REVIEW

Biosynthesis and Secretion of Pituitary Hormones: Dynamics and Regulation

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Abstract

Production and secretion of hormones by the pituitary involve highly orchestrated intracellular transport and sorting steps. Hormone precursors are routed through a series of compartments before being packaged in secretory granules. These highly dynamic carriers play crucial roles in both prohormone processing and peptide exocytosis. We have employed the ACTH-secreting AtT-20 cell line to study the membrane sorting events that confer functionality (prohormone activation and regulated exocytosis) to these secretory carriers. The unique ability of granules to promote prohormone processing is attributed to their acidic interior. Using a novel avidin-targeted fluorescence ratio imaging technique, we have found that the trans-Golgi of live AtT-20 cells maintains a mildly acidic (~pH 6.2) interior. Budding of secretory granules causes the lumen to acidify to <pH 6.0, which is both necessary and sufficient to trigger SPC3-mediated proteolytic conversion of proopiomelanocortin to ACTH. Investigation of the pH regulatory mechanism indicates that the trans-Golgi and secretory granules maintain different pH values by distinct sorting of key membrane transporters. Mathematical modeling of our data suggests that the decreasing pH values of organelles of the regulated secretory pathway is established by gradually increasing the density of active H^+ pumps from the ER to Golgi while concomitantly decreasing the H^+ permeability from ER to Golgi to secretory granules. An *in vitro* assay was developed to study the formation of processing-competent secretory granules from their processing-incompetent precursor trans-Golgi compartment. Our data suggest that ARF1-mediated sorting of proton pumps and leaks during early stages of granule formation confers processing competency to the resulting organelle. Once formed, these young granules continue to undergo membrane remodeling which results in dynamic changes in their exocytotic behavior. Two SNAREs, VAMP4 and synaptotagmin IV, enter newly formed granules but are removed from the maturing granule membrane by vesicle budding. Sorting of these proteins is correlated with the acquisition of Ca^{2+}-triggered exocytosis and a decrease in unregulated exocytotic rate. Thus, biosynthesis and secretion of pituitary hormones are dynamically regulated by intracellular sorting events that govern the functions of their secretory carriers.

Keywords: Corticotropin secretion, prohormone, sorting, membrane remodeling, post-translational protein processing, pH regulation, membrane transport, Golgi apparatus, ADP-ribosylation factor 1, secretory granules.

Abbreviations used in this paper

ACTH, adrenocorticotropic hormone; ARF, ADP-ribosylation factor; BFA, brefeldin A; ISG, immature secretory granule; MSG, mature secretory granule; PLD, phospholipase D; POMC, pro-opiomelanocortin; SPC, subtilisin-like proprotein convertase; Syt IV, synaptotagmin IV; TGN, trans-Golgi network; V-ATPase, vacuolar H^+-ATPase.

Introduction

Protein secretion is an essential function of all cells. While simple eukaryotic organisms carry out only constitutive mode of secretion, animal cells such as those found in the pituitary gland utilize two pathways for secretion (Gumbiner & Kelly, 1982; Moore et al., 1983b). The first pathway of constitutive secretion is similar to that found in yeast cells and serves a housekeeping function. It mediates the continual transport of lipids, membrane proteins, and soluble

cargoes to the cell surface independent of extracellular signals. The second pathway of regulated secretion mediates the release of hormones and other chemical messengers. Special secretory products are sorted and stored within secretory granules whose fusion with the plasma membrane is triggered only in the presence of extracellular signals. Often, these proteins are synthesized as inactive precursors that are cleaved into their functional forms during transit through this pathway. The regulated secretory pathway thus serves two crucial functions: (i) activation of hormone precursors, and (ii) control of hormone secretion.

The co-existence of two secretory pathways within the same cell requires correct sorting of both secretory cargoes and vesicle machinery to each pathway. Sorting of cargoes into secretory granules has been a subject of intensive studies and readers are referred to several excellent reviews on this topic (Arvan & Castle, 1998; Teter & Moore, 1998; Thiele & Huttner, 1998; Tooze et al., 2001). More recent studies have focused on the sorting of secretory granule membrane machinery. It is now clear that secretory granules are dynamic structures whose functions can undergo rapid changes. These changes in carrier behavior directly influence biopeptide secretion, and are brought about by dynamic trafficking of membrane components constituting the vesicle machinery. An understanding of the mechanism controlling secretory carrier dynamics is important to provide an explanation of the hypersecretion phenotype associated with several diseases. Many tumors, for instance, synthesize growth promoting factors which act in an autocrine fashion upon their own cell surface receptors to enhance proliferation (for a review, see Rozengurt, 1999). Under normal conditions secretion of these factors is under tight physiological control, for most growth factors possess granule sorting signals and are targeted to the regulated secretory pathway (e.g., Schmidt & Moore, 1994). Cancerous cells lose this regulation and secrete large amounts of bioactive growth factors constitutively. Dysregulated secretion of bioactive molecules (such as cytokines, proteases, and mucus) also contributes to a variety of lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease (reviewed in Barnes, 1998). It has been assumed that hypersecretion of these molecules results from their mis-sorting from the regulated to the constitutive secretory pathway (Burgess & Kelly, 1987). Our recent studies indicate that the constitutive pathway is not the major source of hypersecretion in endocrine tumors (Fernandez et al., 1997). Instead, the heightened secretion results from peptides that have been targeted correctly to the secretory granules but are released from this locale in an unregulated manner. It appears therefore that hypersecretion of biomolecules results from dysregulation of the secretory carriers rather than from mis-sorting of the cargoes themselves. Thus, in addition to studying how secretory cargoes are sorted into their correct carriers, one must also consider how these carriers are made and what regulates their dynamic properties in normal and diseased cells. These topics are the focus of this review.

