CHAPTER 10

Biogenesis of Dense-Core Secretory Granules

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Contents

Abstract

Dense core granules (DCGs) are vesicular organelles derived from outbound traffic through the eukaryotic secretory pathway. As DCGs are formed, the secretory pathway can also give rise to other types of vesicles, such as those bound for endosomes, lysosomes, and the cell surface. DCGs differ from these other vesicular carriers in both content and function, storing highly concentrated 'cores' of condensed cargo in vesicles that are stably maintained within the cell until a specific extracellular stimulus causes their fusion with the plasma membrane. These unique features are imparted by the activities of membrane and lumenal proteins that are specifically delivered to the vesicles during synthesis. This chapter will describe the DCG biogenesis pathway, beginning with the sorting of DCG proteins from proteins that are destined for other types of vesicle carriers. In the trans-Golgi network (TGN), sorting occurs as DCG proteins aggregate, causing physical separation from non-DCG proteins. Recent work addresses the nature of interactions that produce these aggregates, as well as potentially important interactions with membranes and membrane proteins. DCG proteins are released from the TGN in vesicles called immature secretory granules (ISGs). The

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mechanism of ISG formation is largely unclear but is not believed to rely on the assembly of vesicle coats like those observed in other secretory pathways. The required cytosolic factors are now beginning to be identified using in vitro systems with purified cellular components. ISG transformation into a mature fusion-competent, stimulus-dependent DCG occurs as endoproteolytic processing of many DCG proteins causes continued condensation of the lumenal contents. At the same time, proteins that fail to be incorporated into the condensing core are removed by a coat-mediated budding mechanism, which also serves to remove excess membrane and membrane proteins from the maturing vesicle. This chapter will summarize the work leading to our current view of granule synthesis, and will discuss questions that need to be addressed in order to gain a more complete understanding of the pathway.

Introduction

Overview

In eukaryotes, newly-synthesized proteins destined for secretion are first transferred from the cytoplasm to the lumen of the endoplasmic reticulum, and then progress through the Golgi apparatus to the trans-Golgi network (TGN). At the TGN, the choice of secretory pathways broadens. One route, which appears to be present in all cells, is constitutive in the sense that secretion does not depend on extracellular signals. Such secretion involves the budding of vesicles or tubular elements from the TGN and their subsequent transport to and fusion with the plasma membrane, and is essential for cell growth since, among other functions, it provides new material to expand the cell surface.[1]

In addition to a constitutive route, many cells maintain a secretory mode that is adapted for the tight coupling of protein release to extracellular stimuli. For such regulated exocytosis, the vesicles that carry newly-synthesized protein from the TGN accumulate in the cytoplasm until specific extracellular events trigger their fusion with the plasma membrane, resulting in the release of vesicle contents.[2] The vesicles involved are called dense-core granules (DCGs), the name reflecting the fact that the contents are so highly condensed that they form a large electron-dense plug in the vesicle lumen. A large amount of protein, as well as other molecular cargo, is thus efficiently stored in vesicular reservoirs and later released on demand. This pathway therefore permits larger and more rapid secretory responses than can be generated via constitutive secretion. Classical DCGs in endocrine, exocrine and neuroendocrine cells are responsible for storage of a wide array of signaling molecules (e.g., peptide hormones) and secreted enzymes, and related vesicles are found in metazoan cells of other lineages as well as in numerous unicellular organisms. The secreted proteins and macromolecules play a vast range of functions, from tissue coordination in metazoans to cyst formation in protists.

Regulated secretion also depends upon mechanisms for controlling the timely release of DCG contents, and this is accomplished by regulating the fusion of the vesicle membrane with the plasma membrane. Much of the progress in understanding the mechanisms that mediate this step has been preceded or aided by studies of synaptic vesicles (reviewed in ref. 3), which undergo regulated fusion with the plasma membrane, but differ from DCGs in their biogenesis and acquisition of contents. Comparable work in DCG secretion has shown that many of the molecular components involved in regulating exocytosis and achieving membrane fusion are shared by these two vesicle types.[2] In addition to proteins that appear to be specific for regulated fusion with the plasma membrane, the mechanisms include factors, such as SNAREs and Rab proteins, which are members of families of proteins that are of central importance to vesicular trafficking at multiple stages in the eukaryotic secretory pathway. Thus, regulated exocytosis appears to be accomplished by the coupling of a regulatory mechanism to a universal core of membrane trafficking machinery. Although many of the protein components have been identified, and a more complete understanding of the process remains an important goal for ongoing research. The mechanistic studies of regulated membrane fusion are too extensive to be included in this chapter, but have been covered in many reviews,[4-8] and above.
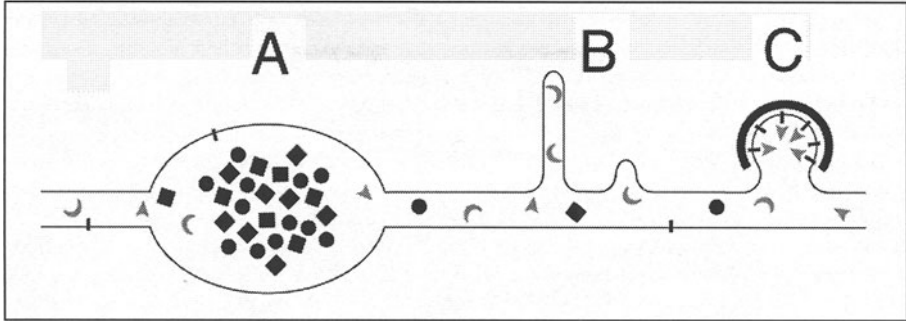
Figure 1. At least 3 pathways diverge at the TGN in neuronal, endocrine and exocrine cells. A) A subset of proteins are destined for dense core secretory granules for release via regulated exocytosis. These are found as aggregates in distended areas of the cisterna. B) Proteins destined for constitutive secretion are transported via vesicles or tubules. C) Proteins destined for lysosomes are concentrated, via the mannose-6-phosphate receptor, into clathrin coated pits and vesicles. Darkly-shaded squares and circles represent proteins that tend to coaggregate under TGN conditions, and that are subsequently stored in DCGs. Lightly-shaded forms represent proteins that primarily exit the TGN via other pathways. Arrowheads represent proteins that are ligands for the mannose-6-phosphate receptor. Crescents represent proteins that are found in a relatively even distribution throughout the lumen. The extent to which some constitutively secreted proteins may be concentrated within specific regions of individual cisternae is not incorporated into this model.

This chapter will instead focus on a part of the pathway that precedes regulated exocytosis, namely the synthesis steps that lead to the formation of DCGs, beginning at the TGN. The TGN is a complex compartment that gives rise not only to the regulated and constitutively released classes of secretory vesicles but also to vesicles that carry hydrolytic enzymes to lysosomes.[9-11] Therefore, we seek an understanding of the signals that guide outbound proteins in a 3-way TGN sorting problem (Fig. 1). DCG protein sorting also continues in a post-TGN compartment, where additional factors come into play. This pathway has been the subject of numerous valuable reviews,[12-19] with a particularly thorough treatment by Arvan and Castle.[20]

The Anatomy of DCG Formation

A number of important insights into the pathway of DCG formation have come from electron microscopy, providing a context for molecular and genetic studies. First, the fact that DCGs appear *dense* implies the existence of mechanisms to drive a degree of macromolecular aggregation that is unusual within the secretory pathway. Many lines of research have led to the conclusion that protein sorting and concentration are intimately linked in this pathway, both relating to the self-aggregating tendency of DCG proteins that will be discussed below.

Rambourg and colleagues have investigated the localization of protein aggregates, using serial thin sections to reconstruct the Golgi apparatus during granule formation.[21-27] In cells producing mucous-containing DCGs, the cis and medial Golgi appear as flat cisternae, and secretory proteins are evenly distributed in their lumina. In contrast, cisterna in trans regions are marked by multiple perforations and are dilated in regions that accumulate aggregates of secretory material. Those dilations grow progressively larger in the more distal regions, while the nondilated portions take on a tubular appearance. At the trans-most cisterna, the dilated regions with their concentrated secretory cargo appear to exist as independent bodies, separate from a residual network of tubular membranes. Several points were established or reinforced by these images. The first is that the visible concentration of DCG proteins begins within Golgi cisternae. A second point is that the TGN, the vesicle donor, appears to be undergoing large-scale changes itself. The images also indicate that the vesicles do not bud conventionally in the manner that is well-established for coat (e.g., clathrin)-mediated steps, since no coats are seen.

