Reconstitution *in vitro* of the pH-dependent aggregation of pancreatic zymogens *en route* to the secretory granule: implication of GP-2

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Regulated secretory proteins are thought to be sorted in the trans-Golgi network (TGN) via selective aggregation. To elucidate the biogenesis of the secretory granule in the exocrine pancreas, we reconstituted *in vitro* the conditions of pH and ions believed to exist in the TGN using the end product of this sorting process, the zymogen granule contents. Protein aggregation was dependent on pH (acidic) and on the presence of cations (10 mM Ca^{2+}, 150 mM K^+) to reproduce the pattern of proteins found in the granule. The constitutive secretory protein IgG was excluded from these aggregates. Zymogen aggregation correlated with the relative proportion of the major granule membrane protein GP-2 in the assay. These results show that the glycosylphosphatidylinositol-anchored protein GP-2 co-aggregates with zymogens in the acidic environment believed to exist in the pancreatic TGN, and thus suggest that GP-2 would function as a membrane anchor for zymogen aggregates, facilitating their entrapment in budding vesicles directed towards the regulated secretory pathway.

INTRODUCTION

The biogenesis of the secretory granule requires the segregation of two kinds of secretory proteins: those that must be tightly packed and concentrated in the granule for release by stimulation of the cell (regulated secretion), and those that are immediately secreted at the cell surface without any concentration (constitutive secretion). The intracellular compartment where this segregation takes place has been identified as the trans-Golgi network (TGN) (Griffiths and Simons, 1986; Pfeffer and Rothman, 1987). In endocrine cells, the sorting process has been shown to depend strictly on the acidification of intracellular compartments since addition of acidotropic drugs totally diverts regulated secretory proteins to the constitutive pathway, suppressing completely the formation of new secretory granules (Burgess and Kelly, 1987). The same phenomenon has been observed in the exocrine pancreas where methylamine also inhibits the formation of granules (De Lisle and Williams, 1987). Furthermore experiments performed *in vivo* have shown that mature endocrine, as well as exocrine, secretory granules are actively acidified (Mellman et al., 1986; De Lisle and Williams, 1987; LeBel et al., 1988). Such an acidification is believed to preserve secretory proteins in a state of condensation (Rothman, 1971; Niederau et al., 1986; LeBel et al., 1988). Indeed the *in vitro* stability of zymogen granules depends strictly on the maintenance of an acidic interior (LeBel et al., 1988). The pH-dependent aggregation of regulated secretory proteins is further supported by the property of soluble zymogens to spontaneously reaggregate upon acidification (Rothman, 1971). As was shown for endocrine granules (Burgess and Kelly, 1987; Pfeffer and Rothman, 1987; Tooze et al., 1987; Gerdes et al., 1989), aggregation is a necessary step in the formation of exocrine secretory granules (Tooze et al., 1989). Accordingly, two concomitant phenomena are associated with zymogen acidification: first condensation and concentration of the secretory material in the TGN, leading to the formation of the granule, and secondly segregation of the regulated secretory proteins from the constitutive proteins by exclusion of the latter from aggregates.

In this report, we have reconstituted an *in vitro* system that mimics the conditions of pH and ions believed to exist *in vivo* in the TGN. Pancreatic zymogens were obtained from purified granules lysed at alkaline pH values. This material constitutes the end-product of the sorting and condensation process that takes place in the exocrine pancreas. Using this zymogen granule content, we have reconstituted *in vitro* the acidic treatment undergone by these zymogens in the TGN. We show that a relatively slight acidification of the medium to pH 6.2 induced an important aggregation of zymogens. Ionic strength and ionic composition of the medium critically affected both the quantity and the identity of the proteins that aggregated. When mixed with zymogens, a typical constitutive secretory protein (IgG) did not co-aggregate upon acidification. This shows that the *in vitro* system of acidic aggregation of zymogens was specific for regulated secretory proteins.

The additional step concomitant or following the aggregation of regulated secretory proteins is the engulfment of these aggregates by vesicles budding from the TGN. To facilitate the phenomenon a membrane-bound receptor that would interact with aggregates has been postulated (Burgess and Kelly, 1987). In the exocrine pancreas, a glycosylphosphatidylinositol (GPI)-anchored protein, GP-2, is common to all granule membranes studied thus far (MacDonald and Ronzio, 1972). It constitutes more than 50% of the protein of the granule membrane in certain species. GP-2 is also found in a soluble form in the granule contents (Paquette et al., 1986). In this paper we show that the amount of zymogens aggregated by acidic titration correlates perfectly with the relative abundance of GP-2 in the reconstituted system. These observations suggest that in the exocrine pancreas, GP-2 is a membrane-bound receptor that routes aggregated zymogens to membrane evaginations of the TGN that pinch off to form nascent secretory granules.

EXPERIMENTAL

Purification of zymogen granules and their subfractions

Rat pancreatic zymogen granules were prepared according to

Abbreviations used: GPI, glycosylphosphatidylinositol; PMSF, phenylmethanesulphonyl fluoride; TGN, trans-Golgi network.

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previously published procedures (Pâquet et al., 1982) by a series of differential centrifugations in sucrose. The pelleted granules were resuspended in 20 mM Hepes, pH 8.0/100 mM KCl/0.5 mM phenylmethanesulphonyl fluoride (PMSF) for lysis. After 30 min lysis at 4 °C, the suspension is called the lysate. The released zymogens (called granule contents) were separated from the membranes by layering the granule lysate over a step gradient made of 2 ml of 0.3 M sucrose and 1 ml of 1.0 M sucrose. After centrifugation at 245000 *g* for 45 min, the zymogens collected on top of the gradient were free from granule membranes as confirmed by assay of marker enzymes (Pâquet et al., 1982). The granule membranes floating at the 0.3 M–1.0 M sucrose interface were resuspended in 0.25 M NaBr to release any adsorbed zymogens. The pelleted membranes were then kept at −20 °C in 5 mM Hepes, pH 8.0, containing 0.5 mM PMSF.