Two distinct functions of the regulated secretory carriers

The hallmarks of secretory granules are their ability to (i) trigger proteolytic activation of packaged prohormones, and (ii) undergo regulated exocytosis. We have developed a model system to study how secretory carriers acquire these two essential functions during biogenesis. The mouse pituitary AtT-20 cell line is ideal for this purpose because it synthesizes the ACTH precursor, pro-opiomelanocortin (POMC),[1] and packages it into secretory granules for regulated secretion. POMC is sulfated and can be pulse-labeled with $^{35}SO_4^{2-}$ in the trans-Golgi (Moore et al., 1983a; Fernandez et al., 1997), providing a specific marker for studying secretory granule biogenesis without complications from earlier steps of the secretory pathway. Furthermore, POMC is proteolytically processed into mature ACTH by the subtilisin-like proprotein convertase, SPC3, also known as prohormone convertase (PC) 1 (Zhou et al., 1993). This allows for the study of both secretory carrier functions (processing and exocytosis) within the same system. Finally, AtT-20 cells are derived from a pituitary tumor and exhibit the hypersecretion phenotype characteristic of many endocrine tumors. Studies of these cells therefore provide insights into the mechanism for altered secretory activities in diseased cells.

Sequential acquisition of carrier functions during secretory granule biogenesis

Kinetic analysis in AtT-20 cells has revealed that newly formed secretory granules, containing protein which was not previously sorted into the constitutive pathway, acquire their carrier functions in two distinct steps (Fig. 1). In the first step, immature secretory granules (ISGs) rapidly form from the trans-Golgi ($t_{1/2} \sim 3$ min). These new granules are already competent to promote proteolytic processing of POMC, but are not yet competent for regulated exocytosis (Fernandez et al., 1997; Eaton et al., 2000). During a second slower step ($t_{1/2} \sim 1$ h), the granules undergo an important switch from unregulated to regulated secretory carriers. Thus, the process of carrier biogenesis can be broadly divided into two sequential steps: (i) acquisition of competence for processing followed by (ii) acquisition of competence for regulated exocytosis.

That granules can acquire processing function before they become bona fide regulated exocytotic carriers provides an explanation for a previous conundrum regarding hypersecretion in diseased states. Since biopeptide activation takes place in the regulated pathway but hypersecretion presumably occurs via the constitutive pathway, it was unclear how these mis-sorted peptides could be activated. We have found that AtT-20 cells secrete over 50% of their newly synthesized hormones constitutively (as in lacking regulation) into the medium, but the majority of these are secreted after targeting to the secretory granules rather than via the classic con-
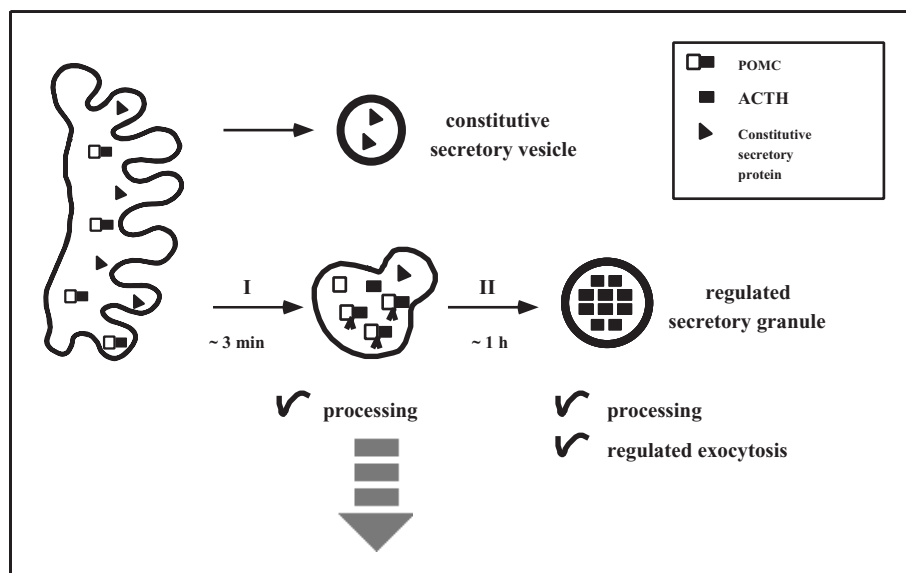
Fig. 1. Model for secretory granule biogenesis in AtT-20 cells. Secretory carriers acquire their functions in two distinct stages. In the first step (I), immature secretory granules arise rapidly from the trans-Golgi. These carriers can already trigger prohormone processing, but they have yet to acquire the regulated exocytotic function. Partially or completely processed peptides can therefore exit the cell from this compartment (thick arrow). Secretion from these ISGs occurs via two mechanisms: direct fusion of ISGs with the plasma membrane and budding of transport vesicles carrying some of the lumenal cargoes to the cell surface (see text). During a second slower step (II), granules then transform from unregulated to regulated carriers. Cells in different physiological states may modulate the kinetics of these two steps to adjust the amount of peptides secreted under stimulated and unstimulated conditions.

