Not All Secretory Granules Are Created Equal: Partitioning of Soluble Content Proteins^D

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Secretory granules carrying fluorescent cargo proteins are widely used to study granule biogenesis, maturation, and regulated exocytosis. We fused the soluble secretory protein peptidylglycine α -hydroxylating monooxygenase (PHM) to green fluorescent protein (GFP) to study granule formation. When expressed in AtT-20 or GH3 cells, the PHM-GFP fusion protein partitioned from endogenous hormone (adrenocorticotrophic hormone, growth hormone) into separate secretory granule pools. Both exogenous and endogenous granule proteins were stored and released in response to secretagogue. Importantly, we found that segregation of content proteins is not an artifact of overexpression nor peculiar to GFP-tagged proteins. Neither luminal acidification nor cholesterol-rich membrane microdomains play essential roles in soluble content protein segregation. Our data suggest that intrinsic biophysical properties of cargo proteins govern their differential sorting, with segregation occurring during the process of granule maturation. Proteins that can self-aggregate are likely to partition into separate granules, which can accommodate only a few thousand copies of any content protein; proteins that lack tertiary structure are more likely to distribute homogeneously into secretory granules. Therefore, a simple “self-aggregation default” theory may explain the little acknowledged, but commonly observed, tendency for both naturally occurring and exogenous content proteins to segregate from each other into distinct secretory granules.

INTRODUCTION

The cellular life of secretory proteins begins with the co-translational translocation of their nascent polypeptide chains into the lumen of the endoplasmic reticulum (ER). These proteins then proceed in the anterograde direction, through the Golgi complex and into the *trans*-Golgi network (TGN), which is considered a “sorting station” for secretory proteins (Farquhar and Palade, 1981). At the TGN, vesicles destined for different subcellular compartments bud while acquiring their cargo. In all cell types, a “constitutive secretory pathway” delivers soluble and membrane secretory proteins necessary for housekeeping functions to the appropriate compartment (e.g., plasma membrane or endosomes/lysosomes; Turner and Arvan, 2000). Besides this constitutive pathway, specialized cell types such as endocrine, exocrine, neuroendocrine cells, and neurons possess a “regulated pathway” in which specialized organelles, the secretory granules, store proteins for release in response to an

incoming signal (Arvan and Castle, 1998). The soluble proteins found in constitutive vesicles and regulated secretory granules differ, and the processes responsible for this segregation are the object of intensive investigation.

Two models, “sorting for entry” and “sorting by retention,” both having experimental support, have been proposed to explain how sorting of soluble content proteins occurs (Arvan and Castle, 1998). The sorting for entry hypothesis postulates the existence of one or more “membrane receptors” able to selectively recruit granule content proteins in the TGN. For example, the interaction of prohormone convertase 2 (PC2) with a lipid raft component in the TGN is essential for its entry into the regulated secretory pathway (Blazquez *et al.*, 2000). Carboxypeptidase E (CPE), through its lipid raft associations with TGN membranes, interacts with prohormone aggregates in the TGN (Rindler, 1998) and is proposed to affect sorting into regulated granules (Cool *et al.*, 1997; Loh *et al.*, 2002; Zhang *et al.*, 2003). Proteins unable to bind to the proposed sorting receptors would then enter the constitutive pathway by default.

Professional secretory cells devote a large fraction of their synthetic efforts to producing the products stored in secretory granules, and the sorting by retention model suggests that bulk flow accounts for protein entry into the immature granules that bud from the TGN. Aggregation and condensation of proteins in the immature secretory granules facilitates retention. Through a remodeling process that allows selective removal of nongranule proteins via vesicular budding, immature granules are converted into mature granules (Kuliawat and Arvan, 1992; Eaton *et al.*, 2000). A role for sorting by retention is supported by the progressive removal of transfected exocrine proteins from granules in AtT-20 cells (Castle *et al.*, 1997) and the transient presence of lyso-

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Abbreviations used: GFP, green fluorescent protein; TGN, *trans*-Golgi network; POMC, proopiomelanocortin; ACTH, adrenocorticotrophic hormone; PHM, peptidylglycine α -hydroxylating monooxygenase; PAM, peptidylglycine α -amidating monooxygenase; NPY, neuropeptide Y; CSFM, complete serum-free medium; PC1, prohormone convertase 1; PMA, phorbol 12-myristate 13-acetate.

Figure 1. Endogenous and transfected proteins. Expression vectors encoding peptidylglycine α -hydroxylating monooxygenase (PHM), PHM fused to GFP (PHM-GFP), monomeric GFP (PHM-mGFP), or DsRed (PHM-DsRed), and pro-neuropeptide Y were constructed (A). Also shown are PAM-3, the naturally occurring soluble form of PAM, proopiomelanocortin (POMC), which is processed into and stored as adrenocorticotrophic hormone (ACTH) in AtT-20 cells, and growth hormone and prolactin, which are both stored in GH3 cells (A). Extracts of AtT-20 cells expressing PHM, PHM-mGFP, or PHM-DsRed were loaded by equal enzymatic activity (375 pmol/h) and separated by SDS-PAGE. Western blot analysis using antibodies to PHM (JH1761) and GFP show that the PHM-mGFP and PHM-DsRed fusion proteins are fully enzymatically active and remain mostly intact in AtT-20 cells and fully intact in GH3 cells, respectively (B).

somal proteins in immature insulin granules (Kuliawat *et al.*, 1997).

Mature granules are competent for exocytosis and secrete their contents in response to extracellular stimuli; they contain very high (90–150 mg/ml) concentrations of their secretory products (Hutton *et al.*, 1983; Oyarce *et al.*, 1996; Dannies, 1999). Insulin crystallizes in β cell granules (Michael *et al.*, 1987). The protein cores of growth hormone and prolactin granules disperse slowly (Angleson *et al.*, 1999; Dannies, 2002). When these aggregates form is a critical part of the sorting process, somewhat blurring distinctions between the models discussed above. In the sorting for entry model, aggregates that form in the TGN could interact with specific membrane receptors, effectively reducing the number of receptors needed (Arvan and Castle, 1998; Tooze, 1998). Electron micrographs of somatomammotrophs demonstrate separation of GH aggregates from prolactin aggregates in the TGN (Fumagalli and Zanini, 1985; Hashimoto *et al.*, 1987). In vitro, several proteins of the regulated secretory pathway aggregate at acidic pH and in the presence of high calcium (Gerdes *et al.*, 1989; Gorr *et al.*, 1989; Colomer *et al.*, 1996). Structural features of the proteins are essential for aggregation of certain proteins as well. N-terminal disulfide-bonded loops have been proposed to be important for routing of proopiomelanocortin (POMC; Tam *et al.*, 1993; Cool *et al.*, 1995), chromogranin B (Chanat *et al.*, 1993), and chromogranin A, and loop-mediated homodimerization of chromogranin A is required for its sorting to the regulated secretory pathway (Thiele and Huttner, 1998). However, not all investigators agree on the importance of the disulfide loops for POMC (Roy *et al.*, 1991).

