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# Biogenesis of secretory granules in the *trans*-Golgi network of neuroendocrine and endocrine cells

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## Abstract

Secretory granule formation requires selection of soluble and membrane proteins into nascent secretory granules, and exclusion of proteins not required for the function of secretory granules. Both selection and exclusion presumably can occur in the compartment where assembly of the secretory granule begins, the *trans* most cisternae of the Golgi complex. Current research focused on the initial stages of secretory granule formation includes a search for the 'signals' which may mediate active sorting of components into secretory granules, and the role of aggregation of regulated secretory proteins in sorting. In addition, the temporal sequence of the sorting events in the Golgi, and post-Golgi compartments has gained much attention, as summarized by the alternative but not mutually exclusive 'sorting for entry' vs. 'sorting by retention' models. 'Sorting for entry' which encompasses the most popular models requires selection of cargo and membrane and exclusion of non-secretory granule proteins in the TGN prior to secretory granule formation. 'Sorting by retention' stipulates that protein selection or exclusion may occur after secretory granule formation: secretory granule specific components are retained during maturation of the granule while non-secretory granule molecules are removed in vesicles which bud from maturing secretory granules. Finally, some progress has been made in the identification of cytosolic components involved in the budding of nascent secretory granules from the TGN. This review will focus on the recent data concerning the events in secretory granule formation which occur, in the *trans*-Golgi network. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Secretion; Regulated secretion; *trans*-Golgi network; Vesicle formation; Immature secretory granule

## 1. Introduction

### 1.1. What is a secretory granule?

Dense core secretory granules are found in all exocrine, endocrine, neuroendocrine, and neuronal cells. Vesicles which are similar to secretory granules are also found in cells of the haemopoetic and immune systems. Formation of these vesicles is initiated in the *trans*-Golgi network (TGN) and results in the secretory proteins being packaged in a concentrated form in the secretory granules. Unstimulated exocytosis of these granules occurs at low levels, but amplified

Abbreviations: TGN, *trans*-Golgi network; ISG, immature secretory granule; MSG, mature secretory granule; CSV, constitutive secretory vesicle; CCV, clathrin-coated vesicle; CgB, chromogranin B; SgII, secretogranin II; hsPG, heparan sulphate proteoglycan; PCs, prohormone converting enzymes; LDCV, large dense core vesicles; DTT, dithiothreitol; ARF, ADP-ribosylation factor; AP-1, adaptor protein-1; PLD, phospholipase D; GH, growth hormone; PRL, prolactin; MPR, mannose-6-phosphate receptor; PIP<sub>2</sub> (phosphatidylinositol 4,5-bisphosphate); PITP, phosphatidylinositol transfer protein; PI, phosphatidylinositol

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secretion from the cell of proteins within secretory granules is regulated exclusively via external stimuli which triggers the fusion of the secretory granule membrane with the plasma membrane.

Secretory granules in endocrine and neuroendocrine cells have a very similar composition. The limiting membrane contains proteins required for secretory granule acidification, transport, targeting and fusion, while the dense core content is composed of a set of soluble proteins, some of which are substrates, some of which are enzymes, others of which have an as yet unidentified function. It is known that there is variation in the content of the secretory granule, depending on the type of tissue the cell is derived from, however, very little is known about the membrane composition and the variation which might be present in the molecules involved in secretory granule biogenesis, targeting and fusion.

Research into the formation of the secretory granules from the TGN is mainly concerned with how the soluble regulated secretory proteins are targeted into the secretory granule, how soluble non-secretory granule proteins are excluded, if at all, what factors determine the membrane composition, and how secretory granule budding occurs from the TGN. This research is complicated by the fact that secretory granule formation could occur in parallel with formation of vesicles directed to endosomes or the plasma membrane. This review will explore the recent progress in these areas and attempt to compare the current models of secretory granule biogenesis from the TGN in endocrine cells and neuroendocrine cells.

## 2. Sorting of soluble proteins in the TGN

Regulated secretory proteins are packaged into nascent secretory granules (which are referred to as immature secretory granules (ISGs)) in a process that is thought to be initiated by selective aggregation of the secretory proteins in the TGN, followed by interaction of the aggregate with the TGN membrane which may involve receptor molecules. It is also possible that selection may be achieved by interaction of individual regulated secretory proteins with a putative membrane receptor prior to aggregation. Proteins which are not present in regulated secretory protein aggregates, or do not interact with the recep-

tor for the regulated secretory proteins, are thought to exit the TGN in constitutive secretory vesicles (CSVs). An alternative hypothesis is that there is very little constitutive secretion originating from the TGN of regulated cells, and that most molecules in the biosynthetic pathway exit into ISGs. In the ISG, the non-secretory granule proteins would presumably be sorted from the regulated secretory proteins via interaction with a receptor and removed from the ISG in constitutive-like vesicles. Aggregation of the regulated secretory proteins would still occur in the ISG and would function to allow retention in the ISG by preventing any interaction with receptors for non-secretory granule proteins. In both models, one could make predictions about the stoichiometry of the receptor relative to the ligand, depending on whether it is a single secretory protein interacting with a single receptor, or an aggregate of regulated secretory proteins interacting with a single receptor. In both models, interaction with a receptor would allow removal of non-secretory granule proteins from the forming or maturing secretory granule. An important point of clarification is that there may well be multiple classes of receptors for both regulated secretory proteins and constitutive secretory proteins in the TGN and the ISG. In addition, interaction of any ligand with its receptor is most probably transient and disrupted, for example, by changes in the pH of the maturing ISG.