stitutive secretory pathway (Fernandez et al., 1997). Secretion results primarily from young granules or ISGs that are already processing-competent but have yet to acquire regulated exocytotic function. Unlike mature secretory granules (MSGs), these ISGs have a much higher basal secretory rate and thus can give rise to unregulated release of partially or fully processed hormones. Studies using brefeldin A (BFA) to perturb vesicle budding indicate that not all peptides are secreted by direct fusion of ISGs with the plasma membrane. Rather, a portion of the molecules are secreted via transport vesicles that bud from the ISGs (Fernandez et al., 1997). Constitutive-like secretory vesicles, described by Arvan and colleagues (Kuliawat & Arvan, 1992), are plausible candidates responsible for this route of secretion (Fig. 1, thick arrow).

The picture is quite different in normal tissues. Studies of primary pancreatic cells have shown that the majority of newly synthesized insulin is stored intracellularly (Kuliawat & Arvan, 1992). Only <5% of hormone is released from ISGs in an unregulated fashion. This quantitative difference between normal and tumor cells may be explained by kinetic differences in granule membrane assembly. In normal cells, the two steps in granule assembly appear to be better coordinated such that new granules quickly become competent for both processing and regulated exocytosis. These two processes are not coupled in tumors, allowing for unregulated secretion of bioactive peptides from immature secretory granules. Thus, cells may modulate the

dynamics of their secretory granule assembly to control the exact amount of peptides secreted in different physiological states.

This sequential model of granule assembly is very different from the mechanism involved in ER vesicle formation. Biochemical and genetic studies have provided a very complete picture of the ER export mechanism (reviewed in Herrmann et al., 1999). It is now clear that ER vesicle formation is achieved by the coordinated sorting of secretory cargoes and vesicle membrane machinery by specific cytoplasmic coat proteins. A sorting complex consisting of a secretory cargo (alpha factor) and vesicle membrane (Sec22p) and cytoplasmic coat (COPII) proteins has been identified in yeast (Kuehn et al., 1998); Sec22p belongs to a family of SNARE proteins involved in vesicle targeting and fusion (reviewed in Jahn & Südhof, 1999). In addition, recruitment of a tethering protein (p115) by rab1 GTPase is thought to coordinate the sorting of several other SNARE proteins (rbet1, syntaxin 5 and membrin) during ER vesicle budding (Allan et al., 2000). Thus, during budding from the ER, the vesicles are already programmed with the correct machinery for subsequent fusion. Such strict coupling may not be desirable in the case of the trans-Golgi, where budded vesicular carriers may need to respond to changing physiological conditions by modifying their destinations and/or fusion characteristics. Thus, the mechanism for vesicle production from the trans-Golgi appears to be fundamentally different from that for ER vesicle budding.

Step one: generation of processing-competent secretory carriers from the trans-Golgi

Essential role of lumenal acidification revealed by *in vitro* reconstitution studies

We have developed an *in vitro* reconstituted system to study the first step of granule membrane assembly: the formation of processing-competent granules from processing-incompetent trans-Golgi membranes (Andresen & Moore, 2001). AtT-20 cells are pulse-labeled with ^{35}S-sulfate and homogenized immediately on ice; under this condition, all labeled POMC resides within the trans-Golgi and is in the unprocessed precursor form. Membranes are then isolated from postnuclear supernatants and used as the donor compartments. *In vitro* reactions are initiated by the addition of ATP, GTP and cytosol and incubated at 37°C. The reaction products are then analyzed in two ways. Firstly, the extent of POMC conversion from unprocessed precursor to processed intermediates and mature ACTH is determined by SDS-PAGE and PhosphorImager. Secondly, the amount of labeled hormones incorporated into budded granules is measured by a differential centrifugation procedure that separates released granules from the donor trans-Golgi membrane. Processing *in vitro* requires vesicle formation from the donor compartment, as it is potently inhibited by BFA. This fungal metabolite interferes with guanine nucleotide exchange on the small GTPase, ARF1, and is a general inhibitor of ARF-mediated vesicle budding (Mansour et al., 1999; Morinaga et al., 1999). Recombinant ARF1 protein strongly stimulates POMC processing and vesicle formation, whereas the GDP-bound ARF T31N mutant potently inhibits both reactions. Based on these findings, we conclude that ARF1 regulates the formation of processing-competent secretory granules from their processing refractive precursor compartment.

