REVIEW ARTICLE

Sorting and storage during secretory granule biogenesis: looking backward and looking forward

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Secretory granules are specialized intracellular organelles that serve as a storage pool for selected secretory products. The exocytosis of secretory granules is markedly amplified under physiologically stimulated conditions. While granules have been recognized as post-Golgi carriers for almost 40 years, the molecular mechanisms involved in their formation from the *trans*-Golgi network are only beginning to be defined. This review summarizes and evaluates current information about how secretory proteins are thought to be sorted for the regulated secretory pathway and how these activities are positioned with respect to other post-Golgi sorting events that must occur in parallel. In the first half of the review, the emerging role of

immature secretory granules in protein sorting is highlighted. The second half of the review summarizes what is known about the composition of granule membranes. The numerous similarities and relatively limited differences identified between granule membranes and other vesicular carriers that convey products to and from the plasmalemma, serve as a basis for examining how granule membrane composition might be established and how its unique functions interface with general post-Golgi membrane traffic. Studies of granule formation *in vitro* offer additional new insights, but also important challenges for future efforts to understand how regulated secretory pathways are constructed and maintained.

INTRODUCTION

This review focuses on sorting processes involved in the formation and maturation of secretory granules, which represent one limb of the membrane-bounded intracellular transport pathway that subserves a unique function in the storage of proteins destined for cellular export. Under resting conditions in the steady state, the cytoplasm of professional protein-secreting cells accumulates mature secretory granules, which can represent the dominant morphological feature of some cell types. These granules play several essential roles in animal physiology related to communication, digestion and defence. First, they can provide for acute export of secretory proteins in excess of immediate biosynthetic capacity. Secondly, they can provide a compartment for sustained proteolytic processing and other post-translational modifications (required for synthesis of peptide hormones). Thirdly, they can enable quantal secretion of numerous bioactive peptides that act on tissues regulated not only by intensity but also by frequency of stimulation.

Most secretory granules contain a proteinaceous dense core, representing the culmination of protein assembly processes that proceed throughout the secretory pathway [1,2]. We now recognize that initial assembly begins in the endoplasmic reticulum [3] (ER; the common point of entry for newly synthesized exportable proteins [4]), where oligomeric associations and other post-translational modifications may be monitored by quality control machinery that regulates ER export [5]. Because ER exit may be rate-limiting, it is under conditions where relatively rapid ER exit

is favoured that storage in secretory granules becomes apparent [6]. With few exceptions [7], ER assembly reactions, such as protein dimerization, are prerequisite for the further Golgi/post-Golgi assembly that contributes to proper packaging within granules [8–11]. However, a detailed understanding of ER associations for most secretory proteins remains obscure.

Only after protein traffic passes through the Golgi complex do the newly synthesized proteins separate for delivery to distinct destinations [12]. Indeed, the *trans*-Golgi network (TGN) portion of the Golgi has come to be recognized as a major branchpoint from which distinct ‘anterograde’ membrane traffic pathways emanate [13], including constitutive traffic via small vesicles to the plasma membrane, lysosomal biogenesis via the endosomal system and the regulated secretory pathway via secretory granules, although the latter organelles are used only in certain specialized cell types.

Delivery of different proteins to different destinations involves processes collectively known as protein sorting and targeting. There is general agreement that proper delivery of exportable proteins is controlled both by information encoded in the structures of cargo proteins, as well as specific components in the vesicular carriers of this cargo. For example, mannose-6-phosphate-receptor (MPR)-mediated sorting of newly synthesized lysosomal hydrolases into clathrin-coated vesicles that are targeted to endosomal membranes, represents the classic post-Golgi sorting/targeting model [14]. The elegance and simplicity of this paradigm led many to conclude that all Golgi/post-Golgi sorting and targeting is similarly initiated. However, results

Abbreviations used: ER, endoplasmic reticulum; TGN, *trans*-Golgi network; MPR, mannose 6-phosphate receptor; IGs, immature granules; CPE, carboxypeptidase E; POMC, pro-opiomelanocortin; ACTH, adrenocorticotrophic hormone; CgB, chromogranin B; ELH, egg-laying hormone; vWF, von Willebrand factor; SNARE, SNAP (soluble NSF attachment protein) receptor; BFA, brefeldin A; ARF, ADP-ribosylation factor; PAM, peptidylglycine α -amidating mono-oxygenase.

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described in this review suggest that this need not be so, including new evidence suggesting the likelihood of selectivity for entry of certain proteins into constitutive secretory vesicles and the growing trend to consider molecular mechanisms that do not exclude soluble proteins from entry into forming secretory granules. Thus, since our last review of the topic [15], views of protein sorting in relation to secretory granule biogenesis have undergone considerable revision.

The contents of secretory granules are released by regulated exocytosis, although under unstimulated conditions [16], regulated secretory proteins (i.e., the subset of secretory proteins stored in granules) are released at low levels [17]. While a number of important features of the regulated secretory pathway may be shared by other exocytotic routes [18–20], the current definition of the secretory granule includes the criterion that luminal protein contents are delivered to this organelle directly from the cell's biosynthetic apparatus (i.e., these contents have not immediately traversed subcompartments within the endosomal system).

Upon physiologically stimulated exocytosis, the extent of degranulation can range from total (as in mast cells [21]) to only a minor fraction (in many endocrine cells [22]). Importantly, in most regulated secretory tissues, in the hours following termination of protein discharge in response to physiological [23,24] or pharmacological [25,26] stimulation, the cells enter a period of positive protein balance so that they re-accumulate granules back to established steady-state levels. The mechanisms (i.e. genes) underlying the maintenance of intracellular storage capacity are largely unknown, yet are essential to the existence of the regulated pathway. During and even after granule restitution, most new secretory protein (which can represent a large fraction of total protein synthesis [27]) is channeled into storage granules rather than being deployed along other post-Golgi routes [28,29].

Formation of the nascent storage granule begins as an outpouching of the TGN to create a condensing vacuole [30,31] that initially is continuous with the TGN's tubular network [32,33]. Existing data suggest that condensing vacuole formation involves progressive luminal dilation [1,34] (i.e. a variation of 'tubular transport' [35]), although it might conceivably also involve discrete fusions of vesicles originating from elsewhere in the *trans*-Golgi region. Interestingly, systematic morphological analysis of condensing vacuoles in relation to the rest of the TGN has shown that there is an inverse relationship between the size of the forming granules and the remaining tubular network [33]. These observations lead to two related possibilities. (1) Specialized mechanisms or locations may have developed for conventional TGN-derived sorting in regulated secretory cells, in order to accommodate co-existence with secretory-granule formation. (2) The possibility must be considered that outpouching of condensing vacuoles might initially be constructed using 'hybrid membranes' comprised of granule-destined and non-granule-destined components, before further segregation in the plane of the membrane takes place. Such a possibility fits with the known recycling of granule membrane components [36–38].

Condensing vacuoles may differ substantially in their dimensions relative to their mature granule counterparts. While many may approximate to spheres, others may have large irregular profiles or even sausage-like boundaries enclosing multiple cores of condensed secretory protein (as in 'progranules' of pituitary lactotrophs) [33]. The size and shape reflects complex factors that vary within and between cell types, including the intrinsic biophysical properties of the secretory products, which influence the kinetics and extent of their macromolecular assembly [39,40]. The electron-dense core of mature granules is routinely [41], but not always, observed, perhaps in some cases because of difficulty

in fixation [26] and in others because mature granules might possess luminal activities that cause disintegration of the core [42]. In addition, the sizes of immature granules (IGs) and mature granules tend to be altered following pharmacological secretagogue stimulation, resulting in subnormal-sized IGs [25,43] or supranormal-sized IGs [26,44]. Nevertheless, under physiological conditions, condensing vacuoles are generally larger than, or of comparable size to, the mature granules [45–47]. Thus maturation appears to be a net subtractive process in which organelle dimensions diminish as the stored products achieve an increase in dry-mass concentration [48]. Not only does there appear to be a reduction in volume and surface area, but also a major decrease in the numbers of membrane proteins [46].

While condensing vacuoles are closely related to IGs, these compartments should be distinguished. First, condensing vacuoles but not IGs are physically contiguous with the TGN [49]. Second, IGs but not condensing vacuoles have acquired competence for stimulus-dependent secretion [50–52]. What molecular events transpire, over and above detachment from the TGN, to endow IGs with competence for regulated exocytosis, remain unknown at present. While some reports have suggested preferential secretion of newly synthesized regulated secretory proteins in the presence of secretagogues [53–55], usually this preferential secretion is observed under unstimulated conditions [28,29]. Indeed, in most cases (but not all [56]), as a function of increasing stimulation, especially to high levels, the preferential behaviour is either diminished [57] or is actually preferential for previously synthesized granule proteins [58–61]. As the IG is the first stimulatable compartment of the regulated secretory pathway, these data suggest that subsequent granule maturation might include negative modulation (or removal) of constitutive fusion machinery from the granule membrane [62].