Purification of GP-2 and enrichment of the 70 kDa protein

GP-2 (with anchor) was purified by affinity chromatography on *Helix pomatia* agarose packed in a HR 5/20, 200 mm × 5 mm glass column (Pharmacia), equilibrated in 20 mM Tris/HCl, pH 8.0/150 mM NaCl/0.8% octylglucoside and run at 0.2 ml/min. Elution was carried out with 50 mM *N*-acetyl-D-galactosamine. The 70 kDa protein was enriched using a 75 mm × 7.5 mm Spherogel TSK DEAE-5PW column (Beckman) with a 72 min (flow rate of 1 ml/min) linear gradient from 5 to 200 mM potassium phosphate, pH 7.5, supplemented with 1 mM diisopropyl fluorophosphate. The load consisted of 5–6 mg of zymogen granule contents, equilibrated in 5 mM potassium phosphate, pH 7.5, containing 0.3 mM *o*-phenanthroline. A Model 126 h.p.l.c. system and a Model 166 detector (Beckman) were used throughout.

Protein concentration and ionic composition

Granule contents and lysate had a protein concentration of about 3 mg/ml in 20 mM Hepes/100 mM KCl. The aggregation assay required different ionic compositions at a protein concentration of approx. 10 mg/ml. These adjustments were made by ultrafiltration using a Centriprep 30 (Amicon). The procedure did not modify the protein composition of samples.

In vitro aggregation assay

In all assays, samples were slightly buffered with 5 mM Hepes and supplemented with 0.5 mM PMSF. Protein concentrations and ionic composition are mentioned in the text. Samples (200 μl) made of granule contents were slowly titrated to the desired pH value with dilute HCl. The pH value was monitored with a pencil electrode. After incubation for 15 min at room temperature, samples were spun at 4 °C on a Beckman Microfuge at 12000 *g* for 10 min. The supernatant (S_1) was collected, titrated back to pH 8.2 and kept for analysis, or for further acidification. The pellet (P_1) containing aggregated proteins was resolubilized in the same volume of the buffer used for the starting sample with a plastic pestle and kept for analysis or for further acidification. When the granule lysate was used in the aggregation assay, the acidified sample was centrifuged for 10 min on a 100 μl 1 M sucrose cushion in order to separate any free membranes from the aggregated zymogens. After centrifugation, when membranes were present on top of the sucrose cushion, they were collected along with the supernatant. Adjusting the pH value to lower than 5.0 induced irreversible protein precipitation. Care was therefore taken during the experimentation to avoid this complication.

The selectivity of the aggregation was tested by addition of non-immune rabbit IgG to the assay. Resulting pellets and supernatants were tested for the presence of IgG on polyacrylamide gels under non-reducing conditions.

Analytical procedures

GP-2 was assayed using a competitive e.l.i.s.a., a procedure described previously (Leblond et al., 1989). Proteins were determined using the bicinchoninic acid (BCA) protein assay (Pierce) using BSA as the standard. SDS/PAGE was performed using the discontinuous buffer system of Laemmli (1970) (6–15% linear gradients). Except for assays where IgGs were added, samples were reduced with 1% (v/v) 2-mercaptoethanol and heated at 90 °C for 3 min. All chemicals were of analytical grade. Electophoresis reagents were from Bio-Rad.

RESULTS

All the tests were performed at protein concentrations of 10–12 mg/ml in order to eliminate any effects due to the concentration of proteins. It has indeed been reported that the efficiency of condensation is affected by protein concentration, and that, below a certain concentration, the aggregation was not effective (Chanat and Huttner, 1991). However, the concentration used is far from 100 mg/ml, the concentration in the TGN that one can calculate from the protein concentration in the granule (260 mg/ml) (Rothman et al., 1989), and from the concentration factor of 2.5 for the zymogens between the TGN and the mature granule (Bendayan, 1984; Posthuma et al., 1988). It is also far from 75 mg/ml, the protein concentration found at times in stimulated rat pancreatic secretions. One fact is certain, however, the protein concentration in our assay is less than that inside the TGN. Thus we believe that the efficiency of interactions between proteins in the *in vitro* assay presented here are much lower than that expected to actually take place *in vivo*, in the TGN. It must be pointed out that the maximal amount of protein aggregating differed slightly from batch to batch of granule contents. However, this parameter was the only one to have an inconsistent behaviour and, as discussed later, can be reasonably interpreted.

Time course of pancreatic zymogen aggregation

Maximal aggregation had already been achieved after 15 min of incubation at all pH values. There was no additional aggregation even after 60 min of incubation. While a shorter incubation period would have been safer, in order to avoid proteolytic degradation during the assay performed at room temperature, it was, however, not feasible due to the time required to adjust precisely the pH value (about 10 min) in such small volumes. Proteases were controlled with PMSF.

pH dependence of pancreatic zymogen aggregation

In order to establish optimal conditions for zymogen aggregation, the effect of pH on the phenomenon was tested between pH 5.0 and pH 8.2. Except for salt-free conditions, maximal aggregation was observed between pH 5.9 and pH 6.2 (Figure 1). For salt-free samples, the optimal pH value was slightly lower at pH 5.7. No differences in the pH profile were observed when the assay was performed with the total lysate instead of the contents. Since pH 5.9 gave optimal aggregations under all ionic conditions tested, it was adopted for all further assays.

