Protein Composition and Biogenesis of the Pancreatic Zymogen Granules

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1. Introduction

The exocrine pancreas produces and secretes multiple digestive enzymes and has been the model in which the structure and functional organization of the mammalian secretory pathway was originally discovered (56) and intensively studied thereafter. The acini of the exocrine pancreas are the functional units of digestive enzyme synthesis, storage and regulated secretion (79). The pancreatic acinar cells exhibit one of the highest protein synthesis rates among mammalian cells. More than 90% of the newly synthesized proteins are targeted to the secretory pathway (68) and packaged into large secretory granules, called zymogen granules (ZG). In contrast to the relatively small neuroendocrine and endocrine granules, ZGs have an averaged diameter of around 1μm. ZGs are responsible for transport, storage and secretion of digestive enzymes and has long been a model for studying the mechanism of secretory granule biogenesis and regulated exocytosis. Stimulation of the acinar cells by secretagogues such as acetylcholine and cholecystokinin triggers fusion of ZG membrane with the apical plasma membrane and the release of digestive enzymes into the pancreatic ductal system. In the duodenum, trypsinogen is converted to trypsin by proteolytic cleavage via enterokinase and activated trypsin then proteolytically activates the other zymogen enzymes (8, 55).

Physiological stimulation of acinar cells by secretagogues triggers local apical Ca^{2+} spiking, fusion of ZG membrane with the apical membrane and exocytosis (50, 57, 58, 81). In contrast to the physiological condition, supramaximal CCK stimulation elicits sustained elevation of cytosolic $[Ca^{2+}]$ and leads to mistrafficking of digestive and lysosomal enzymes, inhibition of apical secretion and abnormal exocytosis redirected to basolateral plasma membrane all of which are believed to contribute to the pathogenesis of experimental acute pancreatitis (21, 65). The ZG content contains primarily the digestive enzymes and associated proteins which are the major protein components of the pancreatic juice secreted into the duodenum. The ZG membrane carries at least part of the molecular machinery responsible for digestive enzyme sorting, granule trafficking and exocytosis. For example, digestive enzyme sorting and packaging will, at least to some extent, depend on interactions between ZG content and ZG membrane components exposed to the lumen of ZGs. The cytoplasmic surface of ZG membrane must contain vesicular trafficking proteins including Rabs, SNARE proteins as well as molecular motors to interact with cytoskeleton. Defective ZG biogenesis and trafficking can result in various pancreatic diseases such as acute and chronic pancreatitis (21, 29, 65). A comprehensive understanding of the protein composition of the ZG content and membrane will provide critical insights in the biogenesis and regulated secretion of pancreatic ZGs.

2. ZG Protein Composition

Zymogens and digestive enzymes

The major secretory products of the acinar cells, namely the content of ZGs, are digestive enzymes which belong to five functional groups of hydrolytic enzymes including endo- and exo-proteases, lipases, glycosidases, and nucleases. In contrast to endocrine cells which often produces a predominant peptide or protein product such as insulin, acinar cells synthesize, package and secrete a mixture of nearly 20 different enzymes and isoenzymes including amylase, trypsinogens, chymotrypsinogens, carboxypeptidases, esterases, lipases and ribonucleases. Most pancreatic proteases are synthesized as inactive precursors, zymogens, which only become activated by a cascade of limited proteolysis within the intestinal lumen. Because of the importance of ZG to digestive enzyme storage and regulated secretion and as a general model for secretory vesicles, the identification and characterization of both the soluble and membrane proteins of ZGs have been of great interest in the field. In an early pioneering study, the secreted ZG contents from the guinea pig exocrine pancreas were analyzed by two-dimensional gel electrophoresis which resolved 19 distinct high molecular weight proteins. Thirteen of the 19 proteins were identified by actual or potential enzymatic activity (66). In more recent studies using modern mass spectrometry-based proteomics analyses, the identities of these enzymes were confirmed and additional isoforms of these digestive enzymes were found (11, 60). The ZG content contains primarily the digestive enzymes and associated proteins which make up the major protein components of the pancreatic juice secreted into the duodenum. Therefore, the identification of ZG content proteins also has a significant impact on biomarker studies in the pancreatic juice (15).