Enlargement/expansion of IGs has been postulated to include homotypic IG fusion or IG-coated vesicle fusion, as suggested by diameter measurements from isolated IG and mature granule fractions derived from PC12 cells [52], as well as from a cell-free system intended to reproduce IG formation [63]. However, the prospective contribution of such fusions to mature granule biogenesis remains an open issue. In recent years, despite elegant electrophysiological measurements that can provide accurate estimates of granule surface areas [64], there are actually few measurements that can distinguish IG–IG fusion from other models of granule formation [46]. Indeed, older electron-microscopic studies supporting IG–IG fusion (e.g. [65]) pre-date acknowledgement of multi-cored 'fusion figures' as a part of the TGN (recently illustrated by [33]). Further, as more has become known about the function of clathrin-coated membranes and their coated-vesicle counterparts [66], it has become clear that uncoating must occur before fusion with an acceptor membrane compartment [67]. Thus images interpreted as fusion figures between clathrin-coated vesicles and either condensing vacuoles or IGs [12,30,31] are now most easily interpreted as the budding off of selective components from these organelles (see below).

In the ensuing discussion, we consider processes involved in the sorting of regulated secretory content proteins, the composition of granule membranes, and newer assays of granule formation that contribute to the analysis of proteins recruited to, or activated within, this compartment. Unfortunately, due to space limitations, we do not directly consider distinctions between secretory granules docked and undocked with the plasma membrane [68–70], nor do we consider promising developments in the analysis of genetically tractable model systems for understanding sorting to the regulated secretory pathway [71–79]. One major theme of this review is the increased realization of significant

protein sorting that occurs beyond the TGN, underscoring heretofore unrecognized relationships between maturing secretory granules and other post-Golgi vesicular carriers.

SELECTION OF CONTENTS FOR STORAGE IN SECRETORY GRANULES

In highly differentiated regulated secretory cells, typically in exocrine glands, high intragranular concentrations (≈ 0.1 g/ml) of a diverse array of secretory proteins co-exist within a single granule (endocrine granules often contain the highest levels of only a few dominant proteins, with others at much lower levels)

[80]. The stoichiometric ratio of proteins may vary between individual granules [81] through transcriptional or translational mechanisms [26,82], although granule composition tends to cluster around a mean value. Less commonly [83,84], the production of distinct granule types occurs simultaneously, and this needs to be explained (below).

The view that sorting of proteins for regulated secretion occurs by a single conserved mechanism derives from transfection and transgenic mouse studies showing that exocrine proteins and non-mammalian secretory proteins can enter the regulated secretory pathway of neuroendocrine cells (e.g. [6,85–89]), while neuroendocrine proteins can enter the regulated pathway of

Scheme 1 Cartoon depicting encompassing variations on the sorting-for-entry hypothesis

In this traditional, textbook-type view [393], the TGN compartment (at top) acts as the sole operator for biosynthetic protein sorting and contains a complex mixture of luminal proteins for vesicular delivery to three outbound routes, shown as compartments (doors) A, B and C that each represent distinct trafficking pathways. The 'a-type' molecules are segregated in vesicles that exclude molecules of the 'b-' and 'c-type', for routing to door A. Similarly, 'b-' and 'c-type' molecules are routed to doors B and C respectively in a manner that excludes the entry of unselected proteins. The designations of the three pathways varies among cell types. For example, in many simple epithelial cells, pathway A leads to the apical plasma membrane, pathway B to the basolateral plasma membrane and pathway C utilizes clathrin coated vesicles for protein targeting to lysosomes (via endosomes). In other epithelial cells (e.g., hepatocytes) doorways B and A may be organized in series, such that all plasma-membrane proteins travel first to the basolateral surface and only then is a subset of proteins sorted secondarily for apical delivery by transcytosis; such a variation is important because it emphasizes the precedent of post-TGN sorting in the biosynthetic pathway [170]. In regulated secretory cells, one pathway has been said to be a constitutive route to the cell surface, a second leads to secretory granules and the third is the AP-1/clathrin coated vesicle route (coat in red) to the endosome/lysosome system (also in red). However, the designation of three anterograde transport pathways may actually be too simple in such cases, because of the presence of separate apical and basolateral branches of the constitutive secretory pathway in regulated exocrine cells and the discovery that cells without obvious surface polarity, such as endocrine secretory cells, are nevertheless likely to contain cognates of the apical and basolateral branches of the constitutive pathway [171]. Given these recent realizations, it is unclear that any of the routes shown can be truly considered as a default pathway for soluble proteins, although the traditional view has considered 'the constitutive secretory pathway' in this context [41,91].

exocrine cells [90]. In the past 15 years, two different hypotheses, described in the following paragraphs, have emerged to explain the selection of content proteins for storage in secretory granules. Importantly, these models are not mutually exclusive.

Sorting-for-entry hypothesis

The sorting-for-entry hypothesis is based on the proposal that the TGN acts as the primary operator for protein sorting in the biosynthetic transport pathway. Importantly, this view, based upon the sorting paradigm for lysosomal hydrolases, is not limited to cells that exhibit regulated secretion [13]. A generic version resembling that used previously in textbooks is shown in Scheme 1.

With regard to regulated secretory proteins, the sorting-for-entry view originally postulated that one or more conserved TGN-associated sorting receptors capable of binding all manner of regulated secretory proteins [91] would 'usher' them [92] or 'zipper' them [93,94] into IGs. Since no receptor is sufficiently abundant to sort regulated secretory proteins with a 1:1 stoichiometry, more recent versions of sorting-for-entry suggest that, within the TGN, regulated secretory proteins either become specifically bound to receptors in the nascent granule membrane or bound to other regulated secretory proteins that are already bound [95–100]. In the latter case, regulated secretory proteins in the TGN have been proposed to form insoluble aggregates [101] that exclude the entry of constitutive secretory proteins [102]. Thus an essential feature of sorting-for-entry models is the apparent 'gatekeeper' function in which only selected secretory proteins (singly or as aggregates) are allowed to pass the 'gate' for entry into IGs, while other proteins are efficiently excluded [103]. Proof of sorting-for-entry to the regulated secretory pathway requires demonstration of partitioning of the selected proteins into IGs in amounts greater than that which would be expected by volume partitioning alone. For this purpose it is necessary to actually know the partitioning of luminal volume into the regulated versus the constitutive secretory pathway, which may vary between cell types and for which extensive analysis has still not been designed. However, according to the sorting-for-entry theory, proteins failing to interact either with a receptor or a protein aggregate bound to the nascent granule membrane, would follow a 'default' route (i.e., one with no gatekeeper) into the constitutive secretory pathway [91,94,103,104]. Curiously, soluble sulphated glycosaminoglycan chains (attached to a xyloside acceptor), proposed as a bulk-flow marker, have been found to enter both constitutive secretory vesicles and immature secretory granules [105,106].

Sorting-by-retention hypothesis

The sorting-by-retention model considers that the IG, an important post-TGN sorting station, serves as a functional extension of the TGN, adopting some of the TGN-sorting mechanisms in addition to the selection of proteins that are ultimately stored, or not stored, within secretory granules [15]. According to this view, the entrance of content proteins into the regulated secretory pathway is not dependent upon gatekeeping within the TGN (Scheme 2A). Instead, luminal proteins not associated directly or indirectly with the limiting membrane may be available to enter the free fluid volume of all vesicle trafficking pathways that are derived from the TGN, again dependent upon the partitioning of available volume into outbound pathways. Sorting of regulated secretory proteins is then thought to occur passively, concurrent with the removal from IGs of a subset of protein components via efficient (receptor-mediated) or inefficient (fluid-phase) means of vesicular egress. Thus, the three defining

features of this model are that: (a) protein entrance into IGs may utilize, but does not require, participation in an insoluble complex (aggregate); further, it requires neither direct nor indirect binding to the nascent granule membrane or to a sorting receptor therein. Because luminal traffic into IGs cannot efficiently exclude entry of soluble proteins, such entry is not restricted to regulated secretory proteins; (b) high-order intermolecular associations cause participating regulated secretory proteins to be efficiently retained within maturing granules; and (c) an exit route actively ushers certain ligands (including lysosomal proenzymes) out of the IG compartment by receptor-mediated sorting, accompanied by the removal of a fraction of secretory proteins that have inefficient retention properties.

Sorting-by-retention has been studied in detail in pancreatic β -cells (Scheme 2B). Here the predominant secretory proteins entering IGs are soluble proinsulin hexamers [10], although they are accompanied by other proteins, including an unsorted fraction of lysosomal prohydrolases [47,107]. Proinsulin hexamers do not close pack into higher-order complexes [8,107]; however, processing to insulin and C-peptide in IGs allows newly synthesized insulin to condense, increasing the efficiency of its storage in granules [51]. By contrast, newly synthesized C-peptide, which remains soluble, shows increased predisposition to escape from maturing granules [108], via the constitutive-like secretory pathway (see the section entitled 'Evaluation: issues and approaches to the sorting-by-retention model'). During the same time period, lysosomal hydrolases appear to be co-transported out of maturing granules [47,107]. Possibly analogous size-fractionation events appear to occur within the endocytic pathway [109,110].