There is a great deal of interest in the mechanisms governing secretory granule biogenesis and exocytosis. Early studies demonstrated that exogenous proteins could be modified so that they would be stored in secretory granules when expressed in a professional secretory cell. For example, α -globin, a cytoplasmic protein, is targeted to regulated secretory granules when fused to the signal sequence plus propeptide of somatostatin (Stoller and Shields, 1989), as is the green fluorescent protein (GFP) when appended to the signal sequence of neuropeptide Y (NPY; El Meskini *et al.*, 2001b). Fluorescently tagged granules are now widely used to study granule maturation and trafficking, the cytosolic machinery interacting with granules, and regulated release (Kaether *et al.*, 1997; Levitan, 1998, 2004). A variety of proteins and prohormones have been coupled with derivatives of GFP to generate fluorescent reporters targeted to secretory granules, including tissue plasminogen activator (Lochner *et al.*, 1998), insulin (Pouli *et al.*, 1998),

atrial natriuretic factor (Burke *et al.*, 1997), oxytocin (Zhang *et al.*, 2002), and vasopressin (Zhang *et al.*, 2005).

GFP is not normally a granule protein. Whether granule entry involves sorting for entry or sorting by retention, the ability of foreign, nongranule proteins to gain access to secretory granules is surprising (Moore and Kelly, 1985). In corticotrope cells, secretory granules typically contain 3000–10,000 copies of POMC product (Oyarce *et al.*, 1996). We wanted to determine whether GFP-tagged secretory proteins expressed in AtT-20 cells were homogeneously mixed with endogenous hormone.

The secretory protein peptidylglycine α -hydroxylating monooxygenase (PHM) functions in granules at a late stage in the biosynthesis of amidated peptides. Because PHM is efficiently targeted to granules (Milgram *et al.*, 1992, 1994), we attached GFP to the C-terminus of PHM (Figure 1A). The PHM-GFP chimera was stably expressed in both AtT-20 and GH3 endocrine cells, and its sorting, localization, and secretion were evaluated relative to that of the endogenous hormones and enzymes produced by these cell types. Our findings indicate that secretory granule populations are heterogeneous with regard to their soluble contents, with the intrinsic biophysical properties of the proteins governing their differential sorting.

MATERIALS AND METHODS

Antibodies and Reagents

The following rabbit polyclonal antisera were used for immunostaining: POMC/adrenocorticotrophic hormone (ACTH; JH93, 1:1000) directed against the N-terminal of ACTH (Zhou *et al.*, 1993), ACTH (JH44, 1:4000) directed against the C-terminal of ACTH (1-39; Marx *et al.*, 1999), and neuropeptide Y (JH3, 1:5000; Marx *et al.*, 1999). ACTH mAb (Novocastra, Newcastle upon Tyne, United Kingdom; 1:500) was also used; based on an ELISA, this mAb recognizes ACTH(1-39) and CLIP [ACTH(18-39)], but not ACTH (1-24). For immunoblot analyses, rabbit polyclonal antisera against PHM (JH1761, 1:1000; El Meskini *et al.*, 2001a) and GFP (Abcam, Cambridge, MA; 1:2000) were used. Polyclonal antiserum to prolactin (IC-5; 1:1000) was obtained from the National Hormone and Peptide Program (NHPP), National Institutes of Diabetes, Digestive and Kidney Diseases (NIDDK), and Dr. Parlow. The LAMP-1 antibody was a rat monoclonal, 1D4B, from cells grown in our laboratory and purchased from the Hybridoma Bank at the University of Iowa (<http://www.uiowa.edu/~dshbwww/1d4b.html>), used at 1:50 dilution with Cy3-labeled anti-rat IgG (1:2000).

Constructs

An expression vector encoding PHM fused to GFP was generated by first removing PHM from the pBluescript PAM-1 vector by digesting with XmnI and HindIII. The PHM fragment was inserted into the pEGFP vector (Clontech, Palo Alto, CA) digested with BamHI and blunted with Klenow, followed by HindIII digestion, yielding PAM-1 residues 1-407 fused in reading frame with EGFP. The construct was verified by DNA sequencing. A vector encod-

ing PHM fused to DsRed-Monomer was generated by inserting the same PHM fragment from pBluescript PAM-1 into the pDsRed-Monomer vector (Clontech), which was digested with EcoRI and blunted with Klenow, followed by HindIII digestion.

To create PHM-GFP A²⁰⁶K (PHM-mGFP), the Stratagene QuickChange method (La Jolla, CA) was used with mutagenic sense primer: 5'-CAG TCC AAG CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT CAC-3' and mutagenic antisense primer: 5'-GTG ATC GCG CTT CTC GTT GGG GTC TTT GCT CAG CTT GGA CTG-3' (Zacharias *et al.*, 2002). The mutagenic codon is underlined. PCR products were verified by DNA sequencing.

Cell Cultures and Generation of Stable Cells Lines

AtT-20 and GH3 cells were initially transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable cell lines were generated by maintaining and selecting transfected cells in DMEM-F12 containing 10% fetal calf serum, 10% NuSerum, penicillin/streptomycin, and 0.5 mg/ml G418. GFP fluorescence was evaluated to obtain clonal lines. Six independent clones for AtT-20 (3 with PHM-GFP and 3 with PHM-mGFP) and two for GH3 PHM-mGFP cell lines were examined. The level of PHM enzymatic activity contained in and secreted by the stably transfected cells ranged from 30 to 70 times higher than the PHM activity in nontransfected cells. Transient transfection of AtT-20 cells was accomplished using 1 μ g DNA per coverslip with 2 μ l Lipofectamine 2000 (Invitrogen). The expression level of PHM-mGFP compared with POMC was determined using a 20-min pulse incubation in medium containing [³⁵S]methionine, followed by immunoprecipitation, SDS polyacrylamide gel analysis, and autoradiography (Milgram *et al.*, 1992, 1994); corrected for the number of methionine residues, the expression of PHM-mGFP was 30% of the molar level for POMC. Similarly, NPY-expressing AtT-20 cells express NPY at levels equimolar to POMC, higher than the levels using the metallothionein promoter for NPY expression (60%; Dickerson *et al.*, 1987).

Immunofluorescence and Image Quantification

Cells plated onto poly-L-lysine-coated 0.17-mm glass coverslips (Fisher Scientific, Pittsburgh, PA) were maintained in DMEM-F12 for 2 d and fixed in prewarmed 4% formaldehyde in PBS for 30 min at room temperature. Cells were permeabilized in 0.075% Triton X-100 and 2 mg/ml BSA in PBS, blocked in 2 mg/ml BSA in PBS, incubated in primary antibody at 4°C overnight, washed three times with PBS, and incubated with fluorescein isothiocyanate (FITC) or Cy3 (Jackson ImmunoResearch, West Grove, PA) conjugated secondary antibodies. Coverslips were mounted on glass slides with Prolong Gold antifade reagent (Molecular Probes, Eugene, OR).

To examine colocalization of PHM-mGFP and ACTH in granules, AtT-20 cells were maintained in culture as described. Cells were examined directly; in some experiments, on the day of the experiment, cells were treated with 10 μ M cycloheximide for 1 h to reduce fluorescence derived from the ER (Sans *et al.*, 2005) and then fixed in 4% formaldehyde.