Effect of ionic strength on zymogen aggregation

Ionic conditions prevailing in the TGN where secretory protein aggregation is believed to take place (Tooze and Tooze, 1986), as

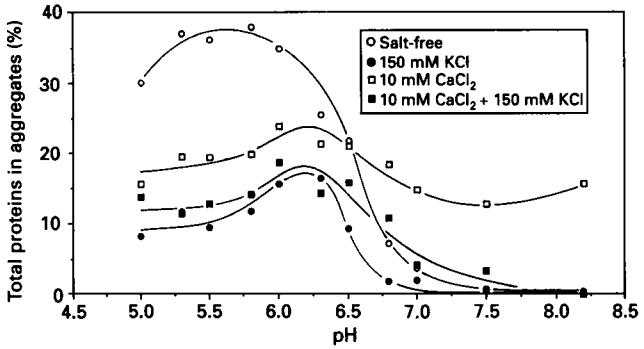

Figure 1 Effect of pH on the aggregation of pancreatic zymogen granule content

Pancreatic zymogens were obtained from purified zymogen granules by lysis at pH 8.0. After concentration of the proteins to a concentration of 10–12 mg/ml in working buffer (5 mM Hepes), the solution was carefully titrated to the desired pH value with HCl, microcentrifuged for 10 min, and the pellet assayed for proteins. Four ionic conditions were tested: working buffer without salts, adjusted at the indicated pH values (○); buffer + 150 mM KCl (●); buffer + 10 mM $CaCl_2$ (□); and buffer + 150 mM KCl and 10 mM $CaCl_2$ (■). Aggregated zymogens are expressed as a percentage of total proteins before acid titration.

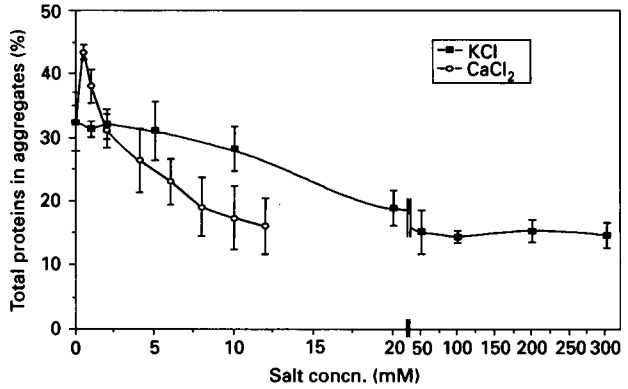

Figure 2 Effect of KCl (ionic strength) and Ca^{2+} on the pH-dependent aggregation of pancreatic zymogen granule content

Zymogens were titrated to pH 5.9 as described in the Experimental section in 5 mM Hepes containing increasing concentrations of KCl or $CaCl_2$. The amount of protein aggregated (expressed as a percentage of total protein before acidic titration) decreased upon addition of up to 50 mM KCl. $CaCl_2$ induced a slight increase at a concentration of 1 mM. At higher $CaCl_2$ concentrations, protein aggregation decreased to plateau at 10 mM $CaCl_2$.

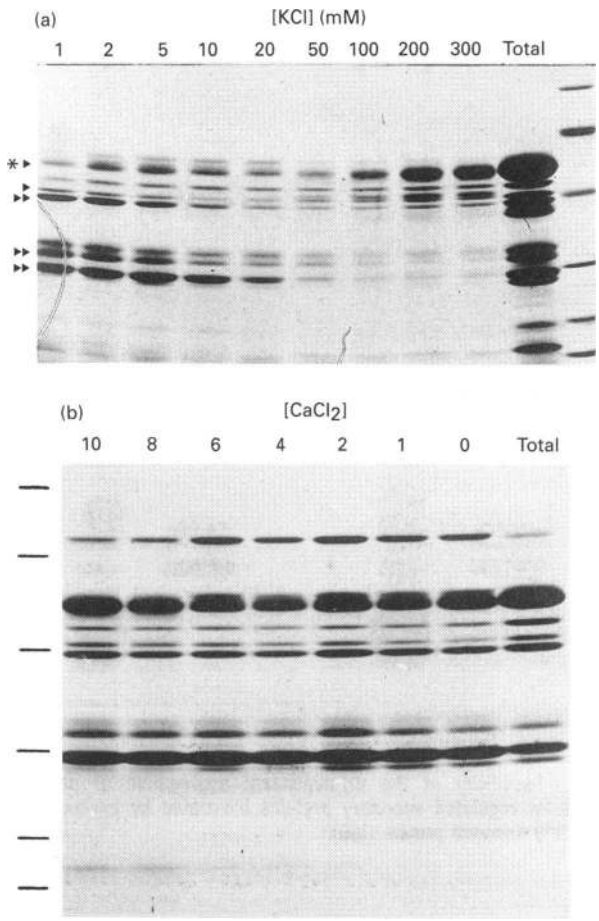

Figure 3 SDS/PAGE profile of the effect of KCl and Ca^{2+} on the proteins aggregating upon acidic titration of pancreatic zymogens

(**a**) SDS/PAGE of the proteins found in the pellet of pancreatic zymogens titrated to pH 5.9 in 5 mM Hepes with various concentrations of KCl, as described in the Experimental section. One of the most remarkable effects is the increase of amylase (*) aggregation upon addition of KCl. Amylase was a minor component of aggregates in KCl-free conditions (lane 1). Increase of the KCl concentration induced the more efficient aggregation of some bands (single arrowheads), and the decrease of others (double arrowheads). Each track was loaded with 100 μg of aggregated proteins. Molecular mass standards were run in the right-hand lane. (**b**) SDS/PAGE of the proteins found in the pellet of pancreatic zymogens titrated to pH 5.9 in 5 mM Hepes with various concentrations of $CaCl_2$, as described in the Experimental section. No selective aggregation of proteins was observed. At all Ca^{2+} concentrations, the pattern of aggregated proteins was identical to the starting zymogen granule content. Each track was loaded with 100 μg of aggregated proteins. Molecular masses of standards in (**a**) and (**b**) [indicated by bars on the left in (**b**)] were 94, 67, 43, 30, 20 and 14 kDa.

