Secretory granules are the cellular organelles mediating storage and regulated secretion of proteins. Their biogenesis involves sorting of secretory protein cargo and membrane constituents, which takes place at two distinct levels, the trans-Golgi network and the immature secretory granule. At both levels, sorting is accomplished by cargo aggregation and cargo-membrane recognition. Given not only the aggregative properties of the regulated secretory proteins but also the ability of lipids to form distinct membrane microdomains, self-organization of both lumenal and membrane constituents is proposed to play a crucial role in secretory granule biogenesis.

Key words: aggregation / granins / lipids / secretory granules / trans-Golgi network

Secretory granules are cellular organelles serving as storage compartments for proteins destined to undergo secretion regulated by external stimuli. A wide variety of cell types are able to secrete proteins via a regulated pathway, and numerous types of secretory granules exist, often characterized by an electron dense core of condensed protein.1 Secretory granules, including those from neuroendocrine cells on which this review will focus, are derived from the trans-Golgi network (TGN).3 They are formed as immature secretory granules (ISGs)4 and usually undergo maturation. The latter includes (i) processing and condensation of the secretory cargo; and (ii) the removal of excess volume containing non-aggregated proteins and of certain membrane constituents. This removal is achieved by the formation of the ISG-derived vesicle (DIV),5,6 resulting in a mature secretory granule (MSG) that is smaller than the ISG.7 However, MSGs may also be larger than the ISGs they are derived from, and hence maturation in addition can involve homotypic fusion of ISGs.4,8,9

In-depth reviews on secretory granule biogenesis and the underlying sorting events are available,6,9–12 and we encourage readers to consult these for a comprehensive view. Here, we will focus on mechanisms and concepts of sorting to ISGs and of ISG formation, with special emphasis on possible interactions between regulated secretory proteins (RSPs) and membrane lipids.

Sorting compartments

Ever since it was found that the regulated pathway of protein secretion coexists with the ubiquitous constitutive pathway of secretion in the same cell,13 which implies segregation of regulated and constitutive secretory proteins from each other, it became a challenging scientific problem to elucidate the sorting process directing the two types of secretory cargo and specific membrane constituents into their respective vesicles. Sorting takes place at two levels in the secretory pathway, the TGN and the ISG.6,14

Sorting at the level of the TGN

The TGN is a major branching point of vesicular transport,15 and the two principal pathways of protein secretion, the regulated and the constitutive pathway, both originate from this compartment. By studying the flow of material from the TGN into ISGs versus constitutive secretory vesicles using pulse-chase analysis with radioactive sulfate combined with subcellular fractionation, it was demonstrated that the TGN is the major site of secretory protein sorting in the neuroendocrine cell line PC12.3 While this is thought to hold true for many neuroendocrine cells in tissues and in particular for neurons,14 it may not be true for all endocrine cells (see below ‘Sorting at the level of the ISG’).
Sorting at the level of the TGN involves two main steps: the first is the selective aggregation of the RSPs in the milieu of the TGN,\(^{16}\) this is thought: (i) to segregate RSPs from constitutive cargo by exclusion of the latter from the aggregates,\(^{17-19}\) and (ii) to enhance the efficiency of the second step. This is the recognition between the RSPs and the membrane of the forming ISG. In an early concept of sorting to secretory granules,\(^{10}\) this recognition was assumed to be mediated by a receptor protein (the ‘sorting receptor’). According to this concept, sorting to the regulated secretory pathway follows the paradigm of the sorting of lysosomal proteins from the TGN to endosomes. The latter involves: (i) recognition between the sorting signal on the cargo, mannose-6-phosphate, and a transmembrane protein, the mannose-6-phosphate receptor; (ii) formation of a clathrin-coated vesicle; and (iii) recycling of the receptor protein.\(^{20}\) By analogy, the existence of a transmembrane protein acting as RSP sorting receptor was postulated, which would bind to a sorting motif common to, and specific for, RSPs. However, despite considerable efforts in several laboratories, neither a transmembrane sorting receptor nor a specific, common sorting motif have been identified so far.\(^{6,14}\)

An alternative concept of cargo-membrane recognition attributes a key role in sorting to a membrane constituent that does not traverse the lipid bilayer: the membrane-associated forms of RSPs.\(^{21}\) Virtually all neuroendocrine RSPs are present not only in soluble, but also in a tightly membrane-associated form.\(^{21-26}\) As will be discussed below (see ‘A unifying view of granule biogenesis and sorting’), RSPs exhibit various modes of homo- and heterophilic interactions, and the ones between soluble and membrane-associated forms of RSPs are good candidates to mediate the binding of secretory cargo to the granule membrane.