### 2.1. Role of aggregation in sorting

One mechanism for sorting soluble secretory proteins into secretory granules is via a selective aggregation of a subset of soluble proteins to the exclusion of other soluble, non-aggregating secretory proteins. For efficient aggregation, the local concentration of the secretory proteins should be high in the sorting compartment. Soluble regulated secretory proteins are for the most part very abundant in cells with a regulated secretory pathway. In particular, a family of proteins known as the granins [1] are highly expressed in most endocrine and neuroendocrine cells. The members of this family of proteins include chromogranin A (CgA), chromogranin B (CgB), secretogranin II (SgII), secretogranin III, and secretogranin IV (also known as 7B2). Based on the primary sequence of the family members, it is thought they

have similar structural features which may enable them to efficiently aggregate in the TGN [2].

Although the exact function of the granins is not known, they might be acting as ‘helper proteins’ [3]. If sorting only occurs via aggregation and does not rely on a specific and stoichiometric receptor-ligand interaction, then efficient sorting of prohormones which either have a less than optimal ability to condense, or are present at very low concentration, might be enhanced by ‘helper proteins’ that aid aggregate formation. Recently, Natori and Huttner [4] have demonstrated that increasing the amount of CgB in AtT20 cells (a cell line derived from the pituitary corticotroph) indeed promoted efficient sorting of the 23 kDa POMC (pro-opiomelanocortin) fragment into secretory granules, resulting in increased storage of the 23 kDa POMC fragment and ACTH.

Aggregation of SgII has been shown *in vitro* as well as in permeabilized TGN membranes, under conditions (low pH, high  $\text{Ca}^{2+}$ ) believed to be present in the luminal environment of the TGN [5,6]. Other soluble secretory proteins, other members of the granin family and other proteins found in secretory granules, have also been shown to aggregate under these low pH, high  $\text{Ca}^{2+}$  conditions (see e.g. [7,8]). Indeed, morphological evidence demonstrating the presence of aggregates of regulated secretory proteins in the TGN [9,10], but not in earlier compartments under normal conditions supports both the location of the initiation of aggregation and its role in sorting. Experiments performed several years ago with weak bases, such as chloroquine and ammonium chloride argued that perturbation of the pH in the TGN caused missorting of regulated secretory proteins into the constitutive pathway (see for example [11]). Although the data did not gain complete support [12] these experiments were the first to suggest that pH may have a role in the sorting of soluble proteins. More recent results obtained with streptolysin O-permeabilized PC12 cells support the idea in that pH may be crucial for sorting of regulated secretory proteins in the TGN [13]. Concerning the exact pH and  $\text{Ca}^{2+}$  requirements *in vivo*, values for the pH in the TGN of cells lacking a regulated secretory pathway have been proposed in two recent studies ( $6.17 \pm 0.02$  and  $6.45 \pm 0.03$ ) [14,15] supporting the notion that the TGN is slightly more acidic

than previous Golgi compartments, but there are still no data available on the free  $\text{Ca}^{2+}$  concentration in the lumen of the TGN. Interpretation of all these results to determine the precise role of pH and  $\text{Ca}^{2+}$  in the TGN requires accurate measurements for pH and  $\text{Ca}^{2+}$  in the lumen of the TGN in cells with a regulated secretory pathway.

Some controversy exists concerning the role of the TGN-localized aggregation event in sorting which may relate to differences between the cellular systems studied.  $\beta$ -Cells of the endocrine pancreas secrete large quantities of insulin [16]. Insulin forms insoluble, close packed crystalline arrays in the presence of zinc, whereas proinsulin has distinct physical properties which prevent its condensation into insoluble crystals. Processing of proinsulin to insulin, which requires two processing enzymes, prohormone converting enzymes (PCs) 1 and 2, acting at two distinct sites (for review see [17]), is crucial for the condensation of insulin. However, the intracellular location where processing of proinsulin and other prohormones begins is unresolved. There are reports which demonstrate that processing of prohormones by PC enzymes can begin in the TGN [4,18,19] whereas other studies indicate that processing is initiated in the ISG [20–22]. Proinsulin conversion has been demonstrated to occur only in secretory granules ([23–25] and references included therein). Therefore, if sorting of regulated secretory proteins into dense cores relies on exclusion of soluble non-granule material through aggregation, or condensation, insulin can only be sorted in a post-TGN compartment. This result also implies that proinsulin cannot be sorted by aggregation in the TGN or any post-TGN compartment. These results have provided the basis for the ‘sorting by retention’ hypothesis [26] (Fig. 1). This hypothesis suggests that regulated secretory proteins are sorted largely by retention in a post-Golgi ISG. Non-secretory granule proteins, or processing remnants of regulated secretory proteins, such as the C-peptide (which is derived from proinsulin and not found in mature insulin), can be in part removed by constitutive-like vesicle budding from the ISG, although the efficiency of this removal is likely to be related to whether a selection process (i.e. receptor-mediated) process is employed. Thus, the efficiency for removal from ISGs might be expected to vary for different proteins (see below).

