makes use of the characteristic of the apical membrane to be highly enriched in glycolipids [42] and N-acetyl glucosamine residues [26] by comparison with the basolateral membrane. Making use of the affinity of WGA for N-acetyl glucosamine, affinity chromatography was used to enrich the membrane fraction. Conditions were chosen to produce membrane vesicles in a right-side-out orientation. Taking γ-GT as the best marker enzyme for the apical membrane [13, 41] and Na,K-ATPase [43] for the basolateral membrane, we have been able to obtain a fraction highly enriched in γ-GT and totally devoid of Na,K-ATPase. Being the site of fusion for secretory granules, acquisition of an apical membrane preparation is an essential step in the development of an in vitro assay for exocytosis with purified secretory granules. This membrane is also of prime importance for chloride transport in secretory epithelia. It could be advantageously used in the study of apical chlo-

ride transport, a process that is dramatically impaired in cystic fibrosis subjects [35].

Materials and Methods

Purification of Pancreatic Apical Membranes

Male Sprague-Dawley rats from our colony, weighing between 200 and 275 g were fasted overnight before decapitation. Pancreases were removed within 2 min, trimmed free of fat and blood vessels, and kept at 0°C in homogenization buffer. All subsequent steps were done at 4°C. For a typical preparation, 6 g of tissue were minced with scissors, suspended in 10 volumes of 10 mM $MgCl_2$, 150 mM NaCl, 50 mM MES,[1] pH 6.0, 0.28 M sucrose with protease inhibitors, 0.1 mM PMSF and 0.1 mg/ml SBTI. Homogenization was done with ten strokes of a Teflon-glass homogenizer. After filtration through four layers of cheesecloth, the homogenate was centrifuged twice for 15 min and 25 min, respectively, at 120 × *g* to remove all gross cellular debris. The supernatant was centrifuged 15 min at 13,000 × *g* to remove granules and mitochondria. This 13,000 × *g* supernatant was recentrifuged at higher speed (25,000 × *g*) to pellet membranes. A great proportion of the tissue γ-GT (65%) was found in this 25,000 × *g* pellet. This fraction was finally washed with 0.2 M KCl and 0.25 M NaBr in 50 mM Tris/Cl, pH 7.5, and constituted the *washed plasma membrane* fraction that was subsequently used for affinity chromatography. The latter fraction was loaded on a WGA Sepharose column pre-equilibrated with 50 mM, Tris/Cl, pH 6.8. After washing with the loading buffer, 0.1 M N-acetylglucosamine in 50 mM Tris/Cl, pH 6.8 was used to elute bound membranes.

Enzyme Assays

Amylase was assayed by the method of Bernfeld [6]. ATP diphosphohydrolase was determined as previously described [22] by monitoring the release of P_i from ADP [3]. TPPase and alkaline phosphatase were assayed by measuring the release of P_i from thiamine pyrophosphate and nitrophenol from *p*-nitrophenyl phosphate, respectively. γ-GT activity was assayed by measuring the release of *p*-nitroaniline at 410 nm from 2.5 mM γ-glutamyl-*p*-nitroanilide in a buffer made of 0.1 M Tris/Cl, 10 mM $MgCl_2$, pH 8.0, with 20 mM glycylglycine as the acceptor [28]. One unit of activity was defined as the amount of the enzyme required for the release of 1 μmol of *p*-nitroaniline per min at 37°C. When the activity was determined in presence of detergent, 10 μg of Triton X-100 per μg of protein were added. This ratio was determined as the lowest to reveal all latent γ-GT without affecting the intrinsic activity of the enzyme, which is susceptible to membrane perturbing agents. Na,K-ATPase activity was estimated by release of P_i from ATP [3]. The incubation medium (1 ml final volume) contained 50 mM Tris/Cl, pH 7.4, at 37°C, 5 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 0.1 mM EGTA, 0.1 mM Tris-ATP with or without 1.0 mM ouabain [34]. Na,K-ATPase was defined as the ATPase activity that could be inhibited by ouabain. Cytochrome *c* oxidase was estimated using a concentration of 0.033% reduced cytochrome *c* [20].

