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Exocrine granule specific packaging signals are present in the polypeptide moiety of the pancreatic granule membrane protein GP2 and in amylase: implications for protein targeting to secretory granules

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The mechanisms for segregation of secretory and membrane proteins incorporated into storage granules from those transported constitutively have been thought to be conserved in diverse cell types, including exocrine and endocrine cells. However, GP2, the major protein of pancreatic zymogen granule membranes, in its native glycosyl phosphatidylinositol (GPI)-linked form, is incorporated into secretory granules when expressed in exocrine pancreatic AR42J cells, but not in the endocrine cells such as pituitary AtT20. To determine whether the protein moiety of GP2 contains the cell-type specific information for packaging into granules, a secretory form of GP2 (GP2-GPI$^-$), with the GPI attachment site deleted, was generated and introduced into AR42J and AtT20 cells. Like native GP2, GP2-GPI$^-$ localized to the zymogen-like granules of AR42J cells and underwent regulated secretion. In AtT20 cells expressing GP2-GPI$^-$, however, the protein was secreted by the constitutive pathway. Thus, a granule packaging signal is present in the luminal portion of GP2 that is functional only in the exocrine cells. However, this cell-type dependent sorting process is not limited to GP2 or membrane proteins. Amylase, a major content protein of pancreatic acinar and serous salivary gland granules, was also secreted exclusively by the constitutive pathway when expressed in AtT20 cells. The cell-type specific targeting of GP2 to granules correlated with its behavior in an *in vitro* aggregation assay where it co-aggregated more effectively with content proteins from pancreatic zymogen granules than with those from pituitary granules. The results indicate that membrane and content proteins can interact during granule formation and that the packaging of exocrine proteins like GP2 and amylase is likely to be dependent on heterotypic binding to exocrine specific proteins.

Key words: amylase/exocrine cells/membrane proteins/secretory granules

Introduction

Membrane and secretory proteins destined for storage in secretory granules are selectively segregated from those exported constitutively (cf. Kelly, 1985). A number of studies have supported the idea that there is a universal mechanism for the packaging of proteins into secretory granules which is conserved between different cell types, including endocrine and exocrine cells (Burgess *et al.*, 1985; Wolf *et al.*, 1988; Castle *et al.*, 1992; Milgram *et al.*, 1992). For example, the exocrine granule protein trypsinogen was packaged in secretory vesicles in transfected AtT20 cells (Burgess *et al.*, 1985). In addition, in transgenic mice, growth hormone (GH), an endocrine product, was synthesized and stored in the zymogen granules of the exocrine cells of pancreas (Ornitz *et al.*, 1985). Gastrin and pancreatic polypeptide, hormones secreted by endocrine cells, were also reported to be routed to the regulated pathway and proteolytically processed when expressed in AR42J pancreatic acinar cells (Dickinson *et al.*, 1993). These results were interpreted to indicate that the process of granule biogenesis and the specificity of its components are conserved between cell types.

Recent evidence, however, indicates that there also exists diversity among secretory granules derived from different mammalian cell types. von Willebrand's factor (vWF), a secretory granule content protein of platelets and endothelial cells, when expressed in endocrine cells, was found to be excluded from the endogenous granules and instead to enter a structure resembling an endothelial cell Weibel–Palade body (Wagner *et al.*, 1991). We have shown that the major pancreatic granule membrane protein, GP2, does not enter granules in the endocrine cell lines, AtT20 and Rin 5F, although it is packaged in the zymogen-like granules of exocrine AR42J cells (Hoops *et al.*, 1993).

GP2 is a glycosyl phosphatidylinositol (GPI)-linked membrane protein (LeBel and Beattie, 1988; Hoops and Rindler, 1991). To explore whether the polypeptide moiety of GP2 could play a role in the cell-type specific targeting, we generated a construct (GP2-GPI$^-$) encoding a secretory version lacking the GPI anchor and expressed it in AtT20 and AR42J cells. We find that GP2-GPI$^-$, like the native protein, is packaged in secretory granules in AR42J cells and is constitutively transported in AtT20 cells. This cell-type specific packaging is also observed for another exocrine granule protein, amylase. In an *in vitro* aggregation assay, the interaction of GP2 with content proteins is much stronger with pancreatic zymogens than with pituitary, suggesting that its packaging is dependent on heterotypic binding to proteins that are not present in the pituitary cells.

Results

Localization of GP2-GPI$^-$ in permanent transformants of AR42J cells

We have previously observed that the GPI-anchored pancreatic membrane protein GP2 was packaged in secretory granules in AR42J cells but not in endocrine AtT20 or Rin 5F cells (Hoops *et al.*, 1993). To determine whether the cell-type specific packaging information is found in the