POMC and its processing enzyme SPC3 traverse the entire secretory pathway together, but processing is not activated until they reach the secretory granule compartment. Since SPC3 is a calcium- and pH-dependent enzyme, we have investigated the role of ionic milieu in proteolytic activation (Schmidt & Moore, 1995; Andresen & Moore, 2001). In both intact cells and *in vitro* reconstituted system, POMC processing was blocked by inhibition of the vacuolar H$^+$-ATPase (V-ATPase) with bafilomycin A1. This indicates that lumenal acidification is necessary for SPC3-mediated POMC processing. To test if acidification is also sufficient to trigger the processing machinery, we trapped the labeled POMC in the donor compartment (using BFA or by leaving out ATP and cytosol in the reconstituted system) while experimentally acidifying the trans-Golgi lumen. POMC processing can be prematurely activated in this compartment when the lumenal pH is lowered to below 6.0. These data establish that a drop in the lumenal pH during budding is both necessary and sufficient to confer processing competency to newly formed secretory granules.

Mechanism of pH regulation along the secretory patthway studied by targeted avidin

How, then, does vesicle formation cause a drop in lumenal pH? Previous pH studies of fixed cells or isolated organelles *in vitro* suggest that Golgi and secretory granules control their acidity by altering their conductances to Cl$^-$ (Hutton, 1982; Barasch et al., 1988; Barasch et al., 1991). According to this model Cl$^-$ serves as a key counter-ion to dissipate the membrane potential generated by the electrogeneic V-ATPase, allowing the latter to work more fully. Acidic organelles therefore maintain distinct lumenal pH values by maintaining different permeabilities to Cl$^-$. However, recent studies of Golgi and trans-Golgi network (TGN) pH in living cells are not consistent with this view. The membranes of these organelles possess high conductances to several counter-ions (including K$^+$ and Cl$^-$) relative to that of H$^+$, suggesting that the steady-state pH values should not be sensitive to alterations in any single counter-ion conductance. Indeed, removal of Cl$^-$ had no effect on Golgi or TGN pH values measured in living cells (Demaurex et al., 1998; Wu et al., 2000a). Due to the lack of existing methods to target ratiometric, pH-sensitive dyes to secretory granules of living cells, the mechanism of pH regulation in the regulated secretory pathway remained unclear.

To provide insights into this question, we have developed a fluorescence ratio imaging technique based on targeting avidin to individual organelles of the regulated secretory pathway (Wu et al., 2000b). These studies were carried out in collaboration with Dr. Roger Tsien and Dr. Stephen Adams of the University of California, San Diego. Chicken avidin was fused to various organelle targeting sequences and the chimeric DNAs were transfected into AtT-20 cells. A series of membrane permeant, pH-sensitive, fluorescent biotin derivatives with distinct pK$_a$ were chemically synthesized, and specifically loaded into the avidin-containing organelles. Fluorescence ratio imaging of living cells was performed to determine the pH regulatory mechanism in the endoplasmic reticulum (ER), Golgi and secretory granules.

