Efficient binding of regulated secretory protein aggregates to membrane phospholipids at acidic pH

Jean LAINÉ and Denis LEBEL¹

Département de Biologie, Université de Sherbrooke, Sherbrooke, Québec, Canada J1K 2R1

Some regulated secretory proteins are thought to be targeted to secretory granules through an acidic-dependent aggregation in the *trans*-Golgi network. In this report we use pancreatic zymogens, a paradigm of regulated proteins, to test this hypothesis, because they qualitatively aggregate upon acidification *in vitro*. Pig zymogens were found to start to aggregate significantly at pH ~ 6.0, a pH slightly lower than that at which rat zymogens aggregate, but still compatible with the pH of the cell-sorting compartments. When pig zymogen granule membranes were mixed with the zymogens in the aggregation assay, membranes that normally floated on 1 M sucrose were observed to be pelleted by the aggregating zymogens. Rat membranes were pelleted by pig zymogens and vice versa. Igs, typical constitutively secreted proteins, which needed chemical cross-linking to serve as an aggregated protein control, pelleted membranes almost independently of pH. Corresponding cross-linked zymogen-

binding ability and pH dependence was unaffected by the chemical modification. Membranes treated with sodium carbonate, pH 11, or with protease K, were still pelleted by zymogens, suggesting that the aggregated zymogens bound to membrane lipids. This hypothesis was confirmed by the efficient pelleting of unilamellar vesicles composed of granule membrane lipids. Vesicles composed of single classes of phospholipids were also pelleted, but with various efficacies. We conclude that pancreatic zymogen aggregates, formed under the acidic conditions of the secretory pathway sorting compartments, have the capacity to bind firmly to membranes through their phospholipid constituents.

Key words: exocrine pancreas, secretion granule, targeting, zymogens.

INTRODUCTION

Proteins destined for storage in secretory granules must be sorted from constitutively secreted proteins. Two general mechanisms have been proposed to explain sorting into the regulated secretory pathway. The first mechanism suggests that within the *trans*-Golgi network (TGN) a receptor interacts in a pH-dependent (acidic) manner with regulated secretory proteins to direct them to the immature secretory granules where final condensation and proteolytic processing takes place [1]. This mechanism is called the sorting-for-entry hypothesis. The second model suggests that the targeting of regulated secretory proteins to immature secretory granules is less selective, in that aggregation under the acidic conditions of the TGN is sufficient to sort secretory proteins to the immature granules where final segregation from mis-routed constitutive proteins occurs. Further acidification would then lead to tighter aggregation of the regulated proteins, resulting in their exclusion from vesicles carrying non-aggregated constitutive proteins and lysosomal enzymes from the immature granules [2,3]. This mechanism is called the sorting-by-retention hypothesis (see [4] for a review). In a previous report we have shown that rat pancreatic zymogens aggregate under acidic conditions identical with those observed in the TGN where targeting to the regulated pathway is initiated [5]. The next step in the correct targeting of the zymogens is the snagging of the aggregate into a vesicle budding in the direction of the immature granule. Clearly, a receptor for the aggregate on the TGN and the immature granule would ease the process. Interactions with some membrane proteins located on the luminal side of granules have been reported, and argue in favour of such a receptor for aggregated proteins. Chromogranin B [6], chromogranin A [7] and dopamine β -hydroxylase [8] have been proposed as receptors

of this type for regulated protein aggregates in the adrenal medulla. In the exocrine pancreas this role was originally attributed to the granule membrane protein GP-2 for the zymogens [9], because, *in vitro*, it was shown to interact specifically at acidic pH with amylase. Amylase is the major pancreatic zymogen and is consequently the principal component of pancreatic aggregates targeted to the secretory granules. However, Colomer et al. [10] have shown that co-transfected GP-2 does not increase the targeting efficiency of transfected amylase to the regulated secretory pathway in AtT20 cells, raising serious doubts about the involvement of GP-2 as a targeting receptor in the exocrine pancreas. As an alternative to proteinaceous receptors, direct interactions of secretory proteins with membrane lipids could be considered. Such interactions would accommodate the sorting-by-retention hypothesis by eliminating the requirement for specific receptors in the TGN, thereby limiting to acidic aggregation the conditions for the successful targeting of proteins to the regulated secretory pathway. In this report we describe the acidic aggregation of pig pancreatic zymogens that was first demonstrated in the rat, and use these aggregates to investigate their ability to interact in a pH-dependent manner with purified granule membranes. We show that pancreatic zymogen aggregates can efficiently bind to phospholipids of the granule membrane in a pH-dependent manner without the involvement of any membrane proteins.