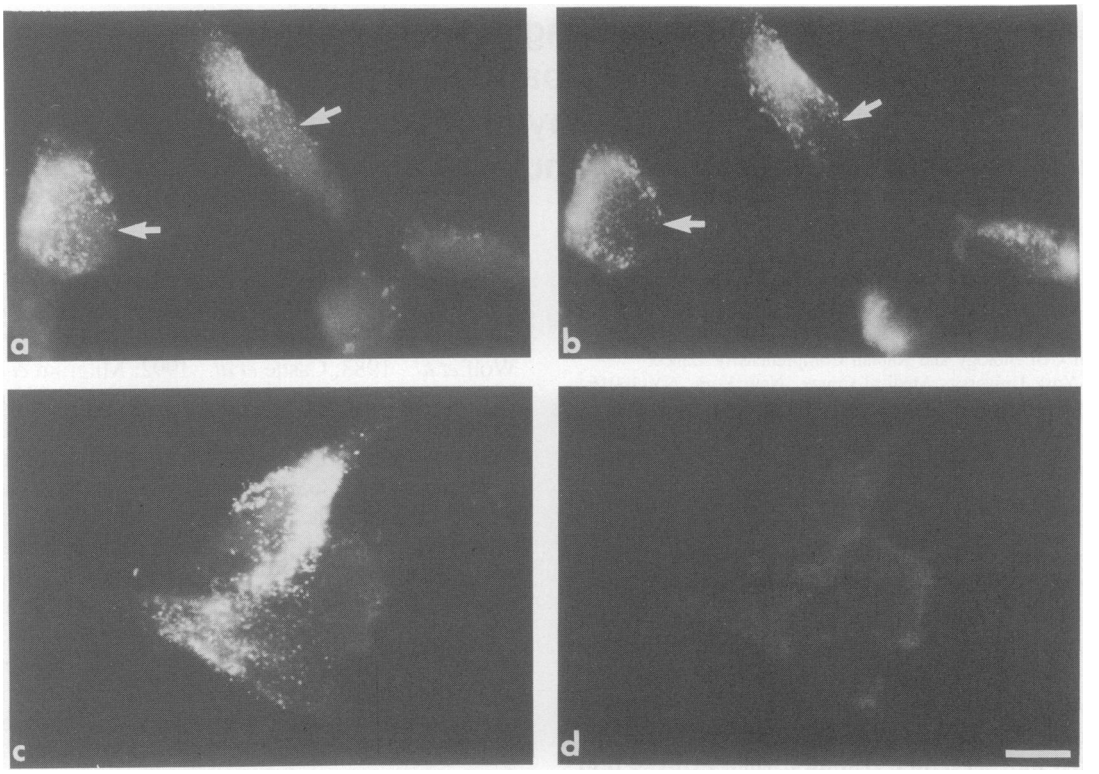

Fig. 1. GP2-GPI$^-$ localizes to the secretory granules of transfected AR42J cells. AR42J cells expressing GP2-GPI$^-$ were grown on poly-lysine treated coverslips and incubated with dexamethasone and Na butyrate according to the protocol described in Materials and methods. After fixation and permeabilization, the samples were stained for immunofluorescence first with rabbit anti-GP2 antibody and then with FITC-conjugated goat anti-rabbit IgG. After blocking with 100 μg/ml rabbit IgG, the coverslips were further incubated in either biotinylated rabbit anti-amylase IgG (**a** and **b**) or biotinylated non-immune rabbit IgG (**c** and **d**), and subsequently with Texas Red–streptavidin. Depicted are matched sets of transformants expressing GP2-GPI$^-$ photographed using FITC (a and c) or Texas Red (b and d) filters. GP2-GPI$^-$ is localized to intracellular vesicles (a and c). These vesicles (arrows, panel a) co-distribute with the marker for zymogen granules, amylase (arrows, panel b). No staining was observed when an irrelevant non-immune biotinylated rabbit IgG was used instead of the biotinylated anti-amylase (d). These results indicate that GP2-GPI$^-$ localizes to the storage granules in AR42J cells. Bar, 15 μm.

luminal portion of the protein, a secretory form of G2 (GP2-GPI$^-$) lacking the GPI attachment signal, was generated (see Materials and methods) and expressed in various cell lines. To confirm that the deletion resulted in a secreted GP2 with no adverse conformational changes, it was expressed in HeLa cells. In these cells, GP2-GPI$^-$, like native GP2, is initially synthesized as a core glycosylated ~70 kDa protein and converted to a terminally glycosylated ~85 kDa polypeptide which is rapidly secreted into the medium (data not shown).

The GP2-GPI$^-$ construct was then introduced into AR42J cells, derived from a pancreatic adenocarcinoma. In the presence of dexamethasone they partially differentiate and form zymogen-like granules where digestive hydrolases such as amylase are stored (Logsdon *et al.*, 1985). We have previously shown that these cells have no detectable GP2 protein (Hoops *et al.*, 1993).

Permanent transformants of AR42J cells were selected for resistance to G418 and isolated colonies were screened for the presence of the protein. By immunofluorescence staining of permeabilized cells, an intracellular vesicular pattern was observed (Figure 1). These vesicles (Figure 1a) corresponded to those that were stained with anti-amylase antibody (Figure 1b), indicating that GP2-GPI$^-$, like amylase, is found in secretory granules. To confirm these results, the cells were examined by immunolabeling at the electron microscopic level (Figure 2). Gold particles corresponding to GP2-GPI$^-$ were found in dense vesicles of transfected (Figure 2a) but not untransfected cells (Figure 2b). These vesicles have the typical appearance of the zymogen-like granules of these cells (Logsdon *et al.*, 1985; Hoops *et al.*, 1993). They also label well with anti-amylase antibodies (data not shown). Label corresponding to GP2-GPI$^-$ was also detected in smaller, translucent vesicles. The origin of these vesicles is uncertain, but they could represent constitutive transport vesicles in the process of transporting GP2-GPI$^-$ to the surface of AR42J cells, which package granule content proteins inefficiently (data not shown). They are unlikely to represent the neuroendocrine vesicles present in these cells since these synaptic-like vesicles lack pancreatic zymogens (Rosewicz *et al.*, 1992).

The prototypical assay to determine whether a protein is secreted by the regulated pathway is to analyze its capacity to be exocytosed upon incubation of the cells with a secretagogue. To this end, the AR42J/GP2-GPI$^-$ cells were labeled with [^{35}S]Met for 30 min, chased for 6 h and incubated for an additional 30 min in the presence or absence of cholecystokinin (CCK) to stimulate exocytosis of storage vesicles (Figure 3b). Amylase and GP2-GPI$^-$ were recovered by immunoprecipitation and analyzed after SDS–PAGE and fluorography. The release of amylase, which is packaged in secretory granules in AR42J cells, was stimulated ~2-fold by CCK (third and fourth columns in Figure 3a). Similarly, the amount of GP2-GPI$^-$ detected in

