REVIEW

Discovery and progress in our understanding of the regulated secretory pathway in neuroendocrine cells

Joëlle Morvan · Sharon A. Tooze

Abstract In this review we start with a historical perspective beginning with the early morphological work done almost 50 years ago. The importance of these pioneering studies is underscored by our brief summary of the key questions addressed by subsequent research into the mechanism of secretion. We then highlight important advances in our understanding of the formation and maturation of neuroendocrine secretory granules, first using in vitro reconstitution systems, then most recently biochemical approaches, and finally genetic manipulations in vitro and in vivo.

Keywords Immature secretory granule ·
Mature secretory granule · Secretogranin ·
Chromogranin · Prohormone convertase

Morphological era of discovery (1950s and 1960s)

Visualization of the cell interior by electron microscopy catalysed both morphologists and biochemists to initiate experiments and make observations to identify, define and understand the complex compartmentalization of specialized cells, most notably including the morphologists G.E. Palade, C. DeDuve, D.W. Fawcett, K.R. Porter, J. Rhodin and F.S. Sjostrand. The exocrine pancreatic acinar was a specialized cell type favoured by GE Palade, and it was with this cell type that the first observations on cells with specialized granule compartments were made.

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The complex morphology of the cell compartments, and the desire to link a morphological observation with basic biochemical functions and pathways known at that time initiated the monumental effort to couple a biochemical approach with EM observations. Indeed in 1956, Palade observed intracisternal granules in the endoplasmic reticulum (ER) which resembled zymogen granules, and postulated that the material in the intracisternal granules was possibly the same as the zymogen granules, and these granules were related to the granules in the cells in the endocrine pancreas, β-cell granules (Palade 1956). Using the pancreatic acinar cell model, experiments by Siekevitz and Palade between the late 1950s and early 1960s defined the role of the ER and other subcellular compartments in the synthesis of zymogen granule proteins, and resulted in a series of papers published in the newly established Journal of Biophysical and Biochemical Cytology, soon to be renamed Journal of Cell Biology (Siekevitz and Palade 1958, 1962). The concept that proteins are transported from their site of synthesis, the ER, through the Golgi complex was also being recognized and visualized using EM autoradiography in the exocrine pancreas (Caro and Palade 1964), first at a low temporal level of resolution. Direct evidence for transport from ER through Golgi to zymogen granules was then obtained using more precise pulse-chase protocols on tissue slices by Jamieson and Palade (Jamieson and Palade 1967a, b). This technical advance was further exploited by Jamieson who applied subcellular fractionation techniques to pulse-labelled slices. Separation of the rough and smooth ER from condensing vacuoles and zymogen granules allowed a quantitative kinetic analysis and a direct demonstration that proteins are transferred from the ER to zymogen granules via condensing vacuoles (Jamieson and Palade 1967a, b).

Parallel studies in the anterior pituitary gland showed that discrete, small individual granules, called immature secretory granules (ISG) in mammotroph cells originated from the Golgi cisternae. These small ISGs coalesced into aggregates surrounded by a single membrane, finally becoming mature secretory granules (MSGs) (Smith and Farquhar 1966). Additional information about the secretory process in mammotrophs was obtained using high resolution EM autoradiography. ISGs (20–100 nm structures) were found to be maximally labelled after 30 min pulse with [3H]-leucine, and mature over the following 2 h to MSGs. This kinetic of maturation correlated well with the observations in the exocrine pancreas which demonstrated the condensing vacuoles were labelled after 37 min (Jamieson and Palade 1967a). The complexity of the secretory granule compartment was revealed in subsequent studies on dispersed pituitary cells where four types of secretory granules were detected (Salpeter and Farquhar 1981). After 15–55 min of chase the labelled protein was found in small (Type I) ISGs, and subsequently in type II and III polymorphic granules, and then larger Type IV MSGs after 55–185 min of chase (see Fig. 1, and Farquhar et al. 1978). A higher degree of resolution was obtained using newly developed fine-grain emulsion which allowed an accurate detection of small structures (20–100 nm), which combined with a more sophisticated analysis of silver grain distribution, led to the discovery that concentration of secretory protein in the ISG compartment was 200 times that of the adjacent Golgi cisternae (Salpeter and Farquhar 1981).