The retention process: condensation

In the subtractive process accompanying granule maturation (see the Introduction), vesicular exit from IGs is viewed as the key component [111]. The driving force underlying sorting-by-retention involves assembly of granule core proteins within IGs. This assembly is promoted by changes in the intraluminal ionic environment that may be general to all regulated secretory cell types, such as mild acidification in the presence of high concentrations of bivalent ions like calcium, or, may involve more specific factors such as proteolytic processing of prohormones, or the presence of other ions such as zinc [10]. Together, these assembly processes are collectively referred to as concentration, aggregation, multimerization, polymerization or condensation [1,31,40,48,97,100,101,107,112–119]. These interactions seem to reflect progressive protein insolubility within the luminal environment of maturing granules.

Condensation of regulated secretory proteins is expected to limit the ability of these molecules to escape from maturing granules in the process of constitutive-like vesicle budding that has now been suggested in multiple different cell types [28,29,51,62,89,108,111,120–123]. Thus manipulations that perturb the environment that normally favours higher-order intermolecular interactions involving the major secretory proteins are likely to lead to enhanced loss of granule proteins into the constitutive-like secretory pathway [124] in parallel with impaired intragranular condensation [107], resulting in deficient protein storage in the regulated secretory pathway [125].

Evaluation: issues and approaches to the sorting-for-entry model

Evidently, 'two sides of the equation' most strongly supporting a sorting-for-entry model would be provided by (i) the identification of a membrane-associated protein ('sorting receptor') that can provide gatekeeping function within the TGN and (ii)

Scheme 2 Cartoons depicting the sorting-by-retention hypothesis

(A) General model. In this view, buds forming from the TGN compartment (at bottom) include selective sorting routes for secretory proteins directed to a cognate constitutive apical pathway, a cognate constitutive basolateral pathway and TGN-derived AP-1/clathrin-coated vesicles targeted to endosomes (coat in red). Although each of these routes is depicted as a route for potentially selective luminal protein sorting, the buds destined to form vesicular carriers for these routes may not efficiently exclude soluble proteins from entry into their lumina. Likewise, formation of the regulated secretory pathway involves significant volume partitioning into the condensing vacuole (at far right). The regulated secretory protein, d, is the most abundantly expressed luminal protein. While some luminal proteins might bind to the condensing vacuole membrane, this need not be the case, as large amounts of soluble d can enter the forming granule compartment. Similarly, to the extent that copies of a-, b- and c- type molecules are not sorted into their respective transport vesicles, these soluble species are also available to enter the condensing vacuole, which buds to form an IG. Further budding of AP-1/clathrin-coated vesicles takes place, removing molecules by specific receptors from maturing granules. To the extent that regulated secretory proteins (d-type molecules) successfully condense within this compartment, they tend to resist exiting the maturing granules by fluid phase loss. (B) The model as applied to pancreatic β -cells. Five specific proteins are shown, with a key at the top. Proinsulin enters IGs and is converted therein into insulin, which begins to condense. AP-1/clathrin-coated vesicle budding (in red) from IGs conveys mannose 6-phosphate receptors (MPRs) with bound lysosomal enzyme precursors (L), in addition to some C-peptide and proinsulin that is inefficiently captured in the fluid phase. The destination of these clathrin-coated vesicles is the endosomal system (also in red), such that lysosomal enzymes are ultimately delivered to lysosomes (also in red), while C-peptide and proinsulin may be exported to the extracellular space, detected as constitutive-like secretion (see the text).

the identification of one or more structural domains on regulated secretory proteins that can be shown to interact with such a TGN gatekeeper. These two issues will now be briefly discussed.

(i) Recent interest has been generated by the proposal that carboxypeptidase E (CPE) serves as a sorting receptor in neuroendocrine cells. The hypothesis is that CPE might control secretory protein entry into granules in a manner that is entirely independent of its enzymic activity [126,127]. This potential role

of CPE is based in large part on studies of neuroendocrine tissues of *Cpe^{fat}/Cpe^{fat}* mice (which express a CPE point mutant that misfolds and fails to be exported from the ER [128]). One of the phenotypic defects in these mice is glucose intolerance that is readily suppressed with exogenous insulin, suggesting a deficiency of processed insulin stored in releaseable granules [129]. Indeed there is decreased proteolytic processing of several different polypeptide hormones of the neuroendocrine system, as well as

an apparent deficiency of peptide hormone storage in the mice [127] and in certain neuroendocrine cell clones transfected with a cDNA encoding antisense mRNA to CPE [126]. The receptor-like properties of CPE were mainly inferred by identification of its binding to the N-terminus of pro-opiomelanocortin [POMC, the precursor of adrenocorticotrophic hormone (ACTH)] and by competitive displacement of this interaction by several regulated secretory proteins (proinsulin, proenkephalin and chromogranin A), but not by constitutive secretory proteins [126]. Thus the prohormone processing defect in *Cpe^{fat}/Cpe^{fat}* mice was postulated to reflect only mis-sorting of prohormones, which fail to enter IGs, the major prohormone conversion compartment [126,127].

While this proposal is certainly intriguing, it is not without problems. In particular, the original report of *Cpe^{fat}/Cpe^{fat}* islets indicated an abundance of proinsulin-rich secretory granules in β -cells and biochemical experiments have confirmed that proinsulin exhibits normal entry into the regulated secretory pathway of islets from *Cpe^{fat}/Cpe^{fat}* mice or β -cell lines derived therefrom [130,131]. Also, an alternative explanation has been put forth to explain the prohormone processing defect seen in cells from *Cpe^{fat}/Cpe^{fat}* mice; namely, because the mutation abolishes enzymic activity of CPE, this may inhibit prohormone proteolytic processing both directly and indirectly [132,133]. These issues argue that the relationship between CPE and secretory protein sorting may be more complex than its direct binding to prohormones within the TGN (see the section entitled 'Evaluation: issues and approaches to the sorting-by-retention model').

(ii) With respect to evidence for sorting ligands, it has been suggested that the N-terminus of POMC and chromogranin B (CgB) contains a conformational motif, specifically a short peptide loop enclosed by a disulphide bridge, that is required for entry into IGs, presumably via specific membrane binding [134–137]. While these findings have engendered strong enthusiasm for the sorting-for-entry model, they must be mollified by several significant considerations including: (a) the potential inaccessibility of putative ligands in the context of polypeptide oligomerization that often occurs in the early part of the secretory pathway [10]; (b) the newly discovered role of the disulphide loop of CgB in dimerization [138]; (c) the absence of N-terminal disulphide loops on many regulated secretory proteins and the presence of such loops on constitutively secreted proteins; (d) the recent demonstration that this disulphide bond is apparently not involved in the membrane interaction of CgB [139]; and (e) the apparent absence of a correlation between the redox state of the disulphide loop *in situ* and the entry of CgB into the regulated secretory pathway in different regulated secretory cell lines, which cannot be explained by cell-line-specific differences in levels of CPE (S. Gorr, X. F. Huang, R. Kuliawat and P. Arvan, unpublished work). A recent publication by Kromer et al. [139a] has reported that human CgB mutagenized to remove the N-terminal disulphide-bonded loop structure exhibits an impaired efficiency of entry into IGs when expressed in PC12 cells as the sole luminal regulated secretory protein using a vaccinia expression system. Although the impaired efficiency has not been explained by a molecular mechanism, the apparent sorting defect is rescued merely by co-expressing wild-type human CgB. Thus luminal proteins other than CgB are not needed for CgB entry into secretory granules in this experimental system, which renders a role for CPE unlikely. It remains to be determined whether the requirement for wild-type CgB to rescue sorting of mutant CgB reflects favourable effects of the full-length protein in *trans* on stabilizing against degradation or on membrane binding or condensation of mutant CgB, but it is interesting that, for wild-type CgB itself, the efficiency of retention in maturing secretory

granules can explain at least half of its apparent sorting to the mature granule compartment [139a]. For all of these reasons we believe that it is presently premature to conclude that CPE plays a role in secretory protein sorting for entry, although its possible role in sorting by retention must still be considered (discussed below).

Evaluation: issues and approaches to the sorting-by-retention model

Two pulse-chase approaches led to development of the sorting-by-retention model (Scheme 2): (i) analysis of the constitutive-like secretion that occurs in parallel with the process of granule maturation and (ii) comparative analysis of the proteins discharged by stimulated exocytosis of immature and mature secretory granules.

