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Biogenesis of secretory granules

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Secretory granules of neuroendocrine cells store and release peptide hormones and neuropeptides in response to various stimuli. Generation of granules from the Golgi complex involves the aggregation of cargo proteins and their sorting from non-regulated secretory molecules. Recent findings on knockout mice lacking individual granule constituents have challenged the hypothesis that an 'essential' protein for the assembly of these organelles exists, while studies on polypyrimidine tract-binding protein and ICA512/IA-2 have provided insight into the mechanisms for adjusting granule production in relation to stimulation and secretory activity.

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Introduction

Since their discovery by electron microscopy more than fifty years ago, secretory granules (SGs, also known as large dense core vesicles) of neuroendocrine cells have attracted the attention of cell biologists. This is because they are the predominant neuroendocrine organelles, have an electron-dense content, and are responsible for the regulated release of peptide hormones and neuropeptides. In this short review we focus on progress in the field of SG biogenesis since 2004. During this time, studies in mice and *in vitro* cultured cells have significantly advanced our knowledge concerning the mechanisms regulating the production of SGs. This information is especially relevant in view of the key role that hormone biosynthesis and secretion have in human health.

Properties of secretory granules

SGs are membrane-enclosed spherical organelles with diameters of several hundred nanometers. When SGs originate from the trans-Golgi network (TGN), they are still immature (Figure 1). The progressive processing and packaging of peptide cargoes leads to the increasing condensation of the SGs' electrodense cores and their conversion into mature SGs [1]. SGs are typically distinguished by their morphological appearance and high immunoreactivity for cell-type-specific peptide hormones and other more widespread cargoes, including chromogranin A (CgA), B (CgB) and secretogranins II–VI [2], as well as prohormone processing enzymes, including prohormone convertases 1/3 (PC1/3) and 2 (PC2) and carboxypeptidase E/H (CPE) [3]. As recently proposed [4], additional minimal criteria for recognizing bona fide mature SGs should also include their prolonged storage in resting cells; the inclusion of specific v-SNAREs proteins that are essential for regulated fusion of SGs with the plasma membrane; and a lack of endosomal or lysosomal markers. Notwithstanding their distinctive properties from other organelles, SGs are a heterogeneous vesicular population even within a single cell type. For instance, they can differ in size and in kinetics of content release [5•,6,7]. Some of these differences may depend on SG aging, since content condensation may progressively reduce granule size and the speed with which cargoes dissolve in the extracellular space. Aging can also affect the probability of SGs undergoing exocytosis, with newly generated SGs being preferentially released [8,9].

Sorting of secretory granule cargoes

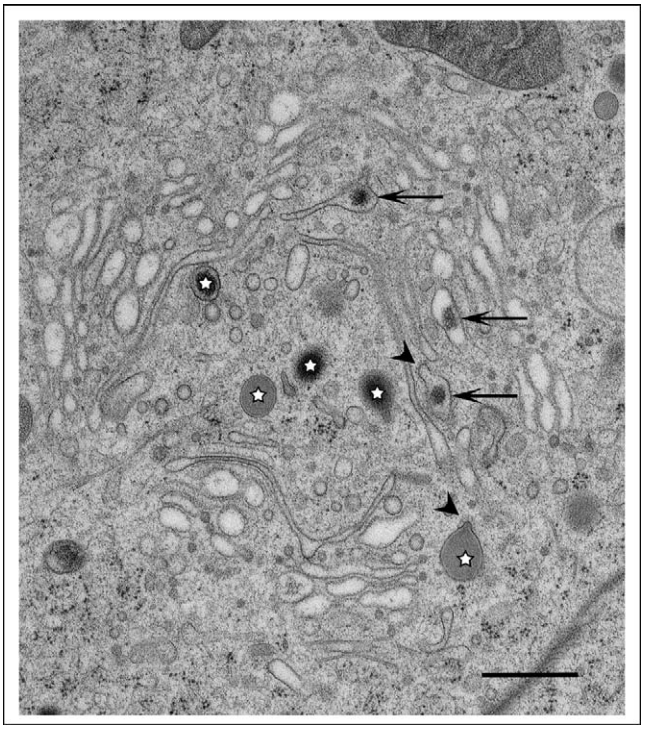
There are currently two models to explain how molecules destined to the SGs are sorted from other secretory proteins; these models are not mutually exclusive [10].