During this period Tartakoff and colleagues outlined the concept that secretory cells may have different ways to secrete newly synthesized proteins; cells such as plasma cells, fibroblasts, and macrophages secrete in a "non-regulated" fashion in contrast to "regulated" cells such as exocrine pancreas, the hallmark of which is the storage of the secretory proteins (Tartakoff and Vassalli 1978). The nature of the secretory pathways in such "non-storage" secretory cells was also explored by the use of drugs that perturbed secretion, such as monensin, a Na^+/K^+ ionophore which causes a neutralization of acidic intracellular compartments, or drugs that alter the energy status of the cell (Tartakoff et al. 1977). The common requirement for energy, cyclic nucleotides, Ca+, and cytoplasmic Na+/K+, in both non-regulated and regulated cells, gave rise to the consensus that there is a common secretory pathway originating in the RER, through the Golgi complex (for review see Palade 1975).

Many of the observations made were obtained using careful morphological approaches and expanded using biochemical techniques, and subcellular fractionation. However, these early biochemical approaches were limited by the inability to manipulate the cell systems in use, typically tissues from mice or rats. The arrival of molecular tools and genetic manipulation provided the next wave of advances bringing the field to our current level of understanding.
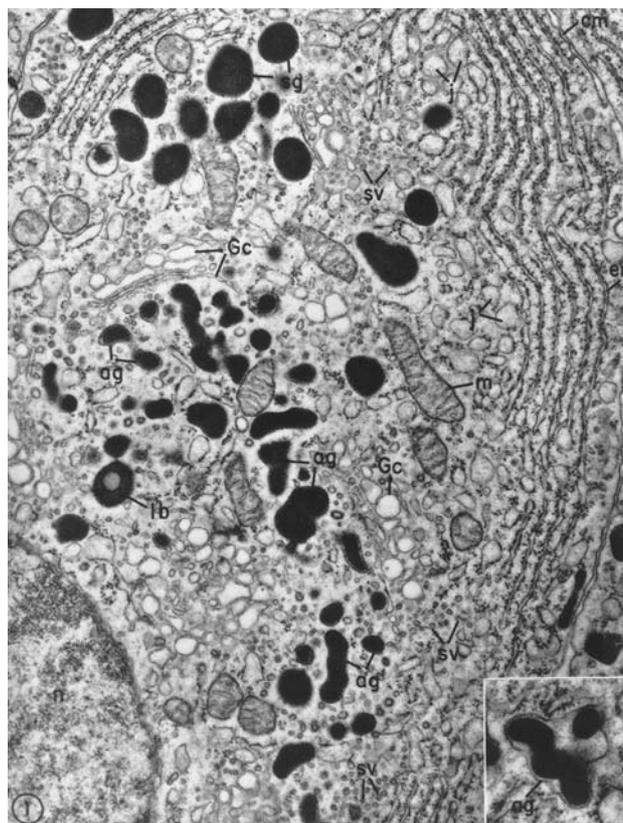
Fig. 1 Mammotroph cell from the anterior pituitary gland of a lactating rat. This micrograph illustrates the morphological complexity of the regulated secretory pathway, and the different types of ISGs. *ER* endoplasmic reticulum; *CM* cell membrane; *SG* secretory granule; *SV* smooth vesicles; *VE* vesicle; *LB* lytic body. Reproduced from the J Cell Biol, 1966, 31:319–347. Copyright 1966 The Rockefeller University Press

Cell line model systems, the molecular age and the sorting problem

Regulated secretory cells, as well as other cell types such as liver cells, have specialized plasma membrane domains, which by definition have a unique composition. Therefore, in addition to classification of regulated versus non-regulated secretion (also called constitutive secretion (Kelly 1985), it was recognized that there must be multiple ways to reach distinct domains from the Golgi complex, in particular for membrane proteins. The first direct demonstration of multiple routes to the plasma membrane was from the work of Gumbiner and Kelly in 1982. Their observations using the AtT20 cell line, a mouse pituitary cell line, showed that the regulated secretory hormone ACTH was secreted with different kinetics from a viral model membrane protein, gp70, a glycoprotein of the endogenous murine leukaemia virus. Surprisingly, the kinetics of the secretion of a proportion of the ACTH precursor, POMC, which escaped the activity of pro-hormone processing

enzymes present in the MSG was the same as gp70 (Gumbiner and Kelly 1982). These experiments solidified "the two pathways hypothesis" (Kelly 1985) which defined distinct post-Golgi pathways for proteins targeted for regulated and constitutive secretion. At this time it was also recognized that the mechanism for segregation of cargo proteins (either soluble or membrane associated), or sorting into a particular pathway was an important issue, as was the identification of the precise location for the initiation of the sorting process. A key technological advance in elucidating both sorting and location was the development of immunoelectron microscopy using thin, frozen sections (Tokuyasu 1980) which allowed the identification of proteins within subcellular compartments.