Staining was visualized by confocal microscopy (AtT-20) or deconvolution fluorescence microscopy (GH3). For confocal imaging, a Zeiss LSM510 mounted on an Axiocvert 100M was used to collect single optical sections (Zeiss, Thornwood, NY). Photomultiplier tube settings were optimized to include the full dynamic range of gray levels. Multitrack scans were used to avoid cross talk between channels. For deconvolution, z-stacks taken at 0.2 μ m were acquired with OpenLab software (Improvision, Lexington, MA) on a Nikon Eclipse TE300 microscope (Melville, NY) and then deconvolved using Volocity deconvolution software (Improvision).

Quantitative colocalization analysis of confocal images of double immunolabeled cells was performed using SimplePCI imaging software (Compix, Cranberry Township, PA). A single workflow was designed to automate identification of all green- and red-labeled granules in each image. This involved processing images through a smoothing filter, thresholding green and red intensities above background levels, and setting inclusion criteria for roundness and area. Mean red and green pixel intensities for all identified granules were calculated by the software. Images were taken so that maximal red and green fluorescence intensities in granules used for quantification were <255, excluding the tips of cells, where granules were too close to each other to distinguish individually. To compensate for different fluorescence intensities in different images, the mean red and mean green fluorescence in the granules in each image was normalized to 1.0, and the ratio of green-to-red fluorescence in each granule in the image was calculated. The green/red ratios were binned to make the histograms shown. Kolmogorov-Smirnov statistics were used to determine the statistical significance of apparent differences in the histograms (Conover, 1999; Howell, 2004). The normal distribution was calculated according to Howell (2004).

Immunoelectron Microscopy

Cells were fixed with 4% paraformaldehyde and 2% sucrose in 0.1 M phosphate buffer, pH 7.2, for 1 h, postfixed with 0.25% tannic acid for 1 h, scraped, and pelleted in gelatin. Polyvinylpyrrolidone/sucrose infiltrated specimens were sectioned at -100°C, and sections were incubated with PHM antibody JH1761 (1:100) followed by protein A-10-nm gold (University of Utrecht, Utrecht, The Netherlands). For double staining, sections were then treated

with fish skin gelatin and bovine serum albumin and fixed again with 4% formaldehyde + 1% glutaraldehyde for 5 min; sections were then incubated with ACTH antibody Kathy (1:1000) followed by protein A-15-nm gold. Control sections incubated without ACTH antibody confirmed that glutaraldehyde fixation abolished binding of protein A-15-nm gold to PHM-stained sections. Stained sections were embedded in uranyl acetate-methyl cellulose and examined with a JEOL 1200 EX II electron microscope (Peabody, MA). The number of 10-nm gold particles (PHM-GFP) and 15-nm gold particles (ACTH) per granule was then counted. Statistical analysis was performed as for the green-red fluorescence; the average number of 10- and 15-nm gold particles per granule was normalized to 1.0.

Secretion Experiments, Enzyme Assays, and Radioimmunoassays

AtT-20 or GH3 PHM-GFP cells were plated on poly-lysine-coated plastic dishes and maintained in culture for 2 d. Before media collection, cells were initially rinsed for three 30-min periods with complete serum free (CSFM) air medium (DMEM/F12 without bicarbonate and supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, insulin/transferrin/selenium from Invitrogen or Mediatech [Herndon, VA], and 0.1 mg/ml fatty acid-free bovine serum albumin). For AtT-20 cells, two 30-min collections were then made for basal secretion, followed by one 30-min stimulation with 2 mM BaCl₂. For GH3 cells, parallel dishes were incubated for 30 min with medium without (basal) or with 2 mM BaCl₂ (stimulated). Medium was centrifuged to remove nonadherent cells, and protease inhibitors were added. Cells were harvested in 20 mM Na-N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), 10 mM mannitol, 1% Triton X-100, pH 7.4 (TMT) or 5 N acetic acid to determine PHM or ACTH content, respectively. TMT extracts were frozen and thawed three times and centrifuged to remove debris. Acetic acid extracts were lyophilized and resuspended in RIA buffer with protease inhibitors. Medium samples from the second basal and stimulation collections, and cell extracts were assayed for PHM activity (Kolhekar *et al.*, 1997) using [¹²⁵I]-labeled α -N-acetyl-Tyr-Val-Gly as substrate. Samples were assayed in duplicate, and reactions were carried out for 2 h. ACTH secretion and cell content were measured by radioimmunoassay using antibody Kathy, directed against the C-terminal of ACTH (Bruzaniti *et al.*, 1999). For quantification of PHM-mGFP secretion from GH3 cells, media, and cell lysates were fractionated by SDS-PAGE, transferred to PVDF, and probed with GFP Ab (Abcam); signals in the linear range were quantified using GeneTools software (Syngene, Frederick, MD).

Hormone Depletion Paradigm

AtT-20 PHM-mGFP cells grown on poly-L-lysine-coated glass coverslips were stimulated for five sequential 20-min periods alternatively with 2 mM BaCl₂ and 1 μ M phorbol 12-myristate 13-acetate (PMA) in DMEM-F12 air medium containing 1 mg/ml bovine serum albumin (Ferraro *et al.*, 2005). After the last stimulation period, cells were fixed in 4% formaldehyde, and immunofluorescence staining was performed as described above.

Before alkalization and cholesterol depletion experiments, cells were subjected to this depletion paradigm in the presence of 10 μ M cycloheximide, followed by a 12-h chase in cycloheximide. Cells were either fixed immediately or were allowed to recover for 36 h in drug-free growth medium, ammonium chloride, or lovastatin.

Alkalization and pH Assessment

The pH gradient in AtT-20 cells was dissipated by treatment with 2.5 mM ammonium chloride for 24 h (Mains and May, 1988). Cells were either fixed and processed for immunocytochemistry immediately after treatment or loaded with acridine orange to check for pH gradient neutralization. Cells were incubated with 2 μ M acridine orange in L-15 in the presence or absence of ammonium chloride for 30 min (Mains and May, 1988), rinsed twice with L-15 without phenol red, and visualized with a LSM510 confocal microscope (Zeiss). Images were acquired identically for control and drug-treated cells to compare acridine orange fluorescence.

Temperature Block and Cholesterol Depletion Experiments

For temperature block experiments, AtT-20 PHM-mGFP cells were incubated for 30 min at 20°C. Cells were fixed and processed for immunoelectron microscopy as described above.

For cholesterol depletion experiments, AtT-20 PHM-mGFP cells were rinsed three times with CSFM before beginning drug treatment. Cells were incubated with 5 μ M lovastatin in CSFM for 36 h and then fixed and processed for immunocytochemistry. Cholesterol levels in AtT-20 cell extracts were determined using the Amplex Red Cholesterol Assay Kit (Molecular Probes), and the reaction product was measured with a fluorimeter.

Figure 2. Validation of quantification paradigm for colocalization. Wild-type AtT-20 cells were visualized simultaneously with polyclonal (A, green) and monoclonal (A, red) antibodies to ACTH. The granules in the overlaid images appear yellow. Colocalization was established quantitatively by plotting the distribution of green/red intensity ratios for all identified granules ($n = 572$; B, ■) and comparing them to a normal distribution (B, line; see *Materials and Methods*). Scale bars, 10 μm (top), 5 μm (bottom).

