Expression of Regulated Secretory Proteins Is Sufficient to Generate Granule-like Structures in Constitutively Secreting Cells*

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The formation of secretory granules and regulated secretion are generally assumed to occur only in specialized endocrine, neuronal, or exocrine cells. We discovered that regulated secretory proteins such as the hormone precursors pro-opiomelanocortin, pro-oxytocin, and pro-opiomelanocortin, as well as the granins secretogranin II and chromogranin B but not the constitutive secretory protein α1-protease inhibitor, accumulate in granular structures at the Golgi and in the cell periphery in transfected COS-1 fibroblast cells. The accumulations were observed in 30–70% of the transfected cells expressing the pro-hormones and for virtually all of the cells expressing the granins. Similar structures were also generated in other cell lines believed to be lacking a regulated secretory pathway. The accumulations resembled secretory granules morphologically in immunofluorescence and electron microscopy. They were devoid of markers of the endoplasmic reticulum, endosomes, and lysosomes but in part stained positive for the trans-Golgi network marker TGN46, consistent with their formation at the trans-Golgi network. When different regulated proteins were coexpressed, they were frequently found in the same granules, whereas α1-protease inhibitor could not be detected in accumulations formed by secretogranin II, demonstrating segregation of regulated from constitutive secretory proteins. In pulse-chase experiments, significant intracellular storage of secretogranin II and chromogranin B was observed and secretion of retained secretogranin II was stimulated with the calcium ionophore A23187. The results suggest that expression of regulated cargo proteins is sufficient to generate structures that resemble secretory granules in the background of constitutively secreting cells, supporting earlier proposals on the mechanism of granule formation.

Endocrine and neuroendocrine cells possess a regulated secretory pathway in addition to the constitutive pathway present in all cells (1). At the trans-Golgi network (TGN), regulated cargo proteins, such as peptide hormone precursors and granins, are sorted into secretory granules where they are stored in a densely packed form. By an external stimulus, the granules are triggered to fuse with the plasma membrane and to release their contents in a controlled manner. The regulated secretory pathway thus requires mechanisms to segregate regulated cargo from constitutively secreted proteins and to package them into specialized vesicles. These membrane-bounded organelles in addition recruit pro-hormone-processing enzymes as well as the components necessary for luminal acidification for transport of the granules to the cell periphery or along the axon and for the controlled fusion with the plasma membrane. So far, little is known regarding the machinery that is required to generate secretory granules. Two non-exclusive models have been proposed on how secretory granules are formed and how specific cargo selection is accomplished (2, 3). The first model, termed “sorting-for-entry,” is analogous to receptor-mediated endocytosis and mannose 6-phosphate receptor-dependent lysosomal transport (4) where cargo binds to receptors, which in turn recruit a cytosolic coat. Similarly, regulated secretory proteins may be selected and other proteins excluded by interaction with receptors in the TGN membrane prior to granule formation. Consistent with this model, the propeptide of prosomatostatin (5) and a disulfide-bonded loop segment of chromogranin B (CgB) (6, 7) have been shown to be necessary and sufficient to mediate granule sorting, suggesting that they constitute sorting signals. An amphipathic loop of pro-opiomelanocortin (POMC) was also found to be necessary for sorting (8). Carboxypeptidase E was reported to bind to this loop and to be required for granule sorting (9). Its proposed role as a sorting receptor, however, is controversial (10). The apparent substoichiometric amount of putative sorting receptors in secretory granules may be explained by the tendency of regulated secretory proteins to aggregate under the conditions of the trans-Golgi (low pH and high calcium concentrations) (e.g. Refs. 11 and 12), which would allow each receptor to sort an entire polymer of cargo molecules.