Lipid Analysis

In order to minimize endogenous lipase activities, 10 mM EGTA was added when membranes were prepared for lipid analysis. Lipids were extracted with chloroform : methanol and separated by thin-layer chromatography on silica gel as previously described [14]. Each class of lipids were extracted from the silica gel for further analysis. Neutral lipids were directly methylated and analyzed by GLC. Phospholipids were further separated by thin-layer chromatography with chloroform : methanol : acetic acid (65 : 25 : 4, vol/vol) as solvent, and glycolipids, by replacing acetic acid by water in the same solvent [38]. After the second separation, each group of lipids were extracted, transmethylated with BF_3 in methanol, and analyzed by GLC [38]. Methylpentadecanoate was used as the standard for quantitation.

Gel Electrophoresis and Immunoblotting

PAGE was performed on 12% gel according to Laemmli [17]. On gels, proteins were stained with Coomassie blue, and glycoproteins were detectd with the periodic acid-Schiff method according to previously published procedures [47]. Electrophoretic transfers from gels were carried out using standard procedures. Renaturation of the blotted proteins, blocking of the nitrocellulose sheet, and incubations with appropriate dilutions of antisera were done according to previously published procedures [7]. Immunoblots were developed with ^{125}I-labeled anti-rabbit Ig $F(ab')_2$ fragment from donkey (Amersham IM1340).

Other Methods

Proteins were estimated by the method of Bradford [10] using BSA as the standard. Triton X-114 phase partitioning of membrane proteins into integral and peripheral proteins was performed as previously reported [21].

Results

The major step in the purification of the apical domain of the pancreatic acinar cell is the sequential differential centrifugations. It led to an 11-fold purification of γ-GT (Table 1). The WGA Sepharose affinity column yielded an additional sevenfold purification. The entire procedure finally led to a 200-μg fraction enriched 75 times in γ-GT, the only known marker for this membrane [12, 25]. As shown on immunoblots probed with anti-γ-GT (Fig. 1*d*), both subunits of the γ-GT were present in the fraction. As a counterpart, Na,K-ATPase, which is strictly confined to the acinar cell basolateral domain [43],

[1] *Abbreviations:* ER, endoplasmic reticulum; γ-GT, γ-glutamyltranspeptidase; GLC, gas-liquid chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; TPP, thiamine pyrophosphate; WGA, wheat germ agglutinin; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor.

Table 1. Purification of pancreatic acinar cell apical plasma membrane by affinity chromatography on WGA Sepharose[a]

Fraction	γ-GT	ATP-diphospho	Total ATPases	Na,K-ATPase	Cyt. *c* oxidase	TPPase	Alk. Pase	A260/A280
Homogenate	388 ± 89	14.1 ± 3.6	17.8 ± 6.7	2.66 ± 0.80	170.3 ± 12.0	1.54 ± 0.34	2.44 ± 0.71	1.61 ± 0.16
Washed plasma membrane	4,337 ± 391	180.5 ± 27.8	125.3 ± 26.5	Not detected	54.2 ± 12.1	2.91 ± 1.03	Not detected	0.92 ± 0.01
Flow through	5,821 ± 852	301.0 ± 29.4	231.0 ± 28.2	Not detected	47.3 ± 13.9	2.85 ± 0.84	Not detected	0.90 ± 0.15
Apical membrane	28,890 ± 3,450	432.1 ± 40.1	445.9 ± 42.3	Not detected	Not detected	2.92 ± 0.95	Not detected	0.83 ± 0.19
(Bound to WGA)	**(74.5)**	**(30.6)**	**(25.1)**					

[a] Specific activities of marker enzymes.
γ-GT activity in nmol · min^{-1} · mg^{-1} protein.
ATP-diphosphohydrolase, ATPases and TPPase in nmol of P_i liberated · min^{-1} · mg^{-1} protein. Limit of detection: 1 nmol · min^{-1} · mg^{-1}.
Alkaline phosphatase in μmol *p*-nitrophenyl produced · min^{-1} · mg^{-1} protein. Limit of detection: 0.1 μmol · min^{-1} · mg^{-1}.
Cytochrome *c* oxidase in nmol of cytochrome *c* oxidized · min^{-1} · mg^{-1} protein. Limit of detection: 10 nmol · min^{-1} · mg^{-1}.
Numbers in parentheses are purification factors.