RESULTS

PHM-GFP and ACTH Are Not Uniformly Distributed in Secretory Granules in AtT20 Cells

To assess whether fluorescently labeled secretory granules accurately mimic granules containing endogenous hormone, GFP was attached to the C-terminus of PHM (Figure 1A). PAM-3, a naturally occurring form of PHM, has a second enzyme in the position chosen for GFP in the PHM-GFP constructs (Prigge *et al.*, 2000; Figure 1A). PHM-GFP was stably expressed in AtT-20 corticotrope tumor cells, which produce and store ACTH and express the prohormone convertase PC1 (Benjannet *et al.*, 1991; Bloomquist *et al.*, 1991). We considered the possibility that the ability of GFP to form low-affinity dimers might create protein-protein interactions sufficient to alter sorting of the fusion protein (Yang *et al.*, 1996; Jain *et al.*, 2001). To ensure that the interpretation of our results was not confounded by dimerization of GFP, a monomeric GFP fusion protein (PHM-mGFP) was created by substituting Ala²⁰⁶ with Lys (Zacharias *et al.*, 2002). Sucrose gradient sedimentation demonstrated that PHM-GFP aggregates at pH 5.5 but PHM-mGFP does not (unpublished data).

Vectors encoding either PHM-GFP or PHM-mGFP were used to generate stable cell lines; PHM-mGFP-expressing cells were used for all experiments unless otherwise indicated. PHM-DsRed was examined after transient transfection. PHM-mGFP and PHM-DsRed are fully active amidating enzymes and remain largely intact in AtT-20 cells (Figure 1B). PHM-mGFP is entirely intact when expressed in GH3 cells. The level

of PHM enzymatic activity contained in and secreted by the stably transfected cells ranged from 30 to 70 times higher than the PHM activity in nontransfected cells. On a molar basis, expression of PHM-mGFP was 30% that of POMC.

Control experiments were first performed to ensure that colocalization would be accurately detected with our confocal setup and quantification paradigm. Wild-type (nontransfected) AtT-20 cells were stained simultaneously with a mixture of polyclonal and monoclonal antibodies to ACTH and visualized with secondary antibodies conjugated to FITC and Cy3, respectively (Figure 2A). Because both antibodies are specific for the unblocked COOH-terminal of ACTH, we expected to see approximately equal red and green intensities in immunoreactive granules. Using an automated workflow designed with SimplePCI (Compix), granules were identified and relative red and green intensities were used to generate a ratio. Data for 572 granules (Figure 2B, ■) are compared with a theoretical normal distribution (line). As expected, most granules had green/red ratios of close to 1. Data are binned to show the distribution within a narrow range of values, almost all of which appear visually yellow. This demonstrated that our quantification paradigm was effective in detecting colocalization and could be applied experimentally to the PHM-GFP cell lines to assess colocalization of the fusion protein and endogenous hormone.

Confocal microscopy of the cell lines demonstrated that both PHM-GFP (unpublished data) and PHM-mGFP were localized in punctate structures collected near the *trans-*

Figure 3. Separation of exogenous and endogenous secretory proteins in AtT-20 cells. Cell lines stably expressing PHM-mGFP were fixed and analyzed for fusion protein (A, green) and for endogenous ACTH (A, red) by immunofluorescence. An enlargement of the merged image shows the lack of colocalization of the two secretory products. Quantification of relative green and red fluorescence intensities in identified granules ($n = 2480$) is plotted as the PHM-mGFP/ACTH ratio in the histogram (B; see *Materials and Methods*). The bars are colored to indicate the visual appearance of granules that fall within that ratio bin. The normal distribution curve (Figure 2B) is superimposed on the histogram. Less than 30% of the granules show colocalization as defined in Figure 2 and visually appear yellow. Based on the Kolmogorov-Smirnov test, this distribution differs significantly from that of the ACTH control ($p < 0.005$). Scale bars, 10 μm (top), 5 μm (bottom).

Golgi network and at the tips of cells (Figure 3A), consistent with targeting of the chimeric protein to secretory granules. Simultaneous visualization of mature ACTH revealed secretory granules similarly localized to the tips of the AtT20 cells (Figure 3A, red image). When the two images were overlaid, it was clear that the two proteins were not distributed identically (Figure 3A, merge and lower panel). The presence of PHM-mGFP but not ACTH at the TGN was expected, because PHM-mGFP fluorescence is visible upon protein folding and does not require additional processing. Mature ACTH is not detected at the same level at this early stage in the secretory pathway; the antibody used for immunolocalization requires endoproteolytic cleavage of POMC to expose the C-terminal of ACTH. Interestingly, when the secretory granules were observed under high magnification (Figure 3A, lower panel), many granules were found to display either intense green or red fluorescence, but not both, indicating a significant lack of colocalization of the two proteins.

The extent to which these soluble proteins are segregated was quantified by comparing the relative intensities of PHM-mGFP green fluorescence and red fluorescence from ACTH immunostaining. The same quantification paradigm used for the ACTH control experiment (Figure 2) was used. For quantitative experiments, cells were treated with 10 μM cycloheximide for 1 h to clear out the ER and ensure that granules were visualized (Sans *et al.*, 2005). In the 11 cells analyzed by this method, 2480 granules were identified. The distribution of green to red fluorescence intensities is plotted

in the histogram in Figure 3B, with granules that are representative of each classification indicated (Figure 3A, lower panel, colored arrows). For example, granules that appear green have ratio values of >2.5 , those that appear red have values <0.4 , and yellow granules have a ratio equal or close to 1. A range of values in between these visually distinct categories was also detected. Only 30% of the identified granules appear yellow, having PHM-mGFP/ACTH ratios close to 1. The remaining 70% contained greater amounts of either PHM-mGFP or ACTH, with more than 50% of the total granules falling into the former category. The majority of secretory granules are not homogenous with respect to their content of PHM-mGFP and ACTH.

Observations made with confocal microscopy were next verified at the ultrastructural level. Antisera to PHM and to ACTH were used to simultaneously visualize exogenous and endogenous protein. Both proteins were localized to vesicles with dense cores, verifying that proper targeting to secretory granules takes place (Figure 4A). The average number of gold particles representing PHM-GFP and ACTH per granule was quantified (Figure 4B). Although some granules contained multiple small gold particles, others contained multiple large gold particles. When normalized to compensate for differences in antibody sensitivity, the ratios failed to yield a normal distribution. Thus immunoelectron microscopy confirmed the partitioning of PHM-mGFP and ACTH detected by confocal microscopy. Both the small number of gold particles per granule and issues of antigen accessibility may contribute to the different distributions

Figure 4. Ultrastructural analysis of secretory protein localization. Cell lines stably expressing PHM-mGFP were fixed and analyzed for fusion protein (10-nm gold particles) and for endogenous ACTH (15 nm gold) by electron microscopy. Quantification of the ratio of PHM-mGFP to ACTH gold particles in identified granules (68 cells; 650 granules; >1200 of each size gold particle) is plotted as the PHM-mGFP/ACTH ratio in the histogram (B; see *Materials and Methods*). Based on the Kolmogorov-Smirnov test, this distribution differs significantly from that of the ACTH control ($p < 0.001$).

observed using immunofluorescence (Figure 3B) and electron microscopy (Figure 4B).