Electron microscopy also suggested that the aggregates undergo progressive changes, and are therefore likely to be dynamic in nature. In pancreatic cells that are synthesizing insulin-storage granules, the proteinaceous cores seen in Golgi dilations appeared less dense than the cores of insulin granules in the cell cytoplasm.[28] Since the latter are derived from the former, this implied that proteins reorganize during an organellar maturation process.

An important conclusion is that DCG formation should be considered as a multi-step process that plays out in sequential compartments. An early phase occurs at the trans face of the Golgi and results in the production of vesicles bearing concentrated secretory proteins. These are called immature secretory granules (ISGs).[29] Subsequently those vesicles are remodeled, as reflected morphologically by cargo condensation and biochemically by changes in protein composition, to become mature DCGs.[30,31] For simplicity, we will refer to the first process as budding, and the second as maturation. Central issues to be considered in this chapter are the mechanisms responsible for protein sorting during those successive steps. Again for simplicity, we will largely confine our discussion to the DCGs found in neuronal and endocrine cells. Many of the same mechanisms are likely to apply to other classes of DCGs, among which are those in hematopoetic cells; for example, see references 32 and 33.

The Genetic Perspective

Genetics frequently offers a natural complement to morphological studies for developing an overview of a pathway. Unfortunately, a weakness in current approaches to analyzing DCG biogenesis is the absence of developed genetic models, although several systems show promise. No human diseases are known to stem from an inability to synthesize neuroendocrine DCGs. Presumably, strong defects in DCG formation would result in embryonic lethality in a complex multicellular organism, since such a defect would preclude regulated secretion of many peptides involved in tissue coordination. However, this has not prevented the generation of regulated exocytosis mutants in more simple systems, such as *Drosophila*. In flies, the null phenotype of a gene called dCAPS (Calcium-Activated Protein for Secretion), is embryonic lethal, but analysis of the larva has shown that the gene product is necessary for DCG exocytosis,[34] as predicted from earlier work in mammalian chromaffin cells.[35,36] Although mutations affecting earlier stages in the pathway (i.e., DCG synthesis) have not been characterized in this organism, the characterization of CAPS mutants in this system provides hope that the earlier steps will be accessible by further mutational analysis. *C. elegans* offers another potentially useful system for the genetic analysis of DCG synthesis, and several mutations affecting regulated exocytosis have been identified in this organism (reviewed in ref. 37).

Currently, the only examples of DCG synthesis mutants are found in single-cell systems: the unicellular ciliates *Tetrahymena thermophila* and *Paramecium tetraurelia*, in which the mutations were chemically induced, and spontaneously-arising clones of the rat pheochromocytoma line PC12.[38-46] The viability of these mutants substantiates the idea that regulated exocytosis, unlike constitutive secretion, is not involved in basal cell growth. That is, DCGs are essential for organismal survival in metazoans, but not for individual cell viability. In the PC12 lines, some mutations appear to disrupt the transcription of numerous granule protein genes.[45,46] In the ciliate mutants, which appear to be due to single recessive alleles, the cargo genes are still expressed though no granules are synthesized. In one *Tetrahymena* line, normal granule cargo appears to be shunted to the constitutive secretory pathway.[47] This phenotype indicates that DCG cargo proteins are not sufficient to direct granule formation, a result which was particularly interesting in the context of experiments in which mammalian DCG cargo proteins were expressed in tissue culture cells that do not normally make DCGs.[48-51] Such cells make vesicles with dense cores, presumably because cargo proteins expressed in nonspecialized cells can induce the formation of their own carriers from the TGN. These results implied that the capacity to make DCGs was inherent in the basic organization of the Golgi/TGN since it could also occur in such nonspecialized cells. Since this capacity appears to have been lost in the *Tetrahymena* mutant, the defect in that line may point to an aspect of Golgi/TGN function that is critical for regulated but not constitutive secretion.

The full relevance of the ciliate or PC12 cell mutants to DCG biosynthesis will only be known when the mutations themselves have been identified. Such genetic approaches provide an unbiased method for the identification of novel genes, and may prove critical in broadening our understanding of the granule synthesis pathway. Although many of the DCG cargo proteins themselves have been cloned and characterized, much less is known about the mechanisms that control protein sorting and condensation. Genetic systems may help to identify the regulatory factors that are involved in these processes.

Protein Sorting into ISGs

Protein Sorting in DCG Biogenesis: An Overview

Protein sorting takes place in the TGN and during maturation. In each case, a single compartment gives rise to multiple pathways, and the challenge is in understanding how DCG proteins, both in the lumen and the membrane, are cosorted from a larger cohort that includes proteins destined for other pathways. The relevant contributions of TGN vs. ISG sorting are likely to be cell-type specific and are generally difficult to quantify experimentally. However, the mechanisms for controlling sorting at both stages may be fundamentally similar. In particular, the considerations that arise from protein aggregation are relevant for both compartments.

A long-standing issue is whether the primary mode of DCG protein sorting is active or passive. The model of active sorting was initially inspired by the paradigm of sorting to lysosomes, in which sorting derives from recognition of a set of soluble lumenal proteins by a transmembrane receptor. Extending this to DCG biogenesis, the model posited that a subset of proteins have positive sorting signals for inclusion in ISGs.[52,53] In this scheme, proteins in the TGN lumen that lack targeting signals are presumed to follow an alternative, default pathway of constitutive secretion. This model has been called "sorting for entry" (Fig. 2A).
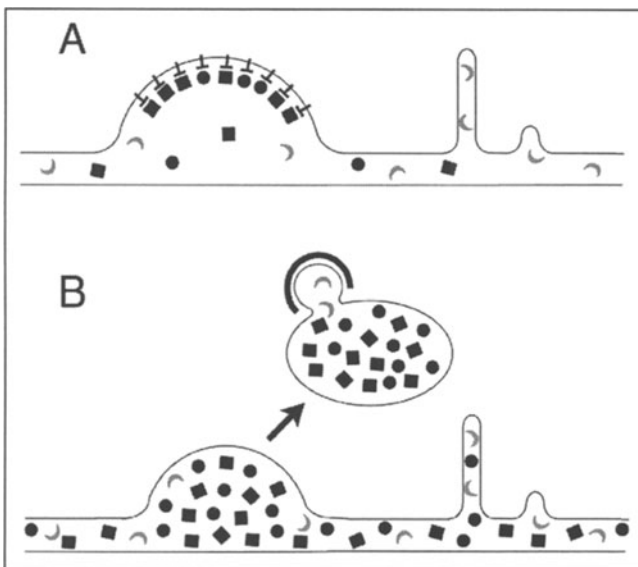

Figure 2. Models for sorting in DCG biogenesis. A) Sorting by entry. A subset of proteins in the TGN is selected for inclusion in budding ISGs because they contain positive sorting signals that interact with an unspecified receptor in the budding ISG membrane. B) Sorting by retention. Budding ISGs include a large fraction of the total TGN volume, and proteins (both aggregated and soluble) are included by random partitioning. The sorting of DCG proteins from others takes place primarily at a subsequent stage, by selective withdrawal of membrane and lumenal proteins from ISGs.

An alternative model posits that newly synthesized proteins can be targeted to ISGs by default, even in the absence of specific targeting signals, if the flux of bulk membrane traffic toward ISGs is greater than that to constitutive or lysosomal carriers. This may indeed be the case for cells that are highly committed to regulated exocytosis.[54,55] In this case, the major sorting events occur in the ISG, which becomes a functional extension of the TGN. Proteins that are retained as ISGs undergo maturation end up as the contents of mature granules. Nongranule proteins can be selectively withdrawn from ISGs during this period, and this model is termed "sorting by retention" (Fig. 2B).

In evaluating either model, the sorting of DCG proteins cannot be considered in precisely the same terms that apply in other pathways, because the tendency of such proteins to self-aggregate facilitates a unique mode of targeting. Among other things, it allows a large group of proteins to be sorted together in a single step. One implication is that sorting receptors, if present, could presumably function at concentrations that are dramatically sub-stoichiometric to their DCG protein ligands. Furthermore, such receptors would only have to recognize some subset of DCG proteins, since the remainder could be sorted indirectly via aggregation. In fact, no receptor has ever been unambiguously identified in this pathway. This does not by itself eliminate a "sorting for entry" model, because a second unusual feature of many DCG proteins is a tendency to bind to membranes. This has implications for sorting that will be discussed in a later section.

