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Phosphatidylinositol Bisphosphate Mediated Sorting of Secretory Granule Cargo

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1. Introduction

Every cell must sort and transport proteins. This is true for soluble proteins as well as proteins that are in membranes, each of which need to be directed to appropriate subcellular or extracellular destinations in order to perform their essential functions. In eukaryotes, selective trafficking contributes to maintaining the different compositions of different membranes such as apical and basolateral plasma membranes, as well as directing appropriate proteins to lysosomes, endosomes, multivesicular bodies, or other intracellular compartments. The normal physiology of the cell is critically dependent on selective trafficking of proteins and membranes between different transport pathways within the cell. Other chapters in this book focus on the mechanics of transporting cargo membranes, including the molecular aspects of vesicle fusion to specific target membranes. This chapter will focus on the importance and mechanisms of sorting luminal cargo into different pathways, i.e., the "selective" aspect of selective trafficking, particularly with respect to exocrine secretion.

Selective trafficking of new proteins is largely achieved by budding of vesicles from the trans-Golgi network (TGN) for transport to specific organelles or to specific regions of the plasmalemma. Different terminology is used for these vesicles depending on their size, histological appearance, contents, or cell type. Granules (including dense-core secretory granules, DCSG) are secretory vesicles present in endocrine, exocrine, immune, and neuroendocrine cells, responsible for both storage and secretion of proteins. Lymphocytes, dendritic cells, and natural killer cells also contain secretory lysosomes for the release of lysosomal enzymes (Stanley and Lacy 2010), and neurons contain peptidergic synaptic vesicles (Park and Loh 2008; Park et al. 2011). However, all of these types of vesicles serve the same broad purpose of transporting specific cargo to specific destinations by an appropriately regulated pathway.

The lipid membranes of these vesicles carry tightly associated cytoplasmic proteins (termed coat proteins) which not only help form the vesicle, but also direct the vesicle to the correct destination (De Matteis and Luini 2008; Santiago-Tirado and Bretscher 2011; Wilson et al. 2011). The matrix of coat proteins on the cytosolic face of the membrane contributes to the bending of the TGN membrane during budding of the vesicle. This matrix is formed by multiple interactions, including binding of coat proteins to phosphatidylinositol phosphates

(PtdInsPs) in the TGN membrane, interactions between the coat proteins, and binding to integral proteins of the TGN membrane. Importantly, populations of vesicles are distinguished by the presence of specific combinations of coat proteins, such as clathrin, Adaptor Proteins (AP1-4), FAPP1/2, GGAs, ARF, v-snares, and synaptotagmin. In the parotid gland, VAMP2, VAMP8, syntaxin4/6, and synaptotagmin decorate the cytoplasmic side of secretory granules (Fujita-Yoshigaki et al. 2006; Wang et al. 2007). These different coat proteins on different vesicles direct the vesicles to the correct target membranes. For example, FAPP2 is critical for constitutive apical trafficking, whereas FAPP1 directs basolateral trafficking (Vieira et al. 2005). The coat proteins also mediate interactions with other proteins, including actins, to mediate transportation of that vesicle.

Having vesicles destined for different targets raises the central question of how does the correct cargo get put into just the correct type of vesicle? These post-TGN vesicles carry integral membrane proteins, which are one type of cargo delivered by this process. Transmembrane cargo proteins (such as MPR300) are localized by direct interactions with coat proteins (such as GGAs) on the outside of the forming vesicle as it buds from the TGN (Ghosh et al. 2003). Sorting sequences which mediate these interactions have been identified on the cytosolic tails of many transmembrane cargo proteins (Folsch et al. 2009). Hence, the problem of sorting membrane cargo proteins to the correct vesicle has an elegant solution based on direct interactions of transmembrane cargo with the coat protein complex which identifies that vesicle and targets it to the correct destination (recently reviewed in (De Matteis and Luini 2011)).

Importantly, the lumen of the vesicle contains a different type of cargo composed of specific soluble proteins. Luminal cargo proteins include lysosomal enzymes, hormones, cytokines, neurotransmitters, digestive enzymes, and salivary proteins. As can be seen from this list, soluble cargo proteins are present in a variety of different secretory cell types. These luminal cargo proteins cannot interact directly with the coat proteins on the cytoplasmic (outer) surface of the vesicle membrane; therefore, other mechanisms must be involved to localize the correct soluble cargo protein into the vesicle destined for the correct target, and not into the incorrect vesicles. This is an information transfer problem, i.e., how to get the information encoded by the cytoplasmic coat proteins (which determine the destination of the vesicle) to select the appropriate luminal cargo proteins.