TGN and post-TGN sorting and processing in neuroendocrine cells

It was recognized in mid-1980s that the trans-Golgi network (TGN) might be the key exit point for proteins destined to the plasma membrane, constitutive secretory vesicles (CSV), endosomes, and ISGs (Griffiths and Simons 1986). It was proposed that sorting receptors might function in this compartment to segregate different cargos into different vesicles (Burgess and Kelly 1987). It also became apparent from studies on the mannose-6-phosphate receptor (M6PR), which with its bound lysosomal enzyme is sorted to endosomes, that sorting to endosomes from the TGN utilized clathrin and clathrin-coated vesicles (Geuze et al. 1985). At this time it was also proposed that CSVs were formed without the aid of clathrin coats (Griffiths et al. 1985). However, clathrin-coated regions were detected on the surface of β-cell secretory granules associated with the TGN after monensin treatment (Orci et al. 1984) or on ACTH-containing ISGs in AtT20 cells (Tooze and Tooze 1986) raising the possibility that 1) ISGs formation is through clathrin-coated regions of the TGN, or 2) that the biogenesis of ISGs involved a clathrin-coated vesicle dependent pathway.

Acidification of the TGN and post-TGN secretory compartments was increasingly recognized to be important for both transport (see Tartakoff et al. 1977) and sorting. A key contribution at this time was the direct demonstration, using DAMP labelling (Anderson et al. 1984), that the TGN and post-TGN compartments were acidic, and there was a gradient of acidification in the secretory pathway. It was proposed that acidification could play role in receptor-ligand uncoupling and recycling, thereby providing directionality to transport (Anderson and Pathak 1985). Although the concentration of DAMP, was thought to be proportional to the extent of acidification, later studies provided accurate measures of pH of the TGN and ISG (see review Moore et al. 2002).

Neutralization of acidic compartments caused mis-sorting of regulated secretory proteins to the constitutive pathway (Moore et al. 1983), a result which gave rise to the possibility that regulated secretory proteins may have sorting signals, and could be sorted by a receptor-ligand interaction in the TGN, or in a post-TGN compartment. The most controversial observation in support of this hypothesis was the proposed role for carboxypeptidase E as the sorting receptor (Cool et al. 1997), although this was immediately refuted (Irminger et al. 1997). Evidence in support for a sorting signal was the demonstration that regulated secretory proteins have transferable sorting signals, the first being in 1986 (Moore and Kelly 1986). An alternative hypothesis was that aggregation of regulated secretory proteins, best demonstrated for the Granins but also shown for a variety of other regulated secretory proteins, and favoured by low pH on the TGN, drives lumenal segregation of regulated secretory proteins. The segregation of the regulated secretory proteins maybe further enhanced by a homophilic interaction with membrane associated population of Granins (Gerdes et al. 1989). Furthermore, evidence for formation of multiple secretory granule populations with different hormone content supports the idea that aggregation drives sorting (Hashimoto et al. 1987). Some of these results form the basis for the "sorting for entry" hypothesis, which proposes that regulated secretory proteins are sorted into ISGs in the TGN, while constitutively secreted proteins enter CSVs (see review Tooze 1998).

Another model to explain sorting in the regulated pathway was developed from experiments in β-cells, where it was shown that insulin-containing secretory granule formation is driven by the hexamerization and condensation of insulin in the ISGs (Arvan et al. 1991). These studies revealed an essential difference between neuroendocrine cells and β-cell insulin granules, giving rise to the "sorting by retention" hypothesis (see review Arvan and Castle 1998) which proposes that rather than an active sorting in the TGN, the crucial sorting step occurs in the post-TGN ISGs. Both the "sorting for entry" and "sorting by retention" hypothesis agree on that non-regulated (possibly missorted) secretory proteins are removed by ISG-specific clathrin-coated vesicles in a pathway called "constitutive-like secretion", after secretory granules have formed from the TGN.

Most, if not all of the soluble regulated secretory proteins are processed by the endopeptidases, the pro-hormone convertases (PCs). Their discovery and final molecular characterization was driven by the original pioneering work of Steiner and colleagues working in the β-cell (Steiner et al. 1974), and reviewed by (Seidah et al. 1993). These enzymes are present in ISGs and MSGs, and are subjected to the same sorting machinery as are proteins such as the Granins. As their activity is pH-dependent, a large number

of studies used the kinetics of their activation as monitors for studying where and when the sorting of regulated proteins occurred (see also below). A direct demonstration that the site of prohomone processing of proinsulin was the clathrin-coated ISG was obtained by immunocytochemistry (Orci et al. 1985b), which were subsequently shown to be acidic (Orci et al. 1986).