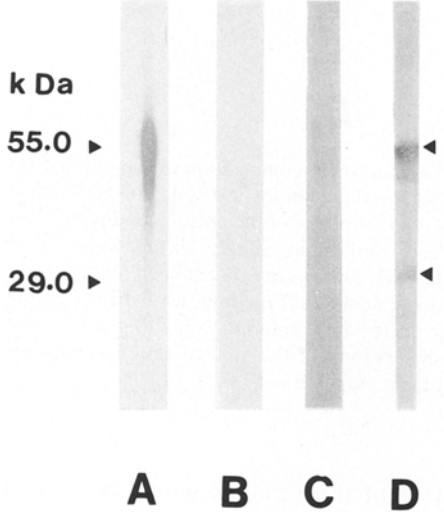

Fig. 1. Detection of amylase, keratin and γ-GT in rat pancreatic acinar cell apical membrane with specific antibodies. Three specific antibodies were reacted with Western blots of proteins of the pancreatic acinar cell apical membrane fraction (200 μg) (lanes *B*, *C* and *D*) and with proteins of pancreatic secretions (100 μg) (lane *A*): anti-amylase in *A* and *B*, anti-keratin in *C*, and anti-γ-GT in *D*. The two subunits of γ-GT (M_r 55,000 and 29,000) are indicated by arrowheads on the right. Use of anti-amylase was tested with the apical membrane fraction (lane *B*) to assess the identity of the major protein (M_r 55,000), since amylase has also a 55,000 M_r. *Lane A:* Pancreatic secretions, where amylase is the major constituent, served as a positive control for the anti-amylase reaction. The M_r 55,000 protein is not amylase. Antisera dilutions used were 1 : 5,000 for anti-amylase, 1 : 2,000 for anti-keratin, and 1 : 5,000 for anti-γ-GT.

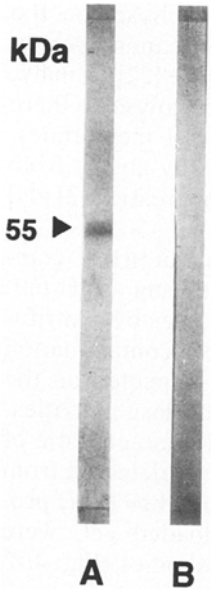

Fig. 2. Anti-Na,K-ATPase β1 subunit reactivity of the rat pancreatic acinar cell apical membrane. *Lane A* shows an immunoblot with kidney crude membranes (100 μg) as a positive control for the reactivity of the antibodies against the β1 subunit of the Na,K-ATPase. M_r of the β1 subunit is 55,000 (arrowhead). *Lane B* shows the lack of reactivity with proteins of the pancreatic acinar cell apical membrane fraction (200 μg). Antiserum dilutions used were 1 : 500.

could not be detected by activity (Table 1), nor by immunoreactivity on Western blots (Fig. 2*b*). Contaminations from other sources were also investigated. For the rough endoplasmic reticulum, the ratio of absorbance at 260 and 280 nm was an indicator for the presence of ribosomes. A factor of 1.9 is indicative of a crude rough endoplasmic reticulum fraction [46]. The ratio for the purified fraction was 0.83 (Table 1). This is close to 0.80, the ratio that we obtained for red blood cell ghosts (*results not shown*). The mitochondrial contamination, monitored by cytochrome *c* oxidase, could not be detected. Nucleotide hydrolases, however, were very markedly enriched in the final fraction. ATP diphos-