Components and topology of the ZG membrane proteins

It is believed that the integral and peripheral ZG membrane proteins serve critical functions for zymogen sorting/packaging, vesicular trafficking and regulated exocytosis. Therefore, a comprehensive identification of ZG membrane proteins is expected to shed new lights on our understanding of ZG biogenesis and secretion. In early studies (20, 35, 49), characterization of rat ZG membranes by SDS-PAGE indicated a relatively simple protein composition of about 10 components with the glycoprotein GP2 accounting for 40% of the proteins. In the past decade, several studies have been carried out to characterize ZG membrane proteins using two-dimensional gel electrophoresis. These efforts led to the identifications of two major ZG membrane components, GP3 (75) and membrane dipeptidase (33), by N-terminal amino acid sequence analysis. However, due to the lack of sensitive tools for protein identification, the identities of many spots resolved on the 2D gels remained unknown. In another study, fourteen spots corresponding to small GTP binding proteins were resolved on a 2D gel of ZG membrane proteins by [^{35}S]GTPγS blotting analysis (26). However, the identities of the spots were undetermined. Different from the above abundant ZG membrane proteins, a number of low abundant regulatory proteins were identified on ZG membrane by immunoblotting and immunocytochemistry. Examples of these proteins included small GTPase, Rab3D (52, 73), and SNARE proteins, VAMP2 (23) and syntaxin 3 (23, 32). More recently, VAMP 8 was found on ZG membrane and to play a major physiological role in regulated exocytosis (77). Despite of the significant amount of knowledge on ZG membrane proteins accumulated in the past decades on an individual basis, a comprehensive characterization of the membrane protein components of this organelle was not achieved until the recent decade. The more recent organellar proteomic analyses using modern mass spectrometry revealed a much more complex makeup of the ZG membrane (10, 11, 60).

Organellar proteomics represents an analytical strategy that combines biochemical fractionation and comprehensive protein identification. Initial purification of organelles leads to reduced sample complexity and links proteomics data to functional analysis (5, 76, 86). In the past decade, organellar proteomic analysis has been carried out for virtually every subcellular compartment in the mammalian secretory pathway including Golgi, ER and secretory granules (76). The first comprehensive analysis of rat ZG membrane were carried out as a natural combination of modern mass spectrometry-based proteomics technologies and a well-established protocol of ZG purification (11). Using this protocol (outlined in Figure 1, *left*), a crude granule pellet (P2) was prepared by two consecutive low speed centrifugations and then further purified by an ultracentrifugation in a self-forming Percoll gradient. A heavy white band, containing highly purified ZGs, was observed and collected just above the bottom of the tube. The ZG membrane and content proteins were then separated by osmotic lysis of ZGs with the ionophore, nigericin, followed by ultracentrifugation. The membrane pellet was washed first with 0.25M KBr and then with 0.1M Na_2CO_3 (pH 11.0) to remove soluble content proteins and loosely associated proteins. The known ZG membrane marker such as Rab3D was highly enriched in the purified ZG (**Figure 1**, *right*).

These new results indicated a much more complex protein composition of the ZG membrane. By combining multiple separation strategies including one-, two-dimensional gel electrophoresis and two-dimensional HPLC with tandem mass spectrometry, over 100 proteins were identified from purified ZG membrane (11). Most of the known ZG membrane proteins were identified, including high abundance matrix proteins such as GP2, GP3, ZG16 and syncollin which are likely involved in ZG sorting and packaging, and low abundance proteins such as
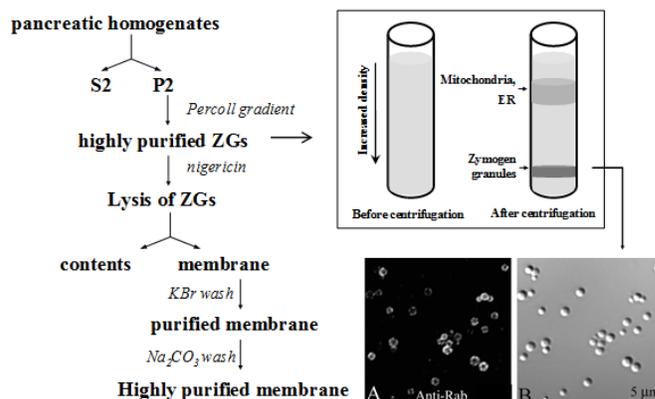

Figure 1. Outline of ZG membrane purification. *Left*, rat pancreata were homogenized and then centrifuged in two consecutive low speed steps to generate a crude particulate fraction (P2) enriched in ZGs. The particulate was resuspended, mixed with equal volume of Percoll, and ultracentrifuged. The dense white ZG band was then collected and washed. To purify ZG membrane, the isolated ZGs were lysed with nigericin and ultracentrifuged to separate contents and membranes. The membrane pellet was then washed sequentially with 250 mM KBr and 0.1 M Na_2CO_3 (pH 11.0). *Right*, Top shows a cartoon to illustrate the Percoll gradient ultracentrifugation; at the bottom are Nomarski and fluorescent images of purified ZGs to demonstrate the purity of ZGs and the positive staining of a ZG marker, Rab3D. Reproduced from reference (11).