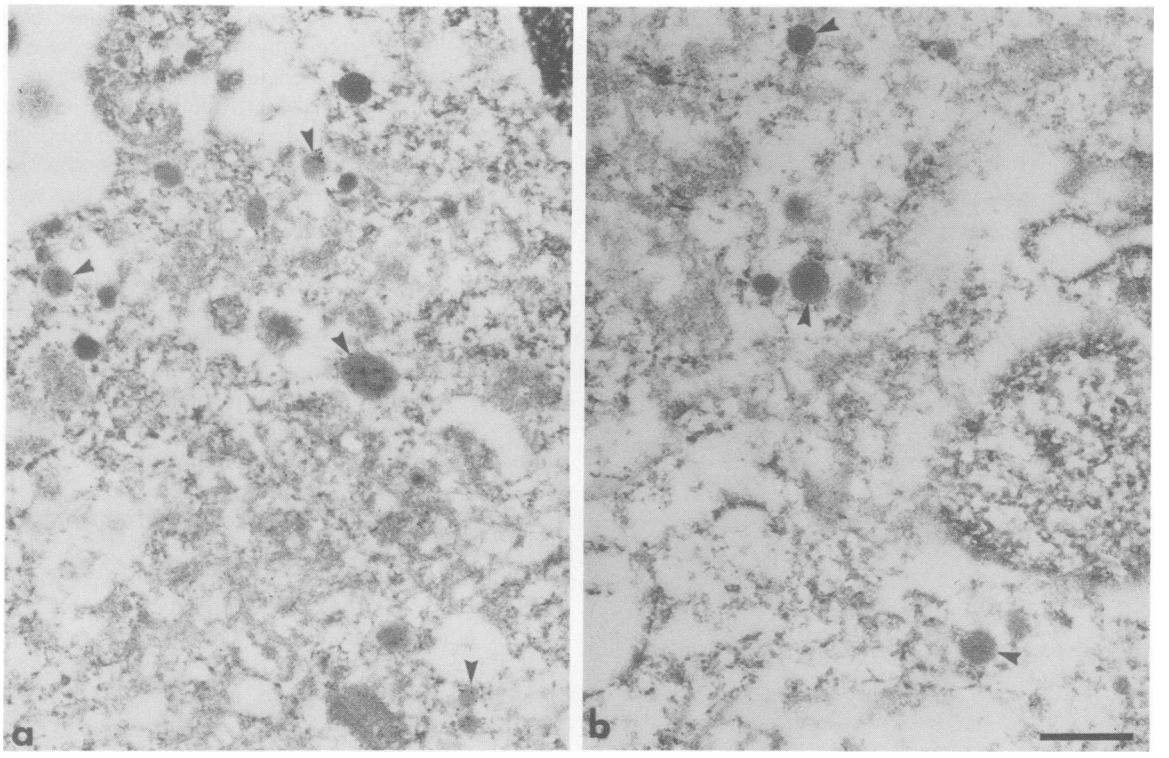

Fig. 2. By immunoelectron microscopy, GP2-GPI$^-$ localizes to secretory granules in transfected AR42J cells., AR42J cells grown on 60 mm dishes were incubated as in Figure 1. The cells were fixed and embedded in LR White (see Materials and methods). Sections on grids were incubated in anti-GP2 antibody followed by 10 nm protein A−gold. In cells expressing GP2-GPI$^-$, gold particles representing GP2-GPI$^-$ (arrowheads) are found primarily associated with secretory granules (**a**). Untransfected AR42J cells have no specific label over their granules (**b**). Bar, 0.5 μm.

the medium was increased by 2- to 3-fold (first and second columns in Figure 3a). Thus, GP2-GPI$^-$ is being secreted by the regulated pathway in transfected AR42J cells.

Localization of GP2-GPI$^-$ in permanent transformants of AtT20 cells

Having found that GP2-GPI$^-$ is packaged in granules in AR42J cells we proceeded to investigate whether, like native GP2, this behavior was cell-type specific. For this purpose the construct was expressed in the endocrine pituitary cell line, AtT20, which has secretory granules containing adrenocorticotropic hormone (ACTH) (Gumbiner and Kelly, 1982). Clones of permanently transformed AtT20 cells that expressed GP2-GPI$^-$ at high levels were isolated and characterized further. By immunofluorescence microscopy of permeabilized cells labeled with anti-GP2 antibodies (Figure 4a), a weak intracellular staining that had the appearance of endoplasmic reticulum was detectable. However, it did not correspond to the typical vesicular pattern observed using the anti-ACTH antibodies (Figure 4b). This result would be expected if GP2-GPI$^-$ were not stored in the secretory granules in AtT20 cells.

To confirm these observations, the exocytosis of GP2-GPI$^-$ was examined after incubation of the cells with 8-Br-cAMP, a secretagogue for these cells (Moore *et al.*, 1983). For the purpose of comparison, an AtT20 clone expressing human growth hormone (hGH) (a gift of Dr R.Kelly, UCSF) was included in this experiment (Figure 5). The cells were biosynthetically labeled for 1 h, chased for 5 h in medium containing excess methionine, and incubated with or without 8-Br-cAMP for 1 h. The amounts of GP2-GPI$^-$ detected in the medium in the presence or absence of 8-Br-cAMP (lanes c and d in Figure 5B) were similar (mean secretion ratio = 1.07 in two experiments). By contrast, the secretion of hGH (lanes g and h in Figure 5B) was stimulated 4.37-fold in this experiment (mean = 5.31-fold in two experiments). As a control, the activity of carboxypeptidase E (CPE), a marker of the regulated secretory pathway in these cells (Bloomquist *et al.*, 1991; Klein *et al.*, 1992), was assayed in the same media samples. Its activity was stimulated 6- to 10-fold (Figure 5A, second and fourth columns). We conclude that GP2-GPI$^-$ is segregated by the regulated pathway of secretion in the exocrine pancreatic cell line but not in the endocrine AtT20 cells. Native membrane-bound GP2 exhibits similar cell-type specific targeting to secretory granules (Hoops *et al.*, 1993), indicating that at least part of the sorting information is found in the protein moiety of the molecule.

Constitutive secretion of the truncated VSV G protein in AR42J cells

In pancreatic acinar cells, virtually all of the known proteins transported along the secretory pathway are packaged in secretory granules. Thus, one possible explanation for our results is that all secretory proteins are incorporated into granules by default. To test this possibility, a secretory form (G^-) of vesicular stomatitis virus (VSV) G, a protein known to be constitutively secreted in AtT20 cells (Moore and Kelly, 1986), was expressed in AR42J cells. Permanent transformants expressing G^- were isolated. By immunofluorescence microscopy, many cells in the population were clearly labeled with antibodies to VSV G (Figure 6a and c). However, this staining consisted generally of indistinct cytoplasmic staining reminiscent of endoplasmic reticulum.

Some highly expressing cells had in addition strong labeling in the Golgi region that did not correspond to the pattern observed using antibodies to amylase, a marker for secretory granules (Figure 6b and d).

The immunofluorescence results suggested that G^- did not accumulate in granules. To confirm these results, the AR42J/G^- cells were subjected to pulse–chase labeling and subsequent secretagogue stimulation (Figure 7). G^- was found to be secreted efficiently. Little G^- could be detected in the medium after a 6 h chase period (Figure 7B, top), and there was no increase in the presence of CCK (Figure 7A, first column). By contrast, amylase stimulation was readily observed in the CCK-treated samples (Figure 7B, bottom). In three similar observations, the ratio of G^- secretion in the presence and absence of CCK averaged 0.9 ± 0.4, while amylase in the same dishes was stimulated 3.6 ± 1.0-fold. These results indicate that G^- is secreted exclusively by the constitutive pathway. Thus, not all secretory proteins enter the regulated pathway in AR42J cells. Consequently, GP2-GPI^- must have specific information for sorting to secretory granules.

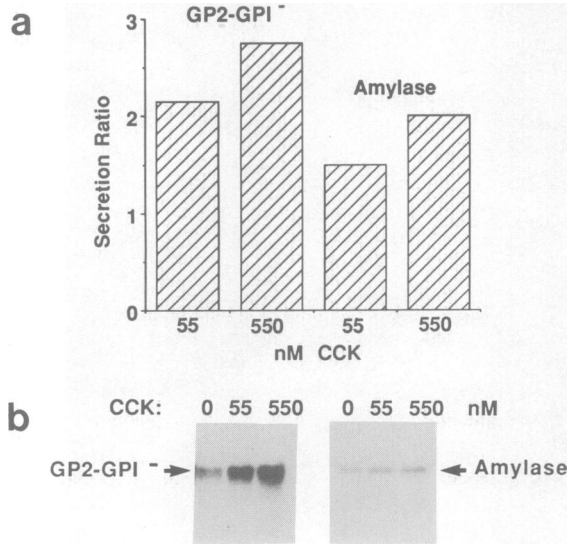

Fig. 3. GP2-GPI^- release into the medium is enhanced after incubation of AR42J transformants in the presence of CCK. AR42J cells expressing GP2-GPI^- were grown on 12-well plates and incubated as described in the legend to Figure 1. The cells were labeled with [^{35}S]Met for 30 min and chased for 6 h. They were then washed and incubated for an additional 30 min in the presence of the indicated concentration of CCK, to stimulate exocytosis of storage vesicles. Half of each medium sample collected was immunoprecipitated with anti-GP2 antibody (**b**, left) and half with anti-amylase antibody (**b**, right). After the precipitates were subjected to SDS–PAGE and fluorography, the amount of radioactive GP2 was quantified on a phosphorimager. The results are represented graphically in (**a**). The secretion ratio corresponds to the ratio of values obtained in the presence of secretagogue over those obtained in its absence after correction for total incorporation of [^{35}S]Met in each sample. The release of amylase, which is packaged in granules in AR42J cells, was stimulated significantly by CCK (third and fourth columns, panel a). Similar results were observed for GP2-GPI^- (first and second columns, panel a), indicating that it is also being secreted by the regulated pathway.