phohydrolase, which has been characterized in granule and plasma membranes [29, 33] as well as localized in the apical plasmalemma of the pancreatic acinar cell [5], is enriched more than 30-fold in the affinity purified fraction. Since the contribution of ATP diphosphohydrolase against ATP cannot be subtracted by use of any specific inhibitor, genuine ATPases would not account for more than 4 nmol · min^{-1} · mg^{-1} protein (Table 1). ATPases are consequently not major components of our membrane fraction. TPPase, a Golgi marker, was not notably enriched. Care must also be taken with this activity since ATP diphosphohydrolase has an affinity for TPP 1/10 the one for ATP [22]. In conclusion, most phosphatases measured in the apical membrane fraction could be attributable to ATP diphosphohydrolase. This interpretation is further supported by the absence of nonspecific phosphatases or alkaline phosphatase (Table 1). P-nitrophenyl phosphate, the substrate used for these activities, cannot be hydrolyzed by ATP diphosphohydrolase [22]. Finally, the absence of alkaline phosphatase shows that there is no contamination from ductal cell membranes. Alkaline phosphatase is indeed totally absent from acinar cells. It is only present in pancreatic [12] and parotid [2] ductal cells.

Though precautions were taken not to lyse granules during homogenization by buffering at pH 6.0, and by getting rid of any intact granules by centrifugation at 13,000 × *g* [29], possible contamination by granule membranes could be suspected on the ground of high ATP diphosphohydrolase activities. However, GP-2, the protein most characteristic of the granule membrane, could not be detected from Coomassie stained gels (Fig. 3), nor when these proteins, transferred from an overloaded gel, were probed with specific anti-GP-2 antibodies (Fig. 4*b*). Moreover, the specific γ-GT activity for pure zymogen granule membranes was 2 μmol · min^{-1} · mg^{-1}, 14.5 times less than the apical membrane fraction (28.9 μmol · min^{-1} · mg^{-1}). Contamination by zymogen granule membranes would therefore be limited, if any.

In order to bind to the affinity column, membrane vesicles should have the right orientation, with carbohydrates externally oriented. Consequently, inside-out vesicles would not bind to the affinity matrix. The effect of high ionic strength during the purification procedure was examined with the aim of maximizing the yield of right-side-out vesicles. Activation of latent γ-GT activity by addition of Triton X-100 was used to monitor the orientation of these vesicles. Under high ionic strength (150 mM NaCl), the membrane fraction would be in the form of unsealed vesicles or open sheets, while under low ionic strength (0 mM NaCl), 36 to 41% of the vesicles would be sealed in an inside-out orientation.

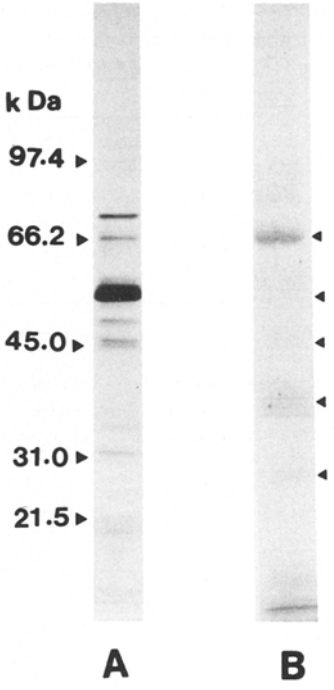

Fig. 3. Protein and glycoprotein composition of the purified rat pancreatic acinar cell apical membrane. *Lane A:* Polyacrylamide gel electrophoresis of the proteins of the apical membrane of rat pancreatic acinar cells purified by affinity chromatography on WGA Sepharose. *Lane B:* same as in *A* but stained for glycoproteins using the periodic acid-Schiff method; arrowheads on the right show location of the pink colored bands that were most evident by direct observation of the stained gel. The position where GP-2 would have migrated is 80 kDa.

Table 2 shows the lipid composition of the membrane fraction. The ratio of proteins to lipids was very high. Proteins constituted more than 75% of the membrane by weight. On the other hand, glycolipids made up more than 37% of the total membrane lipids, phosphatidylcholine only 14%. An important constituent of the lipids was phosphatidic acid which accounted for 32% of the lipids. It is worthwhile to mention that no lysophospholipids were detected.