dynactin2 (45) and VAMP2 which are involved in ZG trafficking and exocytosis. A large number of novel ZG membrane proteins were also identified, including the SNARE protein, SNAP 29, the small G proteins Rab27B, Rab11A and Rap1 and the molecular motor protein, myosin Vc. Indicative of the interest in understanding the ZG membrane proteome, a later study (60) using 1D SDS-PAGE coupled with 1D LC-MS/MS identified, in a more redundant database, 371 proteins from both ZG membrane and content. A large degree of overlap between proteins was found in these two independent studies. The overlap is higher (nearly 100%) for high score protein identifications and major new observations including all the new vesicular trafficking proteins, whereas the disagreement increases in low score identifications. Representative ZG proteins found in multiple proteomic analyses (10, 11, 60) as well as in some individual studies are summarized in **Table 1**.

As a second step towards a comprehensive architectural model of ZG membrane, a systematic topology analysis of ZG membrane proteins was performed by combining a global protease protection assay with iTRAQ-based quantitative proteomic technology (10). In this study, isolated ZGs were incubated with or without proteinase K. The control and proteinase K treated ZG membrane proteins were digested with trypsin and the resulting peptides were labeled with iTRAQ reagents to compare the relative abundance of each peptide in the two samples. Because proteinase K treatment removed cytoplasm-sided ZGM proteins or domains, the iTRAQ ratios (proteinase K treated vs. control) from all peptides fell into two clusters. The peptides from cytoplasm-sided proteins or domains showed reduced iTRAQ ratios (<<1.0) whereas those luminal ZG proteins showed little change (ratios around 1.0) (**Figure 2**). A threshold was determined using a training set of ZG membrane proteins with well-characterized membrane topology. The category with iTRAQ ratios below the threshold included the cytoplasm-sided peripheral membrane proteins, such as synaptotagmin-like protein 1 and Myosin Vc, and membrane proteins with single transmembrane structure or post-translational lipid modification, such as VAMP 2, 8, Syntaxin 7 and all the Rab proteins. The second category with the ratios above the threshold included the lumen-sided peripheral membrane proteins, such as GP 2, GP 3, syncollin and all of the digestive enzymes. Altogether, this analysis was able to successfully assign the membrane topology for 199 identified ZG proteins (10). The advantage of this technique was to analyze a large number of endogenous proteins simultaneously without the necessity to exogenously express fusion proteins. In addition, this technique was also able to map both cytoplasm- and lumen-sided domains from the same transmembrane proteins.

The overall goal of these recent proteomics studies is to build a quantitative, architectural
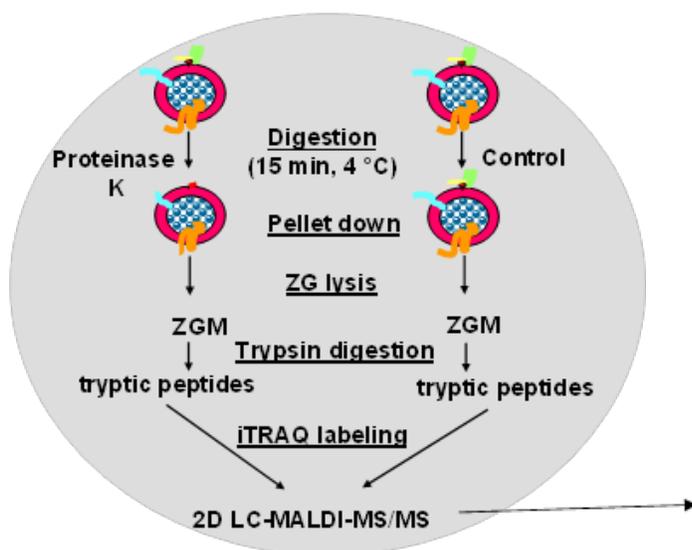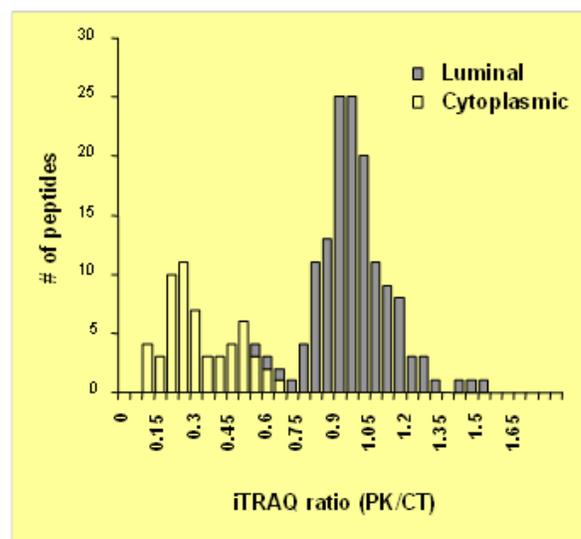