Studies thus far suggest that the mechanism for acidification of the secretory pathway differs from that previously proposed for the endocytic pathway (Fig. 2). Previous experiments have demonstrated an essential role of Na$^+$/K$^+$-ATPase in regulating endosomal pH's (Cain et al., 1989; Fuchs et al., 1989). This electrogenic transporter produces an interior positive membrane potential in early endosomes, impeding maximal acidification by the electrogenic V-ATPase. During formation of late endosomes from early endosomes, Na$^+$/K$^+$-ATPases are sorted away allowing the V-ATPase to acidify the interior more fully. By contrast, membrane potential is not the major determinant of organelle acidity in the secretory pathway (Schapiro & Grinstein, 2000; Wu et al., 2000a; Wu et al., 2001). According to our data, the most important parameters influencing steady-state pH's along the regulated pathway are the rates of H$^+$ pumping
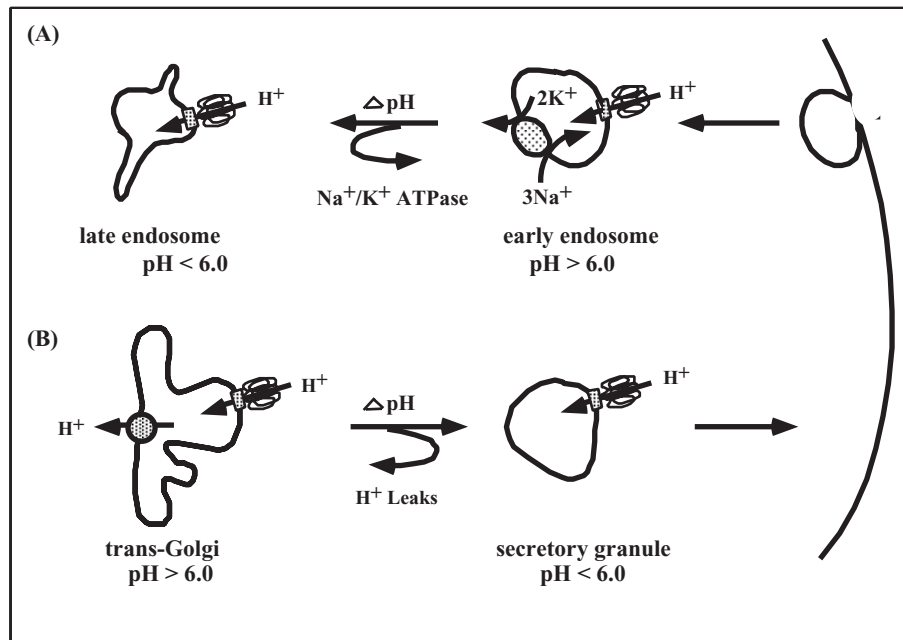
Fig. 2.   Distinct mechanisms for acidification in the endocytic and the exocytic pathways. (A) In the endosomal system, steady-state pH values of individual organelles are thought to be determined by the distributions of V-ATPase and the electrogenic Na^+/K^+-ATPase. Transition from early to late endosomes is accompanied by sorting out of Na^+/K^+-ATPase, allowing the latter organelles to become more acidic. (B) In the biosynthetic or exocytic pathway, counter-ion conductances across organelle membrane are large compared to proton conductance. Membrane potential is therefore not the most important determinant of organelle pH. Instead, transition from Golgi to secretory granules is accompanied by a reduction in the membrane permeabilities to H^+, suggesting that sorting out of proton leakage pathways is an important step in the formation of acidic granules. The dotted oblong, rectangular and circular areas indicate the transmembrane domains of Na^+/K^+-ATPase, V-ATPase, and proton leak pathway, respectively.

and the rates of H^+ back leakage. We have measured the proton leak rates across ER, Golgi and mature secretory granules, and calculated the intrinsic H^+ permeabilities across each organelle by applying the leak data to a mathematical model that takes into account differences in organelle geometry and buffering capacities (Grabe & Oster, 2001; Wu et al., 2001). These data show that the ER membrane exhibits a 2-fold higher H^+ permeability than the Golgi, which in turn has a 4- to 7-fold higher H^+ permeability than mature secretory granules. The results support the idea that acidification from ER to Golgi to secretory granules is accomplished by progressively increasing the density of active H^+ pumps while decreasing the density of H^+ leaks (Fig. 2).

A two-step mechanism regulating the formation of processing-competent secretory carriers

Based on the above studies, it is clear that sorting of membrane transporters during granule formation is a key step in prohormone activation. Where does this sorting take place? Since its first biochemical description (Matlin & Simons, 1983), the Golgi export site has been modeled as a single cisterna – the TGN – where various secretory vesicles are produced. Therefore, a logical assumption is that sorting must occur coincidentally with the production of new secretory granules from the TGN. This view, however, is not consistent

with numerous elegant 3-dimensional reconstruction EM studies by Rambourg (Rambourg et al., 1987; Rambourg et al., 1988; Rambourg & Clermont, 1990) showing that the Golgi export site is made of multiple cisternae rather than a single cisterna. According to this model, cisternae in the cis and medial portions of the Golgi are seen as flat membranes containing evenly dispersed secretory material. At the start of the trans-Golgi which is comprised of four to six cisternae, secretory material starts to condense in dilated portions of the cisternae or 'progranules'. The cisternae become perforated at the same stage, making them appear fenestrated. In more distal cisternae, the progranules appear larger, having swelled with more secretory material, and the flattened portions appear increasingly tubular, with expanded perforations in between. At the last cisterna, the nascent ISG pinches off, leaving behind a residual network of tubular membranes. A summary of these morphological changes is depicted in Figure 3.