Protein Aggregation as a Sorting Mechanism

Many isolated DCG proteins will self-associate under in vitro conditions believed to approximate the TGN; namely, a slightly acidic pH and high calcium concentration relative to earlier compartments in the secretory pathway.[56 57,58] This can serve as a mechanism for sorting because it is selective: proteins that are constitutively secreted tend to remain soluble under conditions that promote DCG protein aggregation. This first sorting step can therefore be imagined as the evolutionary version of ammonium sulfate precipitation, with the collective behavior based on the proteins' individual biophysical properties, for example their surface charge. While the ability of individual proteins to aggregate is variable,[59] mixtures of proteins may show cooperativity in vitro, thereby increasing the efficiency of the step (Fig. 3A).[60]

Efficient protein aggregation might be expected to show concentration-dependence, and indeed isolated DCG proteins only self-associate above a threshold concentration.[57] This in turn suggests that minor constituents of DCGs may depend for their efficient sorting on coassociation with more abundant species, whose concentrations must be sufficiently high to drive their independent self-aggregation. The sorting efficiency of individual proteins can be experimentally measured as the fraction that is stored in DCGs as opposed to being mistargeted to the constitutive pathway. As expected from coassociation models, the sorting efficiency of a protein may vary widely between different cell lines. One would also predict that the sorting efficiency of a protein could be boosted by increasing the expression level of other proteins with which it coaggregates, particularly those which are most abundant. Chief among the abundant metazoan DCG proteins are the chromogranin/secretogranins, a group of proteins with shared physical characteristics despite their very limited sequence similarity.[61,62] Indeed, the overexpression of Chromogranin B (CgB) in the AtT-20 neuroendocrine cell line increased the sorting efficiency of a second DCG protein, pro-opiomelanocortin (POMC).[63]

Nonetheless, it is inherently difficult to test the proposition that self- or coaggregation is a primary sorting determinant using conventional structurefunction analysis, since aggregation is thought to be directed by gross biophysical properties of DCG proteins, and there are no clear "aggregation signals" at the amino acid sequence level. However, recent studies have shown that sorting efficiency can be increased by providing an artificial aggregation signal. Heterologous expression of a 6HIS-tagged secretory protein enhanced the aggregation and DCG storage, in a calcium-dependent fashion, of CgA.[64,65] The authors speculate that the tag functions as an "aggregation chaperone" by providing a local site for the binding of divalent cations, thereby nucleating the aggregation process. Curiously, the 6HIS tagged protein itself was not
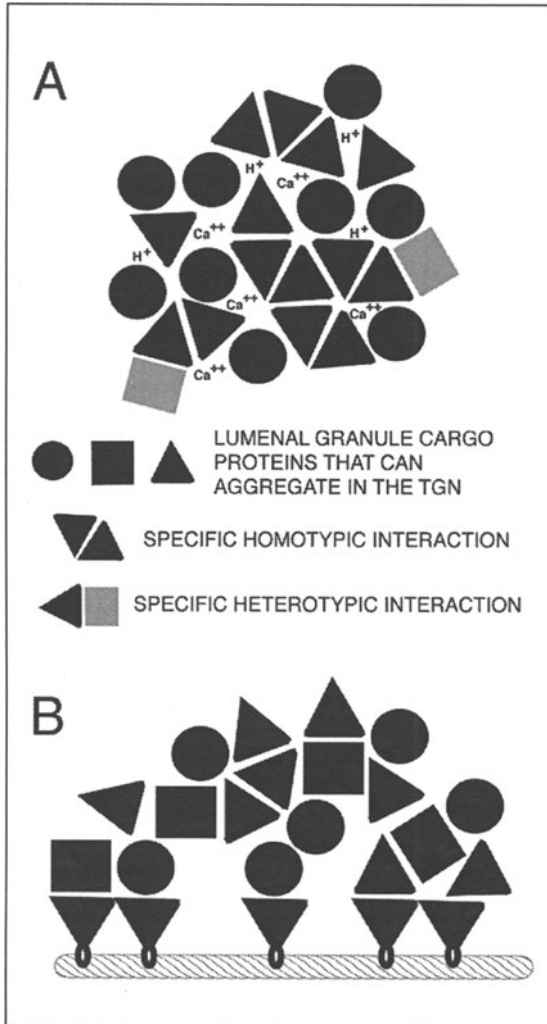
Figure 3. Multiple types of interactions hold aggregated DCG proteins together. A) Aggregates that are sorted to ISGs may be characterized by a heterogenous set of interactions, both specific (homo- and heterotypic) and non specific (e.g., low affinity binding based on charge interactions that are stabilized by low pH and high Ca^{2+}). A small number of protein species may form the bulk of the aggregate, with others present at lower concentrations. B) Multivalent interactions of chromogranin B. CgB (depicted as triangles) has an N-terminal loop-shaped domain that can promote unconventional tight association with the lumenal membrane leaflet, and ~10% of CgB is in this state. The high concentration of CgB immobilized at the membrane may stabilize interactions that are unstable in solution. Specific and nonspecific interactions of the membrane-bound CgB with lumenal proteins can promote high avidity association of a large protein aggregate with the membrane.

stably incorporated into the aggregates, suggesting that DCG proteins in their aggregated form interact more strongly with other DCG proteins than with the HIS tagged peptide. Whether endogenous proteins have similar nucleation-promoting properties remains to be determined.

Identifying the role of any single protein or protein domain in DCG sorting is complicated by the high degree of cooperativity that is hypothesized to exist within DCG protein aggregates.

Colomer et al took advantage of the observation that two exocrine DCG proteins, amylase and GP2, do not coaggregate with neuroendocrine DCG proteins in solution,[66] to study the sorting of DCG proteins the absence of coaggregation. When expressed in the neuroendocrine cells, the exocrine proteins were not stored in DCGs but instead secreted constitutively.[67] In similar experiments, an endothelial DCG protein, von Willebrand factor, was expressed in neuroendocrine AtT20 cells.[49] This resulted, however, not in the constitutive secretion of von Willebrand factor but instead in the formation of two morphologically-distinct classes of granules. One contained endogenous chromogranins, while the other contained von Willebrand factor. A possibility is that two sets of proteins aggregate independently in the TGN, which could be determined by a number of factors. For example, the two sets could precipitate at relatively distinct pH and/or calcium concentrations and thus be spatially or temporally separated. Specific aggregate formation can also arise from conventional protein-protein interactions. In pituitary and pancreatic islet cells, for example, efficient sorting of CgA to DCGs depends on its association with secretogranin III, and an essential targeting sequence in CgA has been determined by gene truncation.[68] CgA sorting in PC12 cells also depends on a specific sequence in the protein, which overlaps with, but is not identical to, that region which is required in pituitary cells.[69] This difference suggests that CgA may be interacting with a different partner in PC12 cells, and indeed these cells do not express secretogranin III. One possibility is that different surfaces of a CgA domain can interact specifically with a range of partners, like a good host at a cocktail party.

In summary, the data indicate that the aggregation of a particular protein depends on a number of factors, including its attraction to other potential binding partners within the aggregate, and the physiologic qualities of the lumenal environment, such as pH and calcium concentration, which affect the strengths of those interactions. The expression of proteins that are differentially sensitive to lumenal conditions or that form exclusive sets of protein-protein interactions can potentially result in the formation of multiple distinct aggregates in the same TGN compartment, each comprised of different proteins. These mechanisms could underlie the natural ability of some cell types to produce more than one class of DCGs, as is observed in *Aplysia* bag cell neurons, bovine pituitary cells, as well as some protozoa.[70-72]

Defining the Sorting Aggregates

Though the model of sorting-by-aggregation is well established, the actual nature of the molecular interactions within such aggregates is difficult to define. The process of aggregation must be reversible so that the contents can be released into solution following exocytosis, and moreover, it must be dynamic enough to permit the reorganization of their substituents during maturation.[73] The latter is particularly clear in pancreatic β-cells, in which the insulin-containing DCGs exhibit a crystalline ultrastructure, observed by electron microscopy, that is not found in ISGs. In comparison to the production of insulin crystals, which involves the assembly of a single protein, the formation of DCG ultrastructure in protozoa may be significantly more complex. In these cells, the lumen of mature DCGs is filled by a crystalline core that consists of multiple varieties of proteins.[74,75] Indeed, the localization of different proteins within the cores of *Paramecium* DCGs has revealed that the crystals contain at least two distinct layers, each with a different set of protein components.[76] Images of ISGs reveal that the components of the two layers are interspersed in this compartment, indicating that the layers are formed during a subsequent reorganization phase. Thus, there is a significant amount of reorganization that must occur during crystal assembly. Overall, the term "aggregation" may be misleading insofar as it suggests a phenomenon based on "stickiness", as for example for misfolded proteins in the endoplasmic reticulum.[77] Instead, the interactions that occur between individual proteins in an aggregate may be transient and weak, stimulating formation of aggregates in the TGN due to stabilizing effects provided by multivalent interactions while also allowing for reorganization of the proteins during crystallization, as in Figure 3.