Endogenous and Exogenous Secretory Products Are Released in Response to Secretagogue

Because both exogenous and endogenous soluble proteins were localized to what appeared to be secretory granules, we asked what consequences the existence of these different vesicle populations would have on regulated secretion of the two products. One possibility was that PHM-GFP, although properly targeted to granules (defined microscopically), might not be in a regulated secretory compartment. To evaluate this possibility, nontransfected wild-type AtT-20 cells, AtT-20 PHM-GFP cells (three independent clones), and AtT-20 PHM-mGFP cells (three independent clones) were stimulated with the calcium-mimicking secretagogue BaCl_2 ; secretion of PHM (Figure 5, top) and ACTH (Figure 5, bottom) were measured by enzymatic assay and radioimmunoassay, respectively. For PHM-GFP and PHM-mGFP, BaCl_2 stimulated secretion of PHM and ACTH an average of 3.5-fold over the basal rate. Importantly, although the selected clones expressed different levels of PHM-GFP, the percent of cell content of both PHM (~17%) and ACTH (~38%) secreted upon secretagogue challenge was comparable across all of the clones (Figure 5). The specific activity of PHM was increased 30–70-fold in extracts of AtT-20 PHM-mGFP cells compared with wild-type AtT-20 cells. Nevertheless, expression of PHM-mGFP did not elevate basal secretion of PHM activity or ACTH compared with nontransfected AtT-20 cells, indicating that most of this exogenous product is efficiently stored in granules.

Figure 5. Storage and secretion of PHM-GFP and ACTH. Basal and stimulated secretion of PHM (top) and ACTH (bottom) were measured in AtT-20 cell lines expressing PHM-GFP or PHM-mGFP (3 independent clones each) and in wild-type AtT-20 cells. Media collected during a 30-min period of basal secretion were compared with 2 mM BaCl_2 challenge. Secretion is expressed as percent of cell content. All clones examined store both exogenous and endogenous secretory products and respond to BaCl_2 by secreting ACTH with a fold stimulation comparable to wild-type cells. Overexpression of PHM-GFP does not impair storage or secretion of this exogenous product, nor does it affect the ability of cells to store endogenous ACTH in secretagogue-responsive granules. Error bars, SD.

Figure 6. Segregation of PHM-mGFP from endogenous hormone is not unique to AtT-20 cells. Stable GH3 lines expressing PHM-mGFP were used to analyze the localization and secretagogue-responsiveness of endogenous hormones and PHM-mGFP. (A) GH3 PHM-mGFP cells were immunostained for growth hormone; a 2D projection of deconvolved images encompassing the entire cell is shown; right, magnification of the inset. (B) PHM-mGFP secretion was analyzed. Cells were either incubated without secretagogue (basal) for 30 min or challenged with medium containing 2 mM BaCl₂ (stim). Cell lysates and media (a 10-fold larger fraction) were analyzed by Western blot using a GFP antibody and the signals quantified are plotted; error bars are SD for three separate determinations. (C) Secretion of prolactin (PRL) from wild-type (WT) and PHM-mGFP GH3 cells was analyzed as in B.

Segregation of Secretory Products Is Not a Cell-Type-specific Effect

To determine whether partitioning of endogenous and exogenous secretory products was a characteristic peculiar to AtT-20 cells or a general feature, we examined GH3 cells. Unlike AtT-20 cells, GH3 cells have a roughly spherical cell morphology, express primarily PC2 instead of PC1 (Friedman *et al.*, 1996), and produce growth hormone and prolactin (Ostlund *et al.*, 1978). Stable GH3 cell lines expressing PHM-mGFP were created. Immunocytochemistry and visualization by deconvolution fluorescence microscopy showed an even more dramatic segregation of secretory products in GH3 cells than observed in AtT-20 cells (Figure 6A). Although PHM-mGFP was localized to vesicles, there was virtually complete separation of PHM-mGFP from endogenous growth hormone.

Secretion of PHM-mGFP and prolactin from wild-type and PHM-mGFP-expressing GH3 cells was next assessed by challenging the cells with BaCl₂ (Figure 6, B and C). Secretion of PHM-mGFP was increased about twofold in response to secretagogue; although largely separated from endogenous growth hormone, PHM-mGFP is stored in secretagogue-responsive granules. Under both basal and stimulated conditions, prolactin is secreted at a rate that is ~10-fold higher than the rate of PHM-mGFP secretion. Interestingly, when compared with wild-type cells, the basal rate of prolactin secretion by PHM-mGFP cells is decreased twofold (Figure 6C, bar graph). Although BaCl₂ challenge increases prolactin release from wild-type cells by 30%, it increases prolactin release from PHM-mGFP cells more than 100%. Although it is not clear how introduction of exoge-

nous secretory product increases storage of endogenous hormone, it is clear that partitioning of PHM-mGFP from endogenous secretory products is not a feature unique to AtT-20 cells.

Separation of Granule Contents Is Detectable during Secretory Granule Maturation

We next asked where PHM-mGFP is sorted from endogenous ACTH. Proteins targeted to the regulated secretory pathway are synthesized in the ER, transported through the Golgi complex to the TGN, and packaged into immature secretory granules, which must undergo a series of maturation steps to become stimulus-competent exocytotic carriers (Eaton *et al.*, 2000). To determine at what stage of granule biogenesis separation of soluble proteins occurs, AtT-20 PHM-mGFP cells were stimulated sequentially and repeatedly with BaCl₂ and PMA to deplete their hormone content (Ferraro *et al.*, 2005). After depletion, newly synthesized PHM-mGFP was detected in the vicinity of the Golgi complex and in the cisternae of the TGN, as indicated by colocalization with TGN38 (Figure 7A). POMC products were also observed in this area using an antibody that recognizes POMC and any smaller products containing the N-terminal region of ACTH. Partial colocalization of POMC and PHM-mGFP is observed in tubuloreticular structures (Figure 7B). Immediately adjacent to areas of colocalization, PHM-mGFP and POMC products are found in separate vesicles, suggesting that segregation occurs early, either during vesicle budding from the TGN or during the maturation of immature secretory granules.

Figure 7. Separation of PHM-mGFP and POMC occurs during secretory granule maturation. To clear the cytoplasm of secretory granules, AtT-20 PHM-mGFP cells were subjected to a hormone depletion paradigm during which they were sequentially stimulated for five periods (20 min each) with 2 mM BaCl_2 (periods 1, 3, and 5) and 1 μM PMA (periods 2 and 4; Ferraro *et al.*, 2005). PHM-mGFP accumulates in and adjacent to tubuloreticular structures immunoreactive for TGN38 (A). Appreciable colocalization and juxtaposition of POMC and PHM-mGFP are visible in the Golgi and TGN areas (B; left: higher magnification of the inset in the right panel). Processed ACTH does not colocalize with PHM-mGFP (C). At the end of a 20°C block, conventional electron microscopy shows condensing vacuoles in the juxtanuclear region (D, left); analysis of serial sections would be required to determine whether some of these condensing vacuoles had lost their connection to the TGN and become immature granules. Immunoelectron micrographs demonstrate extensive colocalization of POMC (15-nm gold) with PHM-mGFP (10-nm gold) in condensing vacuoles (D, right), indicating that segregation does not occur without concentration and aggregation of secretory products. Scale bars, 10 μm (A–C, left), 5 μm (A–C, right), and 500 nm (D).