Sorting 'by entry'

One model, known as 'sorting by entry', proposes that high Ca^{2+} concentration and a pH of <6.5 in specialized parts of the TGN lumen favor the progressive aggregation of SG cargoes such as the granins. These aggregates would, in turn, act as 'seeds' for the precipitation of other regulated cargoes, thereby driving SG formation and maturation. Multiple interactions among granins, prohormones and their processing enzymes further enhance the sorting, processing and retention of regulated secretory proteins within SGs. Anchoring of several granins and prohormone processing enzymes to cholesterol-rich membrane domains is an additional critical step for the segregation of SG cargoes from other secretory proteins within the TGN lumen [11,12].

Since the discovery of sorting receptors for the delivery of hydrolases from the TGN to lysosomes, investigators have searched for an equivalent receptor for the sorting

Figure 1



Assembly and maturation of secretory granules (SGs). Electron micrograph of a high pressure frozen islet cell embedded in epon. Several SGs in various maturation states around the Golgi apparatus are visible. Arrows point at condensing SG components within Golgi cisternae. Arrowheads mark clathrin coats on buds at cisternae and immature SGs. Stars mark SGs at several stages of maturation. Scale bar, 500 nm.

of proteins into the regulated secretory pathway. This hunt culminated in 1997 when CPE was proposed to be the sorting receptor for SG cargoes [13]. This hypothesis remains highly controversial [12,14], since it has been shown that retention of the catalytically inactive mutant Cpe^{fat} in the endoplasmic reticulum precludes the processing of prohormones, but not their routing to SGs. It has been disputed, however, that a fraction of Cpe^{fat} escapes degradation and still reaches SGs [15]. A resolution of this contention has come from the analysis of $CPE^{-/-}$ mice, whose phenotype is similar to, albeit more severe than, that of the Cpe^{fat}/Cpe^{fat} mice [16]. Despite the lack of specific information about the sorting of secretory proteins in $CPE^{-/-}$ mice, the viability of these animals strongly suggests that delivery of prohormones into SGs is not grossly compromised.

Sorting 'by retention'

A second model for SG biogenesis, known as 'sorting by retention' [10], envisions that all secretory proteins not destined for regulated secretion, being excluded from the dense core, are progressively removed from immature SGs via clathrin-coated vesicles, and re-routed toward the constitutive secretory pathway. Recently, it has been shown that GGA (Golgi-associated γ-ear-containing ADP-ribosylation-factor-binding protein) is the adaptor protein recruiting clathrin to immature SGs [17•]. This second model does not exclude the first one, but refines it by clarifying that immature SGs can still act as a more advanced sorting station relative to the TGN. In addition to these models, homotypic fusion of immature SGs involving synaptotagmin IV and the SNARE protein syntaxin 6 could contribute to the maturation process in some endocrine cells [18]. Since mature SGs are typically smaller than immature ones, this remodeling step may not be obligatory. Once a mature SG is formed, it retains its identity throughout the exo-endocytic cycle. Following exocytosis, in particular, SG membranes are recaptured mainly intact [19], although some transmembrane proteins like synaptotagmin-1 and the V-SNARE VAMP2/synaptobrevin2 can diffuse from sites of exocytosis [20], while the cytosolic tail of ICA512/IA-2 can be shaved (see below).

The quest for a 'master' gene for secretory granule biogenesis

A long-standing question concerns the existence of an essential or 'master' gene for SG biogenesis. The opinions on this important subject have also been discordant. On one hand, evidence that antisense-mediated downregulation of CgA expression in pheocromocytoma PC12 cell clones [21] and in mice [22] causes a profound loss of SGs points to CgA being essential for SG biogenesis. CgA, in particular, may protect SG proteins from being degraded within the Golgi complex by up-regulating the expression of protease nexin-1, a serine protease inhibitor [23]. On the other hand, single deletion of various SG genes in mice, including CgA [24•,25•], neither precludes the expression of other SG genes nor the biogenesis of SGs, although processing of cargoes and/or their secretion can be affected. Moreover, expression of granins or prohormones in heterologous cells has not been shown to induce the expression of other SG components [21,26,27•]. On the contrary, absence of CgA or functional CPE in mice is accompanied by the compensatory overexpression of other granins [25•,28]. Taken together, these data argue against the hypothesis that CgA or any other SG component so far considered is essential for the biogenesis of SGs [14,29]. This conclusion does not conflict with the observation that single overexpression of CgA [21], CgB [26] or various prohormones [27•] can induce the formation of vesicles with an electron-dense core in cells lacking the traditional regulated secretory pathway. These data, indeed, corroborate the notion that multiple regulated secretory proteins share the ability to self-aggregate and segregate into post-Golgi organelles distinct from other TGN-derived vesicle carriers. Moreover, they indicate that the display of these properties does not depend on an environment that is unique to the TGN of regulated secretory cells. It

remains to be shown, however, that such SG-like vesicles indeed have all the key properties of bona fide SGs.