The progress from the 1950s until the early 1990s was the result of many researchers' effort, most of which have not been covered in this brief review. The reader is directed to comprehensive reviews from the period (Burgess and Kelly 1987; Mains et al. 1987), and more recently (Borgonovo et al. 2006; Dannies 2001; Kim et al. 2006; Meldolesi et al. 2004; Solimena and Gerdes 2003). The main questions which we focus on in the next section are the molecular machinery of secretory granule formation and maturation, as addressed by the work started in the laboratory of W. Huttner and continued in the author's laboratory which is largely based on in vitro reconstitution assays using isolated TGN, and post-TGN subcellular compartments, in particular ISGs as first developed using Golgi membranes by Rothman and colleagues (Fries and Rothman 1980).

In vitro reconstitution and biochemical analysis of secretory granule biogenesis

Cell-free reconstitution of ISG budding from TGN

While MSGs from a variety of tissues had been extensively characterized morphologically as well as biochemically, little was known about ISGs beyond their morphological appearance (Smith and Farquhar 1966). The cell-free reconstitution of secretory granule budding from the TGN brought new insights to understanding the formation of ISGs (Tooze and Huttner 1990). This assay was based on (1) the high fidelity sorting properties of PC12 cells which, unlike AtT20 cells, target greater than 90% of their regulated secretory protein to ISGs and MSGs, (2) the selective labelling of a regulated secretory marker, secretogranin II (SgII), and a constitutively secreted protein, heparan sulfate proteoglycan (hsPG) with radioative sulfate by sulfotransferases present only in the TGN, and (3) the ability to separate the TGN, ISGs and CSVs from one another by sequential velocity and equilibrium gradients centrifugations. This assay demonstrated that SgII and hsPG were present in two vesicle populations, SgII-containing regulated secretory vesicles (ISGs), and hsPG-containing CSVs. These results provided the first demonstration that regulated secretory proteins and constitutive secretory proteins were sorted into two distinct vesicle populations directly upon exit from the TGN, in support of the "sorting for

entry" hypothesis. The formation of both ISGs and CSVs required GTP-binding proteins (Tooze et al. 1990), possibly heterotrimeric G-proteins (Leyte et al. 1992). The rate of formation of the ISGs was indistinguishable from the CSVs, occurring with a t1/2 of 15 min.

In addition, the subcellular fractionation protocol developed to distinguish ISGs and MSGs allowed the biochemical and morphological characterization of ISGs, in comparison to MSGs, isolated from PC12 cells (Tooze et al. 1991). ISGs were shown to have several components of the clathrin-coat machinery, including AP-1 (Dittié et al. 1996), and ARF1 (Austin et al. 2000), in addition to nongranule proteins such as the M6PR and furin (Dittié et al. 1997, 1999). In addition, it was possible to determine the size of ISGs and MSGs, results which provided direct support for the early EM autoradiography (Smith and Farquhar 1966) and became the basis for the experiments directed towards a molecular understanding of the change in size of the ISG through homotypic fusion and subsequent remodelling of the ISG through clathrin coats (see Fig. 2).

Determination of the pH of ISGs using an in vitro approach

While it was known from earlier studies that in endocrine cells MSGs are acidified it was not known what the precise pH of the ISG was, and if the pH of the ISG differed from
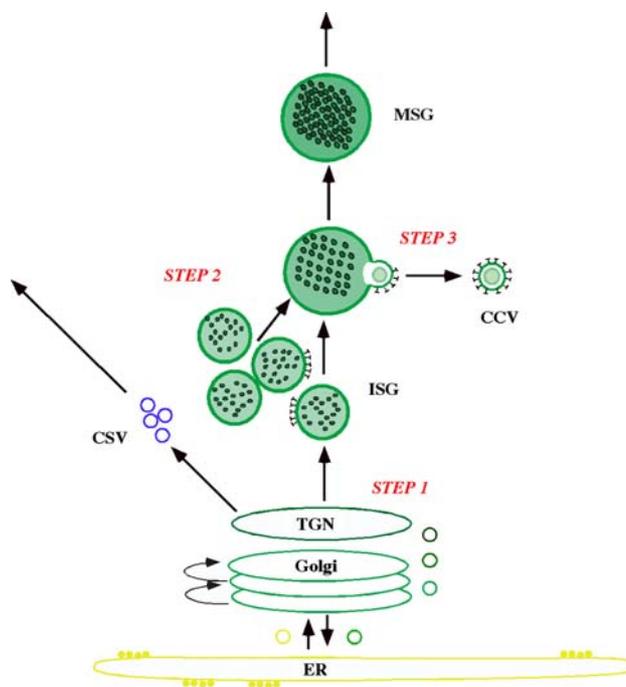
Fig. 2 Key steps in secretory granule biogenesis. Model based on data discussed above, in addition to contributions from many researchers which have not been mentioned, but whose contributions are noted. *Step 1* depicts the formation of ISGs from the TGN, *Step 2* the homotypic fusion event between ISGs, and *Step 3* the clathrin-mediated membrane remodelling event. See text for abbreviations