An interesting example which reinforces the importance of the precise location of prohormone processing on sorting comes from experiments done on *Aplysia californica* [27] and *Lymnaea stagnalis* [28]. These two model systems have been used to demonstrate that sorting of proELH (egg-laying hormone) can be influenced by the location of cleavage, which in turn presumably affects the hormones ability to

aggregate or condense. In *Aplysia*, the proELH is cleaved in the Golgi and the two N-terminal and C-terminal intermediates are sorted into two distinct populations of large dense core vesicles (LDCVs) [27]. *Lymnaea* presents a more complicated scenario in that there are two different types of neurones which produce ELH: in one type (type I neurones) processing occurs in the TGN and results in the appearance of two morphologically distinguishable condensed protein cores, containing either the N-terminal or C-terminal region of ELH, which are sorted to different populations of secretory vesicles, LEG (large electrondense granule) and LDCVs [28]. In the second type of neurone (type II), the ELH is not cleaved in the TGN and is sorted into the LDCVs as a prohormone, after which it undergoes cleavage to mature ELH. This raises an interesting question concerning how proteins exit from the Golgi in type I and type II neurones. It would be of great interest to compare the sorting of true ‘constitutive’ proteins in type I and type II neurones to see if in type I neurones constitutive secretory proteins are secreted constitutively, implying that CSVs are formed directly from the Golgi, and likewise if in type II neurones if these proteins are secreted in a constitutive-like fashion, implying secretion originates from ISGs. Interestingly, the LDCVs from the two types of neurones differ in size, the LDCV from the type I neurone having a size of approximately 150 nm whereas the LDCVs in the type II neurone was 90 nm [28] suggesting that the composition of the content has an influence