The alternative model, “sorting-by-retention,” proposes that selective aggregation of regulated cargo in the TGN results in an immature granule. Captured non-granule molecules are subsequently removed in vesicles budding from maturing secretory granules by clathrin-coated vesicles and by so-called constitutive-like secretion, whereas specific granule cargo is retained (13). This explains the presence of the mannose 6-phosphate receptor, clathrin, and AP-1 adaptors on immature secretory granules (14, 15).
Recently, it has been proposed that a single protein of endocrine and neuronal cells, chromogranin A (CgA), controls secretory granule biogenesis (18). Expression of CgA was even found to induce granular structures in transfected CV-1 fibroblast cells. The interpretation of these observations has been discussed controversially (17–19). In the alternative view, condensing CgA might act as an aggregation or assembly factor specific for the sorting-by-sequestration model (17).

In this study, we report that several other cargo proteins of the regulated secretory pathway of endocrine cells, peptide hormone precursors as well as granins, induce the formation of granule-like structures when expressed in cell lines normally lacking regulated secretory expression. Expression of cargo is sufficient to drive segregation of regulated and constitutive secretory proteins and the formation of dense membrane-bound accumulations with similar ultrastructural appearance as secretory granules, suggesting that initial granule formation requires no additional machinery specific to regulated secretory cells besides the regulated cargo itself.

EXPERIMENTAL PROCEDURES
cDNAs for regulated secretory proteins were gifts by G. Boeke (porcine POMC, University of Montreal), H. Gerdes (human secretogranin II (SgII) and CgB, University of Heidelberg), and M. Ito (human vasopressin, Northwestern University, Chicago, IL). The coding sequence of human oxytocin was assembled from the exons amplified by polymerase chain reaction from the gene (a gift by J. Amico, University of Pittsburgh). The cDNA of human α2-proteinase inhibitor (A1Pi) was from J. L. Brown (University of Colorado, Denver, CO). To C-terminally tag proteins with a C-Myc epitope, a KpnI restriction site was introduced by polymerase chain reaction in place of the stop codon for ligation to the Myc epitope sequence encoding EQKLISEEDLN. In the same way, the C1 epitope ETELDKASQEPLLSTOP corresponding to the C-terminal sequence of the human ayalogycin-preproprotein (aPPI), for which we have a rabbit anti-peptide antiserum (20), was fused to CgB and SgII. The cDNAs were subcloned into the SV40-based expression plasmids pECE or pCB6. For expression in COS-1 cells without plasmid amplification, SgII-Myc was also cloned into pcDNA5 (Invitrogen), which lacks SV40 sequences.

Cell Culture and Transfection—Madin-Darby canine kidney strain II and HepG2 hepatoma cells were grown in minimal essential medium, COS-1 Chinese hamster ovary (CHO)-K1, human embryo kidney (HEK293), and NIH-3T3 cells in Dulbecco’s minimal essential medium supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum at 37 °C with 7.5% CO2. Cells were transfected using Lipofectin (Invitrogen) with 2 μg of plasmid DNA/10-mm dish at ~40% confuency and processed after 48 h. To reduce expression levels of SgII-Myc in COS cells, the SgII-Myc expression plasmid was gradually diluted with pEGFP-N1 (Clontech), whereby the total amount of plasmid was kept constant at 2 μg/35-mm plate. Expression levels were assayed by immunoblot analysis using rabbit anti-secretogranin antisemur (21), a gift from R. Fischer-Colbrie (University of Innsbruck), at a dilution of 1:1000. To generate stable cell lines, HEK293 cells were transfected with pcCB6 containing the vasopressin precursor cDNA and subjected to selection with 1 mg/ml G418-sulfate (Invitrogen). Resistant lines were cloned and analyzed by immunoblotting and immunofluorescence microscopy.