the TGN. The establishment of a PC12 stable cell line expressing the PC2 allowed us to characterize the endopeptidase PC2 activity during granule maturation (Dittié and Tooze 1995). In this cell line, PC2 is correctly targeted to the ISGs and co-sedimented with SgII in fractions containing ISGs and MSGs. [^{35}S] sulfate labelling demonstrated that SgII was proteolytically processed by PC2 in ISGs after approximately 30 min of chase into several lower-molecular-mass proteins, the major ones being a 18 and 28 kDa sulphated fragment.

As the efficiency of processing of SgII by PC2 was pH dependent, we used the ability to quantitate the extent of processing at different pHs to determine the pH in the ISGs (Urbé et al. 1997). Isolated ISGs and TGN, containing [^{35}S] sulfate pulse-labelled SgII were incubated in presence of ATP at physiological pH and the extent of SgII processing in these compartments was compared to a standard curve prepared using ISGs equilibrated at a defined set of pHs. This allowed us to determine that the ISGs intra-granular pH was 6.3, similar to the TGN pH and clearly higher than the pH of MSGs (pH 5.5–5.0). Interestingly, no processing of SgII could be observed in the membrane fraction highly enriched in TGN under conditions for which processing was readily obtained in isolated ISGs. This data represent further evidence that the ISG is indeed a functionally distinct organelle from the TGN. Furthermore, the rate of SgII processing was strongly dependent on the intragranular pH, demonstrating that processing of SgII can be used as a pH indicator for granule interior.

ISG-ISG homotypic fusion

As shown originally by Farquhar (Smith and Farquhar 1966) in the anterior pituitary, and more recently in PC12 cells (Tooze et al. 1991), ISGs increase in size during maturation, and the increase in size was proposed to reflect homotypic fusion of ISG. To demonstrate that ISG–ISG fusion occurred, we developed an assay that provided the first biochemical evidence for such a fusion event and allowed us to dissect the molecular requirements of this process.

The cell-free assay to reconstitute homotypic fusion was performed by mixing two populations of ISGs, one containing the pro-hormone convertase PC2 and the other containing [^{35}S]sulfate-labelled secretogranin II (SgII) (Urbé et al. 1998). The fusion was then measured by quantification of the 18 kDa PC2 cleavage product of SgII. This in vitro reconstitution of ISG-ISG fusion revealed that homotypic fusion is dependent on NSF (Urbé et al. 1998), α-SNAP and on the SNARE Syntaxin 6 but not on Syntaxin 1 or SNAP-25 (Wendler et al. 2001). More recently, Synaptotagmin IV (Syt IV), a member of the Synaptotagmin family of proteins involved in membrane fusion, has also been shown to be required for this step of granule maturation (Ahras et al. 2006).

ISG membrane remodelling

In addition to ISG-ISG homotypic fusion, ISG content and membrane remodelling is another important step during granule maturation. This remodelling is performed via budding of clathrin-coated vesicle from the maturing granule membrane, and is the pathway for non-regulated constitutive-like secretion, or possibly sorting to endosomes. This clathrin-mediated remodelling step is a common feature in neuroendocrine and endocrine secretory granules, and is thought to provide a mechanism for proof-reading the content and membrane composition of the maturing ISG to ensure the production of MSGs which contain biologically active hormones and can undergo efficient exocytosis.

ISGs in endocrine and neuroendocrine cells were shown by morphological techniques to be partially clathrin coated (Orci et al. 1985a; Tooze and Tooze 1986) but the recruitment mechanism and the composition of this clathrin coat were unknown until a biochemical approach was used. Our laboratory developed a fourth cell-free assay whereby the recruitment of the clathrin adaptor complex AP-1 to ISGs was reconstituted by the addition of bovine brain cytosol (Dittié et al. 1996). These experiments showed that AP-1 recruitment to ISGs was ATP-independent but GTP- and, ARF1-dependent. This study demonstrated that it is the AP-1 complex that is involved in the clathrin binding to ISGs. Cross-linking experiments demonstrated the direct interaction of AP-1 and ARF1 on the ISG (Austin et al. 2000). The M6PR and furin are likely part of the cargo targeted for removal from the ISGs by clathrin-coats as they possess sequences in their cytoplasmic domains which interact with AP-1, and are present on ISGs, but absent from MSGs (Dittié et al. 1997, 1999).