Figure 3*a* shows an electrophoretic profile of the purified fraction. It is composed of six major proteins. The principal constituent is a 55-kDa protein that makes up more than 50% of the Coomassie blue staining. Other notable constituents are at 70, 67, and 48 kDa and a doublet at 45 kDa. Gels were also stained for glycoproteins by use of the periodic acid-Schiff method (Fig. 3*b*). Five bands stained at 66, 54, 45, 35 and 29 kDa, the principal glycoprotein being at 66 kDa. Phase separation of integral and peripheral proteins in Triton X-114 solutions (Fig. 5)

Table 2. Lipid composition of the rat pancreatic acinar cell apical membrane

	Washed plasma membrane		Apical membrane	
Class of lipids		% (weight)		
Total phospholipids	85.7	54.1		
Phosphatidylcholine	51.2 ± 1.1		13.9 ± 0.9	
Phosphatidylethanolamine + phosphatidylserine	13.6 ± 4.3		4.9 ± 0.4	
Phosphatidylinositol	11.8 ± 5.4		3.3 ± 1.1	
Phosphatidic acid	9.1 ± 1.6		32.0 ± 15.7	
Total glycolipids	8.4	37.5		
CMH	7.8 ± 1.4		11.2 ± 1.8	
CDH	Not detected		20.4 ± 7.4	
CTH	0.6 ± 0.1		5.6 ± 1.5	
Neutral lipids	5.9 ± 1.6		7.8 ± 2.0	
Lipids/proteins (mg/mg)	0.70 ± 0.08		0.24 ± 0.05	

CMH, CDH and CTH, ceramide mono-, di- and trihexosides.

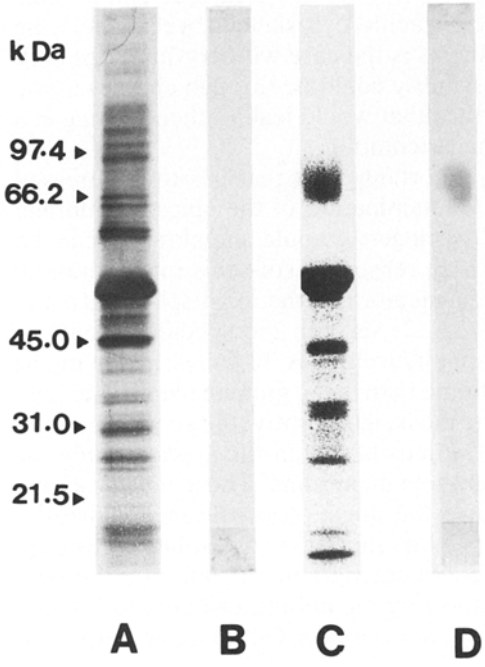

Fig. 4. Absence of GP-2 in rat pancreatic acinar cell apical membranes. The absence of GP-2 in Coomassie blue stained gels overloaded with rat pancreatic acinar cell apical membrane (lane *A*) was confirmed on Western blots by incubation with anti-GP-2 antibodies (lane *B*) (antiserum dilutions 1 : 50,000). Lane *D* shows the positive reactivity of the anti-GP-2 antiserum on blots of zymogen granule membrane proteins (lane *C*).

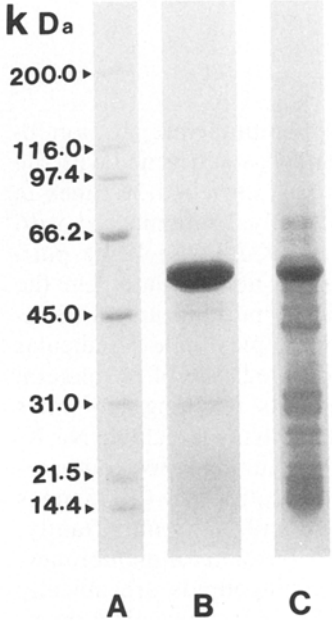

Fig. 5. Triton X-114 phase partitioning of integral and peripheral proteins of the pancreatic acinar cell apical membrane. Pancreatic acinar cell apical membrane proteins were subjected to phase partitioning in the Triton X-114 detergent system. Lane *B* shows proteins that partitioned in the aqueous phase (peripheral proteins), and lane *C*, in the detergent phase (integral proteins). There are two possibilities, the 55-kDa band is composed of two proteins, an integral and a peripheral protein, or the 55-kDa protein in *B* has lost a hydrophobic component. Lane *A* shows M_r standard proteins.