In AtT-20 cells, PC1 cleaves POMC to produce ACTH biosynthetic intermediate and β -lipotropin; earlier immunoelectron microscopic studies demonstrated that this cleavage is initiated in the TGN, but occurs primarily in immature granules (Schnabel *et al.*, 1989). Immunostaining with antisera specific to these POMC cleavage products detected very little immunoreactivity in the reticular structures of the TGN (Figure 7C); most of the granules identified by antisera

specific for the COOH-terminal of ACTH do not contain PHM-mGFP. This suggests that PHM-mGFP is mixed with POMC precursor in the TGN and at earlier stages of the secretory pathway, but tends to part company from POMC and its mature products during packaging into or maturation of immature secretory granules.

To distinguish between these two possibilities, a 20°C temperature block was used to accumulate secretory prod-

ucts in the TGN and immature secretory granules, and localization of PHM-mGFP and POMC was evaluated by immunoelectron microscopy (Saraste and Kuismanen, 1984; Griffiths *et al.*, 1985). In conventional electron microscopic sections, immature secretory granules in AtT-20 cells are characterized by an electron dense core surrounded by a clear halo (Tooze and Tooze, 1986; Alam *et al.*, 2001). When AtT-20 PHM-mGFP cells were incubated for 30 min at 20°C, large condensing vacuoles were observed in the TGN area (Figure 7D, left). The lack of an electron-dense precipitate in these vacuoles indicates that aggregation of secretory products was inhibited by the temperature block. The milder embedding used for immunoelectron microscopy better preserved the protein content of these structures, revealing extensive colocalization of POMC and PHM-mGFP (Figure 7D, right). Whether these organelles containing unsorted cargo are immature granules or still maintain a connection to the TGN (a distinction that cannot be made by examining individual sections), it is clear that the segregation of soluble cargo proteins does not occur at the level of the TGN. This demonstrates that segregation requires concentration and aggregation of secretory products and therefore occurs during the process of secretory granule maturation.

Neither Acidification nor Normal Membrane Cholesterol Is Essential to the Partitioning of PHM-mGFP from ACTH

Low pH-induced aggregation is one mechanism proposed for sorting of soluble granule content proteins (von Zastrow *et al.*, 1989; Laine and Lebel, 1999; Jain *et al.*, 2000). To test whether acidic pH were required for sorting of PHM-mGFP and ACTH from each other, the pH gradients across the membranes of acidic compartments in AtT-20 PHM-mGFP cells were dissipated with the alkalinizing agent ammonium chloride (Mains and May, 1988; Wu *et al.*, 2000, 2001). Ammonium chloride effectively disrupts acidification in the ER, Golgi, TGN, and other acidic organelles due to rapid entry of membrane permeable NH₃ (Wu *et al.*, 2000). The existing granule pool was first depleted by sequential and repeated stimulations with BaCl₂ and PMA in the presence of cycloheximide, followed by a 12-h incubation in cycloheximide (Figure 8A, left). When the cycloheximide is removed and replaced with growth medium, cells recover and the granule pool is replenished within 36 h (Figure 8A, right). To ensure visualization of new granules, alkalization was preceded by this depletion/cycloheximide paradigm. Localization of PHM-mGFP and ACTH was assessed after 36 h of ammonium chloride treatment. As in control cells (Figures 2A and 8A, right), the two secretory products are largely in distinct green and red puncta (Figure 8A). Effective disruption of the pH gradient was confirmed by labeling control and ammonium chloride-treated cells with acridine orange, a weak base that accumulates in acidic compartments. Control cells demonstrate intense labeling of acidic organelles with this dye (Figure 8C, left), primarily at the tips of cells, where secretory granules accumulate. This fluorescence is significantly diminished by ammonium chloride pretreatment (Figure 8C, right). It is important to note that granules formed in the presence of ammonium chloride are authentic secretory granules. As observed in control cells, PHM-GFP does not colocalize with lysosomal-associated membrane protein LAMP-1 after ammonium chloride treatment (Supplementary Figure S1) and basal synthesis and regulated secretion of β -endorphin are not altered by ammonium chloride treatment (Mains and May, 1988).

In addition to low pH, cholesterol has an essential function in secretory granule biogenesis, and association of regulated

secretory proteins with cholesterol-rich membrane microdomains has been proposed as a mechanism by which selective entry into granules occurs (Wang *et al.*, 2000; Dhanvantari and Loh, 2000). If cholesterol normally contributes to the sorting of soluble content proteins, its depletion could preclude separation of PHM-mGFP from ACTH. This hypothesis was tested by depleting the existing granule pool and inhibiting protein synthesis as above, followed by inhibition of cholesterol synthesis with 5 μ M lovastatin and resumption of protein synthesis for 36 h. This treatment resulted in an $\sim$ 50% reduction in cellular cholesterol as determined by quantification in cell extracts (unpublished data). This level of cholesterol reduction has been shown to be sufficient to inhibit secretory granule biogenesis (Wang *et al.*, 2000), and lovastatin treatment reduced the total number of PHM-mGFP- and ACTH-containing secretory granules observed (Figure 8D). The granules that were present, however, exhibited the same type of segregation of PHM-mGFP from ACTH observed in untreated cells, suggesting that cholesterol does not play an essential role in the separation of these regulated secretory products. That PHM-GFP synthesized in the presence of lovastatin is not mistargeted to lysosomes is shown by its lack of colocalization with LAMP-1 (Supplementary Figure S1D).

Partitioning of Soluble Granule Proteins Is a Common Occurrence

The previous results raised the possibility that partitioning of endogenous protein from PHM-mGFP might be caused by the presence of GFP, which was not evolutionarily designed to be a regulated secretory protein. For this reason, we next examined AtT-20 cells stably expressing PHM (without GFP) or NPY. PHM is normally produced from its membrane precursor by endoproteolytic cleavage and was previously shown to be efficiently stored in granules in AtT-20 cells (Milgram *et al.*, 1992, 1994). Although not normally produced in AtT-20 cells, pro-NPY was previously shown to be cleaved into mature NPY, which was efficiently packaged in AtT-20 cells (Dickerson *et al.*, 1987).

Confocal microscopy indicated that PHM and ACTH were largely present in separate granules (Figure 9A). The presence of distinct green and red fluorescent puncta representing immunoreactive PHM and ACTH is comparable to the segregation observed when comparing PHM-mGFP and ACTH. Thus the presence of GFP is not responsible for the partitioning of soluble regulated secretory proteins. Interestingly, cells expressing NPY at levels equimolar to ACTH did not exhibit this phenomenon, and the two secretory products, NPY and ACTH, were localized in the same granules (Figure 9B). These data strongly suggest that separation of soluble products into distinct granule populations is not an artifact of overexpression of secretory products. Overexpressed PHM partitions from endogenous ACTH, regardless of the presence or absence of the GFP moiety, while overexpressed NPY does not.