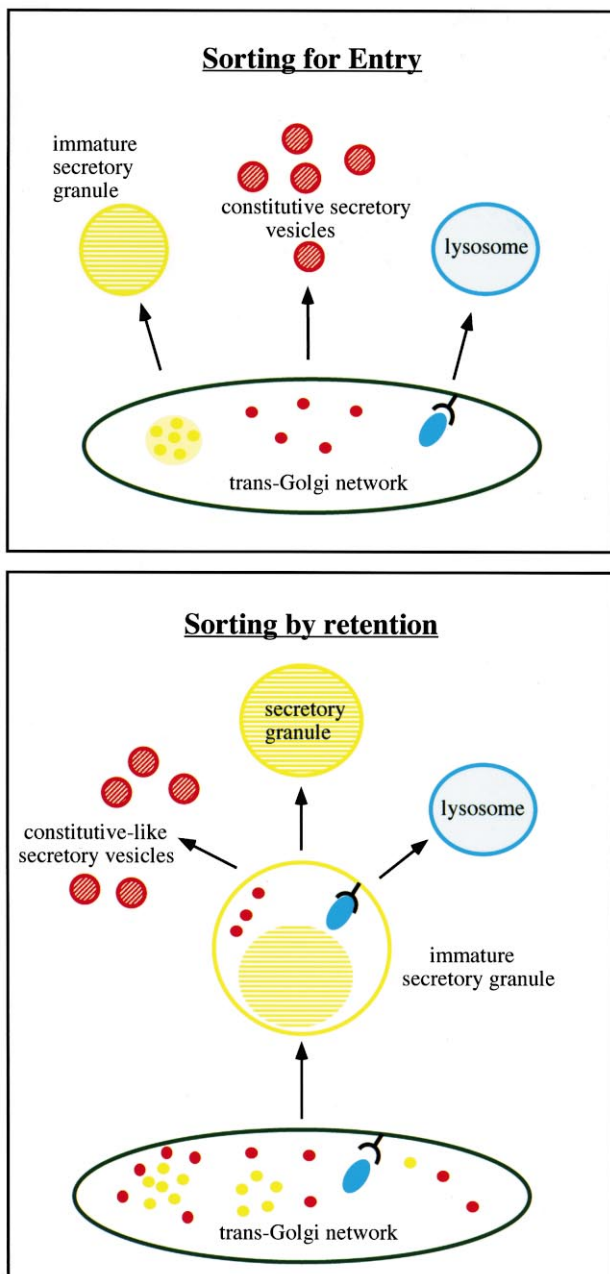


Fig. 1. Depicted in a very simple fashion is a scheme of the sorting mechanisms in the secretory pathway of neuroendocrine and endocrine cells proposed by Arvan and colleagues [26] to explain the different models for formation of regulated secretory vesicles from the TGN, i.e. sorting for entry vs. sorting by retention. Also included are other vesicle classes, such as CSVs and constitutive-like secretory vesicles, as well as a pathway for lysosomal enzymes (blue circle) and MPR (black cup-shaped object). The low amount of missorted proteins, such as furin [62], and MPR found in the ISG in the top panel have not been illustrated for simplicity. The regulated secretory proteins are shown as yellow circles, and the constitutive secretory proteins are shown as red circles. For simplicity, all coats and coat proteins have been omitted, as well as any putative interactions of the regulated secretory proteins with the membrane.

on the final size. In support of this idea, a size difference has also been seen in the secretory granules in endocrine cells of the pituitary (for review see [29]).

While the data to support ‘sorting by retention’ [30] is compelling, the data to support the ‘sorting for entry’ hypothesis, where the bulk of sorting has been demonstrated to occur in the TGN prior to ISG formation, is equally compelling (see above). To reconcile these two hypotheses, it has been suggested [22,31] that whether sorting is predominantly TGN-based (sorting for entry) or ISG-based (sorting by retention) might depend both on the propensity of the major prohormone species to aggregate, or not, and the relative rates of prohormone synthesis and vesicle formation in the TGN.

## 2.2. *Sorting receptors for regulated secretory proteins*

The paradigm for sorting of soluble proteins into vesicles is receptor-mediated endocytosis at the plasma membrane: a receptor binds a ligand and sequesters the ligand for transport to a different compartment in a coat-dependent fashion. This model has been extended to a variety of intracellular transport events including sorting of regulated secretory proteins in the TGN [32]. Regardless of whether a single molecule or a molecular aggregate is sorted, this paradigm has remained attractive enough for investigators to continue to pursue the putative ‘sorting receptor’. The putative sorting receptor could then interact directly or indirectly with the cytosolic machinery involved in the vesicle formation. The search for a sorting receptor has been approached from both the ligand side, i.e. identification of sorting signals in the ligand, and the receptor side, i.e. identification of membrane proteins which bind regulated secretory proteins.

Recent experiments have focused on an N-terminal disulphide loop structure found in POMC, provasopressin, pro-oxytocin, prodynorphin, proenkephalin, CgA and CgB [1,33]. The N-terminal 26 amino acids of POMC have been shown to contain a signal which sorts a reporter molecule into secretory granules in AtT20 cells [34]. It has been further demonstrated that a disulphide bond, within this sequence of 26

amino acids, forms an amphipathic loop structure which may play a role in sorting POMC to ISGs [33]. This data is supported by experiments in which the analogous disulphide bond of CgB was disrupted by incubation of PC12 cells with DTT, leading to constitutive secretion of CgB [35].

In pursuit of a receptor which would recognize the sorting motif researchers have used assays based on the binding of solubilized secretory granule membrane components to immobilized regulated secretory proteins. As some granule ‘matrix’ proteins such as CgB exist as membrane-bound isoforms [36], it has been suggested that the membrane bound forms of such proteins would act as nucleating receptors to recruit soluble regulated secretory proteins [3]. Indeed, recent experiments have demonstrated that the membrane associated form of carboxypeptidase E, a granule specific carboxypeptidase [37], can function as a sorting receptor [38]. These observations are the subject of some debate [31]; in particular, in light of a recent report by Halban [39] where the absence of carboxypeptidase E in a mouse mutant *CPE<sup>fat/fat</sup>* [40] does not result in abnormal insulin processing. However, the principle behind the observations follows a common theme: soluble regulated secretory proteins appear to interact in a homo- or heterophilic manner with membrane associated forms of regulated secretory proteins. At this stage, however, it could also be that other unrelated bona fide membrane proteins act as receptors for the aggregate, such as the IP3 receptor [41,42] identified using the same approach with immobilized, soluble CgA.

A variety of exogenous soluble proteins, such as renin, normally expressed in the kidney [43] to trypsinogen, normally expressed in pancreatic exocrine cells [44], have been expressed by transfection in endocrine and neuroendocrine cell lines. These exogenous molecules have been found to be secreted in a regulated fashion from the transfected cell lines, suggesting that these proteins have been stored in secretory granules. From these data it was hypothesized that a putative sorting signal, a common sequence or structural feature, appears to be conserved across a variety of systems. This signal is not, however, dominant as fusion of proinsulin with the trans-

membrane and cytoplasmic domain of CD5 resulted in targeting of proinsulin to the constitutive pathway and appearance on the cell surface [45].