**Sorting at the level of the ISG**

Once formed at the TGN, the ISG can also be a site of sorting. Recent studies have shown that certain proteins may enter the ISG which are not retained during granule maturation but instead are removed with the IDV.\(^{27,28}\) In particular, lysosomal enzymes together with the mannose-6-phosphate receptor have been found to follow this pathway,\(^{29}\) consistent with the presence of a patchy clathrin coat on ISGs at the site of the budding IDV.\(^{5,6,9,30,31}\) IDVs not only remove lysosomal enzymes bound to mannose-6-phosphate receptors but also are thought to be involved in the transport of some of the soluble cargo from the ISG to the cell surface via the constitutive-like secretory pathway.\(^{6}\) Specifically, IDVs have been postulated to fuse with the early endosome,\(^{6,12,32}\) this would result in constitutive-like secretion via the vesicles that recycle from the endosome to the plasma membrane. This sorting of secretory cargo at the level of the ISG has been referred to as ‘sorting by retention’ because the RSPs are retained with the maturing secretory granule.\(^{6}\) As is the case at the level of the TGN, segregation of RSPs from other secretory proteins at the level of the ISG is mediated by aggregation/condensation of the RSPs,\(^{6}\) and the retention of the granule-specific membrane constituents with the maturing granule is likely to involve their interaction with the RSPs.

**Sorting in different regulated secretory cell types**

While secretory protein sorting is governed by the same mechanistic principles, i.e. aggregation and cargo-membrane recognition, at either level, the TGN and the ISG, the extent of sorting at these levels seems to differ between the various regulated secretory cell types. It is likely that sorting at the level of the ISG plays an important role in cell types where the flow of material into these organelles is massive, as in the exocrine pancreas and in the insulin-producing β-cell,\(^{6}\) although the contribution of sorting at the level of the TGN in these cell types has not been investigated in as much detail as in neuroendocrine cell lines. In contrast, sorting at the level of the TGN will be of major quantitative significance in cells where the flow of material into the constitutive secretory pathway is massive, such as neurons which are characterized by an extensive dendritic tree and a single axon.\(^{14}\)

**Sorted molecules**

Before addressing recent developments in the concept of granule formation, we will briefly review the various classes of granule constituents that become sorted, as exemplified by the chromaffin granule, the secretory granule of the adrenal medulla (for a comprehensive review see Apps\(^{33}\)).

**Luminal molecules**

The lumen of chromaffin granules contains proteins and low molecular weight molecules. By far the most
abundant protein in bovine chromaffin granules is chromogranin A, followed by its paralog chromogranin B. Both are acidic proteins, which are characterized by their ability to aggregate in the milieu of the TGN and secretory granules, and which are thought to assist in the packaging of other cargo. Other luminal proteins, present in lower amounts than the chromogranins, are the enzymes (pro-)hormone convertases (PC 1r and 2, carboxypeptidase E (CPE), peptide amidating monooxygenase, dopamine β-hydroxylase (DBH)) mediating either the processing of peptide precursors (pro-enkephalin, pro-neuropeptide Y) or the production of catecholamines. In line with its function, the mature granule, but not the nascent ISG, contains large amounts of catecholamines and ATP thought to osmotically neutralize the catecholamines. Chromaffin granules contain high levels of calcium ions and protons, keeping the chromogranins aggregated and providing the driving force for the import of catecholamine precursor.

Membrane proteins

Compared to other biological membranes, the protein-to-lipid ratio of the chromaffin granule membrane is relatively low, which is a general characteristic of secretory granule membranes. Transmembrane proteins are especially low abundant, the exception being the electron carrier cytochrome b561. Other transmembrane proteins with known function are transporters (V-type proton ATPase, monoamine transporter), peptide amidating monooxygenase, and proteins functioning in exocytosis (syntaxin, VAMP/synaptobrevin, synaptotagmin).

Interestingly, most other prominent membrane proteins are membrane-associated forms of soluble proteins, a fact that is relevant for granule biogenesis and sorting as will be discussed below. While mDBH is anchored by an uncleaved signal peptide, mCgA, mCgB, mPC1r/3, mPC2, mCPE and mGPIII are probably all anchored by hydrophobic interaction with the lipid bilayer. In the case of mCgA, such an interaction is suggested by the observations that mCgA is the most abundant membrane protein of bovine chromaffin granules and is not extracted by high salt, consistent with its binding to a non-proteinaceous integral membrane constituent. mCPE has been proposed to function as a receptor essential for the sorting of certain regulated proteins in the TGN, a claim which has been highly disputed and, because of space limitations, will not be further discussed here.