Some of the nonspecific, low-affinity interactions that occur in aggregates are likely to be mediated by the effects of calcium and pH in charge neutralization, leading to intermolecular

interactions of acidic proteins by coordinate association with calcium ions (Fig. 3A). It is noteworthy that the chromogranins/secretogranins contain a preponderance of acidic amino acids, which endow these proteins with the capacity to bind large numbers of calcium ions with low affinity.[61] Acidic calcium-binding proteins also form the core of some protist DCGs, though they show little overall sequence homology with mammalian proteins.[78,79] An attractive explanation for the similarities is that they reflect a common aggregation-based DCG synthesis mechanism between protozoa and multicellular organisms, and that the amino acid sequences have evolved under similar constraints.

Targeting Aggregates to Membranes

Following DCG synthesis, the regulated secretion of DCG cargo proteins is dependent on mechanisms that bring the vesicles to the cell surface and control their fusion with the plasma membrane. These activities are dependent on the activity of DCG membrane proteins, for example those that interact with cytoskeleton-based motors for intracellular transport[80] and those that mediate regulated exocytosis.[2] It follows that the aggregation of core proteins during DCG synthesis cannot by itself be sufficient to form functional DCGs, and that there must be specific, though not necessarily direct, interactions between the lumenal proteins and the membrane constituents in order to ensure efficient sorting of these proteins to the same vesicles. These interactions have been difficult to detect, although some possible examples are discussed in a later section. What is clear, however, is that many lumenal proteins can themselves associate with membranes in unconventional ways. However, the nature and the functional significance of those associations are largely unsettled.

Five to ten percent of CgB adheres tightly, in a calcium and pH sensitive manner, to membranes.[81] Whether this fraction is in dynamic equilibrium with the remaining ~90% is not known, but there is no known chemical difference between the two cohorts. The membrane binding of CgB is associated with an N-terminal domain defined by a disulfide-anchored loop, which is sufficient to confer membrane association when linked to an otherwise soluble protein.[82] Importantly, the chimeric protein was sorted to DCGs in spite of the fact that it did not appear to aggregate, suggesting that the N-terminal domain constitutes an independent targeting signal. That same domain may promote homodimerization at neutral pH, implying that it may mediate different interactions in sequential secretory compartments.[83]

CgB, as discussed earlier, also shows a strong tendency to aggregate in a controlled fashion. The coexistence in a single protein of domains that facilitate both protein-membrane binding and homo- or heterotypic protein-protein aggregation, offers the potential to generate cooperative networks with physiologically-useful properties (Fig. 3B). First, the total concentration of DCG proteins needed to reach the aggregation threshold in the TGN may be reduced for any proteins that interact with the membrane, since the local concentration may be increased depending on local membrane geometry. Secondly, the avidity of a CgB aggregate for the membrane will be greater than that of a monomer, since multiple N-terminal domains are available for independent membrane binding. Validation of this came from an extension of the experiments with CgB chimeras outlined above. While a single N-terminal CgB domain was able to direct sorting to DCGs, efficient sorting only occurred when two such domains were present.[82] This suggests that the membrane affinity of a single domain may be only marginally sufficient, but is more than adequate if two or more such domains are linked, as would be the case in a CgB aggregate.

In a nonconventional sense, CgB could be considered as a DCG sorting receptor: a membrane-associated protein that is itself targeted to DCGs, and that can potentially cotransport any proteins with which it associates. A similar argument has been made for the enzyme Carboxypeptidase E (CPE), which is targeted to DCGs by a C-terminal amphipathic alpha helical domain.[84,85] In addition to acting as an enzyme to modify DCG cargo, CPE can also bind a subset of DCG proteins, for example the hormone precursor pro-opiomelanocortin (POMC).[86] The CPE recognition site involved is different from the enzymatic cleft,[87] and binding may be important for efficient sorting of POMC, a conclusion based on experiments with CPE knockout mice and from CPE-deficient cell lines.[86,88] CPE has been called a receptor for POMC and

perhaps for other cargo proteins, though use of the term "receptor" has remained contentious since CPE can also aggregate with POMC, chromogranins, and other cargo proteins in a conventional Ca^{2+} and pH-dependent fashion.[89,90]

Membrane association of CgB and CPE may be a property that has arisen convergently in these proteins, albeit by different mechanisms, reflecting the importance of this activity in DCG cargo sorting. An N-terminal disulfide bonded loop such as that found in CgB is found in several DCG proteins, including POMC and chromogranin A (CgA), though the homology does not extend beyond the structural level,[84] and evidence to date suggests that its role in sorting may be protein-specific. As in CgB, N-terminal disulfide loop domain in POMC is both necessary and sufficient for sorting, but surprisingly, it appears to interact with the membrane indirectly, through interaction with CPE.[84] The disulfide loop in chromogranin A was not necessary for the sorting of this protein in PC12 cells,[69] and instead an interior domain is essential for sorting in these cells, via interaction with membrane-associated secretogranin III.[68,91] These studies find no evidence for a conserved DCG targeting signal, but they do indicate that specific protein-protein interactions can be important for efficient sorting of lumenal cargo.

Precisely how CgB, CPE, secretogranin III, and other ostensibly soluble lumenal proteins associate with membranes is not resolved. There is some evidence that they associate preferentially with cholesterol-rich membranes, so-called lipid rafts.[91,92] Consistent with this, depletion of cholesterol from tissue culture cells decreased the sorting efficiency of both CPE and CgB, though it is difficult to distinguish direct from indirect effects in such experiments.[19,93] In addition, because both constitutive and regulated secretion were inhibited by cholesterol withdrawal, the results do not demonstrate a specific role for cholesterol in DCG formation. The experimental limitations notwithstanding, these data suggest that the association of CPE and CgB with specific membrane sub-domains could be an important aspect of sorting. If lipid rafts are indeed involved in this pathway, it could add another level of complexity to the cooperative mechanisms that may pertain (Fig. 4). Interestingly, CgB is also differentially sorted between the apical and basolateral pathways in polarized epithelial cells, which do not make
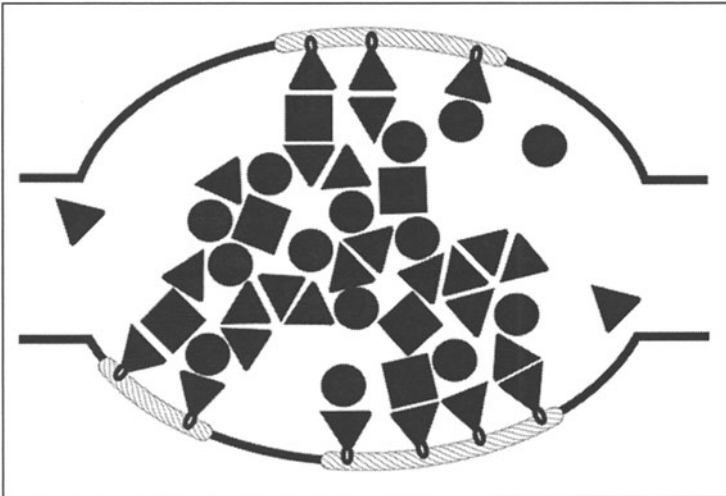

Figure 4. Selective association of DCG proteins with lipid rafts in the TGN. Implications of such association include the following possibilities: 1. Independent association of proteins with a single raft would promote protein-protein aggregation. 2. Protein aggregates could stabilize rafts with which they associate. Large aggregates could lead to formation of extensive rafts. In principle, this process could be sufficient to generate DCGs with a highly biased lipid composition, which is indeed observed.[191] The thickened, patterned regions of the cisternal membrane represent putative lipid subdomains.

DCGS, and this also requires signals within the N-terminal domain.[94] This may suggest a similarity in sorting mechanisms used in epithelial and regulated secretory cells.

A final complication in dissecting DCG sorting signals is that the requirement for the disulfide loop in CgB depends on cell type. Disulfide bond reduction led to the constitutive secretion of newly synthesized CgB in PC12 cells.[95] As expected, this treatment did not affect the sorting of Secretogranin II, a protein that undergoes aggregation but does not contain cysteine residues. However, in GH4C1 cells, the same treatment did not perturb the sorting of CgB.[96] Similarly, CgA sorting also appears to exhibit cell type specificity: a C-terminal truncation was correctly sorted in PC12 but not in GH4C1 cells,[97] and an N-terminal region, which does not contain a disulfide loop, was important for sorting in PC12 cells.[69] Thus, the sorting requirements for CgA, CgB, and granule proteins more generally, may depend on the cell type, specifically because the efficiency of any protein's sorting will depend on the available interacting partners. In some cases, a protein's interacting partner could be a membrane raft, whereas in other cases, the same protein may be delivered to DCGs by virtue of its ability to aggregate with other lumenal cargo proteins.