Immunofluorescence and Antibodies—We used rabbit anti-neurophysin II antibodies against neurophysin II (NPN II) which also recognizes neurophysin I of pro-oxytocin (ICN); rabbit anti-human adrenocorticotropic hormone (ACTH) to detect POMC (Sigma); sheep anti-human TGN46 (Serotech); rabbit anti-A1Pi antisemur from Jerry L. Brown (University of Colorado Health Sciences Center, Denver, CO); rabbit anti-human EEA1 (early endosome antigen 1) antisemur from H. Stenmark (Norwegian Radium Hospital, Oslo, Norway); goat anti-protein-disulfide isomerase from H. P. Hauri (Biozentrum, Basel, Switzerland); monoclonal antibodies against the C-Myc epitope (9E10) (22) against giantin from H. P. Hauri, against lysosom-associated membrane protein-1 (LAMP-1) from J. Rohrer (FMI, Basel, Switzerland), and against Rab5 from R. Jahn (MPI, Göttingen, The Netherlands); and a rabbit antipeptide recognizing the C1 epitope (20). Antibodies were used at a dilution of 1:100 with the exception of anti-ACTH (1:2000) and anti-Rab5 (1:800). As secondary antibodies, non-cross-reacting Cy3-labeled goat anti-mouse, Cy2-labeled goat anti-rabbit, Cy3-labeled donkey anti-sheep, and Cy2-labeled donkey anti-rabbit immunoglobulin antibodies (from Jackson Immunoresearch and Amersham Biosciences) were used as appropriate according to the manufacturers’ recommendations.

Cells were grown on 14-mm glass coverslips, fixed with 3% paraformaldehyde for 15 min at room temperature, washed in phosphate-buffered saline (PBS), quenched with 50 mM NH4Cl in PBS, and permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific antibody binding was blocked with PBS containing 1% bovine serum albumin. The fixed cells were incubated at room temperature with primary antibodies for 1 h, washed with PBS with albumin, and stained with fluorescent secondary antibodies in PBS with albumin for 30 min. After several washes with PBS with albumin, PBS, and water, the coverslips were mounted in Mowiol 4-88 (Hoechst). Staining patterns were analyzed using a Zeiss Axiospl 2 microscope with a KH Series imaging system (Apogee Instruments) or a Zeiss Axiosvert 200 M confocal LSM 5 Meta microscope.

Electron Microscopy—To enrich transfected cells for ultrastructural analysis, COS-1 cells were cotransfected with an expression plasmid encoding pro-vasopressin or SgII-Myc and a plasmid pEGFP-N1 encoding green fluorescent protein (GFP). The cells were brought into suspension by trypsinization and were sorted for GFP fluorescence using a MoFlo cell sorter (Cytomation, Fort Collins, CO). They were then fixed with 3% paraformaldehyde, 0.5% glutaraldehyde in PBS, pH 7.4, for 1 h at room temperature, washed with PBS, incubated with 1% osmium tetroxide for 1 h, washed with water, and dehydrated by successive 10-min incubations with 50, 70, 90, and 100% ethanol followed by 1 h with Epon/acetone 1:1, 1 h with Epon/acetone 2:1, and twice for 2 h with Epon and 24–48 h at 60 °C. Thin sections were stained with 6% uranyl acetate for 1 h and with lead acetate for 2 min.

For immunogold labeling, GFP-positive cells expressing SgII-Myc were fixed with 3% paraformaldehyde, 0.5% glutaraldehyde in PBS for 1 h, washed with PBS, fixed again with 0.5% osmium tetroxide for 1 h, washed with water, and incubated 15 min each with 50 and 70% ethanol, 1 h with ethanol/LR White 2:1, 1 h with LR White (from Polysciences, Warrington, PA), and 24–48 h at 60 °C. Thin sections were then blocked twice for 5 min with 2% bovine serum albumin in PBS, incubated with anti-Myc antibody 9E10 in 2% bovine serum albumin for 3 h, washed three times for 10 min with PBS, and again twice for 5 min with 2% bovine serum albumin, incubated for 1 h with goat anti-mouse immunoglobulin (from British Biocell) coupled to 10-nm gold, washed with PBS and with water, and contrasted with 6% uranyl acetate for 1 h and with lead acetate for 2 min.