(i) Constitutive-like secretion [15] was first detected in exocrine tissues as a pathway of protein discharge attributable to neither constitutive nor regulated exocytosis [28,29,120], appearing developmentally before the machinery of stimulus-secretion coupling is operational. The distinguishing features of this secretion are: (1) its qualitative composition is similar to the spectrum of proteins stored in granules; (2) its quantitative contribution is minor (< 15% of newly synthesized secretory protein), making it detectable only under unstimulated conditions where direct granule exocytosis is largely suppressed; (3) its stoichiometric ratio of different content proteins is not identical with that discharged by granule exocytosis; and (4) its kinetics implicate a process that begins at a stage in intracellular transport after the TGN. These differences suggested an origin of the constitutive-like pathway from maturing granules. Subsequently, analogous results were obtained from the β -cells of pancreatic islets [51,108], providing evidence for the generality of the constitutive-like secretory pathway.

Because of compartment-specific activation of prohormone processing enzymes [140–145] and the luminal dwell time necessary to complete endoproteolytic reactions, IGs are the predominant (albeit not necessarily exclusive [84,146–149]) site of prohormone conversion into mature peptide hormones. In β -cells, insulin and C-peptide are formed exclusively within IGs and under stimulated conditions they exhibit amplified release in a 1:1 ratio [150–153]. However, under unstimulated conditions, direct exocytosis of IGs as well as mature granules is suppressed to low levels, while constitutive-like secretion is not suppressed [51]. Indeed in the absence of any β -cell secretagogues or pharmacological perturbants, secretion is enriched in newly synthesized C-peptide in molar excess of newly synthesized insulin [51,108], proving that vesicles derived from IGs (rather than IGs themselves) must be involved in this secretory pathway. Recently, constitutive-like secretion of newly synthesized processing intermediates of POMC by the AtT20 cell line has also been suggested [62].

A potential morphological correlate of the vesicular origin of the constitutive-like pathway can be observed in the form of clathrin-coated patches and buds that are characteristically seen on IGs [47,49,150,154]. Indeed, pharmacological agents blocking the loss of clathrin from maturing β -granules [155] also inhibit the appearance of labelled secretory proteins in the constitutive-like secretory pathway [108]. As no other kinds of vesicles have been reported to emanate from IGs, a clathrin-coated vesicle-mediated escape from maturing granules (see the section entitled 'Removal of membrane proteins during granule maturation') is currently the best candidate for the origins of the constitutive-like pathway (Scheme 2A). Accordingly, a fraction of newly synthesized secretory proteins may be carried from IGs as fluid-

phase cargo in the clathrin-coated vesicle pathway leading to endosomes and upon reaching this destination there is further branching such that some of these newly synthesized proteins can be diverted to the cell surface [156,157] while other traffic heads to lysosomes (Scheme 2B).

(ii) By stimulating cells to undergo exocytosis at times when biosynthetically labelled proteins are either in IGs or mature granules, it has been possible to provide complementary evidence that some newly synthesized proteins may enter IGs yet fail to remain within the regulated secretory pathway. For example, separate from the fraction of unsorted lysosomal proenzymes that are lost from cells by constitutive secretion [158], growing evidence suggests that a significant additional fraction of newly synthesized lysosomal enzymes may traverse the TGN and enter granules [159] in spite of the fact that (a) these molecules are not known to interact with CPE and (b) the regulated secretory pathway is not even their final destination.

The transient localization of lysosomal proenzymes in IGs was elucidated only recently [47,107,111]. Interestingly, both high-affinity (procathepsin B) and low-affinity (procathepsin L) ligands for MPRs enter β -cell IGs with comparable efficiency. This entry, nearly the same as that of granule proteins (e.g., proinsulin), argues that (1) there is little distinction of luminal proteins at the level of entry into IGs and (2) MPRs are unlikely to serve as a mechanism for entry into IGs [47]. However, with increasing granule maturation, the ability to stimulate exocytosis of proinsulin+insulin remains unchanged, while that for procathepsins B and L varies inversely with the affinity of these ligands for MPRs, indicating that loss from the regulated pathway is very efficient only for those proenzymes with high affinity for MPRs [47]. Following tunicamycin treatment (which prevents addition of N-linked oligosaccharides), newly synthesized lysosomal hydrolases continue to enter IGs to an even greater extent, although subsequent loss from the stimulus-dependent secretory pathway, even for procathepsin B, is now prevented [107]. The evidence now appears overwhelming that vesicles mediate the selective removal of lysosomal proenzymes from maturing secretory granules (see the section entitled 'Removal of membrane proteins during granule maturation').

A similar assay measuring regulated exocytosis of biosynthetically labelled proteins has recently been applied to the study of exocrine amylase and proline-rich secretory protein expressed from cDNAs in AtT20 cells. These proteins, which also are not candidates for CPE association, nevertheless enter AtT20 cell IGs with about the same efficiency as POMC, the endogenous regulated secretory protein [89]. However, at later chase times, the exocrine proteins have largely disappeared from the regulated secretory pathway in AtT20 cells [160] and instead, during the process of granule maturation, these proteins are recovered in the unstimulated secretion [89]. As AtT20 cells are of neuro-endocrine origin, along with accumulated evidence from exocrine and endocrine systems (above), these data support the postulate that vesicular exit from maturing granules (depicted in Scheme 2A) plays a significant sorting role in all regulated secretory cell types.

New aspects to sorting by retention

A corollary of the sorting-by-retention model is that the constitutive secretory pathway originating from the TGN cannot function exclusively as a default route. This might seem a provocative and even troubling proposal, since previous workers in the field of regulated secretion have not seriously considered the possibility of cargo receptors for entrance into constitutive secretory vesicles. However, findings over the past decade may

force reconsideration of those views. Specifically, we now know that, in epithelial cells, there is simultaneous formation of at least two different classes of TGN-derived constitutive export vesicles containing different cytoplasmically associated [161,162], integral membrane [163] and luminal proteins [164,165]. Interestingly, neither of these classes seems to have characteristics of a default route [166,167] (although it must be acknowledged that, in some epithelial cells, not all sorting of newly synthesized exportable proteins occurs at the TGN; e.g., apical plasma membrane proteins can be sorted in post-TGN compartments [168–170]). Importantly, recent information suggests that all cells express distinct sets of constitutive secretory vesicles which pick up specific protein components as they emerge from the TGN [171]. With this in mind, it is worth reconsidering whether any constitutive secretory proteins are truly excluded from entry into the regulated secretory pathway and, if so, might this class of proteins be segregated in the TGN by specifically binding to membranes or proteins destined for the constitutive pathway?

We consider three examples. (1) Immunoglobulins and their derivatives have been considered previously to be dedicated markers of the constitutive secretory pathway [172,173]. However, recent findings in AtT20 cells indicate that both κ light chain and Fc domains may enter IGs, although their storage is limited (A. M. Castle, A. Y. Huang and J. D. Castle, unpublished work). (2) A truncated form of vesicular stomatitis-virus G protein has been reported to be efficiently excluded from secretory granules in AtT20 cells [88,174]. However, recent evidence indicates that this protein behaves as a membrane-bound molecule [175]. (3) Finally, in PC12 cells, a heparan sulphate proteoglycan is frequently cited as a constitutive secretory protein, yet does not distribute as a soluble bulk flow marker and indeed has been demonstrated to be near-quantitatively bound to TGN membranes [101,176]. Thus some luminal proteins that are denied access to the interior of forming granules may so behave because they are actively sorted elsewhere.

By contrast, the sorting-by-retention model predicts that soluble secretory proteins cannot effectively be excluded from entry into IGs. This might provide a rationale by which to understand why numerous proteins that are normally secreted constitutively can nevertheless be found to enter the regulated secretory pathway when expressed in endocrine cells. Such proteins include apolipoprotein A1 [177], insulin-like growth factor-1 [175], parathyroid hormone-related protein [178], certain mutant forms of fibronectin [179] and gp80-clusterin [180]. For that matter, even non-secretory proteins, if directed into the ER as chimeras, have been reported to be delivered to the regulated secretory pathway [136,181]. However, for all these proteins, the efficiency of their ultimate retention in granules may vary, depending upon their ability to associate with endogenously retained granule content proteins [173].

Different granules within one cell type

In light of the foregoing discussion, the sorting of proteolytic products derived from a common precursor into distinct classes of dense core secretory granules needs to be reconsidered. Such is the case for the egg-laying hormone (ELH) precursor in *Aplysia* bag cell neurons [182]. While initial proteolytic cleavage of the precursor may begin in the TGN [84], common processing intermediates related to different final products are found within 'large early granules' [183], indicating that a major fraction of the cleavages occur after entry into IGs, which are likely to be the source of the distinct dense core granules. It should be noted that separate storage of the proteolytic products of the ELH family of hormone precursors is not always detected. Indeed, depending

on the cell type in which the prohormone is expressed, as well as subtle variations in peptide structure amongst ELH-family members, differential storage of distinct cleavage products may either be observed [87] or not observed [184,185]. In AtT20 cells, a portion of the proteolysed ELH prohormone is subsequently stored in granules while other portions are more rapidly secreted from the cells, others appear to be degraded and still others have fates that remain unknown [87]. Such phenomena can be explained either by the sorting-for-entry or the sorting-by-retention hypothesis, but they are more easily reconciled with the latter view.