well as in condensing vacuoles and mature granules, have never been directly measured. In order to keep organelles in osmotic balance with the cytoplasm, it is assumed that the ionic concentration is identical to that in the cytoplasm. By purifying zymogen granules in the presence or absence of 150 mM KCl, followed by determination of their K^+ and Ca^{2+} content by atomic absorption spectrophotometry, we were able to confirm that pancreatic granules are readily permeable to K^+ (Gasser et al., 1988), but not to Ca^{2+} (LeBel et al., 1988). Granules actually lost all their K^+ during the course of the purification in medium lacking K^+. Similar observations have been reported for parotid granules (Izutsu et al., 1991). These observations suggest that the K^+ level, and consequently ionic strength in granules, would be similar to that prevailing in the cytoplasm, that is 150 mM KCl.

When KCl was added to the aggregation assay, a major decrease in the yield of zymogen aggregation was observed. The decrease was already maximal at 50 mM KCl (Figure 2). At higher concentrations, even as high as 300 mM KCl (twice the cytoplasmic concentration), no additional effect was observed. This effect was not ion specific since replacement of K^+ by Na^+ produced the same result. KCl addition modified the electrophoretic pattern of aggregated proteins. Figure 3(a) shows the electrophoretic profile of pellets obtained at different KCl concentrations. It must be emphasized that this gel was loaded with identical amounts of proteins in each track, and does not reflect the yield of protein aggregated. Increasing the KCl concentration induced the amplification of some bands (single arrowheads) in the aggregate and the reduction of others (double arrowheads). These reorganizations were observed at KCl concentrations higher than 20 mM. One of the proteins most affected by KCl was amylase (*), the major constituent of the granule contents (Figure 3a): at less than 50 mM KCl, no amylase aggregated. The protein aggregating at KCl concentrations lower than 50 mM in Figure 3(b) is not amylase but

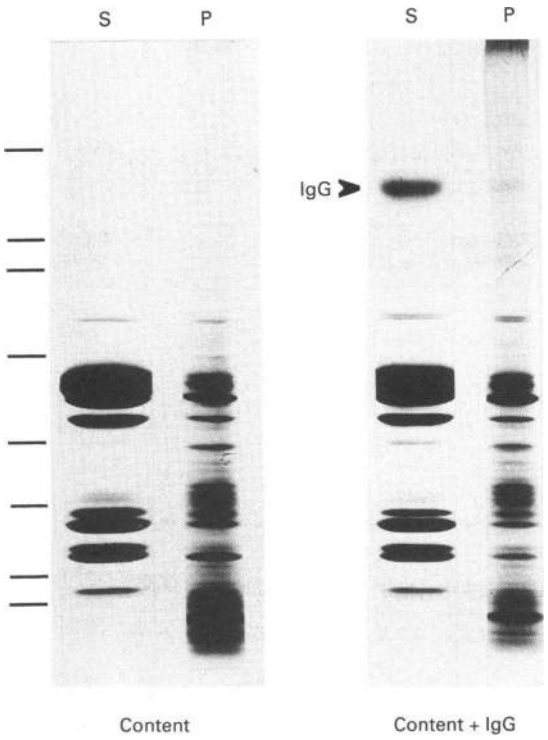

Figure 4 Specificity of the pH-dependent aggregation of pancreatic zymogens for regulated secretory proteins illustrated by exclusion of a constitutively secreted protein (IgG)

The system of pH-dependent aggregation containing 500 μg of zymogens was supplemented with 100 μg of IgG, a protein known to be constitutively secreted. After titration to pH 5.9 and centrifugation, the total pellet (P) and 20% of the supernatant (S) were analysed by non-reducing SDS/PAGE. Quantitatively all the IgG stayed in the supernatant. Only a very faint band of IgG could be observed in the pellet with pancreatic zymogens.

another zymogen migrating slightly more slowly than amylase [see Figure 3(b), 0–6 mM, and Figure 4, P]. In KCl concentrations $\geqslant$ 150 mM, the ratio of amylase to other zymogens became close to the level observed in purified granules. According to these observations, high ionic strength (approx. 150 mM) should therefore prevail in the cellular compartment (the TGN) where sorting of the zymogens takes place via acidic aggregation in order to sort secretory proteins in a ratio corresponding to that found in mature granules. This applies more particularly to amylase, which depends more strongly on KCl for aggregation.

Effect of Ca^{2+} on aggregation

The concentration of Ca^{2+} in pancreatic zymogen granules has been estimated to be 10–13 mM (LeBel et al., 1988). Ca^{2+} has been shown to play an important role in the protein aggregation of chromaffin granules (Gerdes et al., 1989). The precise concentration of Ca^{2+} in the TGN and maturing granules is not known. In the exocrine pancreas, it has been estimated to be approx. 10 mM by X-ray microanalysis (Roos, 1988). In our system, increasing Ca^{2+} concentration to 1.0 mM induced a slight increase in the amount of zymogens aggregating, from 34% to 43%. As was the case for KCl, further addition of Ca^{2+} decreased the efficiency of aggregation to more than half the amount obtained in the absence of salts (Figure 2). At low concentrations, Ca^{2+} had more effect than KCl on zymogen aggregation. The electrophoretic profile of the pelleted proteins was comparable at all Ca^{2+} concentrations, and the proportion of each particular zymogen was very similar to that found in the original zymogen granule contents (Figure 3b). In the presence of Ca^{2+} only, without KCl, the aggregation of zymogens was relatively independent of the pH value (Figure 1). Addition of 150 mM KCl re-established the pH dependence. It must be stressed that in the TGN, the exclusive presence of Ca^{2+} without KCl is highly unlikely. These observations show that ionic composition and pH are two determinant factors in the aggregation process. In the light of these results and of the indirect determination by Roos (1988), we decided to use 10 mM Ca^{2+} in the assay as the best approximation of physiological conditions existing in the TGN.