Membrane Protein Sorting

Our current understanding of signals involved in DCG membrane protein targeting is relatively primitive. In principle, membrane proteins could be targeted by signals in their lumenal, transmembrane and/or cytoplasmic domains; however the characterization of such signals has not been straightforward. A significant obstacle has been the fact that relatively few membrane proteins have been identified that are exclusively localized to granules.[20]

Phogrin (phosphatase homolog in granules of insulinoma) localizes to DCGs in a range of neuronal and endocrine tissues.[98] It is a transmembrane protein with an N-terminal lumenal domain and C-terminal cytoplasmic domain, and is synthesized with a large N-terminal proregion that is later cleaved within ISGs. Either the pro-domain or the lumenal domain of the processed protein can be independently stored in DCGs, indicating that each contain signals sufficient for targeting.[99] One possibility is that these, and by implication the full length phogrin as well, can be sorted by associating with the condensing core of granule cargo in the TGN. This may also be true for two DCG membrane proteins of the anterior pituitary and adrenal medulla, peptidylglycine α-amidating monooxygenase (PAM) and dopamine β-hydroxylase.[60] In these cases, there is physiological evidence that the lumenal domains can sort independently of the transmembrane or cytosolic domains, since both the soluble forms and the transmembrane forms occur naturally in DCGs.[100] Nonetheless, efficient storage of the transmembrane form of PAM also requires signals within the cytoplasmic tail.[101]

The idea that sorting of transmembrane proteins in DCG involves cytosolic signals is also supported by analyses of VAMP2, a widely distributed DCG v-SNARE,[4] and P-selectin, a protein of platelets and endothelial cells.[102] The sorting of VAMP2 to insulin-containing DCGs is impaired by a point mutation in the cytosolic portion of the protein, and the expression of this incorrectly sorted mutant protein is unable to support regulated exocytosis in the absence of wildtype VAMP2.[103] Analysis of P-selectin targeting is complicated by the fact that it can be found in more than one intracellular compartment, suggesting that it contains hierarchical targeting signals.[104] In addition, the DCGs of platelets and endothelial cells share some properties with lysosomes, and mechanisms involved in their biogenesis may differ from those in neuronal and endocrine cells.[102,105-107] Nonetheless, P-selectin expressed heterologously in the neuroendocrine cell line AtT-20 was targeted to DCGs, and this depended on a tyrosine-containing motif in the cytoplasmic domain.[108,109] The same motif is important in the endogenous endothelial cell context, indicating that the targeting mechanisms may be similar. The tyrosine-based motif suggests that this protein can interact with a coat-associated adaptor, and indeed a functional role for AP-3 in the sorting of P-selectin to DCGs has been suggested,[110] but no identified coats are involved in the formation of ISGs in the TGN. One possibility is that conventional adaptor/coat-mediated sorting of P-selectin occurs at a step
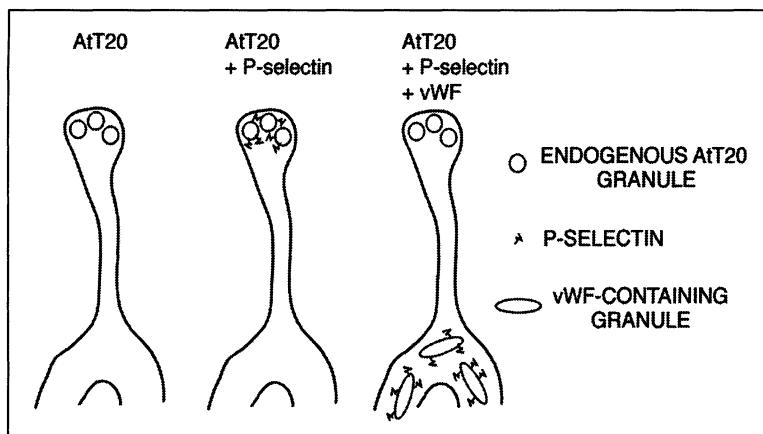
Figure 5. The sorting of P-selectin can be directed by a lumenal DCG protein. CgB-containing DCGs in AtT20 cells are concentrated in neurite-like extensions. When P-selectin was heterologously expressed, it colocalized with CgB. When von Willebrand factor (vWF) was heterologously expressed, it induced the formation of novel granules. When AtT20 cells were cotransfected with vWF and P-selectin, P-selectin colocalized with vWF and not with CgB.

distinct from the known budding and maturation steps in DCG biogenesis; a second is that adaptors may have noncanonical roles unrelated to coat recruitment.[111]

The studies of P-selectin have revealed clear evidence that a cytosolic signal can be important for the sorting of transmembrane proteins to DCGs. Further analysis of the targeting mechanism will likely be an important topic in future research, as it represents an activity that is topologically distinct from the relatively well characterized aggregation-based sorting events in the lumen. In an intriguing set of experiments, Cutler and colleagues found evidence that the function of this cytosolic sorting determinant can be coupled to the expression of a lumenal DCG protein. When the lumenal DCG protein von Willebrand factor (vWF) was coexpressed with P-selectin in neuroendocrine AtT-20 cells, the vWF was stored in vesicles that were distinct from DCGs containing endogenously-expressed CgB,[112,113] a finding that is consistent with previous results.[114] The novel and intriguing finding was that P-selectin was preferentially targeted to the vWF-containing vesicles, indicating that vWF and P-selectin, which are normally expressed in platelet and endothelial cells, could be cosorted in a cell type in which they are heterologously expressed (Fig. 5). There was no indication, however, of a direct interaction between the two proteins, and the sorting of P-selectin in this context was instead dependent on the same tyrosine-containing cytoplasmic motif that had previously been shown to be necessary for targeting to DCGs.

The targeting of one class of membrane proteins, those linked via a GPI-anchor, cannot depend on cytosolic signals, since anchors of this type do not penetrate the cytoplasmic membrane leaflet.[115] For GP-2, the major membrane protein of zymogen granules in pancreatic acinar cells, sorting may occur via a coaggregation mechanism. Its lumenal domain has been found to associate with a lectin (ZG16p), sulphated matrix proteoglycans, and syncollin, the last a lumenal protein that may itself interact with the membrane.[116] These proteins have been postulated to form a membrane-associated matrix that could serve as a sorting intermediary between the membrane and the zymogen core contents.[117] This is a variation on the model described for CgB and CPE as sorting receptors, and suggests by analogy that GP-2 or syncollin might serve as the membrane anchor for the zymogen core. However, DCG assembly is normal in the absence of either protein, indicating that neither is playing a unique role in that regard.[118,119]

In summary, the relatively limited evidence to date suggests that a mechanism similar to that involved in cargo protein condensation is involved in sorting of some, but not all membrane proteins with lumenal DCG contents. In principle, indirect interactions between membrane and core proteins may be equally important in the cosorting of membrane and lumenal cargo. If lumenal proteins like CPE preferentially insert into membrane sub-domains based on their lipid composition, then any membrane proteins that independently partition into the same sub-domains would be cosorted. In support of this hypothesis, recent studies have suggested that prohormone convertases 1 and 2, which are responsible for proteolytic cleavage of lumenal proteins during granule maturation, are sorted to ISGs by virtue of C-terminal membrane raft-associated tails, which are by themselves necessary and sufficient for targeting to DCGs.[110 120] Additionally, cytoplasmic signals on some transmembrane proteins appear to play important roles in sorting, but the mechanisms are unknown.

Vesicle Budding and Maturation

Mechanisms of Immature Secretory Granule (ISG) Budding

The canonical mechanism of vesicle budding, as for example that involved in the emergence of lysosome-bound carriers from the TGN, involves transmembrane receptors, adaptors, and coat proteins. Since there is no evidence that transmembrane proteins or coat proteins are relevant in ISG budding, other mechanisms are likely to apply. There has been some progress in reconstituting this process using cell-free systems, though the field has generally suffered from a lack of in vivo models, for example a well developed genetic system with mutations that affect this step in the pathway. The general approach has been to start with labeled DCG protein in the TGN of permeabilized cells or in Golgi-enriched fractions, then measure the transfer of the label from the relatively large and pelletable Golgi membranes to nonpelletable vesicles, using medium speed centrifugation to separate the two pools. The appearance of label in smaller vesicles is taken as an indication of cargo transfer to ISGs via vesicle budding. Since little is known about DCG biogenesis, it is important to note that "budding" as defined by this assay may include a large number of steps, including the establishment of Golgi/TGN microdomains, and the release of previously budded, weakly associated vesicles. Thus, the results of these experiments could depend upon on the nature of the starting material. In addition, it has not yet been rigorously demonstrated in any system that the released vesicles are bona fide ISGs, for example by testing whether they are competent to fuse with their appropriate target membrane.