Information transfer for sorting is a typical problem in any distribution network and must be solved by companies involved with distribution, such as UPS or FedEx. The need for solutions to such problems is reflected by the growing number of Logistics and Distribution programs at universities. Notably, eukaryotic cells developed solutions to these logistics problems many eons ago. For an exocrine or endocrine cell, the problem is how to get the lysosomal enzymes (soluble cargo proteins) into a forming TGN vesicle destined for the lysosome, and secretory cargo proteins into a different TGN vesicle destined for the plasmalemma. This requires the transfer of information from the cytosolic side of the forming vesicle membrane to the luminal side of the membrane.

While there are some good model systems, we do not have a clear understanding of the molecular mechanisms that direct the sorting of soluble cargo proteins between different vesicles. Nonetheless, this is an important issue since all eukaryotic cells produce several different types of vesicles at the TGN (Folsch et al. 2009), and many cell types secrete proteins by specific pathways such as apical versus basolateral pathways. Changes in

trafficking not only affect the physiology of the cell, but also embryonic development (Shilo and Schejter 2011) and disease. This chapter will focus on sorting (selective trafficking) for secretion of luminal cargo proteins. We will review some of the general aspects of selective trafficking, and then build on that background by focusing on our recent work suggesting a novel mechanism for sorting in the parotid salivary gland.

2. Biogenesis during trans-Golgi network vesicle trafficking

Secreted proteins are translated at the rough endoplasmic reticulum and transit from the ER, through the ER-Golgi Intermediate Compartment (ERGIC) to the Golgi and subsequently the trans-Golgi network (TGN) (Shilo and Schejter 2011). Post-translational modifications such as glycosylation occur in the ER and Golgi. Membranes on the trans side of the Golgi apparatus form dynamic tubular reticular structures having a large surface area (De Matteis and Luini 2008). This network of saccules and tubes is continuously remodeled such that both the structure and size of the TGN varies depending on the secretory activity and the cell type (Trucco et al. 2004). Selective trafficking at the TGN will sort cargo into vesicles or carrier tubules (De Matteis and Luini 2008), and this sorting requires the genesis of carrier vesicles targeted to specific membranes within the cell. As noted above, these vesicles are distinguished by the combination of coat proteins on the cytosolic face of the membrane which determine the target membrane for that vesicle. In some pathways additional sorting occurs at the recycling endosome (reviewed in (De Matteis and Luini 2008; Santiago-Tirado and Bretscher 2011)).

The initiating event in vesicle biogenesis may be driven by the local membrane lipid composition where asymmetry in the types of lipid in the two faces of the membrane bilayer can induce bending (van Meer and Sprong 2004). Initiation may also involve membrane rafts, which are reported to be present in the TGN of all cells (Park and Loh 2008), and on membranes of secretory granules (Hosaka et al. 2004; Lang 2007; Guerriero et al. 2008). While membrane rafts are well characterized to play important roles in endocytosis at the plasmalemma, their role in vesicle formation at the TGN is not as well understood. Many vesicle coat proteins have been localized to lipid rafts on vesicles (Puri and Roche 2006). This has been suggested to be important for the formation of the coat protein complexes on the TGN for the initial creation of vesicle buds and selective trafficking (Simons and Sampaio 2011). For example, SNARE proteins are enriched in cholesterol-dependent rafts in beta-cells and PC-12 cells (Lang 2007). However, technical issues have called into question the validity of some methods for isolation of 'lipid rafts', leading to a more stringent definition and the term 'membrane rafts' (Lang 2007). Nonetheless, even with the more stringent approach, SNAREs such as VAMP2 and VAMP3 are enriched in membrane rafts of vesicles. Membrane rafts can contain different complements of proteins due to specific protein interactions. One model for the formation of vesicle buds is that membrane rafts on the TGN (with associated transmembrane cargo proteins) coalesce creating a lipid domain that is favorable to bending the membrane, and containing transmembrane proteins which can facilitate the decoration of the cytosolic face of the membrane with adaptor and other coat proteins (De Matteis and Luini 2008; Simons and Sampaio 2011). Testing the relevance of this model to selective sorting for secretion in living cells is important, and requires determining whether trafficking vesicles in different pathways (e.g., regulated secretion versus constitutive secretion pathways) contain different types of membrane rafts, or that

some pathways lack membrane rafts. Importantly, Guerriero et al. recently found that raft-independent and raft-associated proteins collect in distinct sites at the Golgi, and likely enter different vesicles (Guerriero et al. 2008).