Specificity for proteins of the regulated secretory pathway

To establish the specificity of the mechanism for proteins of the regulated pathway, proteins known to be secreted by the constitutive pathway were added to the pancreatic zymogens in the aggregation assay. The protein tested was IgG. The choice of IgG was judicious because its isoelectric point is 8.6, close to that of amylase (pI 8.7) (Sanders and Rutter, 1972) which constitutes up to 30% of the zymogens in the granule. Aggregation was performed with 500 μg of proteins from the granule contents to which 100 μg of IgG were added. After acidification, IgG remained soluble. Only insignificant amounts pelleted with zymogens, yielding a very weak band on SDS/PAGE (Figure 4). The *in vitro* pH-dependent aggregation of pancreatic zymogens is therefore specific for regulated secretory proteins. It totally excluded IgG, a well-established constitutive secretory protein.

Evidence for the presence of aggregation-inducing factors in the granule contents

When the pellet (P_1) and supernatant (S_1) (Figure 5) generated by acidification of zymogen granule contents were separately re-adjusted at alkaline pH, and re-acidified, the amount of protein aggregated from the resuspended pellet was 85%, while it was only 13% from the supernatant. As a control, acidified granule contents, not separated into pellet and supernatant but simply redissolved by raising the pH of the whole mixture to pH 8.0, was acidified for a second round. The amount of protein then aggregated was identical to the non-treated content (approx. 20%). This shows that proteins first aggregated (P_1) by acidification of the whole contents had an optimal ratio of aggregating proteins. This interpretation is suggested by the exceptional response (85%) of this pellet to a second acidic treatment. On the other hand, the drastically reduced ability of the supernatant of this first acidification (S_1) to respond to a second acidic titration, suggests that the first pellet (P_1) brought down aggregating factors capable of co-aggregating some zymogens that do not aggregate by themselves upon acidification of S_1. On the gel, only one protein could be seen as being selectively concentrated in the pellet, P_1 (Figure 5). Its molecular mass was 70 kDa. Another protein of different molecular mass (80 kDa), not visible on the Coomassie Blue-stained gel, was also concentrated in the pellet. This protein was GP-2. Quantification by e.l.i.s.a. showed that more than 65% of GP-2 was recovered in the first pellet with 20% of the proteins, while 85% of GP-2 was recovered in the pellet of the second acidification (P_2) with 85% of the proteins. In order to know whether GP-2 and/or the 70 kDa protein had co-aggregating properties, they were separately added in increasing amounts to granule contents before acidification.

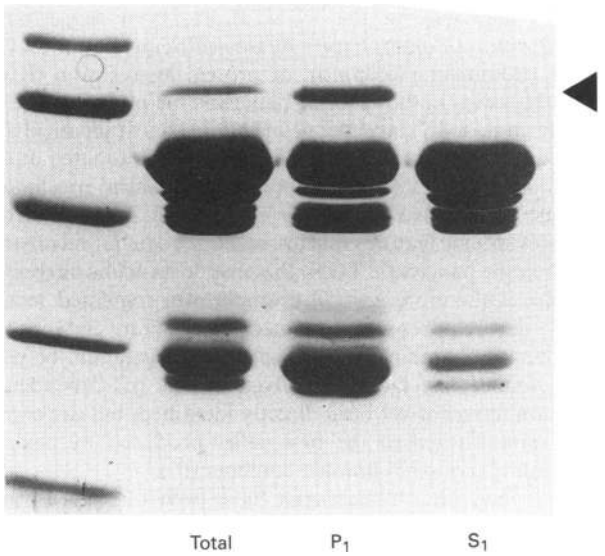

Figure 5 Protein composition of the granule contents, the pellet (P_1) of proteins aggregated at acidic pH, and the supernatant (S_1) containing non-aggregated proteins

Zymogen granule contents were titrated to pH 5.9 in 5 mM Hepes, 150 mM NaCl, 10 mM $CaCl_2$, according to the procedure described in the Experimental section. After centrifugation, 100 μg of the pellet (P_1), the supernatant (S_1) and the granule contents (Total) were analysed by SDS/PAGE under reducing conditions. A 70 kDa protein (arrowhead) was notably enriched in the pellet, and depleted in the supernatant. Molecular masses of standards run in the left-hand lane, are 94, 67, 43, 30, and 20 kDa.