Production of distinct dense core granules can also occur with endogenous proteins that are not derived from a common precursor. For example, growth hormone and prolactin, separate gene products, neither of which undergo proteolytic processing, predominantly are packaged into separate secretory granules of somatomammotroph cells [186]. Likewise, von Willebrand factor (vWF) and tissue-type plasminogen activator are packaged into separate storage granules in endothelial cells [187]. In the case of the pituitary hormones, segregation has been reported to involve mechanisms that operate beyond the Golgi complex [83]. In all of these examples, the different granules generated are each competent for regulated exocytosis.

Potential insight into the storage of vWF (which is packaged in rod-shaped Weibel–Palade bodies) and other elongate multimeric proteins has been gained from transfection studies in which such proteins segregate away from endogenous secretory granules and other established TGN-associated pathways. When expressed in cells with [39] or without [188] a regulated secretory pathway, vWF is incorporated into its own rod-shaped granule, although there is no evidence that this organelle undergoes exocytosis. Similarly, recombinant fibronectin forms a granule distinct from that of endogenous ACTH when expressed in AtT20 cells. Interestingly, when either of two domains that function in high-order fibronectin assembly were deleted, the ability of the mutant polypeptide to be stored in endogenous secretory granules was enhanced [179]. Apparently, the homotypically assembled extended forms of either vWF or wild-type fibronectin exhibit diminished compatibility for packaging with other secretory products, thereby limiting their storage within endogenous secretory organelles.

Packaging agents/helper proteins

In both sorting-for-entry and sorting-by-retention models, protein–protein interactions are viewed to play a central role. However, protein interactions with other packaging agents, such as small molecular components (e.g., ATP, Ca^{2+} and biogenic amines, which are each present at concentrations ranging from 1 to ≥ 100 mM in many granules [80]) are also likely to figure in the condensation process. In addition, interaction of regulated secretory proteins with the luminal leaflet of the granule membrane [103] could contribute to sorting-by-retention. In these processes, helper proteins may conceivably facilitate intermolecular interactions and thereby improve the efficiency of co-packaging and condensation, as part of the differentiated phenotype of regulated secretory cells.

Three different types of helper protein have been considered for such a role. The first is represented by CgB, a member of the granin family that is expressed widely in neuroendocrine cells [189,190] and is co-packaged into secretory granules [173,191]. CgB is a calcium-binding protein and permeabilization of intracellular membranes of chromaffin or PC12 cells at low pH and high calcium (conditions thought to approximate those of the distal secretory pathway) results in recovery of CgB in a

sedimentable form, consistent with the biophysical properties of aggregated secretory proteins [101,116]. Further, a small fraction of CgB has been found to be membrane-adherent [96] (a feature that has also been described for a number of other regulated secretory proteins [100,192–194]), and overexpression of CgB in AtT20 cells appears to enhance the storage of endogenous ACTH [99]. In conjunction with the fact that members of the granin family are expressed in many neuroendocrine cells, the above characteristics suggest granins as possible helper proteins, although this function may be restricted to processing products in neuroendocrine cells [99].

A second prospective type of helper protein in neuroendocrine cells is comprised of prohormone processing enzymes, including CPE. While CPE's role as a TGN-based sorting receptor remains uncertain (discussed above), morphological studies of the pancreatic β -cells of *Cpe^{fat}/Cpe^{fat}* mice suggest impaired condensation in the granule lumen [129]. Whether this is due to loss of CPE activity (which no doubt occurs) or CPE acting in the capacity of helper protein (which is unproved) has not been resolved. In many respects (including the presence of a fraction of molecules that may associate with the luminal surface of granule membranes under mildly acidic 'intragranular-type' conditions [195]), CPE and CgB (above) exhibit similar biophysical properties, again pointing to a co-operative, multi-protein condensation process. Other prohormone processing enzymes may similarly participate, based upon complementary studies examining either the effects of the processing enzymes themselves or the presence of recognition sites within potential prohormone substrates on their subsequent storage [184]. For example, prohormone convertase 1 (PC1, also known as PC3) is an endopeptidase that is able to participate in precursor processing to mature renin [196] or mature insulin [197,198]. Previous studies had suggested that information for granule storage of prorenin is contained within mature renin [199] and more recent studies indicate that the cleavage site itself is required for efficient storage of the hormone [200]. This suggests two possibilities: (1) PC1, like CPE, might serve as a helper protein for intragranular storage and/or (2) cleavage *per se* may assist in the storage of processed hormones. Recently, introducing PC1 (and thus PC1-dependent cleavage) within regulated secretory cells that normally lack PC1 has been found to increase the storage of insulin in secretory granules, while the storage of uncleaved proinsulin in the same cells was not improved (R. Kuliawat and P. Arvan, unpublished work). The complete disruption of functional prohormone convertase 2 (PC2) in transgenic mice [201] causes no apparent defect in proinsulin entry into IGs, although subsequent condensation may be impaired [202]. Thus a quantitative assessment of prohormone entry into IGs, constitutive-like secretion and long-term hormone storage in this model, or in PC1 knockout mice (now being attempted) are likely to yield important information for determining if there is more than one role for these enzymes in the intragranular storage of polypeptide precursors and their mature hormone counterparts.

The third type of prospective helper protein includes sulphated proteoglycans and glycoproteins which are found in a variety of granule types. In mast-cell granules where heparan sulphate proteoglycan is especially prevalent, it has been shown that the packaged content behaves like an ion-exchange gel, swelling and shrinking under different ionic conditions [203]. Also, expression of a basic proline-rich secretory protein has been found to stimulate sulphation of both exogenously expressed and endogenous proteoglycans in AtT20 cells [204]; consistent with the idea that many secretory proteins may co-package with sulphated molecules [106]. Presumably electrostatic interactions between cationic secretory proteins and sulphated proteoglycans are one

of the forces facilitating condensation in secretory granules [113,205]. Further, sulphated glycoproteins and proteoglycans might serve as a bridge between granule contents and the membrane [206]. However, a direct demonstration of this role has not been observed as yet [105].

THE MEMBRANE OF THE REGULATED SECRETORY CARRIER

Sorting-for-entry and sorting-by-retention models also have ramifications for the formation of the membrane of mature secretory granules, which have an uncommonly low density of intramembranous particles by freeze-fracture [46], a lower protein/lipid ratio than most others [207], with a major decrease in the phospholipid/cholesterol ratio [208]. Traditionally, granule membrane components have been assumed to be uniquely designed for the purposes of accumulating secretory proteins at high concentrations and for stimulus-secretion coupling. However, to date, this assumption remains largely unproved. Thus, with only few components being granule-specific (below), the functions of granule membranes may be modulated by relative concentrations and activation states of proteins shared with other membranes as much as they reflect proteins unique to this compartment. Importantly, the property of stimulus-regulated exocytosis is certainly *not* unique to granules: it is shared by other secretion pathways [56,209] as well as regulated exocytosis of specialized lysosomes [210], the recycling limb of the endocytic pathway [211], other endocytically linked pathways (e.g. synaptic vesicles [212], transcytotic vesicles [19], Glut4 vesicles [213], aquaporin vesicles [214]) and some less-well-defined pathways [20,215].

Membrane proteins of secretory granules

Two-dimensional gel analyses of highly purified granule membrane fractions indicate the presence of more than 30 different polypeptides [216,217]. Nevertheless, the list of known granule membrane proteins remains limited. Table 1 presents a summary of many of the membrane proteins that have been found in secretory granules to date. They have been categorized as integral components with transmembrane spans, lipid-anchored components or peripheral components associated with the cytoplasmic surface. The first two categories are further subdivided with respect to where (which surface or within the bilayer) most of the mass of the protein is located and presumed to function. In addition, the suspected functions (if known) and localization(s) within cells where they have been found are listed.

It is immediately apparent from this analysis that the predominant subset of granule membrane proteins are also identified in other kinds of membranes, either within the same cell or in cells not specialized for regulated secretion. While this might possibly reflect multiple distinct isoforms (e.g. GRAMP92, amine transporter), in some cases it is clear that one isoform is distributed to multiple compartments (e.g. syntaxin3, VAMP2). Because IGs bud off vesicles during their maturation, it is possible that some proteins in this subset [e.g. GRAMP92, syntaxin6, peptidylglycine α -amidating mono-oxygenase (PAM)] may function as transients rather than *bona fide* granule residents (see the section entitled 'Removal of membrane proteins during granule maturation' below). Other proteins in this subset (e.g., VAMP2, SCAMPs and PtdIns 4-kinase) appear to represent part of promiscuous trafficking machinery, added to the growing list of protein families whose members may reside and function in more than one type of membrane [218–221], including, but not limited to, granules.