These experiments demonstrate that a diverse set of soluble molecules can be targeted to secretory granules and cast doubts on the existence of a single 'sorting receptor' because it is difficult to reconcile the accurate sorting of such a variety of molecules with a single receptor. Gerdes and colleagues [110] have argued that efficient sorting of these molecules was obtained because the exogenous molecules were sorted by co-aggregation with the endogenous regulated secretory proteins. Therefore identification of a sorting sequence could only be achieved in essentially a null background of endogenous regulated secretory proteins. They have achieved this experimentally using a vaccinia virus expression system which shuts off host protein synthesis, and have shown that exogenous CgB when expressed in vaccinia virus infected PC12 cells can be efficiently sorted to secretory granules but that CgB without the N-terminal disulphide loop ( $\Delta$ cys-CgB) was not correctly sorted. Furthermore, in the same set of experiments the authors demonstrated that  $\Delta$ cys-CgB was correctly and efficiently sorted after conventional transfection protocols providing additional support for their hypothesis. Using this powerful expression system the authors should be able to begin to identify the requirements for sorting of soluble proteins to the secretory granule.

Lastly, supplementary to the postulated role of receptors in sorting regulated secretory proteins or aggregates, sorting receptors may also serve the function of selecting an appropriate membrane for the nascent secretory granule. Indeed, there must be a recognition mechanism between soluble granule proteins and granule membrane proteins or components to ensure that the membrane which finally envelops the dense core contains all the proteins necessary for secretory granule maturation, storage and exocytosis. Again, it is possible that this selection only occurs later in the pathway, in the ISG, and that the proteins not required are removed from the ISG instead of being left behind in the TGN. At present we have very little knowledge about the membrane composition of the nascent ISG which would allow one to speculate on how and where the final selection is achieved.

### 3. Formation of secretory vesicles from the TGN

#### 3.1. Formation of constitutive vesicles

Several years ago it was first shown that AtT20 cells, which have a regulated secretory pathway, have a distinct pathway by which they transport proteins to the plasma membrane, the constitutive secretory pathway [46]. More recently, the vesicles involved in constitutive secretion of proteins from PC12 cells, which also have a regulated secretory pathway, have been identified [47]. It was shown in PC12 cells, using two sulphated proteins (a hsPG and SgII), as markers for constitutive and regulated pathways, respectively, that formation of CSVs occurs concomitantly with formation of ISGs from the TGN. These budding reactions have very similar characteristics to those described below for secretory granules and those identified using other systems which reconstitute vesicle formation from cells without a regulated pathway (see for example [48]). Formation of CSVs appears to be regulated by heterotrimeric GTP-binding proteins [49,50], and be dependent upon ARF1 [51,52].

Although the fidelity of sorting into the constitutive and regulated pathways appears to vary in different PC12 cells clones (compare [47] and [53]) the principle of sorting different populations of proteins in the TGN into distinct vesicle populations remains intact in neuroendocrine cells. In contrast, no one has to date identified a constitutive secretory pathway in endocrine  $\beta$ -cells using a bona fide constitutive secretory protein. The C-peptide, derived from processing of proinsulin, has been shown to be secreted constitutively, however, this might be through a constitutive-like pathway originating from the ISG [54]. It may be that indeed that no constitutive secretory proteins in CSVs are secreted directly from the TGN in  $\beta$ -cells, however, using immunogold labelling techniques CSVs have been identified in  $\beta$ -cells using antibodies to the viral-spike glycoprotein HA of influenza virus [55]. At the same time, experiments were done which demonstrated that corona virus virions, which are released constitutively, were found segregated from dense-cores in AtT20 cells, a neuroendocrine cell line [56]. In retrospect, the ideal experiment would have been to study the transport of coronavirus particles, which have the advantage of

being morphologically identifiable marker for constitutive secretion, in  $\beta$ -cells. However, in the absence of experiments to prove whether secretory proteins are also conveyed by this pathway, the question remains an open one.