Figure. 2. Work flow of iTRAQ-based topology analysis of pancreatic ZG membrane proteins. *Left*, isolated ZGs were treated with or without proteinase K and then lysed. ZG membrane proteins were digested with trypsin, and resulting peptides were labeled with iTRAQ reagents. The peptides were mixed and analyzed by 2D LC-MALDIMS/MS. *Right*, iTRAQ ratio distributions of tryptic peptides from ZGM proteins with known topology. A histogram of iTRAQ ratios (proteinase K *versus* control) from identified peptides illustrates the presence of two distinct clusters of tryptic peptides from cytoplasm-orientated and lumen-orientated ZG proteins, respectively. Modified from reference (10).

Table 1. Representative pancreatic ZG proteins identified from proteomics analysis and their major functional categories

Protein name	NCBI #	MW	pI	Ref
Digestive enzymes				
Alpha-amylase	62644218	51020	8.42	
Anionic trypsin precursor	67548	28363	4.69	
Chymotrypsin C	1705913	30919	5.64	
Carboxypeptidase A1 precursor	8393183	50282	5.38	
Carboxypeptidase A2 precursor	61556903	50269	5.17	
Cationic trypsinogen	27465583	28821	7.45	
Chymotrypsin B	6978717	25934	4.90	
Colipase	203503	13597	8.04	
Elastase 2	6978803	27274	8.81	
Pancreatic lipase	1865644	54494	6.6	
Pancreatic lipase related protein 1	14091772	57122	5.79	
Similar to elastase 3B	62649890	30806	5.47	
Sterol esterase	1083805	72537	5.37	
Small GTPases				
Rab11A	2463536	24509	5.98	(27, 36)
Rab14	420272	24078	5.85	
Rab1A	56605816	25670	5.95	
RAB27B	16758202	27382	5.38	(9)
RAB3D	18034781	26332	4.75	(52, 73)
Rab8A	77748034	23668	9.15	(17)
Rap1	52138628	21201	5.37	
ZG matrix proteins				
Clusterin	46048420	56070	5.53	(51)
GP2	121538	62355	4.9	(20, 59)
GP3	17105374	58695	6.03	(75, 83)
Syncollin	20806121	17780	8.61	(1, 16)
ZG16	19705541	17316	9.79	(44)
Transporters, pumps and ion channels				
Cation-chloride cotransporter 6	13516403	95862	8.09	
Cation-chloride cotransporter 9	23495276	77073	6.2	
Chloride channel protein 3	4762023	90855	5.88	(42)
L-type amino acid transporter 1	12643400	55903	8.18	
Vacuolar-type H^+-ATPase 115 kDa subunit, a1 isoform	13928826	102385	6.04	(63)
Vesicular trafficking proteins				
Cysteine string protein	1095322	24892	4.93	(7)

Dynactin 2	50926127	44148	5.14	(45)
Myosin Vc	62653910	228341	8.17	
SCAMP1[a]	158749626	37999	7.61	
SNAP29	7769720	29000	5.40	
Synaptotagmin-like protein 1	71043698	59471	5.53	
Synaptotagmin-like protein 4	17939356	75900	9.08	
Syntaxin 7[b]	55741787	29851	5.32	
Syntaxin 12	77695930	31187	5.23	
VAMP 2	51704188	12691	7.84	(22)
VAMP 8	13929182	12512	8.93	
Other proteins				
CD47 antigen	55250722	32995	8.91	
CD59 antigen	6978635	13790	8.9	
CD63 antigen	38648866	29617	7.37	
Dipeptidase 1	16758372	48023	5.68	(34)
Ectonucleoside triphosphate diphosphohydrolase 1 (CD39)	12018242	57337	7.47	
Gamma-glutamyl transpeptidase	16758696	66667	8.46	(6)
Itmap1	5916203	72874	6.07	(38)
Polymeric immunoglobulin receptor	27151742	84798	5.07	

Representative ZG proteins from major functional categories are listed. These proteins were identified on purified rat pancreatic ZGs from multiple proteomics studies (10, 11, 60). Proteins potentially co-purified from other subcellular organelles are not included. References for their original discovery or with independent functional characterization or immunostaining are included. Note: a) SCAMP2, 3, 4, and, b) syntaxin 3 were also identified on ZG membrane.