shows that there were two proteins in the 55-kDa band, an integral and a peripheral, or another possibility, that a fraction of the 55-kDa protein lost its hydrophobic moiety. Since the M_r of amylase is also 55,000, Western blots of the total apical membrane fraction were probed with antibodies against rat amylase in order to verify the identity of the soluble 55,000-Da band as amylase or not. No reactivity was detected with anti-amylase (Fig. 1*b*) nor with anti-keratin (Fig. 1*c*), an ubiquitous contaminant in the region of M_r 55,000 in gels [27] that could have made ambiguous the anti-amylase reaction. This shows that the 55-kDa band, both in the detergent and most particularly in the aqueous phase of the Triton X-114 separation, is a protein different from amylase though with an identical M_r. Besides, no amylase activity was detected in the fraction. More likely, the identity of the 55,000-Da band in the detergent phase of Fig. 5, would be the large subunit of the γ-GT. It reacted positively with anti-γ-GT antibodies in Fig. 1*d*. Many other minor bands were also found in the detergent phase, confirming that the apical membrane fraction is rich in integral proteins (Fig. 5*c*) and suggesting that the purification procedure washed out from the membrane most of the loosely bound peripheral proteins.

Discussion

The most enriched apical membrane preparation reported for an exocrine gland was performed with the parotid [2]. The fraction was enriched 26 times in γ-GT activity but was heavily contaminated with basolateral membranes, Na,K-ATPase being purified 20 times relative to the homogenate. On the other hand, the most highly purified apical membrane hitherto is the liver apical bile-canalicular membrane. It has been purified free of basolateral contamination to enrichments reaching 115 times relative to the homogenate with no detectable Na,K-ATPase activity [1]. Our fraction reaches comparable levels of purity with purification of γ-GT 75 times relative to the homogenate and, most importantly, without contaminations by basolateral membranes. In epithelial cells, glycosphingolipids are apically sorted at the expense of PC [42]. Our apical membrane preparation reflects this polar sorting of lipids. More than a third of the lipids in the apical membrane are ceramide mono-, di-, and trihexosides. PC represents only 14% of the lipids. These values are comparable to those reported for the intestinal brush-border membrane [15] and substantiate the apical origin of our membrane fraction. The high concentration of PA in the fraction is interesting. This enrichment is unusual. Two explanations could be advanced. Whether PA derives from the activity of a phospholipase D, or it indicates a contamination by ER membranes since PA lies at the branch point of glycerolipid synthesis in the ER [8]. The latter is less likely due to the very low level of ER contamination measured by the A260/A280 ratio (Table 1). The activity of a phospholipase D is, however, more plausible. Indeed EGTA had to be supplemented when membranes were prepared for lipid analysis. If omitted, more PA was present, suggesting that, during the course of the purification procedure, a hydrolytic activity, assimilable by its product to a phospholipase D, was active in the tissue.

Trying to produce membrane vesicles with a right-side-out orientation has led us to confirm that high ionic strength could have dramatic effects on the direction of the vesiculation process in the pancreatic tissue. This is a serious problem that one faces when purifying a membrane via the properties of its surface. As shown in this report, the proportion of inside-out vesicles could reach 68% of the vesicle population when the ionic strength was low. However, when all the steps were done in presence of 150 mM NaCl, from the homogenization and throughout the centrifugations, all γ-GT carrying vesicles were right-side-out, with their exoplasmic surface accessible. The molecular mechanism involved in the process is not clear but could make use of the contractile cytoskeletal web underlying the membrane as is the case with erythrocytes [44]. Another possibility could be through charges at the vesicles surface that would lead to their closing in a right-side-out orientation.