EXPERIMENTAL

Zymogen preparation and aggregation

Zymogen granule membranes (ZGM) were purified, as previously reported, in parallel with the zymogens (granule content) [11].

Abbreviations used: TGN, *trans*-Golgi network; GPI, glycosylphosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ZGM, zymogen granule membranes; MDP, membrane dipeptidase; GGT, γ -glutamyl transpeptidase.

¹ To whom correspondence should be addressed (e-mail dlebel@courrier.usherb.ca).

Briefly, the granules were purified by a series of differential centrifugations in sucrose. The purified granules were resuspended at pH 8.0 for lysis. After lysis at 4 °C, the released zymogens were separated from the membranes by layering the granule lysate on a step gradient composed of 2 ml of 0.3 M sucrose and 1 ml of 1.0 M sucrose. After centrifugation at 245 000 *g* in a swinging-bucket rotor, zymogens were collected on top of the gradient. ZGM floating at the 0.3 M–1.0 M sucrose interface were washed in 0.25 M KCl to release loosely adsorbed zymogens.

Interaction of aggregated zymogens with ZGM and lipids

Before being submitted to acidification, purified zymogens were supplemented with 0.4 mM Pefabloc® (a non-toxic, potent and irreversible inhibitor of serine proteases from Boehringer Mannheim) and routinely passed through a 0.22 µm filter to remove any preformed aggregates. Zymogen aggregation in the absence of membranes was performed as reported previously at 37 °C [5]. For the interaction of zymogens with ZGM, purified ZGM (250 µg) were mixed with purified granule content (16–18 mg) in a final volume of 1.4 ml. Co-aggregation was allowed to proceed for 5 min at 37 °C, or at the indicated temperature, in the presence of 0.4 mM Pefabloc® after adjusting the pH to the desired value between 4.0 and 7.0 with 140 µl of 1 M sodium acetate/acetic acid buffer. The mixture was then layered on a gradient composed of 400 µl of 1.0 M and 400 µl of 0.3 M sucrose for centrifugation at 200 000 *g* (50 000 rev./min) in a TLS-55 swinging-bucket rotor for 20 min at 37 °C in a TL-100 Beckman ultracentrifuge. Care was taken that the pH of each sucrose layer was adjusted to the pH of the assay. In the established ZGM purification procedure, membranes sediment at the interface of 0.3 M and 1.0 M sucrose, whereas zymogens stay in the overlaid sample. After centrifugation of the membrane–zymogen interaction mixture, the overlaid sample, the 0.3–1.0 M interface and the pellet at the bottom of the tube under the 1.0 M sucrose were recovered and assayed for total proteins, GP-2 and γ -glutamyl transpeptidase (GGT). For the continuous gradient fractionation of the reaction mixture, a 10–50 % continuous sucrose gradient was poured into a 13 ml centrifuge tube and the sample was loaded on top. After a 4 h run at 38 000 rev./min (270 000 *g*) at 4 °C in a SW41 swinging-bucket rotor, the gradient was fractionated from the bottom into 36 fractions of 350 µl. Fractions were weighed for density and assayed for GP-2. When large unilamellar vesicles composed of membrane lipids were used in the assay, 200 nmol of lipids were mixed with 7 mg of zymogens previously desalted on a P6-DG column (Bio-Rad) equilibrated in the same buffer so as to remove any contaminating low-molecular-mass phosphate compounds that could have interfered with subsequent phospholipid determinations.

Cross-linking of zymogens and Igs

Zymogens were cross-linked by adding 25 µl of 50 % glutaraldehyde to 10 ml of 0.22 µm filtered pig zymogen granule contents (~20 mg of protein/ml) supplemented with 0.4 mM Pefabloc®. After a 4 h incubation at 37 °C, 4 ml of 1 M lysine was added to eliminate any reactive glutaraldehyde. The mixture was then incubated overnight at room temperature under constant stirring. Cross-linked zymogens were washed three times in 30 ml of 20 mM Hepes/100 mM KCl/0.4 mM Pefabloc® (pH 7.4) solution by centrifugation/resuspension cycles and homogenized with a Teflon–glass homogenizer for use in the acidic interaction with ZGM. The interaction of the cross-linked

zymogens with the ZGM was performed with 8.3 mg of protein for every 220 µg of membrane.