There is a smaller subset of membrane proteins (e.g., phogrin, dopamine β -hydroxylase, cytochrome b_{561} , SV2, pancreatic

GP-2, γ -glutamyl transferase) that so far have been found to exhibit expression restricted to various kinds of regulated secretory cells. Exocrine- or (neuro)endocrine-specific granule components appear to be an evolutionary specialization. Thus, when exocrine pancreatic GP-2 is expressed in endocrine cells, it does not appear in endogenous granules but instead accumulates in large multivesicular bodies and at the cell surface [160,222], reflecting either exclusion from forming granules or exit from IGs during maturation. Other proteins from this subset, such as P-selectin, as well as phogrin and ICA512, are remarkable because their endogenous subcellular localization is nearly exclusive to granule membranes [111,223–226]. The exocrine granule protein syncollin may be similar [227], although higher-resolution analysis is still needed. Expression/mutation studies of these polypeptides are likely to contribute significantly in further elucidating the mechanisms of granule membrane protein sorting, for which studies to date remain quite limited (see below).

Sorting signals and granule membrane targeting

The best evidence for sorting signals on granule membrane proteins comes from studies of a few model proteins. P-selectin, an integral membrane protein with a short cytoplasmic C-terminus, is a regulated adhesion receptor that is efficiently sequestered in secretory granules in endothelial cells and platelets. When expressed in neuroendocrine cells, P-selectin is mainly concentrated in secretory granules [228] and, following exocytosis in endothelial cells, at least part of the P-selectin recycles from the cell surface to secretion granules [229]. Of particular interest, expression of the cytoplasmic tail of P-selectin linked to the transmembrane and extracellular domains of tissue factor (a plasma-membrane protein) results in targeting of the chimeric polypeptide to secretory granules [230]. While this represents some of the strongest evidence to date for the presence of a granule membrane sorting signal, it is not known whether the localization to granules results from interaction with specialized sorting machinery, selective interaction with other granule membrane components, or even self-interaction.

A second interesting example is PAM (Table 1), which is widely distributed in regulated secretory cells [231]. PAM is produced in integral membrane and soluble forms, both of which follow the regulated secretory pathway, suggesting that the luminal domain contains significant information employed for granule localization [122]. However, the steady-state distribution of membrane PAM in pituitary AtT20 cells [232] features perinuclear tubulovesicles that are distinct from both granules and TGN38, a TGN marker. As this distribution is distinct from that of P-selectin expressed in the same cells, it seems possible that the cytoplasmic segment of integral membrane PAM might control an important, but transient, association with granule membranes. Truncation of the cytoplasmic tail of membrane PAM in particular appears to result in substantial and expeditious redirection to the cell surface [122]. The recent discovery of cytosolic proteins that bind the C-terminus of PAM may be a first step toward identifying cellular machinery that controls its trafficking [233].

A third example which highlights potential differences in targeting signals for proteins residing in endocrine and exocrine secretory granules is provided by pancreatic GP-2, a GPI-anchored protein that may comprise up to one-third of zymogen granule membrane protein [234]. GP-2 is not an essential granule component as signified by its absence from granules during late fetal development [235] or in pancreatic tumour cells [222]. GP-2 is also abundant at the apical surface of pancreatic acinar cells, although biogenesis of apical GP-2 does not appear to involve

Table 1 Membrane proteins of mammalian secretory granules

Protein	Role	Localization(s)	Reference(s)
Integral proteins			
Luminal function			
P-selectin	Cell-surface lectin	Endothelial granules	[228,230]
GRAMP92, GPII	Unknown (related to lamps)	Endocrine, exocrine granules	[250]
		Lysosomes	[249]
GRAMP100	Unknown	Exocrine granules	[308]
PAM	Apical cell surface Peptide amidation Other post-TGN membranes	Endocrine, exocrine granules	[232]
Furin	Post-translational proteolysis	IG entry/exit, recycling	[123]
Dopamine β -hydroxylase	Catecholamine hydroxylation	Endocrine granules	[261]
γ -Glutamyltransferase	Glutathione hydrolysis	Exocrine granules	[309]
	Plasma membrane		[310]
Transmembrane function			
Cytochrome b_{558}	Electron transport	Endocrine granules	[311]
		Synaptic vesicles	[312]
H ⁺ ATPase	Acidification	Granules, endosomes	[313]
		Lysosomes, synaptic vesicles	[314]
Nucleotide transporter	ATP uptake	Endocrine granules, synaptic vesicles	[315]
Amine transporter	Biogenic amine uptake	Endocrine granules	[316]
		Exocrine granules (?)	[317]
		Synaptic vesicles	[318]
Granulophysin/leukophysin	Unknown, synaptophysin relative, CD63	Leucocyte granules	[319]
		Platelet granules	[320]
SV2	Putative transporter	Synaptic vesicles	[321]
	Neuroendocrine granules		
Cytoplasmic function			
Synaptotagmin	Ca ²⁺ sensor, PtdInsP ₂ binding	Neuroendocrine granules	[322,323]
	Syntaxin binding	Synaptic vesicles	[278,324]
VAMP2/synaptobrevin	SNARE protein	Endocrine granules	[325,326]
		Exocrine granules, Plasma membrane	[327,328]
		Synaptic vesicles	[329]
		Glut4 vesicles	[330]
SCAMPs	Unknown	Exocrine, endocrine granules	[326,331]
		Endosomes	[332]
		Glut4 vesicles	[308]
Syntaxin 1	SNARE protein	Chromaffin granules, plasma membrane	[333]
Syntaxin 3	SNARE protein	Exocrine granules, plasma membrane	[334]
		Epithelial plasma membrane	[335]
		Mast-cell granules	Guo and D. Castle, unpublished work
Syncollin	Ca ²⁺ -regulated binding to syntaxin	Exocrine granules	[227]
Syntaxin 6	SNARE protein	Endocrine, exocrine IG	[111]
		TGN	[241]
Phogrin/ICA512	Tyrosine phosphatase homologues of unknown activity	Endocrine granules	[225,226]
Phosphatidylinositide 4-kinase	Phosphorylation of phosphatidylinositide	Endocrine, exocrine granules	[336]; D. Castle, unpublished work
		Glut4 vesicles	[337]
		Coated vesicles	[338]
		Golgi, lysosomes, plasma membrane	[339]
Lipid-anchored proteins			
Luminal surface			
GP-2	Unknown	Zymogen granules, Plasma membrane	[222,234] [236,262]
Dipeptidase	Peptide cleavage	Zymogen granules	[340]
Cytoplasmic surface			
Rab3A	SmG protein, regulated exocytosis	Exocrine granules (late)	[341]
		Endocrine granules	[342]
		Synaptic vesicles (late)	[343]
		Glut4 vesicles	[344]
Rab3B	SmG protein, regulated exocytosis?	Neuroendocrine granules	[345]
		Epithelium, apical plasma membrane	
Rab3D	SmG protein	Exocrine granules	[346]
		Endocrine granules	[347]
		Regulated secretory cognate?	[20]
Rab11	SmG, regulated recycling	Granules, constitutive vesicles, TGN	[348]
		Recycling endosomes	[349]
		Regulated transport vesicles	[350]

Table 1 (cont.)

Protein	Role	Localization(s)	Reference(s)
Heterotrimeric G protein	Regulation of vesicle formation Regulation of cytoskel for exocytosis	Exocrine granules	[351,352]
		Endocrine granules	[353,354]
		Golgi, plasma membrane	[303,355]
		Constitutive secretory vesicles	[356]
		Endosomes	[357,358]
ARF6	Regulation of vesicle formation	Endocrine granules	[359]
SNAP25	SNARE protein	Plasma membrane, endosomes	[360–362]
		Endocrine granules	[363]
Cysteine string protein	Unknown but essential for synaptic vesicles	Plasma membrane, synaptic vesicles	[364]
		Neuroendocrine granules	[365]
		Synaptic vesicles	[366]
c-Src	Tyrosine kinase	Exocrine granules	[367]
		Neuroendocrine granules	[368]
		Plasma membrane, endosomes, synaptic vesicles	[369]
Membrane-associated			
Rabphilin	GTP-dependent Rab3 binding	Secretory granules	[370]
		Synaptic vesicles	[371]
CAPS	Dimeric Ca^{2+} triggering of exocytosis, PIP2 binding	Neuroendocrine granules	[372]
NSF/SNAP	SNARE complex formation	Synaptic vesicles	[373]
		Endocrine granules	[374]
		Endosomes	[375]
		ER/Golgi	[376]
Sec1 homologue	t-SNARE regulation	Neuroendocrine granules	[377]
		Plasma membrane	[378]
Calmodulin	Ca^{2+} regulation	Neuroendocrine granules	[379]
Nucleoside diphosphokinase	ATP $\leftrightarrow$ GTP interconversion	Exocrine granules	[380]

granule exocytosis [236]. While unproved, it seems quite possible that the unusual prominence of GP-2 in the exocrine storage compartment and its exclusion from mature endocrine granules (discussed above) reflects its ability to interact with exocrine granule content proteins [97].