Constitutive secretion of amylase in transfected AtT20 cells

The packaging of GP2 in exocrine as opposed to endocrine cells implies that it interacts with exocrine specific proteins during granule formation. Jacob *et al.* (1992) reported that GP2 binds to amylase at pH 5.5. If the packaging of GP2 were to depend on amylase, its inability to be secreted by the regulated pathway in AtT20 cells could be due to the fact that amylase is not synthesized in these cells. To test this hypothesis, we set out to express amylase and GP2-GPI^- in AtT20 cells.

Initially, permanent transformants of AtT20 cells expressing amylase alone were isolated and screened by radiolabeling techniques for synthesis of the transfected protein. Surprisingly, by immunofluorescence microscopy, no specific staining for amylase above background could be detected in any of these clones (seven) or even in pools of transfected cells, which usually have a high percentage of expressing cells (data not shown). It is unlikely that this could have been the result of low level of expression of the protein since amylase enzymatic activity was readily detectable in the medium of transfected cells (data not shown) and a prominent band of the appropriate size was observed after immunoprecipitation of medium samples from radiolabeled cells followed by SDS–PAGE and fluorography (e.g. see Figure 8). To measure more precisely the amount of amylase

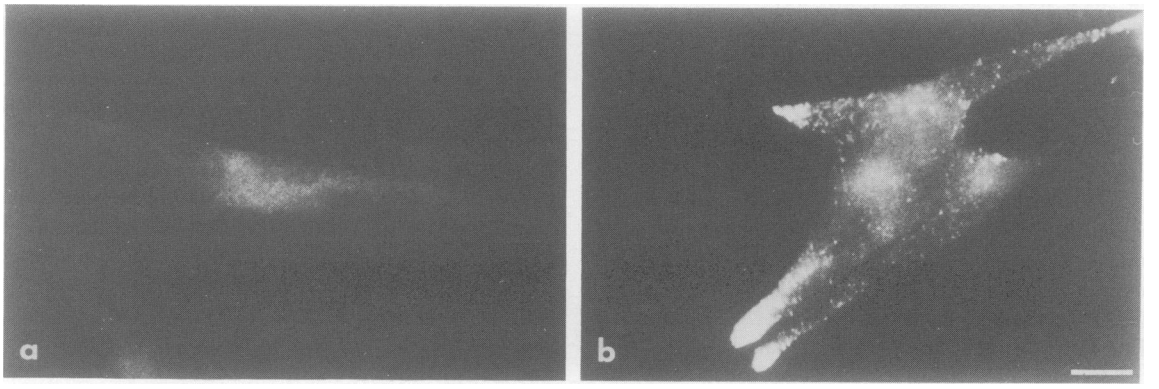

Fig. 4. GP2-GPI^- does not accumulate intracellularly in transfected AtT20 cells. Permanent transformants of AtT20 cells expressing GP2-GPI^- were grown on coverslips, and fixed in 4% paraformaldehyde in PBS. After permeabilization with detergent, the cells were stained for immunofluorescence with rabbit anti-GP2 (**a**) or anti-ACTH (**b**) antibodies. FITC–goat anti-rabbit was used as the secondary antibody. GP2-GPI^- does not accumulate intracellularly and residual staining is diffuse, most likely representing the protein in the endoplasmic reticulum (a). As expected, in the transformants expressing GP2-GPI^-, ACTH localizes to the endogenous secretory vesicles normally found in these cells (b). Bar, 9 μm.

packaged in granules, the secretagogue stimulation assay was employed. As shown in Figure 8, the stimulation of amylase secretion detected after incubation of the cells with 8-Br-cAMP was not significant, only ~1.2-fold in this experiment and averaging 1.04 ± 0.15-fold in three experiments. Using the same media samples, CPE activity was stimulated ±6-fold. Essentially the same results were obtained with permanent transformants of AtT20 cells expressing both amylase and GP2 (not shown). We conclude that amylase, like GP2, is packaged in a cell-type specific manner.

GP2 co-aggregates with zymogen granule content proteins

Protein aggregation under the ionic conditions present in the trans-Golgi network (TGN) is thought to be a critical step in granule biogenesis (cf. Tooze and Stinchcombe, 1992). Secreto/chromogranins have been shown to aggregate in TGN vesicles derived from PC12 and GH4 cells under mildly acidic conditions (Chanat and Huttner, 1991). In addition, the aggregation of pancreatic zymogen content proteins has been reported to occur when the pH is titrated below 6.5 (Leblond *et al.*, 1993), while the constitutive marker, IgG, did not co-aggregate with the content proteins

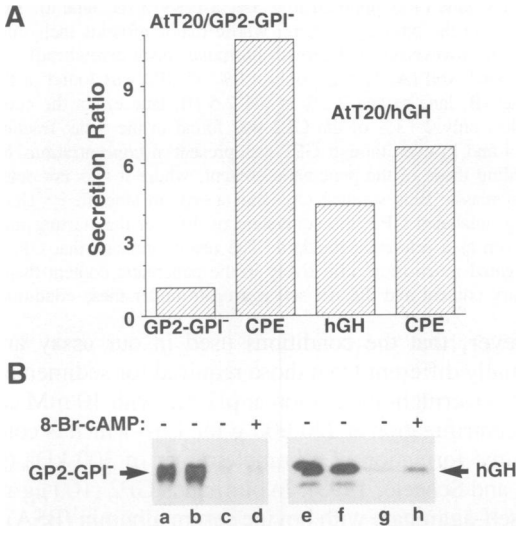

Fig. 5. GP2-GPI⁻ release into the medium is not enhanced after incubation of AtT20 transformants in the presence of 8-Br-cAMP. AtT20 cells expressing GP2-GPI⁻ (lanes a–d) or hGH (lanes e–h) were incubated with [^{35}S]Met for 1 h and chased in the presence of excess unlabeled Met for 5 h. The chase media were collected (lanes a, b, e and f) and the cells were then incubated for an additional 1 h in the presence (lanes d and h) or absence (lanes c and g) of 5 mM 8-Br-cAMP to stimulate exocytosis of storage vesicles. The media from the initial chase and after stimulation were immunoprecipitated either with anti-GP2 (lanes a–d) or anti-hGH (lanes e–h) antibodies and the resulting precipitates subjected to SDS–PAGE and fluorography. The amount of protein immunoprecipitated was quantified by densitometric scanning of the autoradiogram and represented graphically in (**A**) along with the activity of the endogenous granule protein, CPE, measured in aliquots of each medium collected after the stimulation period. The results from one of three similar experiments are shown. The release of residual intracellular GP2-GPI⁻ was very low and did not increase significantly in the presence of secretagogue (first column), indicating that it is not packaged in storage granules in AtT20 cells. By contrast, the release of hGH in transformants expressing it was stimulated significantly (third column), as was the release of CPE from both cells (second and fourth columns).