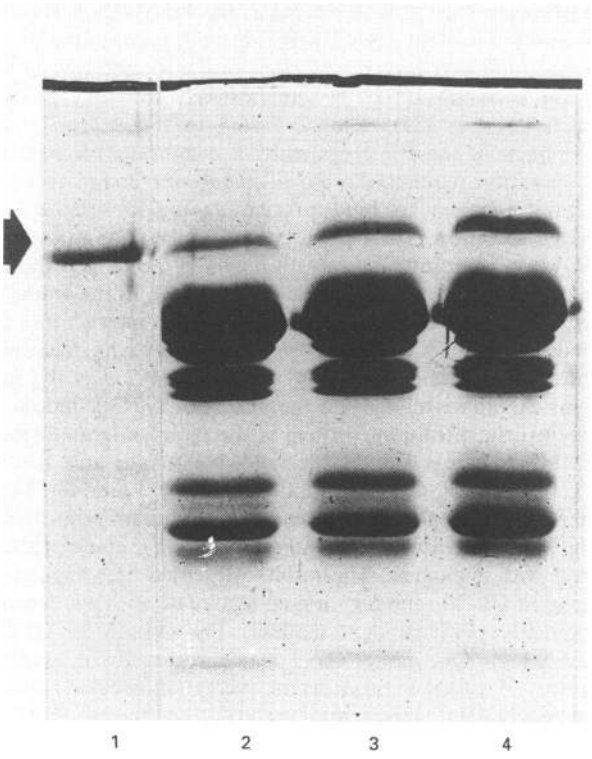

Figure 6 Effect of increasing concentrations of the purified 70 kDa protein on the composition of zymogens aggregating at acidic pH

A purified fraction of the 70 kDa protein (lane 1) was added to zymogen granule contents (lane 2) in ratios of one (lane 3) and two (lane 4) times the endogenous amount. The mixture was submitted to acidification at pH 5.9 and centrifuged. Lanes 2, 3 and 4 show pellets obtained with the control granule contents, the granule contents supplemented with one, or with two times the original amount of 70 kDa protein respectively. The same amount of protein was loaded in lanes 2, 3 and 4.

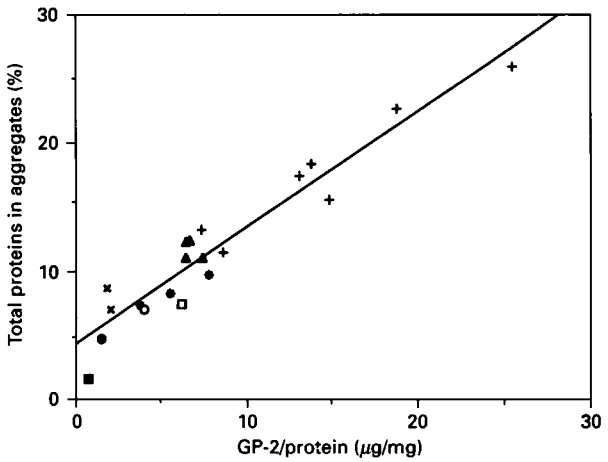

Figure 7 Relationship between the amount of zymogens aggregated at acidic pH and the specific activity of GP-2 in the granule contents

The percentage of proteins aggregated by acidification at pH 5.9 (5 mM Hepes, 150 mM KCl and 10 mM $CaCl_2$) of different preparations of zymogen granule contents was plotted against the specific activity of GP-2 (expressed as μg of GP-2/mg of protein). The results used in the plot were from two different granule contents (×) and four lysates (▲) (total lysed granules with membranes). The amount of GP-2 was also directly altered in contents by different means. Two contents were depleted of GP-2 (before depletion □ and ○; after depletion ■ and ●). Seven contents were supplemented with GP-2 using zymogen granule membranes (+) or the purified protein (*). A correlation coefficient (r^2) of 0.92 was obtained for the plot. GP-2 was determined by e.l.i.s.a. according to Leblond et al. (1989).

Effect of addition of various amounts of the 70 kDa protein and GP-2 on the capacity of pancreatic zymogens to aggregate upon acidification

Supplementing granule contents with one or two times the original content of the 70 kDa protein did not produce any increase in the amount or in the composition of proteins aggregated (Figure 6). We therefore concluded that the 70 kDa protein has a propensity to precipitate upon acidification but did not have any co-aggregating capacity. This lack of effect was predictable since the 70 kDa protein was not consistently present in granule contents (compare Figure 3a with Figure 3b). This behaviour is explained by the extreme sensitivity of the 70 kDa protein to proteolytic digestion. Granule contents had to be treated with 1 mM diisopropyl fluorophosphate and 1 mM *o*-phenanthroline to assure the preservation of the 70 kDa protein during its purification and during the assay.

In order to assess any direct role of GP-2 in the pancreatic zymogen aggregation, the amount of GP-2 in the assay was varied by different means. The amount of protein aggregated could then be plotted against the ratio of GP-2 and total zymogens (Figure 7). The different ratios of GP-2 in various contents and lysates (lysed granules with membranes) were the first source of variation for GP-2 concentration. Secondly, granule contents were depleted of GP-2 by passing preparations through an affinity column made of anti-GP-2 antibodies. This treatment reduced the amount of GP-2 by 75%, and caused a reduction of 55% of the pH-induced protein aggregation. Finally, enrichment of granule contents in GP-2 was performed by two different means: (1) by adding zymogen granule membranes, where GP-2 represents more than 20% of the proteins (LeBel and Beattie, 1984); and (2) by adding chromatographically purified GP-2 (anchored form). In both cases the magnitude of the aggregation increased with the amount of GP-2 added. The very low amount of detergent added with the purified GP-2 did not have any effect on aggregation. As shown in Figure 7, the

proportion of zymogens aggregated was in direct correlation ($r^2 = 0.92$) with the specific activity of GP-2 (i.e. the ratio of GP-2 to the total amount of protein in the sample). From the plot it was calculated that 1 μg of GP-2 induced the aggregation of 13.3 ± 4.8 μg of zymogens, while purified GP-2 aggregated 15.2 ± 5.9 μg. The phenomenon occurred with membrane-bound GP-2 (added membranes) as well as with the soluble protein (anchored or not). It was totally independent of the concentration of proteins in the assay ($r^2 = 0.14$). Variations in GP-2 specific activity between different contents could therefore explain the variability in the extent of aggregation observed from one batch of granule contents to another, even if the aggregation were carried out at identical protein concentrations.