Membrane lipids

Chromaffin granule membranes are rich in cholesterol and lysophosphatidylcholine and contain some sphingomyelin but relatively low levels of glycosphingolipids. (The latter are enriched in constitutive secretory vesicles [see the review by Ikonen and Simons in this issue].) The role of cholesterol was traditionally seen to prevent the leakage of protons from the granule lumen, while lysophosphatidylcholine was proposed to facilitate exocytosis. In light of the ability of cholesterol and lysophosphatidylcholine to interact with each other, these two lipids may also contribute to the formation of microdomains in the secretory granule membrane.

A unifying view of granule biogenesis and sorting

Certain types of membrane vesicles seem to form in a cargo-independent manner, i.e. vesicle formation proceeds irrespective of whether or not the nascent vesicle contains lumenal cargo. This is the case for synaptic vesicles, for the transferrin receptor-containing vesicles that cycle between the plasma membrane and the early endosome, and even for certain TGN-derived vesicles, i.e. the vesicles mediating the mannose-6-phosphate receptor-dependent transport of lysosomal enzymes to endosomes. In contrast, secretory granule formation seems to depend on the presence of RSPs and on their aggregation, as in cells treated with bafilomycin, an inhibitor of the V-type proton ATPase which prevents TGN acidification and hence RSP aggregation, granule biogenesis from the TGN is blocked. Interestingly, the cargo-independent mode of vesicle formation is assisted by clathrin coats. The clathrin coat apparatus, consisting of the cytoplasmic domain of a transmembrane protein (e.g. transferrin receptor, mannose-6-phosphate receptor), an adaptor complex and the clathrin cage provides three key features with regard to vesicle formation: (i) cargo selection—by including certain proteins and excluding others; (ii) cargo concentration—by lateral interaction leading to receptor clustering; and (iii) a scaffold supporting vesicle budding mechanically. As clathrin coats do not seem to be essential for ISG formation from the TGN (but rather
play a role in secretory granule maturation, one may postulate that interactions between RSP cargo and secretory granule membrane constituents functionally substitute for the clathrin coat. This implies that the sorting of both cargo and membrane constituents is mechanistically linked to the formation of the immature secretory granule.

What are the interactions relevant for the formation of the ISG and how do they confer cargo selection, cargo concentration and a scaffold function? We have to consider: (i) interactions between lumenal cargo molecules; (ii) between cargo and membrane constituents; and (iii) lateral interactions within the membrane. The key to the formation of ISGs is found in the particular properties of RSPs. RSPs, as exemplified by the intensely studied chromogranins, undergo various homo- and heterophilic interactions: chromogranin A forms dimers at neutral pH and tetramers at acidic pH, both mediated by the chromogranin loop, a structure necessary for chromogranin sorting. Moreover, chromogranin A aggregates at acidic pH in the presence of calcium. These aggregates do exclude constitutive secretory proteins but are able to include various other granule components including lumenal domains of granule membrane proteins. Furthermore, a fraction (5–10% of total) of the chromogranins exists as a membrane-associated form.

This leads us to the following concept of ISG formation from the TGN (Figure 1). Induced by the milieu of the TGN, aggregation of RSPs will provide: (i) cargo selection by exclusion of constitutive proteins; (ii) concentration of RSPs; and (iii) a lumenal matrix inducing membrane budding that substitutes for the coat-based cytoplasmic scaffold. The consequences of aggregation of lumenal RSPs are transmitted to the level of the membrane by the membrane-associated forms of the RSPs. Lumenal RSPs will, upon aggregation, cluster specific membrane components by coaggregation with either membrane-associated forms of RSPs or lumenal domains of certain membrane proteins. These clusters will also include membrane lipids and lipid microdomains which are bound to the clustered proteins.

![Figure 1](image-url)

**Figure 1.** Sorting of secretory proteins and lipids into constitutive secretory vesicles and ISGs in the TGN. For details, see text.
by thereby mediating sorting of specific lipids into the forming ISG. It should be noted that this cooperative process of mutual enrichment of lipids and proteins is not unique to secretory granules but is a general consequence of lipid–protein interactions and has also been observed in other systems. With respect to the membrane lipids involved, it is of interest that cholesterol, a crucial component of lipid microdomains, is an abundant constituent of secretory granule membranes. One may therefore speculate that cholesterol-rich microdomains, whose function in sorting has previously been demonstrated in epithelial cells (see the review in this issue by Ikonen and Simon, 1994) may also be involved in the formation of ISGs from the TGN.

In conclusion, instead of being driven by cytoplasmic coats assembling on transmembrane receptors, granule formation including the sorting of luminal cargo appears to be a consequence of self-organization: aggregation of the luminal RSPs on the one hand, and lateral interactions at the level of the membrane by formation of lipid microdomains on the other hand, with these two processes being coupled to each other by the membrane-associated forms of regulated secretory cargo.

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References

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