model of the pancreatic ZG which will lead to new hypotheses for subsequent functional analysis of this prototypic secretory granule. These studies have greatly extended our prior knowledge about the protein composition of this organelle. The ZG localization of a number of novel proteins, including Rap1, Rab6, Rab11A, Rab27B, SNAP29 and myosin Vc, were confirmed by immunocytochemistry (**Figure 3**). Several such novel observations have already led to hypothesis-driven functional studies. One example was Rab27B, subsequently demonstrated as an important regulator of acinar exocytosis (9). Other examples included Rap1 (64) and SNAP 29 (78). The fact that Myosin Vc was also identified on ZG membrane in this study led us to hypothesize that Myosin Vc and Rab27B form a complex to tether ZGs at the apical actin web in an analogy to the Rab27A/melanophilin/Myosin Va complex on melanosomes (19, 31, 70, 84, 85). This hypothesis is further supported by the more recent findings of two potential Rab27 effector proteins, synaptotagmin-like protein (Slp) 1 and 4 (10, 60).

A comprehensive model of the ZG requires the absolute quantity of each individual ZG protein as well as the stoichiometry among different ZG proteins. However, to date, this information has not yet been determined for any ZG protein. Very recently, an absolute quantification (AQUA) proteomics strategy (24, 43) using LC-SRM and isotope-labeled synthetic peptides was used to obtain absolute molar abundances for selected mouse ZG proteins, Rab3D and VAMP8 (40). The
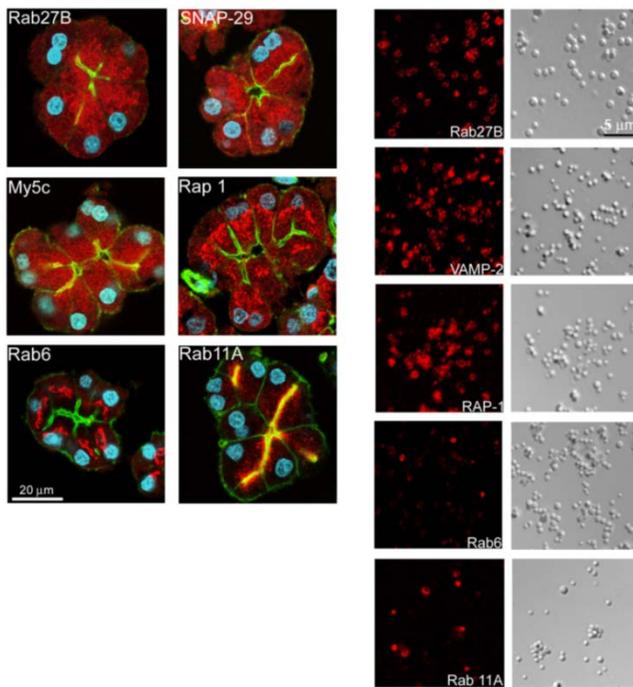
Fig. 3. Immunolocalization of novel small GTPases and SNARE proteins to isolated ZGs. The ZG localizations of some of the novel small GTPases and SNARE proteins were confirmed at isolated ZGs level by immunocytochemistry and confocal microscopy. The immunofluorescent images (red) together with corresponding DIC images for VAMP2, Rab11a, Rap1 and Rab27b are shown. While VAMP2, Rap1 and Rab27b stained every ZGs as indicated by circles outlining individual ZGs, only a portion of ZGs showed positive Rab11a staining. Reproduced from reference (11).

absolute quantities of mouse Rab3D and VAMP8 were determined as 1242 ± 218 and 2039 ± 151 (Mean ± SEM) copies per ZG. The size distribution and the averaged diameter of ZGs (~750 nm) were determined by atomic force microscopy (40). Furthermore, human ZGs were purified from human acini obtained from pancreatic islet transplantation center and the comprehensive constituents of human ZGs were characterized for the first time (40). 180 human ZG proteins were identified including both the membrane and the content proteins. The identification of human ZG specific content and membrane proteins is expected to have a significant impact on translational studies to look for biomarker in pancreatic juice from cancer and pancreatitis patients.