Storage and Stimulation Assays—To analyze the secretion behavior, COS-1 cells transfected with A1PiTS (23), CgB-C1, or SgII-C1 were labeled with [35S]Sulfate (0.5 μCi/ml, from Amersham Biosciences) in sulfate-free medium (from Invitrogen) for 90 min at 19 °C followed by a chase at 37 °C for up to 6 h in medium containing excess unlabeled sulfate. The medium was removed and replaced by fresh chase medium at different times. The labeled protein secreted into the medium as well as the amount endocytosed in the cells during the course of the experiment was immuno-precipitated and analyzed by gel electrophoresis and autoradiography. Signals were quantified by PhosphorImager.

To analyze stimulated secretion, COS-1 cells expressing SgII-C1 were labeled with [35S]Sulfate for 90 min at 19 °C followed by a chase of 3 h, after which the medium was replaced by medium with or without 1 μM A23187 (Sigma; prepared by adding a 100-fold concentrated stock solution in Me2SO or just Me2SO to the medium). Labeled protein secreted into the medium during this time was analyzed by immuno-precipitation, gel electrophoresis, and autoradiography. The total amount of labeled SgII-C1 was determined by immunoprecipitation from the medium and the cell lysate of parallel aliquots of transfected cells after the labeling period. Cell integrity and budding of trypsin blue exclusion was not affected by 1 μM A23187.

To estimate the effect of stimulation on the density of granule-like structures, transfected COS-1 cells expressing SgII-Myc were incubated for 30 min with fresh medium with or without 1 μM A23187 and then fixed and processed for immunofluorescence. 50 random cells of each group were photographed, and the granular and nongranular areas were counted, excluding the Golgi/TGN area of the cells where structures could not be separated from each other by a person unaware of the sample identity. The numbers were normalized for the size of the cells as estimated by measuring the area from tracings of the cell outlines excluding the Golgi area using Adobe Photoshop.

RESULTS

Pre-vasopressin Expressed in COS-1 Cells Accumulates in Granular Structures—The vasopressin precursor protein is
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Regulated secretory proteins expressed in transfected COS-1 cells accumulate in granular accumulations. Transfected COS-1 cells expressing pro-vasopressin (A–C), pro-oxytocin (D), POMC (E), CgB-Myc (F), and SgII-Myc (G) were analyzed by indirect immunofluorescence using antibodies against neurophysin, ACTH, and the Myc epitope, respectively. In panel C, the same field as in C is shown by differential interference contrast microscopy. The micrographs are representative of ~50, ~30, and ~75% of the cells expressing pro-vasopressin, pro-oxytocin, or POMC, respectively. The other cells showed low staining of ER and Golgi as expected for a constitutively secreted protein. Essentially all of the cells expressing CgB-Myc or SgII-Myc showed the granular accumulations as shown in panels A–D, Bar, 20 μm.

Granule-like Structures Are Formed by Various Regulated Cargo Proteins—To test whether the ability to form granule-like structures is unique to the vasopressin precursor, the cDNAs of other granule cargo were transiently expressed in transfected COS-1 cells and analyzed by immunofluorescence. Pro-oxytocin is closely related to pro-vasopressin with 74% identity. In approximately one-third of COS-1 cells expressing pro-oxytocin, a punctate pattern of accumulated protein was detected (Fig. 1D), very similar to the finer structures generated by pro-vasopressin. POMC, the precursor of ACTH and other active hormones, is closely related to pro-vasopressin with 74% identity. In approximately one-third of COS-1 cells expressing pro-oxytocin, a punctate pattern of accumulated protein was detected (Fig. 1D), very similar to the finer structures generated by pro-vasopressin. POMC, the precursor of ACTH and other active hormones, is closely related to pro-vasopressin with 74% identity. In approximately one-third of COS-1 cells expressing pro-oxytocin, a punctate pattern of accumulated protein was detected (Fig. 1D), very similar to the finer structures generated by pro-vasopressin.
Formation of Granular Structures Is Not Specific to COS Cells or to High Overexpression—The formation of these accumulations in COS-1 cells may be due to the latent expression of the putative machinery to generate secretory granules and/or to the high expression levels obtained by T antigen-mediated amplification of the SV40-based expression plasmid used. To test this possibility, SgII-Myc or pro-vasopressin were transfected into HepG2, NIH-3T3, Madin-Darby canine kidney II, HEK293, or CHO-K1 cells. In all of the cell lines, 40–80% of the cells showed granular accumulations of pro-vasopressin without correlation to the expression levels. Bar, 20 μm.