DISCUSSION

Sorting and packaging of regulated secretory proteins to the secretion granule is carried out in an acidic milieu. This condition is postulated to participate in the selective aggregation of regulated secretory proteins, and consequently to segregate them from constitutive secretory proteins. Acidification of the secretory pathway starts in the TGN, at the time when proteins aggregate in coated vesicles, and lasts until they are finally secreted. The most acidic compartment along this pathway was determined to be the condensing vacuole (Orci et al., 1987). In this paper, we have worked with the final product of the pH-dependent aggregation, segregation, and packaging processes performed by the exocrine pancreas, that is the contents of purified secretory granules. With this fraction, we have tried to reconstitute *in vitro* the pH-dependent process that takes place *in vivo*, in the TGN. Conditions in the TGN are believed to be 150 mM KCl, 10 mM $CaCl_2$. Under these conditions, we have shown that pancreatic zymogens aggregate at an optimal acidic pH value of 5.9–6.2. This optimal pH value is very close to that estimated to exist in the TGN (Griffiths and Simons, 1986; Mellman et al., 1986; Anderson and Orci, 1988), that is pH 6.4. However, the actual pH value of the TGN is still uncertain since its determination has never been evaluated directly, but is based on assumptions. It is possible that the pH value is lower, but recent evidence confirms that pH 6.4 would be close to the actual pH inside the TGN (Chanat and Huttner, 1991). Even if the pH value were as low as 5.5, the phenomenon illustrated in the present report, would still take place (see Figure 1).

Ca^{2+} and KCl decreased the magnitude of the aggregation, most probably by inhibiting non-specific ionic interactions. Ca^{2+} was much more potent than KCl. On the other hand, while Ca^{2+} alone induced an aggregation that was independent of the pH value, addition of KCl re-established the strict acidic specificity of the phenomenon. Finally, Ca^{2+} and KCl had two very distinct effects on the pattern of proteins aggregated. The protein pattern changed significantly with KCl concentrations, while the presence of Ca^{2+} made the pattern of aggregated zymogens identical to the original granule composition. Thus, using ionic conditions similar to those believed to exist in the TGN, we could quantitatively and qualitatively aggregate pancreatic zymogens *in vitro*.

This aggregation was shown to be specific for proteins of the regulated secretory pathway. Indeed zymogen aggregates formed *in vitro* excluded the constitutive secretory protein IgG, a protein that is excluded from secretory granules *in vivo* (Rosa et al., 1989). Therefore conditions analogous to those existing in the TGN, where proteins are specifically sorted to the regulated pathway, induce specific aggregation of pure zymogen granule content, and exclude constitutively secreted proteins.

This *in vitro* system with pancreatic zymogens provides us with some indications on the mechanism by which regulated secretory proteins aggregate in the TGN. It has been suggested that neutralization of ionic charges by isoelectric precipitation would be the prominent mechanism of protein aggregation (Koenig, 1974). However in the exocrine pancreas, the pI of amylase is 8.7 (Reddy et al., 1987), and the pI of IgG is almost identical at 8.6. Consequently, both proteins become more protonated at acidic pH values, and would not respond to isoelectric precipitation. Amylase precipitated in the assay. IgG did not. Amylase therefore possesses specific features that are recognized in our reconstituted system in the pancreatic TGN, that enable its acidic aggregation. Amylase is therefore a good example of a regulated secretory protein that requires a specific mechanism, more elaborate than rudimentary charge neutralization, to aggregate and be packed in a granule. The factors involved in this pH-dependent aggregation have not yet been directly identified, but are expected to be concentrated in the first pellet produced by acidic aggregation of zymogen granule contents (P_1).

Until now, three phenomena have been directly associated with sorting of proteins to the regulated secretory pathway. The first one is the acidification of intracellular compartments along the secretory pathway (Tooze and Tooze, 1986). The second is the aggregation, in coated buds, of proteins emerging from the TGN and destined for regulated secretion (Tooze et al., 1987). The third is the preferential concentration of regulated secretory proteins along the secretory pathway (Bendayan, 1984; Posthuma et al., 1988). The final product of this process is a mature secretory granule with a very high internal concentration (Rothman et al., 1989) of osmotically inactive proteins in an acidic environment (Arvan et al., 1984; Niederau et al., 1986; LeBel et al., 1988; Anderson and Orci, 1988; Wong et al., 1991). It has been proposed that protein sorting in the TGN uses a universal mechanism of protein aggregation. This idea is supported by the demonstration that foreign secretory proteins expressed in host cells are correctly sorted to the host-cell secretory granules (Burgess et al., 1985). The next step in the process is the envelopment of these aggregates by a membrane for budding. This operation requires the use of membrane-bound receptors that are predicted to bind protein aggregates destined for regulated secretion. These have been postulated to exist in the TGN, where their interaction with regulated proteins is thought to be pH-dependent (Burgess and Kelly, 1987). In the search for a membrane-bound protein with the ability to interact in a pH-dependent manner with zymogens and serve as membrane receptor for regulated secretory proteins, GP-2 was the most obvious candidate to study in the pancreas. GP-2 is indeed the major intrinsic membrane protein of the zymogen-granule membrane in numerous animal species (MacDonald and Ronzio, 1972). In addition, GP-2 is found in equivalent amounts in the membrane and in the granule contents (Paquette et al., 1986). The latter observation made us suspect that GP-2 could probably interact with zymogens. The results presented here suggest the capacity of GP-2 to interact and co-aggregate with zymogens in an explicitly pH-dependent manner. The efficacy of GP-2 is relatively high. One mol of GP-2 can aggregate about 27 mol of zymogens (if 40000 is taken as an average molecular mass for the zymogens). Both membrane-bound and soluble forms of GP-2 have the same capacity. The mechanism by which GP-2 binds to zymogens and enhances aggregation is unlikely to be through electrostatic interactions, because GP-2 is an acidic glycoprotein (pI of 4.0–6.0), and a decrease of pH would neutralize GP-2's net charge as well as those of zymogens. Indeed most zymogens are slightly acidic, amylase being one of the few exceptions (Scheele, 1975). Hydrophobic interactions would therefore be more likely, satisfying, by the same token, the necessity to dehydrate the secretory protein aggregate for compaction in the secretory