### 3.2. Transport of lysosomal enzymes from the TGN

Transport of lysosomal enzymes from the TGN to endosomes occurs via clathrin-coated vesicles (CCVs), and is mediated by binding of the clathrin-coat proteins to the mannose-6-phosphate receptor (MPR, see review by Hoflack in this issue). It has been assumed that this pathway exists in all cells. Clathrin coats assemble on the TGN via the AP-1 adaptor complex (Robinson, this issue) through an interaction with the cytoplasmic domain of receptors, such as MPR, and other proteins such as furin which traffic to and from the plasma membrane [57–59]. While the distribution of lysosomal enzymes has not been studied in PC12 cells, recent data using both GH<sub>4</sub>C<sub>1</sub> cells [60] and a  $\beta$ -cell line [26] have demonstrated that some lysosomal enzymes are present in ISGs. The appearance of lysosomal enzymes in the regulated secretory pathway provides additional support for the ‘sorting by retention’ hypothesis. These authors [26,60] were able to demonstrate that lysosomal enzymes can be released from the cells by agents used to stimulate regulated secretion. Some of the lysosomal enzymes (cathepsin B) were absent from the MSG, while others (cathepsin L) were detected in MSGs [30]. These results suggest that in the TGN newly synthesized lysosomal enzymes are incorporated into the nascent secretory granule. This could be due to a missorting of the receptors for lysosomal enzymes, MPR, into secretory granules. However, newly synthesized lysosomal enzymes that have diminished or abolished affinity for MPRs continue to enter ISGs in equal or even greater abundance [26,30]. As an alternative explanation, there may be a decrease in the efficiency of sorting of lysosomal enzymes by the MPRs in the TGN of cells with a regulated secretory pathway which allows them to escape from the TGN in other exit pathways, such as the regulated or constitutive secretory pathway, and be secreted or recaptured at a post-Golgi location such as the endosome (see review [61]).

A variety of experiments, including those cited above, have confirmed the transient appearance of lysosomal enzymes in the regulated pathway, suggesting that lysosomal enzymes are being removed from the maturing ISGs. It is assumed that this is occurs via the MPR and CCVs. Indeed, this may explain the transient presence of clathrin coats on the ISGs. Further support for this idea comes from experiments which show that the AP-1 is used on the ISG to assemble the clathrin coat [62], and the MPR has been found in ISGs but not MSGs in isolated secretory granule fractions derived from PC12 cells (A.S. Dittié, submitted). A second protein which is involved in recruiting AP-1 and therefore clathrin onto ISGs is furin, an endopeptidase present in TGN membranes. Furin, like MPR, is found in ISGs but not MSGs [63]. These results suggest that furin, like MPR, is removed from the ISG via CCVs.

### 3.3. Formation of constitutive-like vesicles

The destination of the CCVs, containing either furin, MPR, or both, could be any one of several possibilities, for example, the TGN, early endosomes, or plasma membrane. There is no data at present regarding the content, composition and destination of CCV budding from the ISG, and indeed the CCVs themselves have not been identified. A related question concerns the constitutive-like secretion of proteins which could be considered true markers of the constitutive pathway, and those which are unwanted products from the processing of prohormones, such as C-peptide. It has been proposed that CCVs might be a vesicular carrier for these population of molecules [64]. This raises a very intriguing set of questions: are there multiple classes of CCVs forming from the ISG all with a different cargo?, would these CCVs all have a different destination? or is there a single species of CCVs forming with a mixed cargo? would this CCV deliver its cargo to one destination, for example, the endosome, from where further sorting would occur?

While the mechanism for selection and transport via CCVs is clear when one assumes the cargo associates with a transmembrane receptor, it is more complex for other cargo proteins such as the C-peptide, or those proteins which follow a bulk-flow pathway [65] which has not been thought to be signal-



mediated. How does this signal-less type of cargo enter the CCVs allowing it to be removed? If it is simply a case of exclusion, as formulated by Arvan et al. in their ‘sorting by retention’ hypothesis, one would predict it could not be 100% efficient and some C-peptide would be found in the medium after stimulation of MSG. This has been demonstrated for C-peptide (see [64] and the references therein). Alternatively, it may be that multiple vesicular carriers bud from the ISG with different cargo. Depending on the cargo, distinct mechanisms could be used for selection to increase the efficiency of removal of non-secretory granule proteins from the maturing ISGs.

#### 4. Formation of secretory granules

While the mechanisms involved in the formation of both constitutive and regulated secretory vesicles from the TGN is largely unknown there have been recent reports which suggest that both these budding reactions may be unique in that they function independently of known coat proteins. Morphological data based on high voltage electron microscopy suggests that the formation of constitutive secretory vesicles from the TGN involves a ‘lace-like’ coat [66]. The components of this coat have yet to be identified but may be associated with the p62 complex identified by Howell and colleagues [67] and include p200 [68]. Conventional electron microscopy and immunogold labelling has revealed that patches of clathrin are present on the TGN in the vicinity of forming ISGs and are also found on ISGs [10,69]. However, there is no biochemical data available to date which supports the role of clathrin in secretory granule formation.

Recent results concerning the formation of secretory granules have been obtained primarily from two *in vitro* systems which reconstitute budding from TGN membranes; one using a post-nuclear supernatant derived from PC12 cells [70] and the second semi-intact GH<sub>4</sub>C<sub>1</sub> cells [18]. While in the former assay it is possible to identify two classes of vesicles budding from the TGN, in the latter this has not been possible. However, because there is no data which so far suggests the requirements for formation of an ISG are different from that for a CSV, they will be considered as congruent assays and the data ob-

tained from both, and other assays monitoring constitutive secretory vesicle formation will be compared.