Furthermore, we generated clonal HEK293 cell lines stably expressing different levels of vasopressin precursor (Fig. 3B). In all of the lines, 40–80% of the cells showed granular accumulations without correlation to expression levels. Interestingly, none of the lines showed the massive structures of >2 μm often observed in transient transfections, suggesting that they might be toxic. Although the formation of vasopressin accumulations does not depend strongly on expression level, it is unknown why it is triggered in only a fraction of the cells.

A Constitutive Cargo Protein Is Segregated from Granule-like Structures—When pro-vasopressin was coexpressed with SgII-Myc in COS-1 cells, the two proteins colocalized to the same structures (Fig. 4, A and A’). Similarly, colocalization was observed for coexpressed CgB-Myc and SgII-C1 (SgII tagged with the 10-amino acid epitope C1) (Fig. 4, B and B’). In contrast, when a constitutively secreted protein, A1Pi, was expressed in COS-1 cells, the cells showed reticular ER and perinuclear Golgi staining. Even when expressed in cells producing SgII-Myc, A1Pi (Fig. 4, C and D) was not detectable in

Fig. 3. Granule-like structures are not the result of excessive expression levels. A, COS-1 cells were transfected with pESg, the cDNA of SgII-Myc in the SV40-based expression plasmid pECE undiluted (lane 1) or with 2-, 4-, or 16-times less pESg diluted with pEGFP-N1 (a GFP-encoding SV40-based plasmid, lanes 2–4). In addition, COS-1 cells were transfected with SgII-Myc cDNA in pcDNA5 lacking SV40 sequences (pcDSg, lane 5). 30 μg of cellular protein were analyzed by gel electrophoresis and immunoblotting. For comparison, 10 μg of PC12 cell protein was analyzed in parallel (lane 6). The position of full-size SgII and its processing products are pointed out by large and small arrowheads, respectively. Molecular mass markers are indicated in kDa. In parallel, cells transfected with pESg alone (labeled small of full-size SgII and its processing products) or with pcDSg dilution 4-fold (½) or 16-fold (¼) with pEGFP-N1 were analyzed by immunofluorescence using anti-Myc and Cy3-labeled secondary antibodies. All of the cells expressing SgII-Myc showed granule-like structures. B, stable HEK293 cell lines expressing vasopressin precursor were analyzed by Western analysis (30 μg of cellular protein/lane; –, untransfected HEK293 cells) and by immunofluorescence. For all of the cell lines, 40–80% of the cells showed granular accumulations of pro-vasopressin without correlation to the expression levels. Bar, 20 μm.

Fig. 4. Regulated proteins colocalize in granule-like structures, whereas a constitutive cargo protein, A1Pi, is segregated. Transfected COS-1 cells expressing both pro-vasopressin and SgII-Myc (A and A’), CgB-Myc and SgII-C1 (B and B’), or A1Pi and SgII-Myc (C and D and C’ and D’) were double-stained with antibodies against the two cargo proteins and appropriate Cy2- or Cy3-labeled secondary antibodies. Bar, 20 μm.

was unchanged under these conditions (~10%) and that virtually all of the cells expressing SgII-Myc produced granule-like structures independently of the expression level (Fig. 3A). Correcting the immunoblot signals for transfection efficiency and taking into account efficient storage in PC12 cells, the result suggests that formation of granular structures in COS-1 cells does not require massive overexpression of SgII in comparison to natural secretory granule-producing cells.
the SgII-Myc accumulations (C' and D'). These results suggest that the formation of granule-like structures is specific for regulated cargo and that there is segregation of constitutive and regulated secretory proteins.