A very important point that has to be stressed here is that contamination of the apical membrane fraction by zymogen granule membranes can be ruled out. Three reasons justify this interpretation: first, the apical membrane fraction displays no membrane bound GP-2; second, the specific activity of ATP diphosphohydrolase is 10 times lower in the apical membrane than in the granule membrane [20]; and third, and most significantly, the specific activity of γ-GT is 15 times higher in the apical membrane than in the granule membrane. These observations, however, raise the question of the mechanism by which the apical membrane is assembled. More directly, is the zymogen granule membrane the only vesicle transporting membrane proteins to the cell apex, and if so, how could γ-GT get concentrated in the apical membrane, while GP-2 is withdrawn? The first possibility and simplest mechanism to conceive the biogenesis of the apical membrane would be that it is directly made by fusion of apically targeted specific constitutive secretory vesicles with a high specific activity of γ-GT. The second possibility would be that the recycling of zymogen granule

membranes would not engulf the whole fused granule membrane but, after dispersion of the proteins in the apical membrane, the endocytosis machinery would select some proteins to stay in the plasmalemma from others to be recycled for another round of secretion. γ-GT would then be the example of a protein selectively left in the apical membrane for final residence. This possibility raises some problems due to the very limited time for diffusion of membrane proteins after exocytosis. Endocytosis of the granule membrane is indeed such a rapid phenomenon in the secreting pancreas that it has been difficult to put in evidence an increase in the apical surface as a result of hormonal stimulation [37]. In addition, there is evidence in other tissues that free diffusion of granule membrane proteins does not occur after exocytosis [16, 30, 32, 45]. In these cases, granule membranes were shown to persist as patches on the cell surface. According to these reports and to our observations, the pancreatic acinar apical plasmalemma is therefore more likely to arise from fusion of constitutive secretory vesicles than from the trimming of zymogen granule membranes.

GP-2 is a protein linked to the zymogen granule membrane by a glycosyl phosphatidylinositol anchor [21, 36]. Evidence has been reported that an endogenous phospholipase C is present along the pathway transporting GP-2 to the cell surface, that cleaves its glycosyl phosphatidylinositol anchor, releasing GP-2 in resting secretions [31]. This secretion is thought to originate mainly from the constitutive secretory pathway. Confirmation of the presence of a GP-2 solubilizing phospholipase C in the purified apical membrane fraction has not been possible in our hands. Therefore, if the apical plasmalemma originated from the constitutive secretion, it would most likely be devoid of membrane-bound GP-2 given the evidence that the constitutive pathway is in contact with a phospholipase C activity capable of solubilizing GP-2 [31]. This is exactly what we report here, no membrane bound GP-2 in the apical membrane fraction. Accordingly, the only source of membrane-bound GP-2 at the cell apex would come from the zymogen granule membrane. Since no phospholipase activity has been identified in purified granule membranes [21], this absence of membrane-bound GP-2 in the apical membrane also argues against the zymogen granule membrane as being the source of material to assemble the apical plasmalemma. Finally, retrieval of GP-2 is not promoted by its glycosyl phosphatidylinositol anchor. Indeed when membrane proteins are substituted with such an anchor, their rate of endocytosis is greatly diminished [23, 26]. But GP-2 has been morphologically associated with structures of possible endocytic origin [4]. To explain these observations of endocytosed GP-2, the hypothesis of *en masse* endocytosis of the granule membrane therefore appears the most likely interpretation to bypass the impairment of endocytosis caused by GP-2's PI anchor. It thus appears that the observations with GP-2 also support the hypothesis that the pancreatic apical membrane would be mainly generated by fusion of vesicles from the constitutive secretory pathway.

In conclusion, we believe that this pancreatic apical membrane fraction will be a very precious tool to help define the molecular mechanisms of cellular secretion by the exocrine pancreas as well as the possible implication of this apical plasmalemma in anion transport across the epithelium. Apical anion transport is indeed the process which has been identified as defective in cystic fibrosis subjects [35], and the exocrine pancreas is severely affected by the pathology. However, before these vesicles could be used in a reconstituted system of exocytosis, their orientation should be reverted to inside-out in order to expose the cytoplasmic surface of the apical membrane to interacting zymogen granules.

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