Pig Igs (15 mg/ml), purified according to the method of Tijssen [12] in 10 ml of 150 mM sodium phosphate, pH 8.0, were cross-linked for 2.5 h under constant stirring at 37 °C by the addition of 25 µl of 50 % glutaraldehyde. After homogenization, the reaction was stopped by addition of 4 ml of 1 M lysine with constant stirring for 0.5 h. The mixture was dialysed extensively against 20 mM Hepes/100 mM KCl (pH 7.4), centrifuged and finally resuspended in 11 ml of the same buffer for final homogenization.

Analytical procedures

GP-2 was determined by competitive ELISA as reported previously [13]. GGT was determined in microtitre plates [14] using γ -glutamyl *p*-nitroanilide as substrate. Extraction of lipids from purified ZGM was performed by the standard chloroform/methanol procedure [15]. Large unilamellar vesicles were prepared by mixing membrane lipids and octylglucoside in chloroform. After evaporation of chloroform to dryness under a stream of nitrogen, the mixture was resuspended in 25 mM Hepes (pH 8.0)/200 mM KCl and sonicated for 10 min in a bath sonicator. Decreasing the concentration of octylglucoside to below the critical micelle concentration induced the formation of vesicles by dilution. Vesicles were harvested by centrifugation. Protein was determined using the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.) [16] using BSA as standard. Phospholipid P_i was determined according to standard procedures after mineralization in boiling perchloric acid for 20 min [17]. Unless otherwise mentioned, all chemicals were purchased from Sigma Chemical, Mississauga, Ontario, Canada.

RESULTS

Rat pancreatic zymogens have been shown to aggregate upon acidification to a pH equivalent to that found in the intracellular compartments involved in the sorting of proteins to the regulated secretory pathway [5], specifically the TGN and the im-

Figure 1 Effect of pH on the aggregation of pig pancreatic zymogens

Pancreatic zymogens were obtained from purified pig zymogen granules by lysis at pH 8.0. Acetate buffer solution (1 M) was added to zymogens present at a concentration of 10–12 mg/ml to give a final acetate concentration of 96 mM at the desired pH. After a 5 min incubation at 37 °C and centrifugation at 13 000 *g* for 30 min in a tabletop centrifuge, the pellet was assayed for proteins. Aggregation is expressed as the percentage of total proteins before acidification and represents individual results obtained in three separate experiments performed with three different zymogen preparations (solid line). The effect of Ca^{2+} on the aggregation was tested by addition of 500 µM EGTA to the aggregation assay (broken line).

Figure 2 Effect of temperature on the sedimentation of pig pancreatic zymogens on a sucrose step gradient

Purified pig zymogens were aggregated at acidic pH as described in Figure 1, but at three different temperatures. The sample was overlaid on a step gradient made from 0.3 M and 1.0 M sucrose, and centrifuged at 200 000 *g* for 20 min at the temperature at which the aggregation was performed (4, 25 and 37 °C). The pellet was assayed for protein and the results are expressed as the percentage of total protein in the sample.