**Granule-like Structures Are Post-Golgi Organelles**—In Fig. 5, the colocalization of organelle markers with SgII accumulations was analyzed to test whether the protein might form undegradable accumulations in the ER (e.g., due to misfolding), in lysosomes, or compartments en route. Antibodies against the ER chaperone protein-disulfide isomerase did not colocalize with the punctate structures containing SgII (Fig. 5A). Outside the compact Golgi area, SgII structures were devoid of giantin (Fig. 5B), a marker of the Golgi stacks, and no colocalization was observed with the early endosome markers EEA1 (Fig. 5C) or Rab5 (Fig. 5D) or with Lamp-1 (Fig. 5E), a marker for late endosomes and lysosomes.

Immunolocalization of the TGN marker TGN46 in COS-1 cells expressing SgII-Myc showed the expected strong colocalization in the TGN and some staining of the granule-like structures near the center of the cell but less or no TGN46 staining in peripheral SgII accumulations (Fig. 5, F—F”). This finding is consistent with the model that these structures are derived from the TGN but are losing TGN46 progressively by the budding of vesicles for constitutive-like secretion. The same conclusions were obtained from double-labeling experiments for pro-vasopressin structures and the ER marker p63, giantin, Rab5, transferrin receptor, Lamp-1, or TGN46 (data not shown).

**Ultrastructural Morphology of Granule-like Structures**—To facilitate the analysis by electron microscopy, COS-1 cells were transfected simultaneously with expression plasmids for the vasopressin precursor or SgII-Myc and for GFP. The cells were then trypsinized and subjected to fluorescence-activated cell sorting to isolate the transfected GFP-producing cells. Upon processing of cells expressing pro-vasopressin for electron microscopy, structures of 0.4–1-μm diameter were observed that

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**Fig. 5. Regulated cargo accumulates in post-Golgi structures.** Transfected COS-1 cells expressing SgII-Myc (A and C) or SgII-C1 (B, D, E, and F) were processed for double immunofluorescence microscopy staining for the Myc/C1 epitope and for the organelle markers protein-disulfide isomerase (PDI) and ER, giantin (Golgi), EEA1 (early endosomes), Rab5 (early and recycling endosomes), Lamp-1 (late endosomes and lysosomes), or TGN46 as indicated. The positions of some SgII-positive structures in panels F—F”, which are positive or negative for TGN46, are pointed out by filled or open arrowheads, respectively. Bar, 20 μm.
were quite homogeneously filled with dense material and surrounded by a membrane (Fig. 6, A and B) and were not detectable in untransfected COS-1 cells.

Granule-like structures formed by SgII-Myc had a very similar ultrastructural appearance and an average size of 0.64 μm (±0.16 μm; n = 65). Unlike our anti-neurophysin antibodies, the antibody against the Myc epitope was suitable for immunogold electron microscopy. Anti-Myc antibody in combination with a secondary antibody coupled to 10-nm-gold particles clearly decorated the dense material of the granular structures, demonstrating the presence of SgII-Myc (Fig. 6, C and D). The granule-like structures produced in COS-1 cells are similar in ultrastructural appearance to secretory granules observed in different endocrine tissues but larger because natural granules are typically only 100–400 nm in diameter (27).