If the morphological data are correct, in which step does prohormone activation occur? Studies using our *in vitro* reconstituted system have provided the first biochemical evidence that the Golgi export process is indeed more complicated than previously thought. This became evident when we carefully compared the two parameters measured in our *in vitro* reactions: POMC processing and granule release. If the TGN were a single cisterna, one would predict that sorting
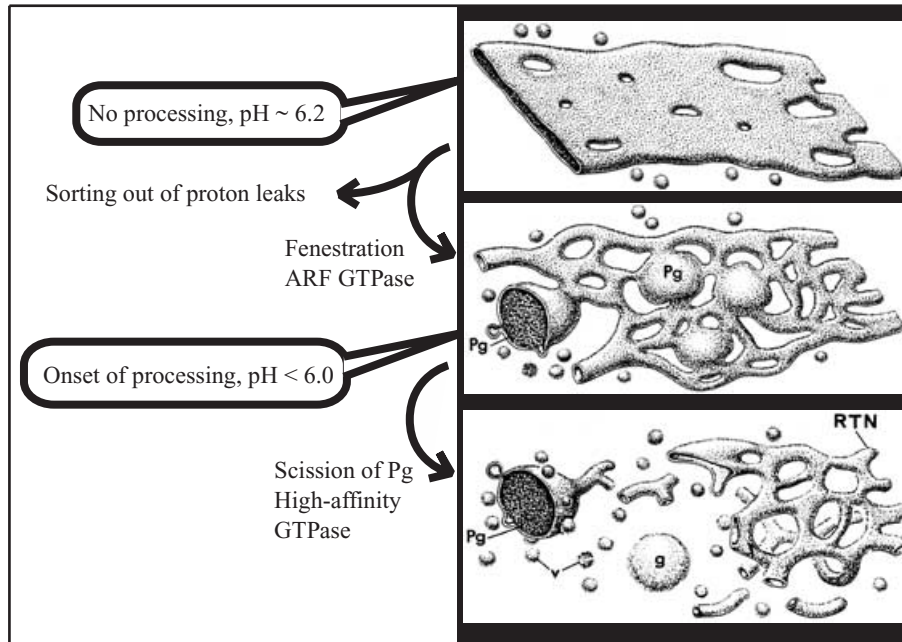
Fig. 3. A two-step model for the formation of processing-competent secretory carriers from the trans-Golgi. (Adapted from Rambourg & Clermont, 1990). The trans-Golgi starts as a flattened cisterna with evenly distributed secretory materials. Binding of ARF1 and effectors to this compartment initiates a series of morphological changes, including fenestration in the membrane and swelling of progranules (Pg) which produce a network of membranes equivalent to the TGN. These morphological changes are accompanied by biochemical sorting of proton pumps and leaks to distinct membrane domains leading to acidification of the Pg lumen. The drop in lumenal pH then activates the granule machinery to initiate proteolytic processing. In a final scission step, progranules are then released from the TGN to yield granules (g) and residual tubular networks (RTN). This step is regulated by a high-affinity GTPase which is distinct from ARF (Andresen & Moore, 2001). This model satisfactorily explains both morphological and biochemical reconstitution data (see text).

of transporters (which activates POMC processing) and granule release occur concomitantly and the two parameters should exhibit identical characteristics. This is not the case. Although proteolytic processing in general correlated well with the appearance of new granules, a significant fraction of processed hormones was still associated with the donor membrane. This was particularly evident when the reaction was carried out in the presence of $100\,\mu M$ GTPγS, which partially inhibited processing but restricted the processed hormones generated in this reaction exclusively to the donor membrane. Further analysis indicates that POMC processing and granule release actually measure two sequential steps during the formation of nascent carriers. Three lines of evidence support this conclusion. Firstly, GTPγS inhibits processing with K_i of $>100\,\mu M$ whereas it inhibits granule release at much lower concentrations (K_i of $\sim 10\,\mu M$). Secondly, while HeLa cytosol and AtT-20 cytosol are equally active in the processing assay, only cytosol prepared from AtT-20 cells supports the granule release reaction. Thirdly, kinetic analysis shows that GTPγS inhibits POMC processing with a $t_{1/2}$ of $\sim 15\,min$, but it inhibits granule release with a $t_{1/2}$ of $\sim 40\,min$. Thus, the onset of processing is an early step whereas granule release represents a late step. Furthermore, ARF T31N inhibits both reactions with $t_{1/2}$ of $\sim 15\,min$, indicating that the ARF1 GTPase regulates the early but not

the late step. Together, these data support the idea that secretory carriers are formed from the trans-Golgi in two steps that are regulated by different GTPases (Andresen & Moore, 2001).

A model consistent with the combined morphological and biochemical data is shown in Figure 3. In the first step, the trans-Golgi membrane begins to fenestrate and hormones are concentrated into numerous budding profiles designated as progranules. These progranules are still connected to the Golgi membrane, but sorting of membrane proteins (i.e., proton pumps and leaks) already takes place allowing for lumenal acidification and onset of prohormone processing. This interpretation is supported by morphological analysis, showing that POMC processing begins at dilated regions of the trans-Golgi (Schnabel et al., 1989). We therefore propose that our POMC processing assay measures the formation of these progranules. ARF1 GTPase regulates the membrane sorting and deformation reactions associated with this step. In the second step, progranules are freed from the network by a membrane scission event which is regulated by a high affinity GTPase. This second step can be measured by our granule release assay. These two assays therefore provide the first *in vitro* system with which both early and late steps in secretory granule formation may be dissected biochemically.