mature secretory granule. The initial behaviour of pig zymogens in response to acidic treatment shows that zymogen aggregation starts at pH 6.2 and reaches a level of 15–20% aggregation at around pH 4.5 (Figure 1). This level is comparable with that attained with rat zymogens at pH ~ 6.0 [5]. The maximum aggregation of pig zymogens is reached at pH 4.0 and remains constant as the pH is further decreased (results not shown). The involvement of Ca^{2+} in this phenomenon was investigated by adding EGTA to the assay. As shown in Figure 1, EGTA impaired aggregation at pH ≥ 5.0 . The addition of millimolar concentrations of Ca^{2+} to the assay did not alter the pattern of aggregation in the physiological pH range (results not shown). Since calcium supplementation did not change the aggregation pattern, all subsequent experimentation was performed without the addition of Ca^{2+} to the purified zymogen fraction. The effect of temperature on aggregation was examined in order to optimize the assay conditions. Figure 2 shows that aggregation is much greater at 37 °C than at 25 °C. For instance, there is an almost 2-fold increase in aggregation at 37 °C as compared with 25 °C at pH 5.0. This effect was even more pronounced at lower pH values, where aggregation was almost totally inhibited by lowering the temperature. These results suggest that zymogen aggregation is mainly due to electrostatic interactions which increase at higher temperatures, as hydrophobic interactions increase at low temperatures [18]. A pH of 5.5 was selected for the acidic interactions between membranes and zymogens, first for a practical reason because in our hands it reproducibly produced a significant level of aggregation, and secondly, because this pH is close to that existing in the two compartments where interaction with membranes takes place, the TGN and the immature granule. The pH measured directly in the TGN in live cells is 5.9 [19] and the pH in immature granules is lower than in the TGN [20].

To monitor the interaction between purified ZGM and pancreatic zymogens, a different assay from that used for zymogen aggregation was required. We decided to use the increased density conferred on membranes by the binding of aggregated zymogens as a means of following the interaction between ZGM and zymogens. ZGM are purified by flotation on a 1 M sucrose cushion [11]. We postulated that significant binding of aggregated zymogens to ZGM would pellet the membranes under the 1 M

Figure 3 Interaction of ZGM with acidic zymogen aggregates

Purified pig ZGM were added to the pH-dependent aggregation assay along with purified pig zymogens. After 5 min at the indicated pH at 37 °C, the mixture was layered on a step gradient made from 0.3 M and 1.0 M sucrose and centrifuged at 200 000 *g* for 20 min at 37 °C. The pellet (A) and the band floating on top of the 1 M sucrose (B) were assayed for the presence of GP-2 and GGT. In (C), the aggregation mixture was overlaid on a 10–50% continuous sucrose gradient that was fractionated from the bottom and assayed for GP-2. The areas under the curve for the right-hand (denser) peak were 0.077, 1.050 and 0.783 for pH 7.33, 5.70 and 5.28 respectively.

cushion, especially if the aggregated zymogens could sediment through 1 M sucrose on their own. Consequently, we chose to use a two-step (0.3 M–1 M) sucrose gradient. The assay was tested by layering membrane-free zymogens, aggregated by acidification, on step gradients and centrifuging at three temperatures: 4, 25 and 37 °C. As shown in Figure 2, 13% of the proteins that aggregated at pH 5.0, and 37% of the proteins that aggregated at pH 4.0, were found in the pellet of the sucrose gradient at 37 °C. These amounts are very close to those measured

Figure 4 Specific pH-dependent interaction of cross-linked zymogens with ZGM

Purified ZGM were added to soluble or cross-linked zymogens as in Figure 3 for interaction at an acidic pH of 5.5 and at the control pH of 7.5. The mixture was layered on a step gradient composed of 0.3 M and 1 M sucrose and centrifuged at 200 000 *g* for 20 min at 37 °C. Cross-linked Igs were used as a control of constitutively secreted proteins. GP-2 immunoreactivity was assayed in the pellet to monitor ZGM and the results are expressed as a percentage of the total GP-2 in the assay. X-linked indicates cross-linked.

in the standard aggregation assay at similar pHs (see Figure 1 for comparison). To monitor membranes in the mixed assay with zymogens, GP-2 was chosen as it is the major ZGM protein [11]. Two fractions were monitored for GP-2: (1) the interface between 0.3 M and 1 M sucrose where ZGM normally float; and (2) the pellet under the 1 M sucrose. As shown in Figure 3, upon acidification of the mixture of ZGM and aggregated zymogens, ZGM that floated on 1 M sucrose at neutral pH (Figure 3B) were quantitatively found in the pellet when the pH was adjusted to 5.2 or less (Figure 3A). Acidification of ZGM alone did not affect their sedimentation behaviour (results not shown). Since GP-2 is a glycosylphosphatidylinositol (GPI)-anchored protein [21] that could be released from the ZGM by phospholipases present in the membranes [22], the suitability of GP-2 as a marker was counterchecked with GGT, a type II membrane protein that is not susceptible to release by phospholipases. Sedimentation of GGT and GP-2 were absolutely parallel in the gradient (Figures 3A and 3B), thus validating the use of GP-2 as the marker for ZGM. The shift in density of the ZGM upon acidification through interactions with the zymogens was also confirmed by isopycnic centrifugation on continuous sucrose gradients (Figure 3C). The continuous sucrose gradient, performed at three different pHs, showed that the density of the membranes increased from 1.065 at pH 7.33, to 1.125–1.145 at pH 5.28. These results ruled out the possibility that membranes were simply dragged down in the pellet of the step gradient by the mass of the aggregated zymogens.