Further investigation into cargo sorting from the ISG showed that VAMP4, a SNARE involved in endosome to TGN vesicle trafficking, which is present on ISGs but removed from the maturing granule membrane, is also able to bind AP-1. The recruitment of AP-1 by VAMP4 is dependent on phosphorylation of the cytoplasmic domain of VAMP4 by the kinase CKII. CKII phosphorylation of VAMP4 allows the recruitment of PACS1 to ISGs enhancing the AP-1 dependent sorting event (Hinners et al. 2003). These data suggest that sorting non-regulated secretory proteins from ISGs, including the SNARE proteins, can be mediated by the recruitment of clathrin coats and may be essential for maturation of ISGs.

More recently, our laboratory demonstrated that the GGAs proteins are also involved in this clathrin-mediated membrane-remodelling step of secretory granule maturation. GGAs (Golgi-associated, γ-ear-containing, ADP-ribo-

sylation factor-binding protein) are involved in recruitment of AP-1 and clathrin coats to membranes (for review see Robinson and Bonifacino 2001). siRNA mediated knock-down of GGA3 and over-expression of dominant negative form of GGA proteins resulted in a retention of the SNAREs Syntaxin 6 and VAMP4 in the MSGs. In addition, an alteration of the PC2 activity was also detected, suggesting that inhibition or alteration of clathrin-coat remodelling inhibited or altered intra-lumenal acidification (Kakhlon et al. 2006). Similar effects on PC2 activity were seen after siRNA mediated knock-down of Syt IV, suggesting that inhibition of either homotypic fusion or remodelling have similar detrimental effects on acidification of ISGs.

It was proposed that secretory granules, like other membranes have lipid micro-domains, or rafts (Tooze et al. 2001), and that the lipid composition of the secretory granule membrane is important for protein sorting during clathrin-coat mediated membrane remodelling. In fact, cholesterol was shown to be an important component in secretory granule formation (Wang et al. 2000). Recent work by Katsumata et al. (2007) has shown that the secretory granule membrane is composed of two kinds of micro-domains and that the proteins expected to be removed during the membrane remodelling (VAMP4 and Syntaxin6) are clustered together in the same micro-domain and separated from the proteins (VAMP2) that stay on the MSG membrane. These experiments validate our observations (see above), as well as the work by H.P. Moore in AtT20 cells (Eaton et al. 2000) and provide new information that opens new avenues for research into the role of lipids in sorting from ISGs.

Recent developments in secretory granule formation and maturation

In this last section we summarize the most recent developments in our understanding of secretory granule biogenesis obtained from biochemical screens, in vivo experiments based on genetic manipulation of cell in culture as well as in vivo transgenic mouse models. Only a small number of recent results are highlighted and readers are directed to recent reviews (Borgonovo et al. 2006; Dannies 2001; Kim et al. 2006; Meldolesi et al. 2004; Solimena and Gerdes 2003). The experiments we highlight show the direction that the field has taken, and the way less tractable questions can be addressed by new biochemical screens and transgenetic mouse models.

Role of cholesterol in secretory granule biogenesis

Secretory granule formation is dependent on lipid raft microdomains that are enriched in cholesterols (Wang et al.

2000). A recent investigation of the role in vivo of cholesterol in secretory granule biogenesis was done using Smith–Lemli–Opitz Syndrome (SLOS) and lathosterolis mouse models (Gondre-Lewis et al. 2006). Both diseases are a result of inborn errors in cholesterol synthesis. SLOS is a disease caused by a defect in the function of the enzyme, 7-dehydrocholesterol reductase (DHCR7), required for the final step of cholesterol biosynthesis. Patients with lathosterolosis lack lathosterol-5-desaturase (SC5D), the enzyme that catalyse the next-to-last step in cholesterol synthesis. Data obtained from DHCR7 and SC5D knock-out mice demonstrated that impairment of the cholesterol biosynthesis results in a significant decrease in secretory granules in pancreas, pituitary and adrenal glands. In addition, abberant zymogen granules were found in the exocrine pancreas, in which there was also observed a decrease in regulated secretion which could be rescued with exogenous cholesterol. The authors conclude that the presence of an elevated quantity of other sterols, 7-DHC and lathosterol, in Dhcr7-/- and Sc5d-/- mice, respectively, could not replace cholesterol in the regulated secretory pathway. They speculate that the abnormal properties of the secretory granule in these mice models may be attributed to the reduced rigidity of membranes containing these sterols instead of cholesterol. Importantly, this study demonstrates that cholesterol is essential during secretory granule biogenesis in vivo.