Functional categories of ZG proteins

The secretory granules in neuroendocrine, endocrine and exocrine cells share fundamental molecular mechanisms in granule formation, intracellular trafficking and regulated exocytosis (3, 30, 69). For ZGs, this multi-step process includes budding of immature granules off the trans-Golgi network, granule maturation and granule transport towards the apical pole in the vicinity of plasma membrane, tethering/docking at the plasma membrane and regulated exocytosis of ZG contents triggered by the rise of local Ca^{2+} concentration upon hormonal and neuronal stimulations (**Figure 4**). In spite of a general model outlined above, the detailed molecular mechanisms are not yet completely understood. The ZG membrane is believed to carry at least part of the molecular machinery responsible for each of these steps. Therefore, the recent comprehensive identification of the ZG protein components has shed new light on ZG biogenesis, trafficking and exocytosis (10, 11, 28, 60). The identified ZG proteins fall into several broad functional categories which link their identities to potential functional importance. These categories include proton pumps and ion channels, enzymes, vesicular trafficking proteins, matrix and glycoproteins, small GTP-binding proteins (**Table 1**).

The ZG matrix protein group includes the highly abundant ZG proteins such as GP2, GP3, ZG16 and syncollin, previously known to be present on the inner surface of ZG membrane and likely involved in zymogen sorting and packaging (more detailed discussion in the next section) (41, 44, 46, 69). In addition to the high abundance structural proteins and enzymes, many of the known functional and regulatory proteins were also identified on the outer surface of the ZGs. These included vesicular trafficking proteins such as SNARE proteins VAMP 2 and VAMP 8, molecular motors myosin Vc and dynactin 2, a subunit of dynactin adaptor complex for the minus end-driven microtubular transport motor, dynein. A significant numbers of small GTPases were

identified including multiple Rabs and Rap1. Among them, only Rab3D was previously reported on ZGs (52, 53, 72, 73). These newly identified ZG-localized small GTPases represented one of the major novel findings from the extensive proteomics analyses of ZG membrane. Ion channels and transporters are usually low abundant proteins and with multi-transmembrane domains which make them hard to detect by proteomics approaches. Within this category, various subunits of the vacuolar H+-ATPase (V-ATPase) have been constantly identified in multiple proteomics studies (10, 11, 60). A few other ion channels and transporters have also been identified in individual proteomics studies (**Table 1**). In addition, functional evidence indicates the presence of several ion channel and transporter proteins in ZG membranes (aquaporins, vacuolar-type H+-ATPase, zinc influx transporter SLC30A2). The evidence for the

K$^+$ channels Kv7.1 and Kir6.1, for ClC Cl$^-$ channels and the vesicular nucleotide transporter SLC17A9 in ZG is less strong. Detailed discussion of channels in ZG can be found in (71).

3. ZG Biogenesis - Sorting and Maturation

Protein sorting at the trans-Golgi Network (TGN) is particularly important for professional secretory cells, such as pancreatic acinar cells. In contrast to most eukaryotic cells that secrete proteins constitutively via TGN-derived vesicles, acinar cells store their regulated secretory product, zymogens, in granules that undergo exocytosis in a stimulus-dependent manner. In acinar cells, a mixture of different zymogens are packaged within the TGN, some zymogens form protein complexes and progressively aggregate in a Ca^{2+}-
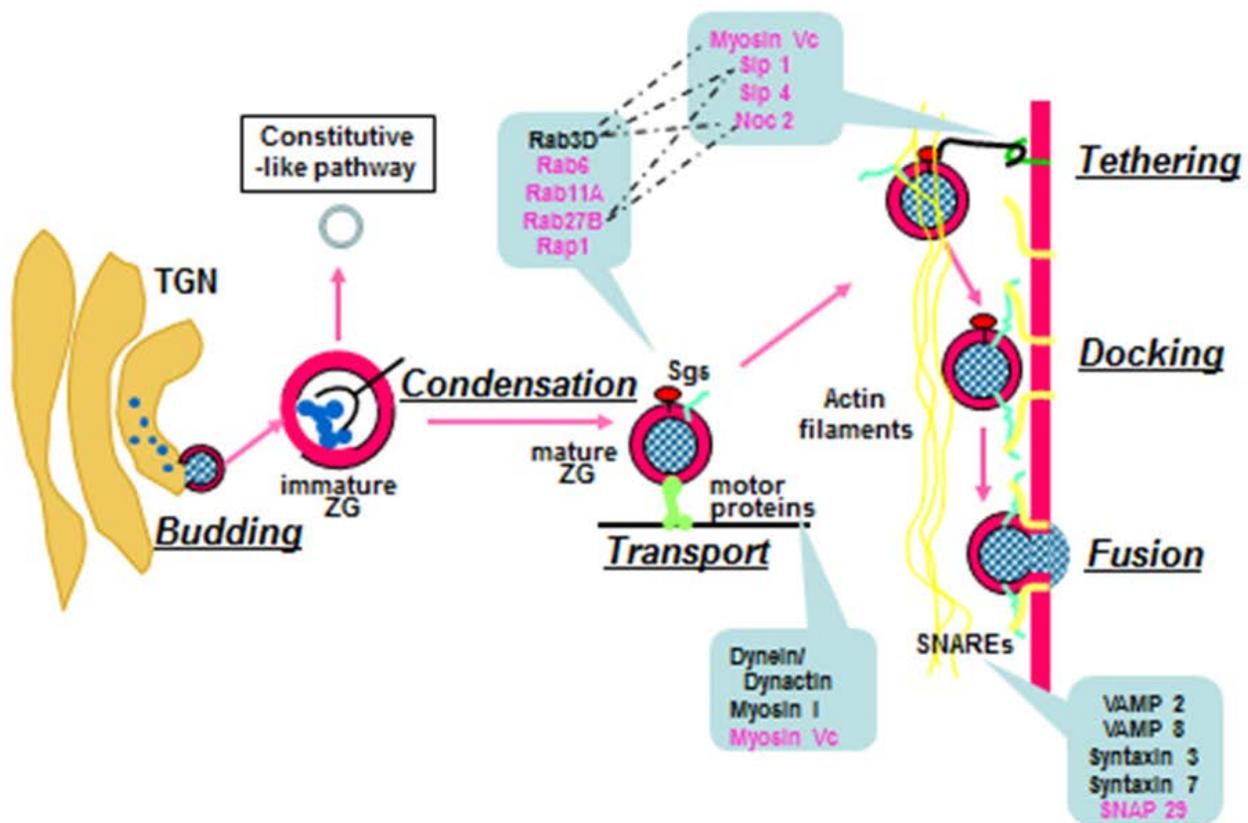