when added together with them. Therefore, the packaging of GP2 in secretory granules is potentially a function of its capacity to co-aggregate along with the granule content proteins. Since the protein composition of the secretory granules in AR42J and AtT20 cells is quite distinctive, the ability of GP2-GPI⁻ to co-aggregate with content proteins might also vary considerably with cell type. To investigate this possibility, we isolated content proteins from acinar pancreas and anterior pituitary, which correspond to the tissues of origin of the AR42J and AtT20 cells, respectively, and used them in an *in vitro* aggregation assay (see Materials and methods).

As shown in Figure 9A, pancreatic zymogens and pituitary granule content proteins form sedimentable complexes when the pH is titrated to 6.0. All of the major proteins in both preparations precipitate to some extent in a pH dependent manner, including those corresponding to amylase in the pancreatic content and prolactin and growth hormone in the pituitary (Figure 9A). When [^{125}I]GP2 was included in the assays, it co-aggregated well at pH 6.0 with the other zymogen granule content proteins but not with the pituitary content (Figure 9B). Quantitation of the amount of GP2 in the pellet indicates that, in three similar experiments, it aggregated 4–4.2 times better in pancreas than in pituitary at pH 6. Since GP2 is normally present in the pancreatic juice in an aggregated form (Rindler and Hoops, 1990), this result could have been a consequence of the homotypic aggregation of GP2 itself, which has been observed previously by Fukuoka *et al.* (1992). It should be noted,

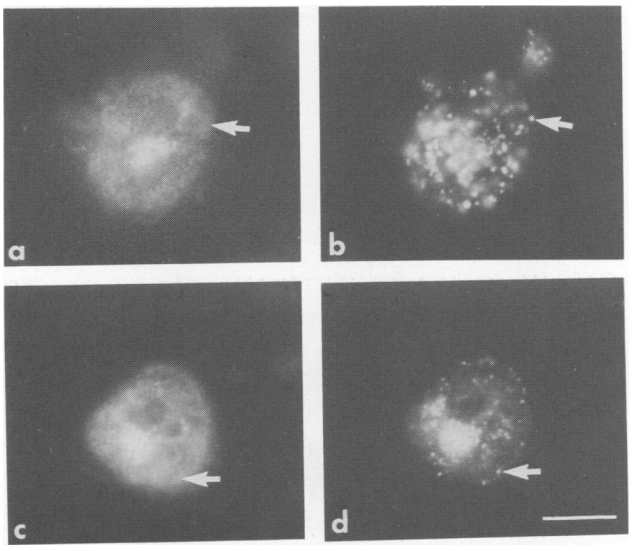

Fig. 6. A secretory form of the VSV G protein does not localize to secretory granules in transfected AR42J cells. AR42J cells expressing G⁻ were stained for immunofluorescence as described in Figure 1. A monoclonal anti-G protein was used together with rabbit anti-amylase antibodies. FITC-labeled anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG were the second antibodies. (**a**) and (**c**) (FITC) show that the label for the G protein is prominent in the Golgi region of highly expressing cells and is also present throughout the cytoplasm in a pattern taken to represent endoplasmic reticulum. The corresponding amylase staining on the same cells is depicted in (**b**) and (**d**) (rhodamine). Amylase is found both in the Golgi and in secretory granules (arrows, b and d). No correspondence between VSV G and amylase staining (arrows, a and c) was observed outside the Golgi region indicating that G⁻ protein did not accumulate in granules. Bar, 8 μm.

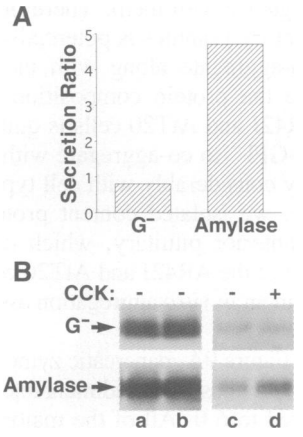

Fig. 7. The release of a secretory form of the VSV G protein is not enhanced after incubation of AR42J cells with CCK. AR42J cells expressing G^- were labeled with [^{35}S]Met for 1 h and then chased for 6 h as described in the legend to Figure 3. The chase medium was collected (lanes a and b) and the cells were incubated for an additional 30 min in the presence (lane d) or absence (lane c) of CCK (5.5×10^{-7} M). Half of each medium sample collected was immunoprecipitated with anti-G antibody (**B**, top) and half with anti-amylase antibody (B, bottom). After the samples were subjected to SDS−PAGE and fluorography, the amount of radioactivity in the appropriate band was quantitated on a phosphorImager. The results of one of three similar observations are represented graphically in (**A**). G^- was found to appear in the 6 h chase medium (B, top, lanes a and b). Very little G^- appeared in the stimulation medium (B, top, lanes c and d), and there was no effect of CCK (A, first column). By contrast, the release of amylase (B, bottom, lanes c and d), a marker for secretory granules, was stimulated significantly in the presence of CCK (A, second column). The results confirm that G^- is not stored in secretory granules in AR42J cells.

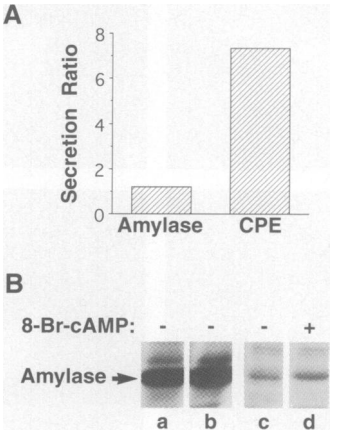

Fig. 8. Amylase release into the medium is not enhanced after incubation of AtT20 transformants in the presence of 8-Br-cAMP. AtT20 cells expressing amylase were incubated with [^{35}S]Met for 1 h and chased for 6 h. The chase media were collected (lanes a and b) and the cells were then incubated for an additional 1 h in the presence (lanes d) or absence (lane c) of 5 mM 8-Br-cAMP with 0.25 mM isobutylmethylxanthene. These samples as well as the medium of the initial chase were immunoprecipitated with anti-amylase antibody and the resulting precipitate subjected to SDS−PAGE and fluorography. The amount of protein immunoprecipitated was quantitated using a phosphorimager and represented (**A**) along with the CPE activity measured in aliquots of medium collected after the stimulation period. The results of one of three similar experiments are shown in the figure. The release of CPE was significantly stimulated in the presence of 8-Br-cAMP (second column). By contrast, there was no increase in the amount of amylase released into the medium under the same conditions (first column), indicating that it is not packaged in granules.