Regulation of secretory granule maturation

Peptidylglycine α-amidating monooxygenase (PAM) is an essential enzyme in the processing of many bioactive peptides and hormones (Prigge et al. 2000). PAM is targeted to the regulated secretory pathway in neurons and neuroendocrine cells. It is a type I membrane protein containing two enzymatic domains within the lumen of the secretory granule, a transmembrane domain, and a cytosolic domain containing sorting signals. PAM catalyses one of the final steps in peptide biosynthesis, and is retrieved from ISGs and the plasma membrane for re-utilization in newly forming secretory granules (Ferraro et al. 2005). Among the proteins able to interact with PAM are two Rho guanine nucleotide exchange factors (GEFs), Kalirin and Trio, identified by yeast two-hybrid screens using the cytosolic domain of PAM as bait (Alam et al. 1996; Xin et al. 2004).

In a recent study, Kalirin and Trio have been shown to be involved in the maturation of secretory granules (Ferraro et al. 2007). Overexpression of their N-terminal GEF domain enhances secretion from ISGs, reducing regulated secretory protein storage through constitutive-like secretion, in the absence of secretagogue stimulation of regulated exocytosis. Conversely, when GEF activity is inhibited the constitutive-like release is inhibited, resulting

in the accumulation of cargo in MSGs above even normal levels. These results indicate that these two Rho-GEFs regulate sorting into secretory granules, constitutive-like secretion from ISGs, and perhaps constitutive secretion, and provide a novel mechanism for the regulation of secretory granule maturation.

Role of Chromogranin A in secretory granule formation

Chromogranin A (CgA) and B (CgB), members of the Granin family, have long been proposed to control the secretory granule biogenesis because of their pH-, calcium- and catecholamine-dependent aggregation properties, and widespread expression pattern. Recent results supports this hypothesis, however other recent results suggest that the regulated phenotype (Day and Gorr 2003; Meldolesi et al. 2004) is not simply conferred by expression of CgA.

In support of the hypothesis, in 2001, Kim et al. demonstrated that specific depletion of CgA, but not of CgB, by siRNA impaired secretory granule production in PC12 cells. In contrast, over-expression of CgA in 6T3 cells lacking CgA and the regulated secretory pathway, or fibroblastic CV-1 cells, not only triggered recovery of secretory granules but also regulated secretion (Kim et al. 2001). However, Malosio et al.(2004) refuted the hypothesis by demonstrating with very similar approaches that the "newly formed" vesicles which contained the CgA were in fact lysosomes, and overexpression of CgA did not induce the appearance of secretory granules, or alter the number of secretory granules.

Three groups used mouse models to determine the role of Chromogranin A in vivo by targeted ablation of the Chromogranin A gene. Mahapatra et al.(2005) confirmed the putative role of CgA in secretory granule biogenesis, in particular a decrease of chromaffin granule size and number. In addition, defects were observed in neurotransmitter storage and release and regulation of blood pressure. Similar results were obtained using anti-sense vectors specific for CgA in transgenic animals which resulted in a reduction of the CgA levels (Kim et al. 2005). In the adrenal medulla of these mice there was a large decrease in the number of secretory granules; in addition it was noted that the secretory granules present appeared to be swollen. This defect may be related to a need to maintain a proportional catecholamine and CgA concentrations.

Lastly, Hendy et al. (2006) found that the CgA null mutant mice had elevated secretion of epinephrine, norepinephrine and dopamine and that mRNA and protein level of other secretory granule proteins were up-regulated. No obvious abnormalities in development or neuronal and endocrine functions have been noticed. The authors suggested then that the increased expression of the other Granin family members is likely to compensate for the CgA deficiency.

Role of prohormone convertases in secretory granule maturation

Both in vitro and ex vivo the activity of the PC enzymes (notably PC1/3 and PC2 which will be the focus of the discussion here) have been extensively studied. PC null mice models have confirmed the in vivo function of the PCs and extended the characterization of their specific substrates and action in different tissues.

The first PC transgenic mice model obtained was the PC2 null mouse (Furuta et al. 1997). Mice lacking PC2 activity develop normally and are fertile, but exhibit a variety of neuroendocrine processing abnormalities in the brain and in pancreatic islets. These mice show elevated levels of pro-insulin (Furuta et al. 1998) and pancreatic islet pro-glucagon processing is completely blocked. Other defects in PC2 null animals include lack of production of α-MSH associated with accumulation of ACTH in the pituitary intermediate lobe (Laurent et al. 2002).