The increased ZGM density at acidic pH in the presence of zymogens could also be explained by a physical entrapment of membrane vesicles in a meshwork created by the aggregated proteins. To assess this possibility, we used in the assay zymogens artificially aggregated by chemical cross-linking. The relative capacity of the zymogens to interact with membranes was estimated using cross-linked Igs, a paradigm of constitutively secreted proteins that do not aggregate under acidic conditions. Cross-linked zymogens interacted with ZGM under acidification as efficiently as native ones but with a much lower background at neutral pH (Figure 4). This low background with cross-linked zymogens could be explained by the neutralization by the cross-linking agent of lysine side-chains that decreased the number of positive charges on the zymogen aggregates. Conversely, acidification did not notably increase (0.6-fold) the capacity of cross-linked Igs to interact with membranes. These observations show

Figure 5 Cross-species interaction between pancreatic zymogen acidic aggregates and ZGM

Purified pig or rat ZGM were added to pig or rat zymogens for interaction at acidic pH. Of the two pHs used, 5.4 is better for pig zymogen aggregation, whereas 5.8 is better for rat zymogen aggregation. After pH equilibration, the mixture was layered on a step gradient composed of 0.3 M and 1 M sucrose and centrifuged at 200 000 *g* for 20 min at 37 °C. GP-2 immunoreactivity was assayed in the pellet to monitor ZGM and the results are expressed as a percentage of the total GP-2 in the assay. Results are the means of two representative experiments.

that pancreatic zymogens responded to a decrease in pH by aggregating spontaneously, and that acidification significantly increased their capacity to bind granule membranes. A comparable increase in membrane binding was not observed with the control aggregated proteins (Igs).

The possibility of cross-reactivity between the pig and rat specimens was also investigated. Using the appropriate pH for zymogen aggregation in each species (pH 5.8 for the rat and pH 5.4 for the pig), acidic interactions were studied between zymogens of one species and ZGM of the other, and *vice versa* (Figure 5). Zymogens of each species could pellet ZGM of the other. In these mixed-species assays, the ability to pellet ZGM was controlled by the conditions of the pig system.

The possibility that zymogens adsorbed to the membrane preparations were responsible for the interactions observed in the sedimentation assay was also investigated. All adsorbed proteins were released from ZGM by washing with 0.1 M sodium carbonate, pH 11.5. This procedure is known to release all adsorbed material, leaving only integral membrane proteins such as GP-2 and membrane dipeptidase (MDP) on ZGM [22]. Carbonate-treated ZGM behaved identically with native ZGM in the step gradient upon co-aggregation with zymogens (results not shown), suggesting that the integral membrane proteins GP-2 and/or MDP alone would be sufficient to interact with the aggregated zymogens and direct the ZGM into the pellet. To test whether proteins (GP-2 and MDP) or phospholipids were responsible for the acidic interaction with the aggregated zymogens, ZGM were treated with proteinase K for 16 h. Proteolysis did not reduce the capacity of zymogens to interact with the membranes upon acidification (results not shown). In addition, since pig ZGM are known to have a very high proportion of phospholipids and a corresponding low ratio of proteins [11], it appeared that the aggregated zymogens could bind directly to the membrane phospholipids. Phospholipids were extracted from ZGM, reconstituted into unilamellar lipid vesicles and used as the source of membranes in the co-aggregation assay with soluble zymogens. Figure 6 shows that pancreatic zymogens have the capacity to pellet the phospholipid component of ZGM as well. To identify which constituent phospholipids were principally involved in the binding to zymogen aggregates, unilamellar vesicles made with various species of phospholipids were assessed in the acidic pH assay with pancreatic zymogens. Phosphatidyl-

Figure 6 pH-dependent interaction of zymogens with ZGM phospholipids and different phospholipid species