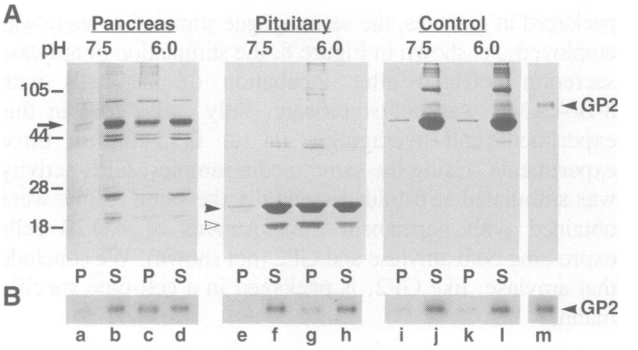

Fig. 9. Differential aggregation of GP2 with pancreatic and pituitary content proteins. [^{125}I]GP2 was added to an *in vitro* aggregation assay together with acinar pancreatic (lanes a−d) or pituitary (lanes e−h) secretory granule content (3 mg/ml), or with BSA (lanes i−l) containing 100 μg/ml unlabeled GP2. The assay was performed as described in Materials and methods using a 20 mM KCl, 5 mM HEPES, 10 mM MES buffer at pH 7.5 (lanes a, b, e, f, i and j) or titrated to pH 6.0 (lanes c, d, g, h, k and l). After a 15 min incubation and subsequent centrifugation, the entire pellet (lanes a, c, e, g, i and k) and 30% or the supernatant (lanes b, d, f, h, j and l) of each sample were subjected to SDS−PAGE, followed by staining with Coomassie Blue (**A**) and autoradiography on a PhosphorImager to visualize the radioactive GP2 (**B**). In the presence of pancreatic content, where all of the major proteins were present in the pellet (A, lane c) including amylase (arrow), 17.0% of the GP2 sedimented at pH 6 (B, lane c) as opposed to 2.3% at pH 7.5 (B, lane a). By contrast, in the pituitary content, whose major proteins including prolactin (arrowhead) and growth hormone (open arrowhead) aggregated well (A, lane g), only 4.0% of GP2 was found in the pH 6 pellet (B, lane g) and 2.2% at pH 7.5 (B, lane e). In the control samples, only 2−3% of the GP2 was found in the pellet fractions (B, lanes i and k) even though GP2 was present at concentrations far exceeding those of the pancreatic content, where it was not detectable by Coomassie Blue staining (A, lanes a−d). In lane m, [^{125}I]GP2 and 1.5 μg unlabeled GP2, the equivalent of 30% of the starting material, were run as a reference standard. The results indicate that GP2 aggregated much more effectively in the pancreatic content than in the pituitary content and did not self-aggregate under these conditions.

however, that the conditions used in our assay are substantially different from those required for sedimentation of GP2—overnight incubation at pH 5.5 with 10 mM calcium and centrifugation at 220 000 *g* for 1 h—which is consistent with the formation of a homotetramer of 300 kDa (Freedman and Scheele, 1993). In our hands GP2 (100 μg/ml) did not self-aggregate with bovine serum albumin (BSA) added as a carrier (Figure 9, lanes i−l). This amount of GP2, which can be observed readily by Coomassie Blue staining of the polyacrylamide gel (Figure 9A, lane m), was far greater than that present in the zymogen granule content, which was below the level of detection (Figure 9A, lanes a−d). Amylase, in contrast to GP2, did partially self-aggregate under the same conditions (data not shown) so it could not be tested. Nonetheless, the differential association of soluble GP2 with granule content proteins from pancreas as opposed to pituitary correlates with its observed incorporation into granules in pancreatic AR42J cells as opposed to pituitary AtT20 cells.

Discussion

GP2, which in the zymogen granule comprises at least 30% of the total membrane protein (Ronzio *et al.*, 1978; Rindler and Hoops, 1990), is, in its native GPI-linked form, targeted to the regulated pathway in transfected AR42J cells but not in AtT20 or Rin 5F cells (Hoops *et al.*, 1993). We now show

that GP2-GPI$^-$, a secretory form of GP2, behaves similarly when expressed in AR42J cells and AtT20 cells. Like native GP2, GP2-GPI$^-$ in AR42J cell transformants localizes to secretory granules and is subject to secretagogue-stimulated secretion. By contrast, a secretory version of the VSV G protein, a marker for the constitutive transport pathway, was not packaged in granules in AR42J cells. Thus, the secretory form of GP2 contains information that allows it to be packaged like a content protein in its native exocrine cell.

The luminal domain of another granule membrane protein, peptidyl α-amidating enzyme (PAM), has granule packaging information as well. Soluble, secretory forms of PAM, generated by alternative splicing of the mRNA, are thought to be incorporated into the secretory granules of their native tissues (Braas *et al.*, 1989; Eipper *et al.*, 1992). Indeed, soluble forms of the PAM encompassing the luminal domain were sorted to the regulated pathway when expressed in AtT20 cells (Milgram *et al.*, 1992). Thus the luminal portion of PAM may have evolved to behave like a secretory granule content protein and have packaging signals. By contrast, GP2 enters granules exclusively as a membrane protein in the pancreas (Havinga *et al.*, 1985) and does not have alternatively spliced forms (Fukuoka *et al.*, 1991). Unlike GP2 and PAM, the granule membrane protein P-selectin has been reported to have a cytoplasmic targeting signal (Disdier *et al.*, 1992). When most of its cytoplasmic tail was deleted, the resulting protein was transported constitutively to the cell surface in AtT20 cells, implying that the luminal portion of the molecule lacks granule packaging information. Thus, granule membrane proteins exist in at least two subtypes, those with luminal sorting information and those with cytoplasmic sorting signals.

The existence of granule membrane proteins with luminal packaging information may be essential for the process of secretory granule formation. Granule biogenesis begins with the partial condensation of the content proteins in the TGN and condensing vacuoles (cf. Tooze and Stinchcombe, 1992). The nascent content protein aggregate must interact with membrane proteins to ensure its appropriate envelopment during granule formation. These associations may be mediated by a receptor that recognizes sorting signals in these molecules, or they may be a consequence of spontaneous co-aggregation of proteins under the ionic conditions and pH present in the TGN, as may be the case for chromogranins (Gorr *et al.*, 1988; Chanat and Huttner, 1991; Yoo, 1993). The fact that soluble GP2 sedimented with zymogen granule content proteins at pH 6 in our assay supports the idea that interaction between membrane proteins with luminal packaging information and content proteins occurs by co-aggregation, as does the precipitation of the content proteins themselves. The condensation process would in this way be extended to and abetted by the membrane proteins, which in turn interact with other granule membrane proteins, such as P-selectin (Disdier *et al.*, 1992), with cytoplasmic sorting signals recognized by a cytosolic sorting machinery.