Figure 4. A working model of ZG biogenesis and regulated exocytosis. For ZGs, this multi-step process includes the budding of immature granules from the trans-Golgi network (TGN), granule maturation through condensation and transport towards the apical pole in the vicinity of plasma membrane, tethering/docking at the plasma membrane and regulated exocytosis of ZG contents through membrane fusion. Modified from reference (80).

and pH- dependent manner. Parts of the Golgi cisternae become dilated and these condensing vacuoles (CV) pinch off to become immature granules (IG) and then mature into ZGs. The selective aggregation of pancreatic secretory proteins has been well documented (13, 18, 48), but the underlying molecular mechanism by which the secretory proteins are sorted into the regulated secretory pathway is still not well understood. Two different hypotheses, not necessarily mutually exclusive, have been developed to explain the selection of content proteins for storage in the secretory granules (4, 30).

The sorting-for-entry hypothesis proposes that the TGN acts as the primary protein sorting station in the biosynthetic transport pathway. This is based on the paradigm for newly synthesized lysosomal hydrolases which are sorted into clathrin-coated vesicles by mannose-6-phosphate receptor (MPR) and then targeted to endosomal membranes. For the regulated secretory proteins, the sorting-for-entry model postulated the presence of TGN membrane associated sorting receptors to facilitate cargo entry into IGs. Through this mechanism, only selected secretory proteins can enter IGs, whereas other proteins such as those targeting to the constitutive secretory pathway are efficiently excluded. Careful immuno-electron microscopy observations have demonstrated the segregation of regulated and constitutive cargo at the level of the TGN (54), supporting an active sorting mechanism.

In the sorting-by-retention hypothesis, the IG serves as an important post-TGN sorting station. In this model, protein entrance into IGs is largely unselective and high-order intermolecular associations allow regulated secretory proteins for efficient retention within maturing granules. Concurrently, a subset of protein components is removed via receptor-mediated sorting or bulk flow. The driving force underlying this subtractive retention involves assembly of granule core proteins within IGs by aggregation/condensation,

the progressive protein insolubility within the luminal environment of maturing granules in a Ca^{2+}- and pH- dependent manner. Condensation of regulated secretory proteins allow them to remain in the maturing granules while the lysosomal proteins are removed by constitutive-like vesicle budding.

In pancreatic acinar cells, membrane proteins involved in the sorting and packaging of zymogens are expected to have their functional domains exposed on the luminal side of the ZG membrane. In the past decades, studies have been focused on several abundant luminal ZG membrane proteins for their potential roles in zymogen sorting and ZG formation (69). GP-2 represents up to 40% of the total ZG membrane proteins in rat ZG (62). The membrane association of GP-2 is via a glycosylphosphatidylinositol (GPI) anchor (47, 67). Since GP-2 can form stable complexes with zymogens at mildly acidic pH but not at alkaline pH (12, 48), it was suggested that GP-2 may act as a sortase for aggregated secretory proteins (39). However, the findings that ZGs in mouse pancreas can form in the absence of GP-2 indicated it is not required for ZG biogenesis (87). Another abundant luminal ZG membrane protein, syncollin, is a component of lipid rafts (41). While the rates of synthesis and intracellular transport of secretory proteins were reduced in syncollin-deficient mice, these mice are viable and showed no detectable changes in pancreatic morphology, regulated exocytosis, or zymogen content (2). So syncollin does not seem to be required for ZG biogenesis either. While several other luminal membrane associated ZG proteins have also been studied in ZG biogenesis, it is still not clear that any of these proteins alone is indispensable for ZG formation.