Large unilamellar vesicles, composed of phospholipids extracted from ZGM or of various classes of phospholipids, were added to soluble zymogens for interaction at the acidic pH of 5.5. Control incubations were carried out with lipids in the absence of any zymogens. The mixtures were layered on a step gradient composed of 0.3 M and 1 M sucrose and centrifuged at 200 000 *g* for 20 min at 37 °C. Phospholipid P_i was assayed in the pellet and the whole 1 M sucrose phase because of the high buoyancy of pure lipid vesicles. The results are expressed as a percentage of the total lipids in the assay and are the means of three ($\pm$ S.E.M.) or two representative experiments.

inositol (PI) and phosphatidylserine (PS) were the lipids with the highest capacity to interact with zymogens (Figure 6). However, these two species of phospholipids are less likely to interact with zymogens *in vivo*, since their localization is almost entirely on the cytoplasmic leaflet of the membrane. Phosphatidylcholine (PC), lysoPC, phosphatidylethanolamine (PE) and sphingomyelin interacted similarly, but at half the level of PI and PS. Controls at pH 7.5 did not induce the appearance of any lipids in the pellet (results not shown). Since the ZGM are mostly constituted of PC, lysoPC, PE and sphingomyelin [11,23], the proportion of ZGM lipids pelleted by the acidic zymogens reflects well the actual composition of the granule membrane. The phospholipids most likely to interact *in vivo* with the zymogens are those localized on the exoplasmic leaflet of membranes, i.e. PC, lysoPC and sphingomyelin. The relative amounts of pelleted lipids in Figure 6 are lower than those in Figure 4, where ZGM were used and GP-2 was assayed in the pellet. This is because of the high amounts of lipids that had to be added in the case of Figure 6 due to the lower sensitivity of the P_i assay. Consequently, a lower proportion of the lipids could be pelleted by a similar amount of zymogens. These observations strongly suggest that pancreatic zymogens directly interact with membrane phospholipids in a pH-dependent manner in the acidic milieu of the intracellular sorting compartments of the secretory pathway.

DISCUSSION

The aim of this study was to assess whether pancreatic zymogens that aggregate under mild acidification could interact with granule membranes. Inherent in this is the ultimate objective of identifying the receptors involved in this interaction. Current theory postulates that in order to be efficiently engulfed in vesicles budding from the TGN in the direction of the regulated secretory pathway, proteins must bind to a membrane receptor (see [24] for a review). GP-2, the major protein of the ZGM, has been proposed to serve as such a receptor in the exocrine pancreas [9]. However, when co-transfected in AtT20 cells with amylase, the principal pancreatic zymogen in terms of mass, GP-2, was not found in the cell granules. These observations raised serious doubts as to the possibility that GP-2 is the receptor which targets pancreatic proteins to the regulated

pathway. In an attempt to identify this putative exocrine pancreatic receptor, we used a system composed of pig pancreatic zymogens and pig ZGM. Using this assay, we found that acidic zymogen aggregates interacted very efficiently with ZGM, as shown by their taking the originally floating membrane into the pellet in sucrose gradients. This interaction was extremely efficient with regulated secretory zymogens, but weaker with cross-linked Igs, the prototype of constitutively secreted proteins. The cross-linked Igs control was very unnatural, but necessary, because Igs (as well as all constitutively secreted proteins) are not known to aggregate upon acidification and could therefore not pellet membranes if bound as monomers. As a result, this control does not provide evidence for any specificity of binding, but rather provides an indication of the specific 'affinity' in acidic conditions of the zymogen aggregate for the membrane. In our system, the strength of interaction between zymogen aggregates and membranes appears strong even at pH 5.5, a pH that is not optimal for pig zymogen aggregation. At such a pH, which mimics the actual pH in the TGN and immature granule compartments, the binding of zymogen aggregates can still pellet membranes very efficiently. The phenomenon was not unique to pig zymogens; it could be reproduced with rat zymogens at a slightly different pH similar to that reported previously for this species [5].