Forward genetic screenings to identify key factors for SG biogenesis have been performed in *Tetrahymena thermophila*. This ciliate unicellular organism contains several hundred granules, which undergo massive and synchronous exocytosis in response to agents inducing secretion [30]. Following random chemical mutagenesis or ribosome-mediated gene silencing, *Tetrahymena thermophila* mutants lacking SGs can be isolated because they swim instead of being entrapped in the capsule formed from the released granule content. Using this strategy, several genes for SG cargoes have been identified, including a family of five proteins all of which are essential for the normal assembly of the granule core [31,32•]. However, a master gene for SG biogenesis has not yet been found.

The isolation of mutants lacking SGs in multicellular organisms has never been reported, because of either lethality or system redundancy. Nevertheless, microarray screenings of mammalian cells in culture provide a means to extend the search for key factors in SG biogenesis beyond the 'candidate' gene approach focused on single SG components. Expression profiling of insulinoma MIN-6 cells stimulated with glucose [33], which promotes SG biogenesis, or pheochromocytoma PC12 cell clones that have spontaneously lost the ability to generate SGs [34], revealed significant changes in the expression of many genes involved in secretion, intermediate metabolism, signaling, transcription, mRNA processing and protein translation. Thus, it appears that neurosecretory competence depends on a very complex program that requires multiple control steps, not only at the level of transcription, but also post-transcriptionally [29,35].

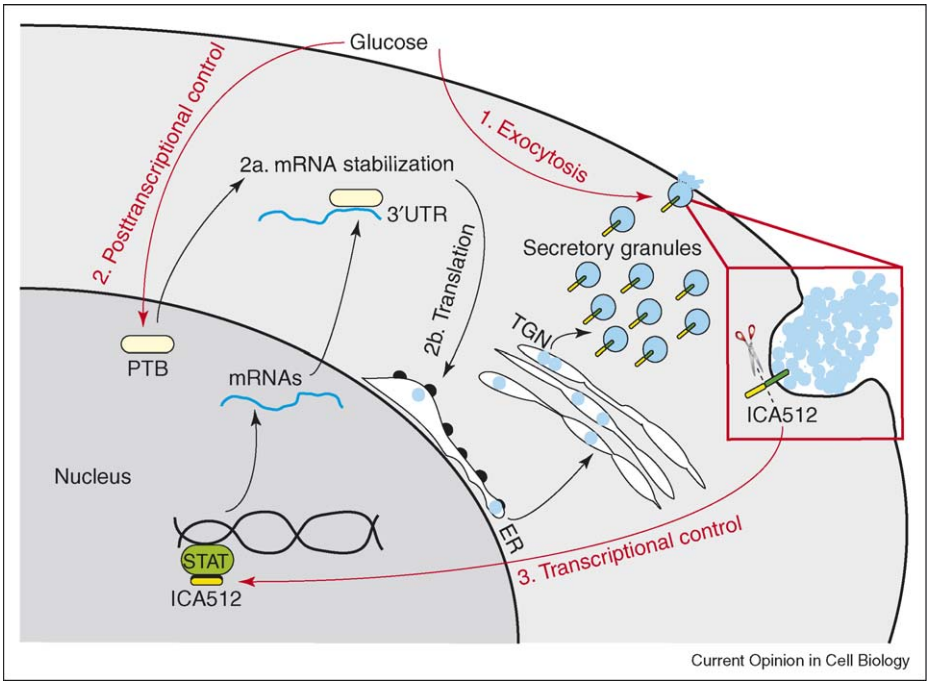
mRNA stability and translation: key steps in secretory granule biogenesis