Removal of membrane proteins during granule maturation

Removal of proteins from the IG membrane was first suggested by the declining density of intramembranous particles during granule maturation as revealed by freeze–fracture electron microscopy [46], an observation that was soon followed by evidence of the disappearance of clathrin coats from the surface of maturing granules [208]. The adaptor protein complexes that facilitate clathrin coat assembly on the surface of membranes, namely, AP-1 in the TGN, AP-2 at the plasma membrane and AP-3 (see below), bind to specific membrane proteins and are well known to participate in selective membrane protein sorting [237]. The role of AP-1 in clustering and relocating MPRs with lumenally bound lysosomal prohydrolases from the TGN is particularly well established [238,239]. More recently, similar functions have been suggested for AP-1/clathrin-coated membrane in regulated secretory cells [176], where lysosomal prohydrolase sorting clearly extends into the IGs (see the section entitled ‘Evaluation: issues and approaches to the sorting-by-retention model’ above). Indeed, immunoelectron-microscopic observations show that cation-dependent and -independent MPRs and AP-1 adaptors emerge from IGs on clathrin-coated vesicular buds [111]. AP-1 adaptors have also been implicated in removing the processing protease furin from IGs [123]. Analogous selective sorting may extend to additional membrane proteins that are largely common to endocrine and exocrine IGs rather than mature granules.

By contrast, proteins such as phogrin, which enter endocrine IGs but exhibit no decline in concentration as granules mature [111], apparently avoid vesicular escape pathways from maturing granules. These findings highlight the current uncertainty about which steps of membrane protein trafficking in the regulated secretory pathway involve specific sorting. Conceivably, membrane protein information could be used for selective entry into IGs (see the section entitled ‘Membrane proteins excluded from granules’ below), retention within IGs, or exit from IGs. The fact that MPRs and furin have signals in their cytosolic tails that stimulate interaction with AP-1 adaptors suggests their active sorting for exit from maturing granules. However, for molecules like phogrin or ICA512, it is unclear whether signals for removal during granule maturation are simply lacking or whether efficient retention in mature granules is signal-mediated.

The presence of export traffic from maturing granule membranes may have interesting ramifications for circulation of members of the SNARE [SNAP (soluble NSF attachment protein) receptor] family of membrane-docking/fusion proteins. For example, syntaxin6 (a protein structurally related to the Pep12 SNARE protein that functions in targeting newly-synthesized proteases to the yeast prevacuolar compartment [240]), has been implicated as a SNARE protein for TGN-to-endosome trafficking in mammalian cells [241]. Interestingly, in both endocrine and exocrine secretory cells, syntaxin6 enters IGs and is efficiently removed from maturing granules in parallel with the loss of MPRs and AP-1 [111]. Likewise in the exocrine pancreas, cellubrevin (a VAMP2 paralogue that functions in constitutive recycling [242]) is concentrated on condensing vacuoles and IGs but not mature granules [243]. Furthermore, apical accumulation of VAMP2, which may participate in docking/fusion of exocrine secretory granules [244,245], might reflect similar trafficking (although alternative pathways that are used in other cell types [246] also remain possible).

Lysosomal-type membrane proteins in secretory granules

The story with subcellular distribution of lysosomal-type membrane glycoproteins [247] is still developing. Acid phosphatase was the first lysosomal-type activity suggested to be present in condensing vacuoles and IGs, but not mature granules [31], a finding that has received recent additional support [248]. Further, at least three different reports indicate that the integral granule membrane proteins GRAMP92 (found in both endocrine and exocrine granules), GP II (found in endocrine granules) and chromomembrin B (found in chromaffin granules) are highly related to lysosomal membrane glycoproteins, especially Lamp-2 [249–251]. In the case of GRAMP92, immunocytochemical analysis indicates that the protein persists in mature granules but at concentrations considerably lower than in lysosome-like organelles [250]. These limited data seem consistent with the possibility that at least a portion of lysosomal-type membrane proteins passes through the regulated secretory pathway *en route* to lysosomes; however, the exit from maturing granules appears less efficient for these proteins than, e.g., for MPRs. Presumably, these differences reflect differences in the efficiency of recognition of sorting information in their cytosolic tails [252]. This leads to two important related issues which remain to be explored.

(1) Failure to remove from maturing granules certain transmembrane proteins with either the tyrosine-containing or dileucine-type sorting motif means that those proteins in particular may become available at the plasma membrane upon regulated granule exocytosis. Studies indicate that secretory granule membrane recycling from the surface, in part or *in toto*, requires clathrin-coated invagination and endocytosis [38,253], presumably mediated by AP-2 adaptors [254]. Thus inefficient recognition of tail motifs at the level of secretory granules, with more efficient recognition at the plasma membrane, could provide a crucial means to trigger the internalization of recycling granule membranes. There is precedent to think that the efficiency of such recognition could be regulated by post-translational modifications such as local phosphorylation or protein oligomerization [123,255].

(2) A new adaptor complex AP-3 has recently been characterized [256] and the possibility has not yet been formally excluded that some transmembrane proteins might be removed from maturing granules via vesicles coated with AP-3 rather than AP-1/clathrin. Interestingly, the distinct distribution of AP-3 from both AP-1 and endosomal markers has suggested a function in the formation of lysosome-like pigment granules [257]. Further, reports suggest that the tyrosine-containing sorting signals on lysosomal membrane proteins may interact with AP-3 adaptors [258].

Membrane recycling and granule formation

Several stereological and tracer-uptake studies performed on cells that had been stimulated to undergo exocytosis have indicated that secretory granule membranes of both exocrine and endocrine cells are recycled [37,229,259–261]. However, proteolysis and phospholipase action within granules may result in loss of the luminal domains of certain polypeptides upon exocytosis [122,262]. While older studies suggested that zymogen-granule membrane proteins generally might have a long half-life relative to stored content [36], these studies now need to be repeated with high resolution of discrete membrane components. Nevertheless, the limited evidence available supports the assumption that granule membrane proteins may recycle back to the *trans*-Golgi/TGN after endocytosis.

The fact that at least two types of granule membrane proteins have been identified, namely (1) those tending to be removed

from granules during maturation (e.g. MPRs) and (2) those tending to remain in mature granules (e.g. P-selectin or phogrin), suggests that at least two types of membrane protein recycling loops are possible. A ‘short loop’ exemplified by MPRs going to and from the endosomal system (largely skipping the cell surface) has been the route proposed in cells lacking an obvious regulated secretory pathway [239,263], and this will not be further reviewed here. A ‘long loop’ would occur only after surface externalization of membrane proteins, upon granule exocytosis [38]. Indeed, P-selectin, which currently is one of the best model proteins, undergoes significant recycling as measured by externally added antibodies [229], even though its half-life is shorter than that of many other recycling cell-surface proteins [264]. Unfortunately, relatively little information is available regarding (1) the pathway of recycling, (2) whether or not granule membrane components remain persistently segregated in microdomains from other membrane components, (3) if and when membrane proteins from the short and long loops might be reunited in the recycling pathway and (4) how stimulation of granule exocytosis might change the dynamics of membrane internalization. However, there is little doubt that, even after compound exocytosis with acute plasma-membrane expansion, membranes are returned to Golgi/post-Golgi compartments without a significant loss of total cellular membrane surface area, within an approx. 3 h period [265,266] that (to some extent) precedes the ER expansion indicative of accelerated protein and lipid biosynthesis. Thus the current view is that, in highly differentiated secretory cells, recycled membrane represents a large fraction of the total [33]. For this reason, the real challenge may be to segregate away those membrane proteins that are destined to reside at sites other than granules. Further work is clearly needed in this area.

Membrane proteins excluded from granules

By analogy to the secretory proteins, it is worth considering whether certain membrane proteins are actively selected as components for entry into forming granules, or conversely, whether other itinerants in the TGN are prevented from entry. Once again, these models are not mutually exclusive. While it must be acknowledged that few of the answers are presently known, it seems appropriate to describe briefly what limited information is available, as this may spark specific investigations into the important question of how certain membrane proteins find themselves excluded from granules.