Storage and Stimulated Secretion in COS-1 Cells—In pulse-chase experiments with transfected COS-1 cells, we have previously observed that ~85% of newly synthesized pro-vasopressin was secreted into the medium within 2 h (24) and, within 4 h, ~10% was retained in the cells (data not shown). Sorting of pro-vasopressin into granule-like structures thus appeared rather inefficient, although it has to be considered that only half of the cells expressing pro-vasopressin form accumulations. In Fig. 7, we determined the retention efficiency of SgII-C1 and CgB-C1, which form granule-like structures in all transfected cells. C1-tagged proteins were analyzed, because they could be more efficiently immunoprecipitated than the Myc-tagged versions. SgII and CgB are naturally sulfated in the ER, which also contains SgII-Myc. Bars, 0.5 μm.

A1PIT was almost completely secreted within the first hour of chase (lanes 1–7 and circles). In contrast, only 70% CgB-C1 (lanes 8–13 and triangles) and less than half of SgII-C1 (lanes 14–19 and filled squares) was constitutively secreted within the first 2 h and further release was slow. After 6 h, ~10% CgB-C1 and ~40% SgII-C1 were still retained intracellularly. The latter corresponds to a level of retention similar to that found for regulated secretory proteins in endocrine cell lines (e.g. exogenous CgB in PC12 cells) (6).

When the cells were labeled at 37 °C for 1 h (Fig. 7, lanes 20–25 and open squares), ~40% of the labeled SgII-C1 was already secreted into the medium during the labeling period. After a 6-h chase, ~25% was still stored intracellularly. Retention of SgII-C1 thus appears to be increased when labeling was performed at 19 °C. This is most probably due to the increased concentration of the protein in the TGN when exit is blocked and/or the extended residence time in the compartment where granule formation takes place.

An obvious question is whether granule proteins stored in COS-1 cells can be stimulated to be released. To test this hypothesis, cells were transfected with SgII-C1 (the regulated cargo that was most efficiently stored), were labeled for 90 min at 19 °C with [35S]sulfate to load granules with radioactive SgII-C1, and chased for 3 h with excess unlabeled sulfate to allow the constitutively secreted protein to be released. The cells were then incubated with fresh medium with or without the calcium ionophore A23187 for 15 or 30 min. As shown in Fig. 8A, >10% of the initially labeled SgII-C1 was stimulated to be secreted by the ionophore after 15 min and ~20% after 30 min. Based on the pulse-chase experiment of Fig. 7, this finding corresponds to ~20 and ~40%, respectively, of the retained pool after 3 h of chase. Stimulated release of SgII was accompanied by a significant decrease of the average density of granule-like structures by ~30% after a 30-min incubation with A23187 (Fig. 8B, p < 0.0001 according to Student’s t test). Secretion of SgII stored in granule-like structures in COS-1 cells can be induced by increased calcium concentration.
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DISCUSSION

Secretory granules of endocrine and neuroendocrine cells typically contain hormone precursors and granins as their main cargo. We have made the observation that the hormone precursors pro-vasopressin, pro-oxytocin, and POMC as well as the granins SgII and CgB are able to generate granule-like structures when expressed in cell lines lacking a regulated secretory pathway. The observed structures resemble secretory granules with respect to several criteria. They contain the protein at high concentrations as judged by immunofluorescence and in densely packed form as is apparent in differential interference contrast microscopy. They are membrane-bounded structures of similar appearance in electron microscopy to that of certain endocrine granules in tissues, although larger. They represent post-Golgi structures devoid of markers of endosomes or lysosomes but containing low amounts of TGN46. These structures are not formed by a constitutively secreted protein. When coexpressed with SgII, A1Pi is not found in the granule-like structures formed by SgII-Myc, which demonstrates sorting between constitutive and regulated cargo. The formation of SgII accumulations is accompanied by significant intracellular storage as observed in pulse-chase experiments. Incubation with the calcium ionophore A23187 induces secretion of stored SgII.