The reconstituted budding reactions utilize ATP, as expected, and most but not all require a cytosol extract.[31,121-124] The small GTPase ARF is required, although the targets for this regulatory protein are not yet clear. One potentially relevant ARF target is phospholipase D (PLD), the binding of which to the membrane can enhance ISG budding.[125] PLD converts phosphatidyl choline to phosphatidic acid, perhaps thereby effecting a change in membrane curvature.[126] This idea is appealing because, in the absence of coat proteins, the membrane curvature required for ISG budding must be induced by other mechanisms.[127] In addition, the indirect products of PLD activity may recruit additional effectors to the budding site, including the unconventional GTPase dynamin-2.[128] Dynamin mediates membrane scission events, such as pinching off vesicle buds. However, PLD does not stimulate budding in all reconstituted systems; the differences may reflect the variety of ways in which donor fractions are prepared.

There is evidence that kinases and phosphatases, heterotrimeric G-proteins, and a phosphatidyl inositol transfer protein (PITP) are involved in ISG budding, but the enzymatic substrates have not been established.[129-133] An important unanswered question is whether any of these activities, the majority of which are as yet unidentified at the molecular level, is specifically required for the formation of DCGs and not other membrane carriers. PLD, for example, has been implicated in TGN tubulation, but the downstream effectors, as for DCG budding,
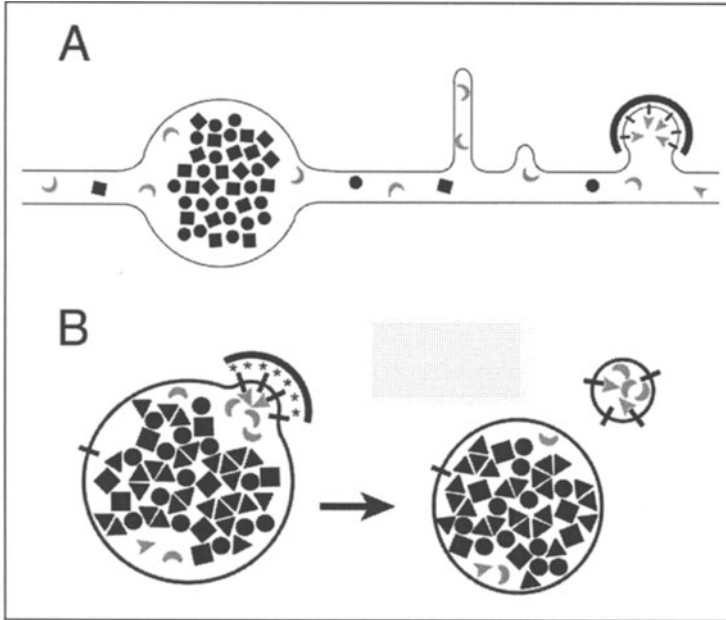
Figure 6. Protein sorting via vesicle budding. A) The "sorting by exclusion" model for the formation of ISGs in the TGN. A subset of proteins in the TGN aggregate to form large cores. Because of their size, these aggregates cannot be accommodated within vesicles or tubules emerging from the TGN. In this model, ISGs arise as the residue of a dispersed TGN, during which most soluble proteins are withdrawn. B) Vesicle remodeling during maturation. ISGs serve as a donor compartment for budding of clathrin-coated vesicles. The reaction depends upon AP-1, which can be recruited by the cytoplasmic tails of furin, the mannose-6-phosphate receptor, and other membrane proteins. The mannose-6-phosphate receptor can in turn bind any soluble lysosomal enzymes in the ISG lumen, so these will also be withdrawn in the budding vesicles. Other soluble proteins may also be included based on random partitioning, but the aggregated DCG proteins will be excluded. At the end of this process, the mature secretory granule is no longer a budding donor compartment, perhaps because it no longer contains membrane proteins that can recruit AP-1 (represented by stars).

are unknown.[134] The noncanonical GTPase dynamin has been implicated in DCG budding as well as in constitutive secretion.[128,135]

One possibility is that these activities are only indirectly involved in DCG synthesis. According to the "sorting by exclusion" model (Fig. 6A), ISGs are created by a passive process, as aggregation prevents DCG cargo from entering into outbound vesicles and tubules that bear lysosomal or constitutively secreted proteins. Instead of being actively budded from the TGN, aggregated proteins would be enriched in a separate subset of relatively large membrane carriers, while non-DCG proteins are removed from the compartment by active coat-dependent processes. Thus, the cytosolic components identified as ISG budding factors by in vitro reconstitution assays may really be parts of the mechanisms for other secretory pathways. According to this model, the so-called sorting receptors need only act as membrane tethers in associating the lumenal aggregates with membrane rafts. As there is no need to transport this material to a new compartment, the receptors do not recruit cytosolic coat proteins for vesicle budding, as the traditional membrane receptor proteins do in sorting proteins to other pathways.

An alternative to the "sorting by exclusion" model proposes that ISG budding is indeed an active process, and that the same mechanism is also involved in driving the budding and tubulation of constitutive secretory carriers from the TGN. Although the two pathways give

rise to vesicles of vastly different sizes, it is possible that the difference is caused by the cargo proteins (large aggregates versus soluble material) and is not a reflection of different cytosolic budding machinery. The formation of constitutive secretory carriers, like ISG budding, differs from clathrin-dependent transport at the TGN in that is not associated with the appearance of vesicle coats. There are similarities between the budding of constitutive and regulated vesicles at the molecular level as well: in addition to Rab proteins, constitutive traffic has been shown to rely on the activity of Dynamin-2,[128] protein kinase D,[136] and heterotrimeric G-proteins ,[130] factors which may also be associated with ISG budding (above). Cholesterol depletion has been shown to inhibit both pathways,[93] however it is difficult to know whether the treatment has a direct effect on both pathways, or whether the inhibition of one pathway could inhibit the second via some indirect mechanism. Thorough testing of this model requires experiments that avoid this problem.

The only concrete indication that there are DCG-specific budding factors is that, at least in one reconstituted system, the cytosol requirement cannot be substituted for by an extract from HeLa cells, which do not make DCGs.[137] One possibility that is compatible with both sorting models is that specific cytosolic proteins are involved in establishing or facilitating Golgi subdomains in which DCG proteins condense. The structural and functional analysis of the TGN is at a very early stage, but the existence of sub-domains is consistent with the observed nonuniform protein distribution within a single cisterna, as well as with live imaging of heterogenous budding structures.[94,138] However, the cisternal dilations involved in ISG budding do not necessarily reflect the active maintenance of sub-domains. A simpler view is that the cisterna are passively stretched around the forming granule protein cores, like the bulges in a pancake around blueberries.

Future work with in vitro systems may provide molecular identification of activities that are required for ISG budding, but the question of whether cells have machinery that is specifically used for this purpose will need to be addressed by other types of analyses. If the budding mechanism is specific to ISGs, and not indirectly required for ISG production, as in the "sorting by exclusion" model, the prediction is that knocking out individual components would inhibit ISG formation without also inhibiting the exit of lysosomal or constitutive proteins from the TGN.