The tentative impression from four examples (below) is that the major mechanism of efficient exclusion may involve specific interactions that render the protein inaccessible for entry into the granule membrane. For example, the cytoplasmic tail of TGN38 encodes both a relative retention signal within TGN [267–269] as well as information specifying interaction with proteins involved in the budding of constitutive transport vesicles [270]. Notably, constitutive transport of TGN38 to the cell surface does not appear to occur by default, as its trafficking in polarized epithelial cells involves selective basolateral recycling [271]. Two viral membrane proteins, the vesicular-stomatitis-virus G protein and influenza haemagglutinin [272,273], do not exhibit retention properties in the TGN, but like TGN38, they are selected for entry into distinct subclasses of constitutive secretory vesicles [171,274]; these molecules also appear to be precluded from entry into the regulated secretory pathway [34,275,276]. A further example is E-selectin (interestingly related to the granule protein P-selectin), which also exhibits a selectively polarized expression at the surface (of endothelial cells [229]), suggesting that its exclusion from granules might occur via analogous mechanisms. Finally, synaptophysin is a remarkable example in this category.

Table 2 A subset of proteins implicated in formation and/or maturation of secretory granules and other post-Golgi vesicles

Component	Function(s)	Reference(s)
ARF	Activation of phospholipase D	[381]
ARF-guanine nucleotide exchange factor	ARF activation by GDP ↔ GTP exchange	[382]
Phospholipase D ₁	Membrane-bound phospholipase D; Generates phosphatidic acid; mediates ARF-dependent coated-vesicle formation by lipid effects	[383] [384,385]
PtdIns(4,5)P ₂	Phospholipid cofactor binds, activates phospholipase D ₁ , ARF-guanine nucleotide exchange factor (?)	[382,386]
PtdIns transfer protein	PtdIns lipid intermembrane exchange; Sec14, required for vesicle formation, possibly supplying substrate for PtdIns kinases and by promoting diacylglycerol accumulation	[299,387–389]
PtdIns 3-kinase	Golgi-derived vesicle formation	[300,390]
Heterotrimeric G proteins	Regulate level of ARF-GTP and vesicle formation; possibly regulate vesicle uncoating	[303,391,392]

It is known to bind VAMP2 in synaptic vesicles [277], yet, unlike VAMP2 (and other proteins whose distribution is shared between secretory granules and synaptic-like vesicles [278,279]), it is largely excluded from dense core granules [280,281]. Although a molecular explanation for these observations has not yet been obtained, the data support the idea that the activity of targeting signals may be regulated along selected pathways [123,255]. However, it must be emphasized that these examples do not exclude the formal possibility that some membrane proteins may reside in granules simply because they lack appropriate targeting signals to direct them elsewhere.

Unlike the foregoing examples, the insulin-responsive Glut4 glucose transporter appears quite unusual in that it is excluded from secretory granules in some instances [278,282] and not others [283,284]. One recent report showing that Glut4 is present in substantial quantities in secretory granules of atrial cardiomyocytes, where it is naturally expressed, was interesting because stimulated exocytosis in these cells did not appear to increase Glut4 at the cell surface, nor did it stimulate glucose uptake [284]. At present it seems possible that Glut4 might behave like MPRs, syntaxin6 or lysosomal membrane proteins in being able to enter forming granules, but tending to exit at some later point. As in the case of MPR-based sorting in pancreatic β -cells [47], the extent to which the pathway of Glut4 includes the regulated secretory granule may vary between cell types or between clones from the same cell line.

Interactions of membranes with content: role in membrane sorting?

While we have already discussed the potential role of peripheral proteins that might function to associate the stored contents of secretory granules with the luminal aspect of the granule membrane, we have not considered whether integral membrane proteins are sorted by binding to granule content proteins, i.e., evidence for luminal domain-mediated sorting. The co-segregation of membrane proteins and content aggregates was envisioned as one of the original models of sorting in the regulated secretory pathway [104]. A possible example of this kind of sorting involves the inclusion of membrane-bound GP-2 in exocrine pancreatic granules, hypothetically as a result of its interaction with soluble zymogens [97]. Further, by *in vitro* assay at low pH with or without high calcium, certain solubilized forms of membrane proteins, such as dopamine β -hydroxylase or PAM, can be sedimented in the presence of aggregates of

regulated secretory proteins [100]. This finding appears consistent with other reports that the luminal domain of PAM may contribute to its sorting in neuroendocrine cells [122]. Obviously, such stabilizing interactions within the lumen could potentially contribute to sorting for both secretory and interacting membrane proteins, using either sorting-for-entry or sorting-by-retention mechanisms. However, no evidence has yet been obtained to actually show that these interactions are essential to the formation of secretory granules.

Assays of IG formation from the TGN

The process of generating stimulus-competent IGs from TGN-associated condensing vacuoles involves a geometric sorting process in which vesicles with a low surface/volume ratio are separated from tubules with a high surface/volume ratio, leaving behind TGN residents. For the detachment of vesicles from the TGN, recent studies have examined potential roles for cytoplasmic scaffolds or coats, analogous to coats that mediate formation of transport vesicles from other cellular compartments [237,285,286]. Although there was a time when it was thought that clathrin coats might be involved in granule formation [49,155], as described in the section entitled 'Removal of membrane proteins during granule maturation', newer studies link clathrin with AP-1 binding [176] and the trafficking of syntaxin6 [111], furin [123] and MPRs [111] carrying lysosomal proenzymes [47] from IGs to endosomes. Thus, a mystery awaiting to be resolved is whether specific coat proteins are directly linked to IG formation.

A relationship between recruitment of cytoplasmic coat proteins and granule formation has been suggested by the finding that brefeldin A (BFA), a fungal metabolite which prevents assembly of COPI, AP-1, myosin II and, perhaps other (?), coats [161,162,287–291], also inhibits formation of IGs [292], leading to increased TGN-based sialylation of CgB [293] and preventing IG-based processing of proinsulin and PoMc [62,153]. (Although currently unexplained, these results have not been obtained in all cell types [147].) Following the discovery that BFA inhibits guanine nucleotide exchange on the ADP-ribosylation factor (ARF) [294,295], a succession of studies has implicated roles for ARF, nucleotide exchange factors and other downstream signal mediators in the formation of post-Golgi vesicles including IGs [296,297]. Although space limitations preclude extensive review, recent results from cell-free budding assays [147,298] have also focused attention on PtdIns transfer protein [299], PtdIns 3-

kinase [300], tyrosine kinase and phosphatase activity [301], phospholipase D [302] and heterotrimeric G proteins, G_s and $G_{i/o}$ [303] as potential regulators of TGN-vesicle budding (see Table 2).

Developments in this field continue to evolve rapidly. However, as new studies suggest that membrane coats and buds can be formed *in vitro* even in the absence of specific membrane protein recruitment [304], a vigilance must be maintained regarding the specificity of the buds and vesicles produced in future *in vitro* studies. Already the possibility exists for the formation of as many as five different kinds of vesicles from the TGN alone, specifically (1+2) two distinct classes of constitutive secretory vesicles [171], (3) IGs [63], (4) vesicles mediating retrograde transport [305] and (5) vesicles representing the homotypic disassembly of the TGN [306,307]. Further, we must remain cognizant of the complexity that, depending upon how the assay is performed, there is potential difficulty in distinguishing buds that originate from distinct Golgi subcompartments, rather than from the TGN alone.

In addition to these specific points, a more general issue is that, in the steady state *in vivo*, the TGN tightly couples a high flux of membrane inflow (with large contributions from membrane recycling as well as *de novo* synthesis) with its function in membrane outflow [35]. It is not certain that *in vitro* systems can faithfully preserve this coupling. Since the normal formation of IGs represents a variation of tubular transport requiring continuous membrane flux [33], the products of *in vitro* budding may be expected to alter the luminal volume and membrane proportioning into IGs versus other TGN-exit pathways. Thus *in vivo* IG formation may not be replicated with perfect fidelity, resulting in abnormalities that may be subtle, or in some cases more severe, e.g., reconstitution of smaller rather than full-sized IGs, or modest alterations of density or even composition, may be expected. Nevertheless, there is no question that the potential utility of these cell-free systems is formidable, in particular, to discover specific molecular components of the TGN vesicle budding machinery, as well as in studying examples of protein sorting for entry. However, based on the reasoning described above, it seems that much of the protein sorting by retention may remain undetected in these systems.

CONCLUSION

The secretory granule is the specialized organelle of regulated secretory cells that facilitates long-term intracellular storage of secretory products at high concentration. Yet the information obtained so far has not established any unique and conserved targeting mechanism for content proteins, relatively few unique membrane proteins and no unique cytoplasmic coat that mediates granule formation. In fact, an accumulating body of information suggests that granules extend a variety of TGN-associated functions and that storage may be determined at least in part by passive condensation of secretory proteins leading to their retention within maturing granules, possibly assisted by helper proteins that may facilitate interactions between luminal polypeptide species, or with the lipid-rich granule membrane. Thus, the storage compartment progressively evolves away from other post-Golgi sorting activities which operate in parallel. On the basis of overlapping components with other recycling vesicular carriers, much of the machinery that functions in granule trafficking may be regulated by post-translational modifications affecting activities on the granule membranes. Clearly much work will still be needed to unravel the mysteries of this specialized secretory organelle.

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