7B2, also known as SgV or SGNE-1, is a small acidic protein exclusively localized to neuroendocrine tissues. This protein has been shown to be associated to PC2 and it functions as a chaperone for PC2 (Braks and Martens 1994), and for review see Mbikay et al. (2001). A 7B2 null transgenic mice model has shown that 7B2 is required for PC2 activation in vivo, having in addition important functions in regulating pituitary hormone secretion (Westphal et al. 1999). 7B2 null mice have no demonstrable PC2 activity, and in general agreement with the PC2 null mouse (Furuta et al. 1997), are deficient in processing islet hormones and display hypoglycemia, hyperinsulinemia, and hypoglucanemia, with generalized islet cell expansion and altered islet cell morphology. However, the most important impairment of PC2-mediated peptide processing in 7B2 nulls involved the synthesis of the corticotrophin ACTH. ACTH is produced by cleavage of POMC through the action of PC1/PC3, further internal cleavage of ACTH occurs by the action of PC2 specifically in the neurointermediate lobe. In 7B2 nulls, ACTH remains intact in the intermediate lobe, resulting in extremely high levels of ACTH in this lobe (Westphal et al. 1999). The overall phenotype suggests that 7B2 may be involved in PC2 activity, as well as secretory granule biogenesis.

Finally, the role of the PC1/3 endopeptidase has also been investigated in a transgenic mouse model. Disruption of gene-encoding mouse PC1/PC3 results in a syndrome of severe post-natal growth impairment and multiple defects in processing many hormone precursors, including hypothalamic growth hormone-releasing hormone (GHRH), pituitary proopiomelanocortin to adrenocorticotropic hormone, islet proinsulin to insulin and intestinal proglucagon to glucagon-like peptide-1 and -2 (Zhu et al. 2002). In summary, the analysis of PC2, 7B2 and PC1/3 null mice has

confirmed that PCs play a crucial role in the processing of many hormone precursors and the highlight the importance of PC2 and PC1/3 in neuroendocrine tissues, and regulated secretion.

Role of Rab3D in secretory granule maturation

Rab proteins are small GTPases that belong to the Ras protein superfamily and that function in membrane traffic. Rab3A is one of the best-characterized Rab protein. In mammals, three additional isoforms are expressed which are referred to as Rab3B, Rab3C and Rab3D. Rab3A, Rab3B and Rab3C are predominantly expressed in the nervous system, where they are localized to synaptic vesicles (Fischer von Mollard et al. 1994; Martelli et al. 2000). Rab3A-deficient mice studies suggested that Rab3A has a function intimately associated with fusion more than a function in vesicle tethering at the synapse (Geppert et al. 1997).

In contrast to Rab3A, Rab3B and Rab3C, Rab3D is predominantly expressed outside the nervous system, in peripheral tissues where the other isoforms either are expressed at low levels or are lacking. Originally identified in fat cells, Rab3D is present in several additional cell types including secretory cells such as pancreatic and parotid acinar cells, mast cells and peptide-secreting cell lines. In secretory cells Rab3D appears to be predominantly localized to secretory granules, thus mirroring the distribution of Rab3A in neurons and neuroendocrine cells. It was hypothesised that Rab3D could have the same function as Rab3A but in secretory cells.

Riedel et al. (2002) investigated the role of this Rab3 isoform by knocking out the Rab3D gene in mice. Rab3D-deficient mice are viable and fertile and showed no obvious defect. In particular, both kinetics and dose response of secretagogue-induced enzyme secretion of the pancreas were normal. However, these mice show a substantially increased size of secretory granules in both exocrine pancreas and the parotid gland with the volume being doubled, while the intragranular protein concentration appears to be unchanged. These data suggest that Rab3D functions in granule maturation but not in exocytic membrane fusion.

Conclusions and future perspectives

The early morphological and biochemical characterization of the regulated secretory pathway provided key insights to the secretory process. More recent experiments have provided a more detailed understanding of the functional properties, and the regulation of the maturation of neuroendocrine secretory granules. Many important issues need to be resolved, for example how the cytoplasmic

events, such as membrane remodelling are coupled with the intralumenal biochemical changes such as PC activation, prohormone cleavage, and acidification. In addition, the biological relevance of the maturation process, as it affects hormone processing and secretion, remains to be determined in light of the differences between endocrine and neuroendocrine granules.

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