Protein Sorting in ISGs

Depending on cell type, the importance of ISGs as a locus of protein sorting may be as important as that of the TGN. Sorting at this level involves the budding of vesicles from ISG membranes, resulting in the remodeling of membrane and lumenal contents by selective withdrawal (Fig. 6B). This targeted removal occurs via clathrin-coat recruitment to ISGs occurs via the AP-1 adaptor, which is recruited by membrane proteins in an ARF-dependent, BFA-inhibitable step.[139] Proteins known to be withdrawn from ISGs include the cargo protease furin and the mannose-6-phosphate receptor, both of which can interact directly with AP-1.[140-143] The mannose-6-phosphate receptor can bind any lysosomal enzymes that may have been incorrectly sorted upon exit from the TGN, and this step therefore leads to selective withdrawal of some lumenal proteins by classical receptor-based sorting.[144] Mature DCGs do not support CCV formation, the simplest explanation for which is that ISGs become progressively depleted of proteins that act in the recruitment of AP-1. Consistent with this, myristoylated ARF1 binds to ISGs but not mature granules in vitro.[139] Recent evidence suggests that the full cohort of ARFs and adaptors present on ISGs includes ARF1, 5 and 6, and AP-1 and –3.[145] These may all be present on a uniform population of vesicles, or may reflect heterogeneity within ISGs.[143]

Coated vesicles budding from the ISG will also withdraw any soluble proteins that randomly partition by diffusion into the vesicle lumen during budding. However, large aggregates of proteins that are condensing in the ISG are too large to fit into the buds, and are therefore selectively retained.[146] The efficiency of this separation is increased by the tendency of

nonaggregating proteins to be concentrated at the periphery of the vesicle lumen, as they are excluded from the dense core forming in the center of the ISG. As a result, the soluble proteins accumulate in a place where they can readily enter the vesicles that are budding from the membrane. These may include proteins that randomly partition at the TGN into budding ISGs, but will also include some soluble products of DCG proprotein processing. The best characterized of these is derived from proinsulin, which is processed into and A, B, and C peptides.[147] The first two are disulfide linked, and crystallize to form the granule core. The C peptide is soluble and is largely excluded from the core, and is selectively withdrawn.[148,149]

A collateral consequence of ISG maturation is the generation of a set of coated vesicles bearing newly synthesized proteins, some of which have undergone processing by ISG-specific enzymes. At least in some cell types, these can deliver their cargo to the plasma membrane, probably via an endosomal intermediate.[150] This has been called "constitutive-like" secretion: constitutive-like in that it is independent of extracellular stimulation, but with kinetics that are slower than those of true constitutive secretion. In pancreatic β cells, the C peptide that is withdrawn from ISGs is secreted via this route.

The model that describes the progressive enrichment of granule cargo during ISG maturation has been given the name "sorting by retention" and essentially posits that sorting in ISGs can be based on a protein's ability to aggregate, rather than depending on specific targeting signals. The concepts are like those of the "sorting by exclusion" model that may apply at the TGN, and the similarity in models may be a reflection of similar molecular mechanisms in vivo. Thus, ISGs may simply be a functional extension of the TGN, which becomes progressively enriched in DCG contents as nonaggregating proteins are actively removed during maturation. Thus, there may not be any mechanistic differences between coat mediated sorting at the TGN versus the ISGs, though the material that is included in the budding vesicles could change as the compartment matures.

Alternatively, modification of the coat mediated sorting machinery may be required in order to facilitate sorting from a compartment that is progressively changing. For example, such modifications may be necessary for the trafficking of proteins that are allowed to enter ISGs but are not stored in mature DCGs, such as proteases (see "Structural Maturation of ISGs" section), or for adapting to differences in membrane composition between the TGN and ISGs. Indirect evidence in support of this possibility comes from the study of the membrane lipid component phosphatidyl inositol 4-phosphate (PI-4-P) and its derivatives. In the TGN, these molecules play important modulating roles, including the recruitment of AP-1/clathrin coat proteins for vesicle budding.[151] The levels on PI-4-P in the TGN are affected by the activity of PI-4 kinase, which is stimulated by myristoylated ARF1-GTP, a part of the coat formation machinery.[152,153] Interestingly, ISGs have been found to contain a PI-4-K activity that is not stimulated by ARF1-GTP.[154] The TGN has two different PI-4 kinases (II and III), and it is possible that ISGs only recruit one of these.[152,153]

Coat recruitment at the TGN vs. ISGs may also be differentially regulated by modification of the vesicle cargo, since the binding of AP-1 to the cytoplasmic tails of both furin and the mannose-6-phosphate receptor is stimulated following their phosphorylation by Casein Kinase II.[141,143] In this regard, a very interesting observation is that newly-budded ISGs are rapidly transported to the cell periphery, at least in some cell types, and therefore primarily inhabit a different cellular microenvironment from the TGN.[155] This may be relevant for differential regulation of similar activities at the TGN vs. ISGs, for example if receptors in ISGs are selectively modified.

Although the data is not yet conclusive, the emerging view of sorting from ISGs is that it is directed by the core elements of a "flexible" AP-1/clathrin dependent sorting mechanism that is differentially controlled at the ISGs versus the TGN. The model holds that the sorting events of ISG maturation are not mediated by a unique vesicle trafficking mechanism, but are instead accomplished by pathway-specific modifications of machinery that is common to all cell types. A similar phenomenon may occur at an earlier stage of the pathway, where the coat-independent

machinery that drives the formation of constitutive carriers from the TGN may be adapted for the budding of ISGs, as discussed in the "Mechanisms of Immature Secretory Granule (ISG) Budding" section. This apparent mechanistic conservation may explain the ability of fibroblast cells, which do not normally make DCGs, to make dense-cored vesicles when expressing heterologous chromogranin genes or vonWillebrand factor.[48,50,51] However, these observations do not preclude the possibility that specialized DCG-producing cells express proteins that specifically modify parts of the conserved cellular trafficking machinery to enhance DCG synthesis.

Structural Maturation of ISGs

The cores of newly-budded ISGs appear less electron-opaque than those in mature DCGs, and are also lower in buoyant density,[30] indicating that granule cargo becomes increasingly condensed during granule maturation. This is one reflection of the larger remodeling of protein and lipid constituents during the maturation process, which includes the selective withdrawal of components that are present in immature, but not mature, granules. This overall process serves important structural functions. The tighter packing offers increasingly efficient storage, and not simply because more material can be contained in a fixed vesicle volume. Protein condensation overcomes an energetic barrier that is posed by a vesicle filled with concentrated soluble macromolecules, which is hyperosmolar when compared to cytosol. Maintaining such a vesicle would require constant pumping of osmolytes to counter vesicle swelling, an expensive cellular proposition. Within DCGs, aggregated proteins are no longer solvated, and are therefore osmotically inert.

The progressive condensation during maturation parallels, and is likely to be controlled by, changes in the lumenal environment.[156] In neuroendocrine cells, the TGN is acidified to pH ~6.4 by vacuolar ATPases.[157] These are also present in the ISG membrane, with the result that the ISG continues to acidify.[158-160] At the same time there is an increase in calcium that, along with other cations,[161] is important for charge neutralization of the largely acidic core proteins. This calcium may be cotransported from the endoplasmic reticulum with calcium-binding DCG cargo proteins, or imported via ISG membrane ion exchangers.[162] The ionic changes can trigger changes in DCG protein conformations or interactions. For example, CgB forms homo-oligomers under the conditions found in ISGs.[56,163] The functional significance is as yet unknown, but these are presumably based on contacts different from those involved in aggregative sorting.

One well-established consequence of ISG acidification, in combination with increased Ca^{2+}, is the activation of proteases that are specifically localized to DCGs. The contents of neuronal and endocrine DCGs are largely synthesized as proteins that are proteolytically processed to generate bioactive peptides, the species that are eventually released during exocytosis.[164] Proteolytic processing involves a variety of enzymes including amino- and carboxypeptidases, and a family of aspartyl proteases called prohormone convertases.[165-168] Members of this family are differentially active over a range of proton and calcium concentrations, and may thus act sequentially on their substrates during ISG maturation, in a cell type-dependent fashion [Davidson, 1988 #572;Laslop, 1998 #2270;Goodge, 2000 #1981;.[169] Though ISGs are considered to be the major compartment of proprotein processing, in some cell types processing may begin in the TGN, and Moore and colleagues have begun to resolve the requirements for ISG budding from those required for the onset of processing.[137,170,171] In their cell-free system, the onset of processing precedes budding. Both require hydrolyzable GTP, but at two distinct concentrations. This difference suggested a model in which the former requires ARF, while the latter depends upon a heterotrimeric G-protein.

In addition to generating mature peptides, proprotein processing may drive the physical reorganization of the core, in cases where mature peptides can pack more tightly than the precursors. The best example of this is found in β-cell granules, in which mature insulin but not proinsulin can assemble into hexagonal crystals, simply because processing relieves a packing constraint[147,172,173] (Fig. 7). The control of assembly via proteolytic processing is strongly reminiscent of mechanisms involved in viral capsid formation.[174]
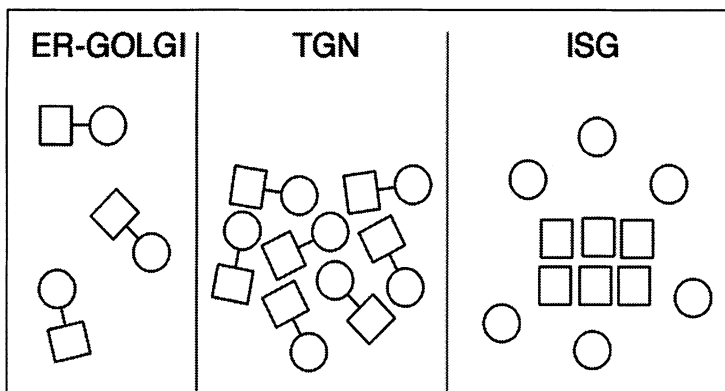
Figure 7. Proprotein processing and structural maturation. DCG cargo proteins transit the ER/Golgi as soluble species. Selective aggregation in the TGN transforms these into a loose aggregate. Proteolytic processing, chiefly in the ISG, can allow reorganization and further condensation.