Biophysical Properties of Cargo Molecules Govern Their Differential Sorting

On the basis of these results, we considered the possibility that separation of secretory products was related to their differing abilities to interact with themselves or with each other in the changing luminal environment of the TGN/immature secretory granule or with their differing abilities to interact with the membranes of the TGN or immature granules. To test this more directly, AtT-20 cells were transiently cotransfected with vectors encoding two different fluorescent PHM fusion proteins: PHM-mGFP and PHM-DsRed. The 45-kDa PHM domains are identical and, based

Figure 8. Neither acidification nor cholesterol is essential for partitioning of soluble contents. The existing pool of secretory granules was depleted by subjecting AtT-20 PHM-mGFP cells to our depletion paradigm in the presence of 10 μ M cycloheximide, followed by a 12-h chase in cycloheximide (A, left). After removing the cycloheximide, the granule pool is replenished within 36 h (A, right). All drug treatments were preceded by this depletion paradigm to ensure visualization of granules formed during the period of the experiment. (B and C) AtT-20 cells expressing PHM-mGFP were incubated with ammonium chloride for 36 h to dissipate pH gradients; treatment efficacy was verified by the reduced fluorescence of the acidophilic dye acridine orange in ammonium chloride treated versus control cells. Luminal alkalinization had no effect on PHM-mGFP and ACTH segregation (A). (C) Lovastatin treatment for 36 h reduced cellular levels of cholesterol by 50% (unpublished data). In these conditions, the number of granules was reduced, but partitioning of ACTH from PHM-mGFP was not prevented. Scale bars, 10 μ m (A; left, B and D), 5 μ m (right, B and D), and 20 μ m (C).

on assessment of enzyme activity, fold into an active conformation. However, the mGFP and DsRed sequences are only ~30% identical. Because PHMcc molecules differing at

a single amino acid crystallized under different conditions (Siebert *et al.*, 2005), PHM-GFP and PHM-DsRed would not be expected to cocrystallize in immature granules. Consis-

Figure 9. Differential sorting may reflect the biophysical properties of the granule proteins. Immunocytochemistry was performed on AtT-20 cell lines stably expressing PHM (A) or proneurotensin Y (B) to evaluate localization with respect to endogenous ACTH. NPY (green, B) and ACTH (red, B) are stored in the same granules, whereas ACTH (red, A) segregates from PHM (green, A). (C) Cotransfection of vectors encoding PHM-mGFP and PHM-DsRed resulted in their segregation into separate vesicles. Partitioning may reflect the biophysical properties of the soluble molecules. Scale bars, 10 μ m (left), 5 μ m (right).

tent with this hypothesis, confocal microscopy shows that the coexpressed PHM fusion proteins are largely present in separate granules (Figure 9C). This result demonstrates that different proteins large enough to form stable structures are likely to partition into separate granules.

DISCUSSION

Our data demonstrate that segregation of soluble secretory granule proteins occurs quite commonly. When stably expressed in AtT-20 and GH3 cells, PHM-mGFP is largely in granules separate from those containing endogenous hormone. PHM-mGFP and PHM-DsRed show partial separation from each other. Despite this segregation, both exogenous and endogenous secretory products are efficiently stored and undergo regulated exocytosis. These observations are consistent with an important role for self-aggrega-

tion in granule biogenesis and must be taken into account when using fluorescently tagged proteins to study granule dynamics and exocytosis.

Self-Aggregation: A Default Mechanism for Cargo Protein Sorting

The simplest interpretation of our data are that the biophysical properties of soluble secretory granule proteins govern their segregation (Figure 10). In granules, luminal protein concentrations are high, 100–150 mg/ml; growth hormone, insulin, and ACTH concentrations range from 10 to 42 mM (Hutton *et al.*, 1983; Oyarce *et al.*, 1996; Dannies, 1999). In exocrine pancreatic cells, granule content proteins show the greatest increases in concentration between the ER and the TGN, with smaller increases occurring as they move from the TGN into granules (Oprins *et al.*, 2001). This suggests that concentrations in the TGN may favor self-association

Figure 10. Self-aggregation default model for protein sorting. The extent to which proteins are segregated is dictated by the biophysical properties of the individual molecules and their level of expression. Peptides with no defined structure (e.g., NPY, ACTH) lack a tendency to self-associate, are mixed in transit through the secretory pathway, and tend to be homogeneously packaged into secretory granules. Homotypic aggregation of structured proteins (e.g., PHM-GFP, GH, PRL) occurs when luminal concentrations are high. Because even a small aggregate will occupy much of the space in a single granule, other content proteins (e.g., ACTH) are excluded and partitioning occurs.

(as in secretory granules). In the cell lines examined, expression of endogenous POMC and exogenous PHM, PHM-GFP, or NPY was roughly equimolar. PHM crystallizes *in vitro* at concentrations around 1 mM (Prigge *et al.*, 2000), meaning that levels reached in the TGN and immature secretory granules could be conducive to self-association. Because each granule contains only 5000–10,000 molecules of its major content protein, any tendency of content proteins to self-associate could lead to their partial segregation.

To exclude the possibility that the presence of a fluorescent tag caused this separation, we compared the localizations of ACTH and PHM. As observed for PHM-mGFP, ACTH and PHM were not uniformly distributed in secretory granules. Despite sharing an identical domain, PHM-mGFP and PHM-DsRed were partially segregated. PHM, mGFP, and DsRed each adopt a stable, folded structure and have been crystallized (www.rcsb.org/pdb: 1RRX; 1ZGO; 1OPM; Zacharias *et al.*, 2002). In contrast, NPY and ACTH, which are unstructured (Mozsolits *et al.*, 1999; Thomas *et al.*, 2005), showed little segregation; neither pro-NPY nor POMC has been crystallized (www.rcsb.org/pdb). The simplest hypothesis consistent with our data is an old one, namely that individual proteins dictate the extent of their segregation in part through their ability to self-associate (Figure 10).

For some proteins, self-association may begin early in the secretory pathway. Numerous electron microscopic and biochemical studies have established that aggregation of soluble cargo can occur in the ER, resulting in homotypic aggregates of secretory proteins. The mRNAs encoding POMC and PHM-mGFP can accommodate up to 25 and 80 ribosomes, respectively (Wolin and Walter, 1988). Polysomes yielding multiple nascent chains in close proximity could promote early aggregation. POMC forms oligomers independent of pH and calcium, and its self-association could occur in the ER (Cawley *et al.*, 2000). Voltage-gated potassium channels are known to form tetramers while still attached to ribosomes (Lu *et al.*, 2001). Magnocellular neurons have regions of ER devoted to the synthesis of vasopressin

versus galanin, which could result in elevated local concentrations (Landry *et al.*, 2003).