granule (Wong et al., 1991). However, we cannot rule out the possibility that proteins other than GP-2 are also involved in the aggregation of regulated secretory proteins. In fact, the intercept on the y axis in Figure 6 suggests that other factors, not yet identified, exist, or that zymogens have an intrinsic capacity to aggregate by themselves at acidic pH values. This capacity manifestly exists but is low, less than 5% according to our observations, and would not be very efficient responding to the discreet acidification of the TGN.

In the model presented here, where care was taken to reproduce as close as possible the conditions prevailing in the TGN, GP-2 behaves as the postulated membrane-bound receptor that carries regulated proteins to budding vesicles. In its membrane-bound form, GP-2 binds to aggregated zymogens, and co-aggregates with them with high capacity in a pH-dependent manner. GP-2 has therefore the capacity to attach aggregated zymogens to the membrane of the TGN in a pH-dependent manner. These bound aggregates could be subsequently engulfed in budding vesicles via a clathrin-dependent mechanism (Griffiths and Simons, 1986). In addition, the property of self-association of GP-2 at acidic pH (Fukuoka et al., 1992) would facilitate the accretion of these complexes in such budding vesicles. Inside the condensing vacuoles and immature granules, the aggregate must be released from the membrane in order to allow completion of zymogen packaging and maturation of the granule. In an analogous system of sorting, lysosomal proteins are released from the mannose 6-phosphate receptor by a decrease in pH. However, the release of GP-2 from its cargo of zymogens is less likely to be efficiently achieved by an acidic environment since its binding is found to be preserved in such acidic conditions in our experimental conditions. However, the peculiar membrane anchor of GP-2 allows its release from the membrane by phospholipases (LeBel and Beattie, 1988; Fukuoka et al., 1992). In fact, the soluble GP-2 found in pancreatic secretions (Havinga et al., 1985; LeBel and Beaudoin, 1985; Rindler and Hoops, 1990) results from the action of an endogenous phospholipase C (Paul et al., 1991). Therefore, it is conceivable that when a vesicle carrying aggregated proteins bound to GP-2 fuses with immature granules, the phosphodiester bond of the GPI anchor could be hydrolysed by a phospholipase yet to be identified, thus releasing GP-2 and its cargo of zymogens into the forming granule. Two predictions of the proposed model are that GP-2 would be released from the maturing granule membrane and that it would be found in a soluble form in the secretion. Both predictions are borne out by experimental results (Geuze et al., 1981; Paquette et al., 1986; Rindler and Hoops, 1990; Paul et al., 1991). The proposed model and existing experimental results are consistent with GP-2 functioning as a carrier of aggregated zymogens to forming secretion granules.

Results reported here are the first evidence of a membrane receptor involved in a pH-dependent interaction with exocrine secretory proteins. An exocrine pancreatic Golgi membrane receptor has previously been reported (Chung et al., 1989). This receptor was shown to bind proteins at pH 7.0 and release them at acidic pH values. Despite its exocrine origin, it was only shown to bind soluble endocrine proteins. The universality of the aggregation-sorting phenomenon suggests that an analogous receptor exists in all secretory tissues with a comparable mode of action. It is then reasonable to expect that a receptor from one tissue could be operative, at least partially, in another tissue. Conversely, the more regulated proteins from two different tissues are related, the higher the chance that the receptor sorting the protein in one tissue could work in the other tissue. Here we have evidence that suggests such a functional equivalence for two related exocrine glands. When GP-2 was added to purified parotid granule contents, the acidic aggregation of parotid proteins increased by the same factor. Its capacity of aggregation for the parotid zymogens was 12.52 ± 3.94 μg of protein/μg of GP-2, almost identical to its capacity for pancreatic zymogens, 15.2 ± 5.9 μg of protein/μg of GP-2. This observation argues in favour of a certain degree of functional universality for the receptors involved in the sorting of regulated secretory proteins in exocrine glands.

Until now, the immunological approach to finding a protein common to the luminal aspect of all secretory granule membranes has been unsuccessful. In this line of thinking, GP-2 is very strictly restricted to the exocrine pancreatic tissue (Scheffer et al., 1980). It is additional proof for a lack of complete identity between membranes of secretory granules of different origins. But here we show that, even though no protein structurally similar to GP-2 exists in the parotid, GP-2 can efficiently aggregate parotid secretory proteins. One is then inclined to ask: If pancreatic GP-2 functions in the parotid, which biochemical structure of GP-2 is common to the protein accomplishing the same function in the parotid or in different glands? Apart from the particular conformation of the polypeptide moiety of GP-2, the common structure shared by proteins with a similar function could be the GPI membrane anchor. As discussed previously, cleavage of the GP-2 membrane anchor would be inherent to its function. The high mobility conferred to membrane proteins by the GPI anchor would indeed be a serious advantage for the diffusion of docked aggregates towards regions of the TGN where budding takes place. It is therefore possible that the GPI anchor is a feature common to proteins accomplishing a function similar to GP-2 in various exocrine or even endocrine secretory tissues. The implication of GP-2 in the phenomenon of aggregation of pancreatic zymogens is further supported by its specific binding to amylase in acidic conditions (Jacob et al., 1992).

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