Step two: conversion from unregulated to regulated secretory carriers

Identification of a sorting pathway responsible for granule membrane remodeling

Once ISGs form from the trans-Golgi, how do they then switch from unregulated to regulated secretory carriers? We hypothesize that this transition is achieved by remodeling of the fusion machinery on nascent granule membrane. Since the SNARE family of membrane proteins is known to control membrane fusion, we have screened members of this family for potential candidates that may be responsible for this switch. Kinetic studies show that the v-SNARE, VAMP4, marks a novel vesicular sorting pathway during granule membrane remodeling (Eaton et al., 2000). VAMP4 is associated with the trans-Golgi and newly formed ISGs, but this association diminishes as nascent granules mature. Removal of VAMP4 from the regulated pathway occurs by ARF-mediated vesicle budding since it is blocked by BFA. BFA also inhibits the unregulated secretion of hormones from ISGs, suggesting that VAMP4 might be the v-SNARE in this transport step (Fernandez et al., 1997). In support of this hypothesis, over-expression of a soluble VAMP4 peptide inhibits unregulated secretion from the ISGs (B. Eaton & H.-P.H. Moore, unpublished). Furthermore, inhibition of the VAMP4 pathway with BFA arrests granules in an unregulated state. Thus, sorting of VAMP4 along with other membrane components is responsible for changes in granule's secretory properties.

Synaptotagmin IV as a key factor in the switch from unregulated to regulated carriers

The above studies support the hypothesis that an inhibitor of regulated exocytosis is removed during the switch from unregulated to regulated carriers. We have identified this inhibitor to be synaptotagmin IV (Syt IV) – a Ca^{2+}-independent member of the synaptotagmin protein family (von Poser et al., 1997). We have found that Syt IV has similar subcellular distributions as VAMP4, and it is removed from the ISG membrane via the same sorting pathway (Eaton et al., 2000). By contrast, synaptotagmin I, a calcium sensor mediating regulated exocytosis, is found on both ISGs and MSGs. Syt IV is known to form hetero-oligomers with synaptotagmin I and by doing so, it interferes with regulated exocytosis (Littleton et al., 1999; Thomas et al., 1999). Indeed, overexpression of Syt IV perturbs proper acquisition of Ca^{2+}-triggered exocytosis during ISG maturation (Eaton et al., 2000).

Together, these studies indicate that sorting of Syt IV and VAMP4 out of the ISGs switches the organelle from an unregulated to a regulated carrier (see Fig. 4). Interestingly, Syt IV expression is tightly regulated during development and by neuronal activities, and Syt IV mutant mice show defects in learning and memory (Ferguson et al., 2000). This suggests that the characteristics of ISGs may be dynamically regulated during synaptic growth and modifications. Recent studies have shown that dense-core granules may participate in transporting synaptic components to the nerve terminal (Jontes et al., 2000), suggesting a link between secretory granule biogenesis and synaptogenesis. Understanding ISG dynamics is therefore an important step towards an understanding of synaptic plasticity.

Summary and future perspectives

Although it has long been known that the trans-Golgi is responsible for producing secretory granules, the mechanism for granule formation from this organelle is still poorly
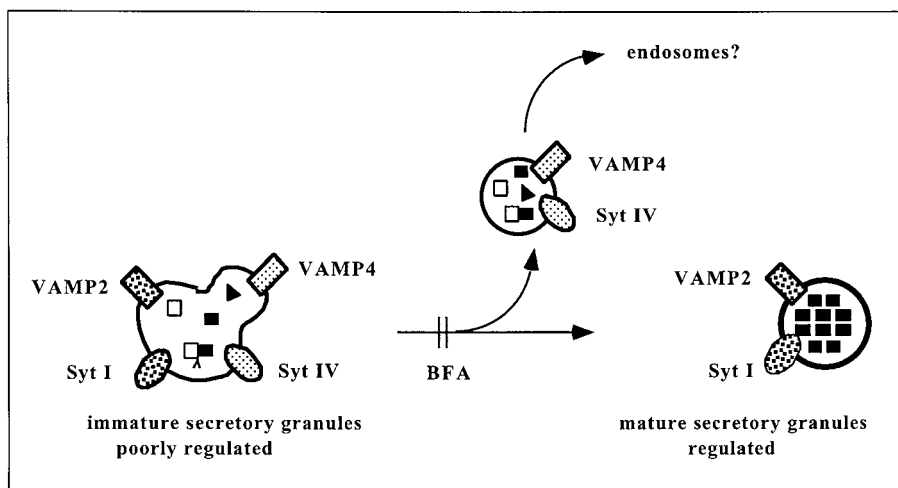