However, the similarity of granule-like structures in COS-1 cells with secretory granules in endocrine cells has its limitations. Storage efficiency, although quite high for SgII (Fig. 7), was lower for CgB and hardly measurable for pro-vasopressin (24). The structures were 2–3 times larger (for pro-vasopressin even more) than natural endocrine secretory granules. The precursor proteins were not processed, because the appropriate enzymes are not expressed. Continued release of SgII-C1 upon stimulation with A23187 during 30 min (Fig. 8) might suggest inefficient docking to the plasma membrane, slow membrane fusion, or slow transport to the periphery of the cell. It is probable that endocrine cells have specialized mechanisms to regulate these processes and to optimize their efficiency. The effect of calcium ionophore on SgII secretion may also be related to calcium-induced exocytosis of membranes previously observed in CHO and 3T3 cells in electrophysiological studies (29, 30), a process that seems to play a role in plasma membrane enlargement in differentiation and membrane repair (31).

CgB has previously been expressed in Vero cells as a GFP fusion protein and visualized in punctate structures (32, 33). They were interpreted as constitutive secretory vesicles, because ~80% of labeled CgB-GFP was secreted within 2 h (33). After submission of our study, CgB was shown to generate granular structures when expressed in COS-7 and NIH-3T3 cells as visualized by electron microscopy (19), in agreement with our own findings.
Our results indicate that expression of regulated cargo proteins is sufficient to perform the initial stages of regulated secretion in the background of non-endocrine cells. The simplest explanation is that granule formation is the result of self-organizing properties of regulated cargo, mainly via their ability to selectively aggregate under TGN conditions of high calcium and low pH (see Refs. 11 and 12) and potentially to interact with the TGN membrane (as reported for CgB) (34). The forming granule may spontaneously detach from the TGN as a result of the dynamics of fusion and fission of the network membranes. Contaminating material destined for endosomes/lysosomes or for the cell surface (such as mannose 6-phosphate receptors or TGN46) is removed by clathrin-coated vesicles and constitutive-like secretory vesicles, respectively, just as it is from the original TGN membrane. This corresponds to the sorting-by-retention model and in the simplest case may not require any additional machinery specific for the regulated pathway up to this point. The situation may be similar to that of von Willebrand factor precursor, a multimeric protein of endothelial cells and megakaryocytes involved in platelet adhesion. Upon expression in monkey kidney CV-1 cells, structures were observed that were morphologically similar to the endothelial-specific Weibel-Palade bodies (35). Whatever machineries are involved in generating granules as observed in COS-1 cells, they are either constitutively expressed also in non-endocrine cells or they are induced upon expression of regulated cargo. The first possibility is supported by a study by Chavez et al. (36), which suggests that constitutive secretory cells such as CHO or L cells have a cryptic regulated pathway. A significant fraction of [35S]sulfate-labeled free glycosaminoglycan chains were stored intracellularly and could be stimulated to be secreted by phorbol ester or by increased cytoplasmic calcium. Supporting the second possibility, CgA has been proposed to play the role of the master control molecule of initiation and regulation of dense-core secretory granule biogenesis (16), even in CV-1 cells. The interpretation of CgA as a on/off switch of granule formation has raised controversy (17, 18), and CgA has been alternatively proposed to act as one of several assembly factors in granule biogenesis (17). Our results indeed show that CgA is not unique in its ability to induce the formation of granules. All five regulated cargo proteins tested in our study induced granule-like structures in COS-1 cells but with different efficiencies. Only a fraction of the cells expressing prosaposin, pro-oxytocin, or POMC generated granules, whereas virtually all of the cells expressing SgII or CgB generated them. This observation supports the proposed functions of granins as assembly factors or helpers of granule formation and as carrier proteins. Similarly, it had been observed that overexpression of CgB increased granule sorting and processing of POMC in AtT-20 cells (37). The peptide hormone precursors share the ability to assemble into granule-like structures by themselves, although to a lesser extent than the granins. Auxiliary granins are certainly not the only factors lacking in COS-1 cells that increase efficiency of sorting or other steps in regulated secretion in professional regulated secretory cells.

The analysis of granule formation and function in non-endocrine cells may be useful to identify such factors and their mechanisms of action.

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