4. ZG Trafficking and Exocytosis

Upon nervous and/or hormonal stimulation (82), ZGs move towards the apical plasma membrane in a microtubule- and actin-dependent manner

(45, 74). Secretagogue stimulation of the cells causes an elevation of the intracellular Ca^{2+} concentration, which in turn triggers granule fusion. Detailed discussion of ZG exocytosis can be found in (14).

For ZG membrane proteins, major new findings from the recent studies came from the vesicular trafficking and small GTP-binding proteins groups (10, 11, 28, 60, 80). Among the small GTPase identified on ZG membrane, only Rab3D was previously reported on ZGs. In addition, punctate subapical staining of Rab11 just deep to the apical plasma membrane was observed in pancreatic acinar cells (27). The ZG localization of the majority of newly identified small G proteins including Rab27B, Rab11A, Rap1 and Rab6 were confirmed by immunocytochemistry at the isolated acini and ZG level (Figure 3). In subsequent functional studies, it was shown that Rab27B localizes on ZGs and plays an important role in regulating acinar exocytosis (9, 37). Furthermore, Rap1 was localized for the first time on pancreatic ZGs although it had previously been localized on parotid secretory granules and it was found that Rap1 activation plays a regulatory role in pancreatic amylase secretion (64). While Rab3D and Rab27B were present on all the ZGs, Rab6 and Rab11A localized to only a fraction of ZGs (**Figure 3**). This could indicate the existence of different subpopulations of ZGs. However, because Rabs can be extracted by corresponding GDIs from the membranes and cycles between membrane and cytosol, an alternative interpretation is that this represents ZGs at different stages in the secretory pathway. In addition to the small G proteins, we also found a novel SNARE protein, SNAP29, on ZGs. The ZG membrane localization of SNAP29 was confirmed by immunocytochemistry. Furthermore, it was found that SNAP29 and VAMP2 formed a complex on ZG membrane (78).

Another important category of molecules critical to vesicular trafficking and exocytosis are the molecular motors and corresponding adaptors. It was reported previously that myosin Vc localized to the exocrine pancreas and largely overlaps with apical F-actin (61). Proteomics analysis identified the presence of myosin Vc on ZG membrane. It is of interest that Rab27a forms a complex with myosin Va on melanosome through a synaptotagmin-like linker protein, melanophilin. This complex is required to tether melanosomes to the actin cytoskeleton. In acinar cells, Rab27B, myosin Vc and at least two synaptotagmin-like proteins, Slp1 and Slp4, are present on ZG membrane. By analogy, Rab27B, slps and myosin Vc could form a complex on ZGs to regulate the tethering of ZGs at the apical membrane. In exocrine pancreas, targeting of ZGs to the apical cell surface requires an intact microtubule system and is then transferred to actin (25). The minus end-driven microtubular transport is thought mediated by molecular motor, dynein rather than kinesin. Purified ZGs were found to be associated with cytoplasmic dynein intermediate and heavy chain and to contain the major components of the dynein activator complex, dynactin. Consistent with this report, proteomics analysis identified a component of the dynactin complex, dynactin2/dynamitin from highly purified ZG membrane. Interestingly, it has been demonstrated that Rab6 functioned as a tethering factor controlling the recruitment of dynactin to membranes.

5. Conclusions and Future Directions

The pancreatic ZG has been a prototypic model for all secretory granules in the regulated secretory cells. Since its discovery and initial morphological characterization by electron microscopy, information has been uncovered regarding its protein compositions and molecular mechanisms to govern its biogenesis, intracellular transport and regulated exocytosis. With the help from modern proteomics technologies, a very detailed molecular model of ZG content and membrane is being established. In the foreseeable future, a comprehensive molecular

architecture of ZG will be developed including protein components, their membrane topologies, their copy numbers per ZG and protein complexes they are associated with. This comprehensive molecular view will hopefully be accompanied by a thorough mechanistic understanding of the detailed steps on how the immature granules are formed from TGN and condensed into mature ZGs, as well as how they are released upon physiological stimulations.

6. References

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