Rapid induction of SG production following prolonged stimulation is critical to sustain hormone secretion, especially because newly generated SGs are preferentially released [8,9]. The critical role of post-transcriptional mechanisms in SG biogenesis emerged in the 1980s, when it was shown that the increased biosynthesis of insulin that occurs shortly after glucose stimulation of pancreatic β-cells was insensitive to actinomycin D, which inhibits transcription [36]. Glucose enhances the stability and translation of proinsulin mRNA through the concerted action of its 5′- and 3′-untranslated regions (UTRs) [37], and stimulates the recruitment of proinsulin mRNA to the ER [38•]. In concert with insulin, glucose prompts the rapid translation of other SG components, including CgA, PC1/3, PC2 [39] and ICA512 [40]. Recent findings have begun to unravel the molecular nature of these post-transcriptional events. Stimulation of β-cells with either glucose or cAMP-increasing agents, such as glucagon-like peptide 1 (GLP-1), triggers the nucleocytoplasmic translocation of polypyrimidine tract-binding protein (PTB) [41••,42], a RNA binding protein involved in pre-mRNA splicing, mRNA stability and localization, and cap-independent translation. Cytosolic PTB, in turn, binds to a degenerated pyrimidine-rich motif in the 3′-UTR, but possibly also in the 5′-UTR, of mRNAs for proinsulin [43] and other common SG components [41••], thereby enhancing in a concerted fashion their stability and translation (Figure 2). Conversely, knockdown of PTB expression in rat insulinoma INS-1 cells by RNA interference downregulates the expression of many SG proteins and causes the disappearance of these organelles, while other cellular features are unaffected [41••]. Although the ubiquitous expression of PTB means it cannot be regarded as a master gene for regulated secretion, it is the first identified factor for post-transcriptional control of stimulus-induced SG biogenesis in β-cells, and possibly other neuroendocrine cells. This hypothesis, however, still requires validation through the generation and analysis of $PTB^{-/-}$ mice.

Coupling production of secretory granules with their consumption

Cells adjust the biosynthesis of SGs not only in response to their stimulation, but also in relation to SG depletion. Evidence for feedback mechanisms promoting the transcription of secretory genes following SG exocytosis has been obtained in *Tetrahymena termophila* [44], but their molecular nature is unknown. In β-cells, insulin and its receptor have been proposed to activate an autocrine signaling cascade that promotes insulin gene transcription [45]. This hypothesis, however, remains controversial. Addition of exogenous insulin to isolated islets, indeed, increased the biosynthesis of neither preproinsulin mRNA nor proinsulin above the levels induced by glucose [46]. Nor did it enhance the glucose-induced activation of kinases such as Erk1/2, PKB and $p70^{S6K}$, which have been implicated in glucose-stimulated insulin biosynthesis [46]. Furthermore, targeted ablation of the insulin receptor in mouse β-cells did not reduce insulin mRNA or protein levels [47]. An alternative retrograde signaling mechanism involves ICA512/IA-2, an intrinsic membrane protein of SGs whose cytoplasmic tail contains a catalytically inactive protein tyrosine phosphatase (PTP) domain [48,49]. On exocytosis of insulin SGs, ICA512 is transiently inserted into the plasma membrane, where its intracellular domain is cleaved in a Ca^{2+}-induced/calpain1-dependent fashion [40,50••] (Figure 2). The resulting ICA512 cytosolic fragment, which contains the PTP-like domain, is then targeted to the nucleus, where it binds tyrosine phosphorylated STAT5 and STAT3, thereby preventing the inactivation of these transcription factors by dephosphorylation [50••,51•]. Through STATs, therefore, ICA512 up-regulates the transcription of its own gene as well as that of insulin and other SG components. This interpretation is consistent with the evidence that deletion of *ICA512* in mice impairs insulin secretion [52], while its overexpression in MIN-6 cells increases the number of SGs

Figure 2



Novel pathways for glucose-induced biogenesis of secretory granules (SGs) in pancreatic β-cells. Glucose stimulates SG exocytosis (1), while promoting the post-transcriptional upregulation of SG biosynthesis (2) by inducing the nucleocytoplasmic translocation of PTB, which increases the stability (2a) and translation (2b) of mRNA for SG proteins. Exocytosis of SGs, in turn, is associated with the calpain-1-mediated cleavage of ICA512 and the translocation of the resulting ICA512 cytosolic fragment into the nucleus, where it enhances the transcription of SG genes (3).

[53•]. Since *ICA512* is widely expressed in neuroendocrine cells, this pathway could represent the first general homeostatic feedback mechanism coupling the biosynthesis of SGs to their consumption.

Conclusions

Traditionally, studies of SG biogenesis have focused on the mechanisms through which proteins destined for regulated release are sorted into post-Golgi secretory vesicles with an electron-dense core. As discussed above, a full description of SG biogenesis should include the post-transcriptional and transcriptional mechanisms responsible for the concerted expression of SG components in response to stimuli and exocytosis. A quantitative description of these mechanisms may eventually explain not only how SGs are assembled at the TGN, but, perhaps even more importantly, how neuroendocrine cells count the size and consumption of their SG stores in order to balance SG production with demand. This knowledge could be especially valuable in medicine. One could indeed imagine that many human disorders ultimately result from neuroendocrine cells that, like incompetent accountants, lose track of their numbers.

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