A major step in the condensation of pancreatic proteins occurs between the ER and *cis*-Golgi (Oprins *et al.*, 2001). Additional condensation occurs in more distal compartments, with differences observed between the behavior of amylase, chymotrypsinogen, and procarboxypeptidase A. Aggregated ANP has been detected throughout the Golgi complex (Slot *et al.*, 1997). Prolactin (Dannies, 1999) and POMC (Schnabel *et al.*, 1989) aggregates are seen in the lumen of the TGN. Although low pH promotes the aggregation of some granule proteins (Gerdes *et al.*, 1989; Gorr *et al.*, 1989; Colomer *et al.*, 1996), segregation of the granule content proteins we studied was not eliminated after alkalization of the granule lumen. Although different proteins will have different tendencies to self-aggregate, increased concentrations of luminal proteins as during secretory granule maturation will promote aggregation.

Endogenous Granule Content Proteins Segregate from Each Other

Segregation of granule content proteins is not an artifact of exogenous expression. Electron microscopic studies of somatomammotrophs demonstrated that growth hormone and prolactin are rarely found equally intermixed in granules (Fumagalli and Zanini, 1985; Hashimoto *et al.*, 1987). Follicle-stimulating hormone (FSH) and luteinizing hormone (LH), produced in the same gonadotrope, reside in distinct secretory granules (Thomas and Clarke, 1997). The bag cell neurons of *Aplysia* contain separate granules with peptides derived from the N- or C-terminal region of the egg-laying hormone precursor (Fisher *et al.*, 1988). Granules containing bag cell peptides are released locally, whereas granules containing egg-laying hormone are released into the circulation (Sossin *et al.*, 1990). Beginning with translation in different domains of the ER, hypothalamic magnocellular neurons segregate their neuropeptide cargoes, with galanin and vasopressin localized to separate granules that are targeted to dendrites (galanin) or axons (vasopressin; Landry *et al.*, 2003). Despite these well-established examples, investigators have generally assumed that their exogenous marker protein mimics endogenous hormone. Based on our observations, high-resolution analysis of colocalization is required before this conclusion can be accepted.

Self-Aggregation and Other Modes of Sorting within the Secretory Pathway

In addition to self-aggregation, luminal pH and calcium, endoproteolytic cleavage, lipid rafts, and heterologous protein–protein interactions clearly affect granule formation. Two models, sorting for entry and sorting by retention, provide a framework in which to consider the contribution of self-aggregation to granule formation (Huang and Arvan, 1994; Arvan and Castle, 1998; Gorr *et al.*, 2001). In the sorting for entry model, the interactions of luminal proteins with membrane proteins (e.g., carboxypeptidase E [CPE]; Cool *et al.*, 1997) or with detergent-resistant lipid rafts (Blazquez *et al.*, 2000; Martin-Belmonte *et al.*, 2000; Taylor *et al.*, 2002) play a key role. In the sorting by retention model, protein aggregates remain in granules, whereas nongranule proteins are removed (Kuliawat *et al.*, 2000). The ability of some granule content proteins to generate granule-like structures when expressed in cells lacking a regulated secretory pathway is consistent with an important role for self-aggregation (Beuret *et al.*, 2004).

Consistent with earlier work showing that corticotropes do not require a low pH step for the entry of POMC into

granules or the occurrence of stimulated secretion (Mains and May, 1988), segregation of ACTH and PHM-mGFP continued to occur when cells were treated with ammonium chloride (Figure 8), chloroquine, or bafilomycin (unpublished data). As in AtT-20 cells, ammonium chloride did not alter the delivery of regulated secretory proteins to granules in parotid cells (von Zastrow *et al.*, 1989). The sorting of other granule proteins is dependent on changes in pH (Gerdes *et al.*, 1989; Gorr *et al.*, 1989; Colomer *et al.*, 1996). Low pH and high calcium concentrations have a synergistic effect on aggregation of the granins, and changing the milieu of the ER to TGN-like conditions is sufficient to trigger their aggregation (Chanat and Huttner, 1991). For secretory proteins that require low pH to aggregate, alkalization would preclude their condensation.

The role played by detergent-resistant cholesterol-glycosphingolipid-rich lipid rafts in granule biogenesis was investigated by partially depleting cholesterol using lovastatin. Although cholesterol plays an essential role in granule biogenesis (Thiele *et al.*, 2000; Wang *et al.*, 2000), depletion of cholesterol to a level that limits secretory granule formation did not prevent the segregation PHM-mGFP from ACTH in the few granules formed (Figure 8C). This observation argues against an essential role for cholesterol or raft-anchored proteins in the segregation process. An association of both CPE and secretogranin III with detergent-resistant rafts has been reported to affect the sorting of POMC, ACTH, and chromogranin A into granules (Dhanvantari and Loh, 2000; Hosaka *et al.*, 2004, 2005).

Work with other secreted proteins supports the importance of intermolecular interactions in cargo sorting and storage. Nonaggregating salivary proteins expressed in AtT-20 cells are not retained in mature granules and are recovered instead from basal medium (Castle *et al.*, 1997). The segregation of salivary proline-rich proteins from ACTH was attributed to differences in the conditions required for aggregation.

Endoproteolytic cleavage clearly affects the packaging of granule proteins; this effect may in part reflect differences in the ability of precursor and product to self-associate. Proinsulin enters immature granules in the soluble phase, and endoproteolytic cleavage is essential before crystallization of insulin can occur (Kuliawat and Arvan, 1994). Granular insulin is largely insoluble, whereas proinsulin and C-peptide are soluble (Kuliawat *et al.*, 2000). PC1-mediated processing of proinsulin into insulin causes a change in its biophysical state, resulting in homotypic polymerization and subsequent retention in granules (Kuliawat and Arvan, 1994). Endoproteolytic cleavage is essential for retention, because GH4C1 cells (which lack PC1) cannot efficiently store insulin (Reaves *et al.*, 1990).

Our data show mixing of newly synthesized PHM-mGFP with intact POMC in the vicinity of the Golgi and TGN, with segregation of PHM-mGFP from ACTH first apparent in vesicular structures adjacent to this region (Figure 7, B and C). Electron microscopy after a 20°C block clearly shows that segregation does not occur without concentration and aggregation of secretory products (Figure 7D). Extensive colocalization of POMC products and PHM-mGFP in immature secretory granules suggests that partitioning requires aggregation during the process of granule maturation.

Pronounced differences in the behavior of closely related fluorescently tagged granule proteins has been noted previously (Michael *et al.*, 2004). Close examination of confocal micrographs showing colocalization of endogenous and exogenous granule proteins often reveals a great deal of heterogeneity, suggesting that a more detailed analysis would reveal partial segregation. Coupled with the effects of controlled changes in the luminal milieu, the effects of con-

trolled endoproteolysis, heterologous protein-protein interactions and the interactions of proteins with lipids, secretory granule formation can be understood in terms of simple biophysical principles.

Our study, along with a large body of evidence from other investigators, shows that the tendency for homotypic aggregation, along with modulators such as pH, calcium levels, and lipid concentrations, are major determinants in the sorting and segregation of soluble proteins in the regulated pathway. In nontransfected AtT-20 cells, secretory granules contain ~1 molecule of PHM for each 1000 molecules of POMC-derived peptides; the tendency of PHM to aggregate is not relevant to the sorting process. In contrast, partial segregation by homotypic aggregation is accentuated in studies that involve the overexpression of marker proteins (Oyarce *et al.*, 1996).

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