The process of DCG maturation, which includes the generation of active peptides by proteolytic processing and the condensation of cargo into a densely packed, osmotically inert form, serves to increase the efficiency of the regulated secretory pathway in several ways. First, the condensation of material allows great quantities of protein to be stored in the vesicles, with the consequence that a small number of exocytic events can generate a relatively large secretory response. Second, proteolytic processing in ISGs allows the cell to combine multiple DCG peptides into a single proprotein, thereby linking the sorting of these proteins at earlier stages of the pathway. In neuroendocrine ISGs, for example, the chromogranin proteins are cleaved into multiple biologically active peptides with different postexocytic functions.[62,175] Furthermore, limiting the site of proteolytic processing to ISGs may provide a failsafe mechanism, ensuring that the active forms of the proteins are only found in a compartment that is under direct control of the regulated secretory pathway, therefore leaving any incorrectly sorted proteins as uncleaved precursors.

Functional Maturation of ISGs

The remodeling of the membrane attending the budding of clathrin coated vesicles does not simply serve to remove proteins that may have been incorrectly targeted at the TGN. Rather, it also underlies differences in the activity of ISGs and mature granules. This was suggested by the observation that ISGs and mature granules differ dramatically with regard to exocytosis: whereas mature DCGs undergo efficient exocytic fusion with the plasma membrane in a stimulus-dependent fashion, ISGs exhibit an increased tendency to fuse with the plasma membrane in the absence of stimulation. In AtT20 cells, unregulated release of DCGs from ISGs proceeds for 2-3 hours after ISG budding from the TGN.[176] These ISGs contain two SNAREs, VAMP4 and Synaptotagmin IV (Syt IV), which are withdrawn during maturation in a brefeldin A-inhibitable step.[159,171,177] During the same period, the maturing granules become responsive to exocytic stimuli, a process also blocked by BFA. That the two phenomena may be linked is suggested by the observation that overexpression of Syt IV itself decreased the responsiveness of maturing granules to secretory stimuli.[171] Syt IV is thought to act as a negative regulator of calcium-induced exocytosis,[178] and the withdrawal of this inhibitory factor from ISGs may foster maturation. A recent study showed that the removal of VAMP4 from ISGs depends upon interactions with AP-1 and the coat protein PACS-1,[179] thereby providing genetic confirmation and molecular detail to this model. However, a complication of this model is that Syt IV is thought to inhibit membrane fusion by forming inactive heterodimers with synaptotagmin I, and the mechanism by which the heterodimers are separated and Syt IV is selectively removed from the ISGs is unknown.

Another functional characteristic that may, in some cell types, distinguish ISGs from DCGs, is that ISGs can undergo homotypic fusion, a reaction that has been more extensively characterized in vitro than in vivo.[177,180,181] The specific function of this reaction is not clear. In some systems homotypic-like fusion might allow for the synthesis of specialized DCG cores in which the contents are not randomly distributed. In *Pseudomicrothorax dubius*, two kinds of ISGs, containing morphologically-distinguishable cargo, fuse during the process of assembling a complex core structure.[182] More generally, consolidation could potentially define the size of the granules, which in many systems appear to be controlled.[183] Disruption of the gene encoding Rab3D, an exocrine granule-associated small GTPase, resulted in a doubling of mature granule volume, and one possibility is that Rab3D acts as a negative regulator of homotypic fusion.[184]

At some level, membrane remodeling must account for the difference in the fusogenic behavior of ISGs vs. mature granules, and attention has focused on the SNAREs, due to their importance in regulating membrane fusion. ISGs from PC12 cells contain syntaxin 6, which must be present on both donor and acceptor membranes for efficient homotypic fusion in vitro.[177] Syntaxin6 is also present in clathrin-coated vesicles which bud from the ISG membrane,[142] consistent with the idea that it is selectively removed during maturation via several likely AP-1 binding sites in its cytoplasmic domain.[140]

As ISG maturation appears to involve the removal of specific factors via the budding of clathrin coated vesicles, it is possible that more thorough analyses of the target proteins and their interacting partners will help to uncover ISG-specific machinery that regulates clathrin-dependent sorting in this compartment. More broadly, the identification of the molecules that define the functional maturity of DCGs by their presence or absence in the vesicle will provide insights into the nature of organelle identity, a topic that is central to an understanding the general principles of vesicular traffic. Finally, recent evidence hints at aspects of granule maturation that have not previously been recognized. Functional maturation of secretory granules may extend beyond the period of morphological change, based on the observation that the distribution and fusogenic activity of granules may change with vesicle age.[185]

Conclusion

The majority of the work on DCG synthesis has focussed on the sorting of the lumenal content proteins in the TGN and ISGs. These studies have, for the most part, supported the nonspecific aggregation-based model for sorting that was proposed by Chanat and Huttner in 1991.[57] Not surprisingly, studies of many granule cargo proteins in multiple systems have revealed some cases that are possible exceptions to this general rule, where specific protein-protein interactions are required for the sorting of a particular protein, as discussed in the "Protein Sorting into ISGs" section of this chapter. Overall, the precise requirements for the sorting of any particular protein is likely to be both context (which other granule cargo proteins are being expressed, and in what quantities) and cell type dependent (protein aggregation is sensitive to physiological properties of the lumen, such as calcium concentration and pH, which may vary between cell types), though it is likely that the general principles of aggregation-based sorting apply in all cells that produce DCGs. Further analysis of the specific sorting requirements for individual proteins may lead to a greater knowledge of the details of aggregation-based sorting, but the next leap forward in our understanding of they system will more likely come from experimental approaches that expand beyond the level of individual proteins and consider the DCG synthesis pathway more broadly. For example, cargo protein aggregation is known to be sensitive to lumenal calcium concentration and pH levels, but the mechanisms that control these physiologic parameters have not been elucidated. Secondly, how are granule cargo proteins sorted to the same destination as other proteins that are essential for DCG function, such as membrane fusion machinery? The answers to these questions may be learned from studies in genetic systems, such as *C. elegans*, *Drosophila*, and ciliated protozoans, which offer promising avenues for further experimentation. These organisms have recently been used to identify elements of the regulated exocytosis machinery,[37,186,187] and similar studies could uncover genes that are involved in vesicle synthesis.

Another major gap in our understanding of the granule synthesis pathway is the extent of its functional relationship with other branches of the secretory pathway. Two decades ago, DGC formation was considered to be one of a small number of distinct, post-TGN secretory pathways. This carried the assumption that vesicles bound for constitutive or regulated exocytosis, or toward lysosomes, would rely on distinct mechanisms for their biogenesis. That view now seems, paradoxically, to have been both too simple and too complex. It was too simple because post TGN traffic cannot be neatly divided into three branches: for example, what was called the constitutive pathway may in fact consist of multiple branches.[188,189] This was initially established for apical vs. basolateral targeting in polarized epithelia, but there is evidence in other cell types as well. Furthermore, the mechanisms for DCG formation are not easily separated from those that are directly involved in other pathways, implying that the secretory pathway cannot be divided into distinct, independently functioning branches. For example, AP-1 dependent sorting of proteins to the lysosomal pathway is associated with ISG maturation, and may also be part of the driving force for the "sorting by exclusion" of DCG contents in the TGN (see "Protein Sorting in ISGs" section).

At the same time, the fact that the DCG synthesis pathway and lysosomal pathway use some of the same machinery argues that the historical view of distinct mechanisms was too complex. Similarly, the historical view that constitutive and regulated secretory carriers are fundamentally different may also be incorrect. The idea that constitutive traffic is based on small vesicles is being modified by the recognition that TGN tubulation may be as, if not more, important in this pathway, at least in some cell types (references in ref. 190). Thus coat-mediated vesicle formation may be the exception rather than the rule for anterograde traffic to the plasma membrane, and the formation of constitutive and regulated secretory carriers may share common mechanisms. In the extreme, the mechanisms may be mostly conserved, and the end products depend upon the behavior of the vesicle cargo.

Addressing these issues directly will require identification of factors required for ISG budding and TGN tubulation. While progress has recently been made toward the latter, details regarding the former are extremely limited. Success in this may depend on further exploitation of cell-free systems, strengthened by development of new genetic models.

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