Fig. 4. Membrane sorting events during the switch from unregulated to regulated secretory carriers. The transition in the carrier's exocytic function is effected by vesicle budding from the ISGs, a process that can be inhibited by BFA (Eaton et al., 2000). This sorting pathway carries away two SNARE proteins – VAMP4 and Syt IV – from maturing secretory granules, but leaves behind VAMP2 and Syt I – the two key constituents of the regulated fusion machinery. The vesicles that remove VAMP4 and Syt IV may be constitutive-like vesicles that fuse with endosomes (Turner & Arvan, 2000).

understood. Granule budding requires ARF1. The exact role of ARF1, however, is less clear. In other transport steps, ARF and its relative SAR1 participate in vesicle formation by recruiting cytoplasmic coat proteins (e.g., COPII coat for transport from ER to cis-Golgi, COPI coat for intra-Golgi and retrograde transport, and clathrin coat for transport from the plasma membrane to endosomes). In contrast, granule formation from the trans-Golgi does not require the participation of known coat proteins (Barr & Huttner, 1996: J.M. Andresen & H.-P.H. Moore, unpublished). Instead, it has been suggested that budding of granules from the trans-Golgi does not require any coat protein and the role of ARF in this reaction is to activate phospholipase D (PLD) activity (Chen et al., 1997). However, in our reconstituted system, addition of bacterial PLD did not stimulate POMC processing (J.M. Andresen & H.-P.H. Moore, unpublished). A coat-less budding mechanism also fails to explain how sorting of membrane transporters and hence lumenal acidification could be accomplished. A more likely explanation is that ARF participates in granule formation by recruiting an as yet unidentified effector. Supporting this view, recent studies of ARF mutants have indicated that the effect of ARF on Golgi morphology is mediated by an unknown effector distinct from COPI or PLD (Kuai et al., 2000). An important future quest is therefore to identify the ARF effector(s) involved, and to learn how it might influence sorting of proton pumps and leaks during production of secretory carriers from the trans-Golgi.

Another area of inquiry is the identity of proton leakage pathway(s) across organelle membranes. Our studies have underscored the importance of these leakage pathways in controlling acidification in the secretory pathway. The identity of these leakage pathways is currently unknown. Recent studies by Grinstein and colleagues have implicated the involvement of a voltage-sensitive, Zn^{2+}-inhibitable proton channel in Golgi pH regulation (Schapiro & Grinstein, 2000). Further characterization of this and other candidate transporters will be important to understand the mechanism of pH regulation along the secretory path.

It is recently appreciated that after secretory granules bud from the TGN, they continue to undergo a series of ill-defined 'maturation' steps that ultimately transform them into bona-fide secretory granules. Several constitutively secreted proteins are first incorporated into ISGs but become removed during the maturation process. Likewise, a fraction of lysosomal hydrolases and endosomal endopeptidases escape sorting at the TGN and are subsequently sorted away from the ISGs. As such, the ISG has sometimes been regarded as the functional extension of the TGN by completing the cargo sorting function initiated at that site (reviewed in Arvan & Castle, 1992). ISGs also differ from MSGs in their size and density. Homotypic fusion between ISGs has been demonstrated, and is likely to contribute to the increase in size as ISGs mature (Wendler et al., 2001). It is thought that following granule-granule fusion, soluble contents condense and excess membranes are removed by

vesicle budding. In this way, MSGs acquire their higher density and larger size after maturation. An important conclusion from our studies is that maturation not only affects granule lumenal contents and their physical properties, but also plays a key role in determining their functional characteristics as regulated or unregulated secretory carriers. Studies of the VAMP4/SytIV sorting pathway we have identified will be important in understanding the dynamics of secretory carriers in normal and diseased states. It is interesting to note that VAMP4 contains phosphorylation-dependent sorting domains and that initial mutational analysis suggests that these sites are critical for proper VAMP4 sorting in AtT-20 cells (B. Eaton, C. Chan & H.-P.H. Moore, unpublished). It will be important to investigate what physiological conditions effect the trafficking of VAMP4 and how these events modulate hormone secretion. An intriguing possibility is that hypersecretion of hormones from tumor cells could be a result of phosphorylation-dependent changes in membrane sorting during granule biogenesis.

Most cells are capable of constitutive secretion, but until recently it was commonly thought that regulated secretion was limited to professional secretory (such as neural, endocrine, and exocrine) cells. Yet recent evidence suggests that many other cell types utilize this process as well. Regulated exocytosis may in fact be used for a variety of purposes, such as mobilization of water channels in kidney, translocation of glucose transporters in adipocytes, movement of H^+/K^+-ATPase to gastric lumen, and regulated insertion of intracellular vesicles during plasma membrane repair in wound healing (reviewed in Teter & Moore, 1998). Indeed, using a fluid tracer to monitor trafficking of all Golgi-derived secretory vesicles, we have detected a regulated secretory pathway in a number of mammalian cell lines which have previously been considered to possess only the constitutive secretory pathway (Chavez et al., 1996). Insights gained from the study of pituitary cells can thus provide a general conceptual framework for similar events occurring in most, if not all, mammalian cells.

Acknowledgements

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