GP2-GPI$^-$ and amylase were found to be incorporated into secretory granules in a cell-type specific manner. Although cell-type specific packaging of granule content proteins has also been observed for vWF (Wagner *et al.*, 1991) and *Aplysia* egg laying hormone (ELH) (Jung *et al.*, 1993), each of these cases differs in important ways from our observations with amylase and GP2. vWF expressed in AtT20 and Rin 5F cells is excluded from the endogenous granules but does enter an organelle resembling a Weibel–Palade body of endothelial cells, where vWF is normally the major granule protein (Wagner *et al.*, 1991). This was taken to mean that vWF, unlike GP2, was still routed to the regulated pathway, but to a different population of granules, similar to what has been described in pituitary somatomammatrophs, where distinct granule subtypes exist in the same cell (Hashimoto *et al.*, 1987). Indeed, *Aplysia* bag cell neurons also have two types of granules (Fisher *et al.*, 1988). A proteolytic cleavage converts the ELH precursor into distinct N- and C-terminal portions in the TGN. The C-terminal segment enters dense core granules that are transported to the processes of these cells, while the N-terminal portion is in granules that remain in the cell body (Fisher *et al.*, 1988). In transfected AtT20 cells, cleavage occurs normally but only the carboxyl portion enters the endogenous secretory granules; the N-terminal portion, like GP2 and amylase, is constitutively secreted (Jung *et al.*, 1993). In the evolutionarily distant *Aplysia*, the granules in which the N-terminal segment of ELH is stored are not well characterized and have not been shown to undergo regulated exocytosis. Furthermore, there is as yet no known equivalent structure in mammalian neurons.

By contrast, mammalian amylase and GP2 are found in well defined exocrine secretory granules which contain a number of content proteins capable of being incorporated into the endocrine granules of transfected AtT20 cells. These include pancreatic trypsinogen, β-NGF from submaxillary gland, proline-rich proteoglycans from the parotid and a soluble PAM resembling the salivary form (Burgess *et al.*, 1985; Wolf *et al.*, 1988; Castle *et al.*, 1992; Milgram *et al.*, 1992; Castle and Castle, 1993). In our own laboratory, we have observed in transient expression experiments that bovine prochymosin, a granule protein of gastric chief cells, is sorted to the regulated pathway (G.Beaudry and M.Rindler, unpublished observations). It should be noted that none of the exocrine proteins routed to AtT20 granules are very abundant in their native granules and none are known to be expressed in more than one exocrine cell type. Amylase, on the other hand, is the most abundant protein of pancreatic zymogen granules and a major constitutent in parotid and serous submandibular gland granules as well (Smith and Frommer, 1975; Cameron and Castle, 1984).

An important clue to the underlying difference between proteins that enter the granules of pituitary AtT20 cells and those that do not comes from our aggregation assay results. The major secretory granule content proteins of the pituitary and pancreatic granules aggregated under the conditions of reduced pH expected to be present in the TGN, a site where the segregation of constitutively secreted from regulated proteins is thought to occur (Tooze and Tooze, 1986; Chanat and Huttner, 1991). GP2 aggregated much more readily with the pancreatic zymogens than with the pituitary proteins or with itself. It is likely that amylase behaves similarly, but we were not able to analyze this directly since amylase aggregates homotypically under the conditions of the assay. How is it then that other exocrine granule content proteins can be packaged in secretory granules in AtT20 cells? An indication as to the mechanism comes from the work of Gorr *et al.* (1992). They reported that among the zymogen granule proteins, chymotrypsinogen and trypsinogen can bind to endocrine proteins that are secreted by the regulated pathway (prolactin, chromogranin A and insulin) but not to

constitutively secreted proteins (BSA and immunoglobulin). Significantly, amylase, the major protein in these preparations, was not one of the binding proteins identified in this way (Chung *et al.*, 1989; Gorr *et al.*, 1992). We postulate that content proteins like trypsinogen may be packaged in the secretory granules of AtT20 cells by virtue of their interaction with other content proteins in these cells during their condensation. Amylase and GP2, on the other hand, interact exclusively with components present in the exocrine cells.

In conclusion, our results indicate that there is no universal recognition mechanism or set of sorting signals that governs the targeting of proteins to secretory granules in every tissue or even acounts for the packaging of different products from the same granules. They strongly support selective co-aggregation as being central in the segregation of constitutive from regulated proteins and in cell-type specific packaging of granule proteins. In addition, our data implicate co-aggregation as a mechanism of membrane–content protein interaction during granule formation.

Materials and methods

Antibodies and cell lines

Antisera against GP2 was prepared and affinity purified as described previously (Rindler and Hoops, 1990). Rabbit antiserum to VSV was raised against purified virus grown in HeLa cells and mouse monoclonal antibody was a gift of Dr T.Gottlieb, NYU Medical Center (Gottlieb *et al.*, 1993). Rabbit antibodies to ACTH and to amylase were purchased from Sigma (St Louis, MO). Rhodamine- or FITC-conjugated secondary antibodies and Texas Red–streptavidin were obtained from Jackson Immunoresearch Laboratories (West Grove, PA) or Organon Teknika-Capel (West Chester, PA). AtT20 cells and AR42J cells were cultured as previously described (Hoops *et al.*, 1993). AtT20 cells expressing growth hormone (Moore and Kelly, 1985) were a generous gift of Dr R.Kelly, University of California, San Francisco, CA).

Generation of constructs

A construct encoding the N-terminal 492 amino acids of GP2 (GP2-GPI$^-$) was generated by introducing a stop codon at base 1593 using standard PCR methodology (Saiki *et al.*, 1988). This modified cDNA, lacking the sequence coding for the GPI attachment signal, was subcloned into the pLNCX vector (Miller and Rosman, 1989), and its entire DNA sequence was confirmed using the dideoxy chain termination method (Sequenase; US Biochemical, Cleveland, OH). The truncated VSV G protein cDNA construct generated by site-specific mutagenesis was a generous gift of Drs T.Gottlieb and D.Sabatini, NYU Medical Center, and was subcloned into pLNCX. This secreted form of the G protein differs from the original truncated G (Rose and Bergmann, 1983) in that it stops right at the membrane spanning segment (the codon in the cDNA encoding amino acid 462 was changed to a stop codon) with no additional amino acids. It is rapidly secreted from transfected cells (e.g. see Figure 6). The cDNA encoding mouse salivary amylase (Schibler *et al.*, 1980) was a gift of Dr P.Wellauer (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). It was subcloned into pcDNAIneo (Invitrogen, San Diego, CA).