##### 4.1. *The role of GTP-binding proteins in secretory granule formation*

The first observations made using the *in vitro* budding assays described above, both of which are ATP and cytosol dependent, concerned the requirement for GTP. The formation of secretory vesicles from the TGN requires GTP and is inhibited by non-hydrolysable GTP analogs [18,47]. In the PC12 cell assay, the same effect was observed with [AlF<sub>4</sub>]<sup>-</sup> and several other lines of evidence demonstrated that heterotrimeric GTP-binding proteins are involved in the regulation of post-Golgi vesicle formation (see [71] for review). The precise function of GTP is not yet clear as there may be several different GTP-binding proteins involved in vesicle formation from the Golgi, including ARF (see below). An intriguing related observation concerns the role of an as yet unidentified cytoplasmic phosphoprotein as a modulator of the heterotrimeric G protein(s) that regulate vesicle formation from the TGN [50]. A similar requirement has been seen in the semi-intact GH<sub>4</sub>C<sub>1</sub> *in vitro* assay [72] in experiments using inhibitors of tyrosine kinases and phosphatases. This observation is supported by several other reports from cell-free budding assays which indicate that phosphorylation regulates vesicle formation [48,73,74]. A role for tyrosine-phosphorylation in secretory granule biogenesis and/or function has also been suggested by two recent reports that have identified two highly homologous proteins in the membrane of secretory granules which appear to be non-functional protein-tyrosine phosphatases, called phogrin and the ICA 512 autoantigen [75,76].

##### 4.2. *The role of coat proteins in secretory granule formation*

Most of the well characterized transport steps in the cell (for example from the endoplasmic reticulum to the *cis*-Golgi, intra-Golgi, plasma membrane to endosome) have been shown to be mediated by vesicles which form by coat-mediated budding reactions from donor compartments. In contrast no

known coat, or coat protein, has been demonstrated by morphological or biochemical techniques to be involved in secretory granule formation. As mentioned above, if a coat were required, the most likely coat protein candidate would be clathrin. Formation of clathrin-coats on the TGN requires AP-1 (see above) and the monomeric GTPase ADP-ribosylation factor, or ARF [77,78] and these components might therefore be thought to function in the formation of secretory granules. However, to date there is no evidence which implicates clathrin in the formation of either constitutive secretory vesicles or secretory granules from the TGN. On the other hand, reports from the two cell-free assays described above have demonstrated that ARF is required for the budding of secretory granules and post-TGN vesicles. Myristoylated ARF1 peptide stimulated both the formation of secretory granules and constitutive secretory vesicles from isolated TGN membranes [51], while in a semi-intact cell system [52] ARF1 peptide was sufficient for stimulation of budding of GH- and PRL-containing vesicles. Importantly, although ARF is known to be involved in the recruitment of COP I coats to membranes (see article by EMR in this issue), Barr and Huttner [51] could demonstrate that depletion of COP I from cytosol did not inhibit post-Golgi vesicle formation.

Several reports have recently demonstrated that ARF may function by activating phospholipase D ([79–81] and see [82] for review). Interestingly, addition of phospholipase D (PLD) has been shown to stimulate budding from the TGN in the semi-intact assay using GH<sub>4</sub>C1 cells [83]. The stimulatory effect of PLD might be attributed to its ability to catalyse the hydrolysis of PC to PA and alter the lipid composition of the membrane. This local increase in charged lipids has been proposed to function to stabilize AP-2 association with endosomal membranes [84]. Alternatively, the production of PA may have several consequences including the production of PIP<sub>2</sub> (phosphatidylinositol 4,5-bisphosphate) in the membrane [85]. PIP<sub>2</sub> has been implicated in a variety of membrane traffic events [86] and has been shown to regulate the nucleotide bound state of ARF [87,88] and to stimulate PLD activity [89] leading to a feedback cycle of interactions between ARF, PLD, PA and PIP<sub>2</sub>. It has been proposed [83] that the effect of ARF and PLD on budding might alter-

natively be to recruit AP-1 to regions of the membrane which are high in PA. However, from more recent experiments we now know that PLD does not play a role in AP-1 recruitment [84] to TGN membranes, it would thus seem most likely that ARF and PLD are functioning to promote budding from the TGN via a signalling cascade. Consistent with this scenario [73] Shields and colleagues had previously shown that PKC can stimulate budding, and suggest that PA could function as a second messenger to trigger intracellular signalling events such as activation of PKC.

#### 4.3. *The role of phosphatidylinositol metabolism and secretory granule formation*

Further exciting results have been obtained with regard to cytoplasmic proteins required for vesicle formation from the TGN. A phosphatidylinositol transfer protein (PITP) has been implicated in the formation of both regulated and constitutive secretory vesicles [90]. The mammalian PITP activity can be substituted for by Sec14p, a yeast protein from *Saccharomyces cerevisiae* (which is, however, not homologous to the mammalian PITP) that is required for normal Golgi function and shares with PITP the ability to exchange phosphatidylinositol for phosphatidylcholine ([91]; see also contribution in this issue by Bankaitis). Additional evidence recently published related to the function of Sec14p, obtained from the study of Sac1p, a sec14-bypass suppressor [92] would suggest that PITP can also function to cause a local production of diacylglycerol which may be involved in coat recruitment in much the same way the PA has been postulated to be involved in ARF and AP-1 recruitment (see review [93]).