Identification of the membrane components responsible for this interaction between ZGM and aggregated zymogens was then investigated. The possibility that the interaction was in fact due to a zymogen-zymogen interaction with zymogens still adsorbed to the membrane was ruled out by the continued interaction of ZGM, left with only its integral membrane proteins, with the aggregated zymogens. Extensive digestion of these washed membranes with protease finally ruled out any significant involvement of membrane proteins in the binding of aggregated zymogens. This finding was not totally unpredictable since ZGM are rich in phospholipids and very poor in proteins [11]. It was then observed that vesicles composed exclusively of lipids extracted from ZGM were as efficient as native membranes in the acidic interactions with zymogens, indicating that the acid-dependent binding of zymogens to the lipid component of membranes is a very significant phenomenon in the exocrine pancreas. This interaction between phospholipids and zymogens could explain the tight association that is habitually seen upon electron microscopic observation of zymogen granules in both pancreatic tissue and purified granule preparations [11]. Most likely this interaction is not universal as, for example, it is not compatible with the core structure of the insulin granules found in the endocrine pancreatic β -cells. However, in a model where sorting to the regulated pathway at the exit of the TGN is less specific and more dependent on aggregation, followed by the further segregation of constitutive proteins in a downstream compartment, a pH-dependent interaction with phospholipids would eliminate the necessity for a specific receptor in the initial targeting compartment. The ability of aggregates to bind membranes could then be viewed as a facilitating mechanism to direct regulated proteins *en masse* to the next compartment of the secretory pathway. Further along the secretory pathway, the strong binding of the aggregates to the maturing granule membrane would render impossible their accidental departure from the compartment. This binding to membranes could help repulse (to the opposite pole of the maturing granule) membrane proteins that have no affinity for the aggregates and that are likely to be involved in the specific targeting of soluble constituents, such as the lysosomal cathepsins which are found at low levels in the pancreatic mature granule, to their appropriate destination [2]. These observations argue in favour of the sorting-by-retention hypothesis [4]. Support for this mechanism in the exocrine

pancreas comes from the observation that the sorting of lysosomal cathepsins takes place in immature granules, which are further along the regulated secretory pathway than the initial aggregation of regulated proteins [2,25]. In this system, where the sorting of a secreted protein to the regulated pathway would be by default, the targeting competence of the protein would rely greatly on its presence in the regulated protein aggregate. The concomitant pH-dependent aggregation and membrane binding that we have observed with pancreatic zymogens would clearly be cost efficient, particularly in cells where huge amounts of proteins have to be secreted, such as is the case in the exocrine pancreas. The acinar cell is a good example of a cell where a sophisticated mode of targeting proteins to the regulated pathway is not necessary because the consequences of mis-sorting are not catastrophic. Indeed, any mis-sorted apically secreted proteins would end up in the duodenum where the main function is proteolytic degradation. Under these conditions the critical step for the targeting of a protein to the regulated pathway would reside in the capacity of that particular protein to aggregate upon acidification, or for it to have an affinity for the aggregate. The phenomenon could then explain the observations that the transfection of proteins into foreign cells possessing a regulated secretory pathway led to correct targeting of some [26], but not all, regulated proteins from the same cell [10,27]. Exceptionally, this phenomenon could also lead to a protein that is constitutively secreted in the original cell being actively accumulated in a foreign cell [28].

Based on these observations, one could conclude that an affinity for membranes would be the property that leads a protein to the regulated secretory pathway. However this is not the case since the pancreatic granule membrane protein GP-2, which is bound to the membrane by a GPI anchor, is not targeted to granules when expressed in non-pancreatic cells [10]. In the pancreas the best hypothesis to explain the targeting of GP-2 to the granules is through binding to amylase [9], the major component of pancreatic acidic aggregates [5]. The targeting of MDP to the pancreatic granule membrane [22], which is also GPI anchored, should satisfy the same rationale. Though, if this hypothesis is incorrect, one would be forced to conclude that there is no distinct apical constitutive secretory pathway in the exocrine pancreas and that all proteins destined for the apex of the cell must be transported via the zymogen granules.

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Note added in proof (received 5 January 1999)

During revision of this manuscript, it was reported that immunoglobulin polypeptides enter the regulated secretory pathway in AtT20 cells [29]. In Figure 6 we report that Igs have a high capacity to bind to membranes at acidic pH. Together, these observations support the conclusion that the binding to membranes at acidic pH is a mechanism of consequential importance for targeting proteins into the regulated secretory pathway.

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