Isolation of permanent transformants

AtT20 and AR42J cells were transfected and permanent transformants selected as previously described (Hoops *et al.*, 1993). AR42J clones were pre-treated for 66–90 h with dexamethasone (10^{-6} M) and with 10 mM Na butyrate for ~48 h to increase expression of the proteins of interest (Gottlieb *et al.*, 1986). In these experiments using AtT20 transformants expressing amylase, the cells were pre-treated with 10 mM Na butyrate for 12–15 h. The butyrate pre-treatment itself did not interfere with the ability of AR42J cells to store newly synthesized amylase (see Figure 3) or with the regulated secretion of CPE from AtT20 cells (see Figure 5).

Immunofluorescence microscopy

Cells expressing the proteins of interest were grown on coverslips which for AR42J cells were pretreated with poly-L-lysine (0.1 mg/ml). They were fixed, permeabilized and stained for immunofluorescence with antiserum at 1:100–1:200 and/or mouse ascites at 1:50. FITC or rhodamine conjugated goat anti-rabbit, donkey anti-rabbit or mouse IgG were used as the secondary antibodies (Hoops *et al.*, 1993). For double labeling experiments using AR42J/GP2-GPI$^-$ cells, coverslips were first stained with anti-GP2 and FITC goat anti-rabbit IgG. They were then blocked with 100 μg/ml rabbit IgG before being further incubated in biotinylated rabbit anti-amylase IgG or non-immune biotinylated rabbit IgG followed by Texas-Red–streptavidin.

Immunoelectron microscopy

Cells grown in the 60 mm culture dishes were fixed for 1 h in paraformaldehyde (3%)–lysine–periodate (McLean and Nakane, 1974), detached from the dishes, pelleted and fixed for an additional 9 h in the same fixative. Cell pellets were embedded in LR white (The London Resin Co., Hampshire, UK). Sections on grids were incubated in affinity purified anti-GP2 antibody followed by colloidal gold (10 nm) coupled to protein A (E-Y Laboratories, San Mateo, CA). They were viewed and photographed on a JEOL-JEM 1200 EXII electron microscope.

Biosynthetic labeling and pulse–chase experiments

Cells were grown to near confluency on six-well dishes. For AR42J cells, the dishes were pre-treated with poly-L-lysine (0.1 mg/ml). The cells were labeled with 125–250 μCi/ml [^{35}S]Met (>1000 Ci/mmol; NEN/DuPont, Wilmington, DE) for 20–60 min and chased for 5–6 h as previously described (Hoops *et al.*, 1993). The cell extracts were collected and sonicated in 1% SDS, 10 mM Tris, pH 8, containing 1 mM EDTA, while 0.2% SDS was added to the collected medium. The samples were boiled and immunoprecipitated as described (Hoops *et al.*, 1993). After analysis by SDS–PAGE and fluorography, using EN^{3}HANCE (NEN/Dupont, Wilmington, DE), the bands corresponding to the protein of interest were quantified by either densitometric scanning of the autoradiograms, using a LaCie scanner (LACIE Ltd, Beaverton, CA) and NIH Image 1.52, or on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In some cases images were imported into Quark Express (Denver, CO) for computerized labeling and printing of figures.

Secretagogue stimulation

Secretagogue-stimulated release from AR42J cells by CCK was conducted as detailed previously (Hoops *et al.*, 1993). Half of each sample collected was immunoprecipitated with anti-amylase antibody and the remainder with anti-GP2 or anti-VSV G antibody. AtT20 cells were incubated for 1 h in the presence or absence of 5 mM 8-Br-cAMP after the pulse and the chase (and 0.25 mM isobutylmethylxanthene when indicated); CPE activity was measured by the dansyl-FAR method of Fricker and Devi (1990). The remainder of the samples were immunoprecipitated with the antiserum indicated and subjected to SDS–PAGE and fluorography as detailed above. All data were corrected for differences in the overall recovery of the radiolabeled proteins in each sample.

Preparation of pancreatic and pituitary granule content proteins

Zymogen granules, content and membranes from rat or dog pancreas were prepared essentially as described (Rindler and Hoops, 1990). Tissue was minced or, for the canine pancreas, forced through a tissue press, before being homogenized in 0.3 M sucrose 0.5 mM EDTA, 2 mM EGTA, 3.3 mM MOPS buffer, pH 6.8, with a cocktail of protease inhibitors. A post-nuclear supernatant was prepared after centrifugation for 10 min at 750 *g* and granules were sedimented at 1750 *g* for 30 min. They were resuspended and layered onto 35% Percoll gradients (0.27 M sucrose, 20 mM MOPS, 5 mM benzamidine) spun at 10 000 *g* for 60 min, and then collected and washed in the same buffer. Granules were incubated in lysis buffer (150 mM KCl, 25 mM HEPES, 0.5 mM EDTA, 2 mM EGTA, 5 mM benzamidine, pH 8.0) and the content and membranes separated as previously described (Rindler and Hoops, 1990). GP2 was released from purified, carbonate-washed dog pancreas membranes (Rindler and Hoops, 1990) with 0.25 units phosphoinositol specific phospholipase C (Boehringer Mannheim, Indianapolis, IN) in 5 mM HEPES, pH 7.5. After centrifugation at 100 000 *g* for 20 min, the supernatant was collected. GP2 was the only detectable protein released. It was iodinated using lactoperoxidase (Kadner *et al.*, 1984).

Bovine pituitaries were decapsulated, minced and homogenized in 0.32 M sucrose with protease inhibitors. After a nuclear spin as above, a crude granule preparation was obtained by centrifugation at 4750 *g* for 30 min. The resuspended pellet was layered into 1.6 M sucrose and pelleted at 150 000 *g* for 2 h. The granule pellets were lysed by sonication in phosphate buffered saline, pH 9, with 100 mM KCl and aprotinin (10 U/ml) added, and the soluble content was recovered after centrifugation at 100 000 *g* for 30 min.

In vitro aggregation assay

Granule contents were de-salted over Biogel P-6 DG columns (Bio-Rad Laboratories, Richmond, CA) equilibrated with 5.5 mM HEPES, pH 7.5. A procedure similar to that described by Leblond *et al.* (1993) was employed. Samples (50 μl) containing granule content or BSA (2.5–3 mg/ml final concentration) in 20 mM KCl, 5 mM HEPES, 10 mM MES, pH 7.5 and [^{125}I]GP2 were titrated slowly to pH 6.0 by adding an aliquot of 0.125 N HCl while vortexing. After incubation for 15 min at room temperature, the reaction mixture was centrifuged for 15 min at 15 000 *g* in an Eppendorf centrifuge. Supernatant and pellet fractions were subjected to SDS–PAGE on 10–15% gradient gels (without reducing agent) followed by staining of the gels with Coomassie Blue and autoradiography.

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