PtdIns is also the substrate for the yeast kinase Vps34 which has PI 3-kinase activity [94]. The synthesis of PtdIns(3)P by Vps34 has been implicated in the transport from the Golgi to the vacuole in yeast and it has been suggested that Vps34 activity is required for the formation of a specific vesicle type from the Golgi [95]. A wortmannin-sensitive PI 3-kinase activity is also required for the sorting of lysosomal enzymes from the Golgi to the endosome in mammalian cells (see [96] for review). In addition, a recent report has shown that a PI 3-kinase activity is required for formation of constitutive secretory

vesicles from isolated rat liver Golgi membranes [97]. Interestingly, this PI 3-kinase regulatory subunit associates with the cytoplasmic domain of a TGN-resident protein TGN38 in a complex with a small molecular weight GTPase, previously identified as rab6 [67]. The authors hypothesis is that the activity of the PI 3-kinase is stimulated by the activation of the GTPase [97]. So far there has been no report implicating a PI 3-kinase in the budding of regulated secretory granules. In fact, incubation of PC12 cells with wortmannin had no effect on either constitutive or regulated secretory vesicle formation, transport or exocytosis of secretory proteins (S. Urbé and S.A. Tooze, unpublished observations). It maybe that the activation of the PI 3-kinase is dependent upon the presence of particular receptors in the forming bud, for example, the presence of TGN38. Data from experiments performed to examine the composition of the nascent ISG demonstrates that there is no TGN38 present in ISGs [63].

With regard to the role of PtdIns-metabolites in vesicle-formation, it is of interest that a 105 kDa protein, oculocerebrorenal syndrome of Lowe (OCRL) [98], belonging to the inositol (Ins) 5-phosphatase family has recently been localized to the Golgi complex [99]. OCRL dephosphorylates Ins(1,3,4,5)P<sub>4</sub>, Ins(3,4,5)P<sub>3</sub> and most importantly PIP<sub>2</sub> in vitro and seems to act preferentially as a lipid-phosphatase in vivo, and could therefore regulate the levels of PIP<sub>2</sub> in the Golgi-membrane [100]. Another inositol-5-phosphatase, synaptojanin, has been implicated in the recycling of synaptic vesicles, a process that involves the formation of clathrin-coated vesicles [101]. Synaptojanin interacts through amphiphysin [102] with the small GTPase dynamin which appears to play an important role in the budding of clathrin-coated vesicles from the plasma-membrane (for reviews see [103–105]). Although the function of dynamin has predominantly, but not exclusively [106], been linked to the function of clathrin, it has been speculated that there might be a dynamin for every budding event in the cell [107]. A protein with immunoreactivity to antibodies raised against conserved regions of the dynamin-family has been localized to the Golgi complex in fibroblasts and melanocytes [108]. As GTP-hydrolysis is required for dynamin function it is possible that the inhibitory effect of GTP $\gamma$ S on the budding of secre-

tory vesicles from the TGN reflects not only an involvement of heterotrimeric G proteins and small GTPase, but also of dynamin.

## 5. Conclusions

Research on secretory granule biogenesis has revolved to a large extent around the granule content selection mechanisms. The only coat-proteins identified so far on the secretory granule, which are present only on ISGs are clathrin and adaptor-complex AP-1, and they function to mediate vesicle budding from the ISG rather than formation of the ISG from the TGN [109]. It has been suggested that budding of nascent secretory granules does not require a coat protein, but that the aggregated core of the granule is able to 'drive' vesicle formation in a way analogous to the budding of a viral particle. This hypothesis predicts that there have to be specific interactions between the granule core and the granule membrane that mirror the binding of viral nucleocapsid proteins to the membrane-bound envelope proteins. In this way the secretory granule core could mould its own membrane, driven only by a thermodynamically favourable binding of granule membrane proteins with the aggregating content. This hypothesis becomes less attractive if the nascent ISG contains largely uncondensed, unaggregated secretory proteins which, along with the membrane proteins, are not sorted prior to budding (see the sorting by retention model above). Work on this sorting mechanism is in progress and new results will no doubt be forthcoming to clarify the inconsistencies present. To address these questions, it would be most interesting to have an in vitro system for formation of ISGs using the cells, or cell lines (i.e.  $\beta$ -cells), from which the sorting by retention hypothesis has been developed. The development of such a system would allow a direct test for common structural and regulatory molecules involved in formation of secretory granules in neuroendocrine and endocrine cells.

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