2.1 Phosphatidylinositol phosphates in biogenesis of secretory vesicles

In all cell types, the earliest events that are strongly linked to the biogenesis of secretory vesicles is the binding of coat proteins to phosphatidylinositol phosphate lipids (PtdInsP) and PI-kinases in the TGN membrane. The phosphoinositides and small GTPases of the Arf and Rab families define the identity of the membrane and recruit additional coat proteins (Di Paolo and De Camilli 2006; De Matteis and Luini 2008; Santiago-Tirado and Bretscher 2011). PtdInsPs are recognized as being critical for selective trafficking of vesicles within cells (Di Paolo and De Camilli 2006; D'Angelo et al. 2008; Vicinanza et al. 2008; Graham and Burd 2011). Phosphatidylinositol comprises less than 10% of membrane phospholipids, and the individual phosphorylated forms total less than 1.5% of lipids (Di Paolo and De Camilli 2006; Roth 2004). Phosphatidylinositol can be phosphorylated on any combination of carbons 3, 4, or 5 of the inositol ring (Fig. 1). The most abundant, PtdIns4P, occurs at approximately 0.05% of membrane lipids, whereas the low abundance forms such as PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 are approximately 0.0001% each (Cullen 2011). Subcellular pools of the 7 different PtdInsPs have diverse regulatory roles in cytoskeleton remodeling, second messenger signaling, endosomal trafficking, membrane trafficking, osmotic stress, nuclear signaling, and other aspects of cell physiology (Godi et al. 2004; Balla and Balla 2006; Di Paolo and De Camilli 2006). Cellular effects can be mediated by signaling through production of second messengers (diacylglycerol, and inositol trisphosphate). However, many cellular effects are mediated by the localized anchoring of cytosolic proteins having specific PtdInsP-binding domains (e.g., PH, FYVE, PX, ENTH-domains) (De Matteis et al. 2005; Balla and Balla 2006). For example, Arf1 directly binds and recruits PI4-Kinases to the TGN. This produces PtdIns4P which aids in recruitment of coat proteins such as AP1, GGAs, VAMPs, and FAPP1/2 most of which interact with PtdIns4P as well as other proteins in the coat matrix (Balla and Balla 2006). This interaction provides identity to the membrane (reviewed in (Santiago-Tirado and Bretscher 2011)). PI3K-C2α is also present on the TGN. Each type of PtdInsP is localized to specific membranes within the cell. The most abundant phosphoinositides, PtdIns4P, and PtdIns(4,5)P_2, are predominantly localized to the Golgi and plasma membrane, respectively, whereas PtdIns3P and PtdIns(3,5)P_2 are predominantly localized to early and late endosomes (Di Paolo and De Camilli 2006; Santiago-Tirado and Bretscher 2011). PtdIns(3,4)P_2 is rare in resting cells, but is present in the plasmalemma and multivesicular and early endosomes (Roth 2004; Di Paolo and De Camilli 2006). PtdIns(3,4)P_2 is not noted as being present in the Golgi or TGN (De Matteis et al. 2005), however, this

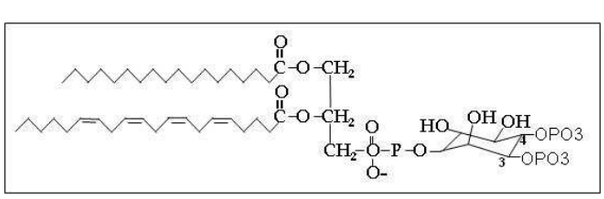

Fig. 1. Structure of Phosphatidylinositol (3,4)bisphosphate. PtdIns(3,4)P_2 is an exceedingly rare membrane lipid that is highly localized to specific subcellular membranes within the cell.

has not been well characterized in any cell type. Recently, PtdIns(3,4)P_2 was found to be transiently synthesized at the plasmalemma as a second messenger of platelet-derived growth factor (PDGF) (Hogan et al. 2004). Importantly, our understanding of the roles of PtdInsPs derives primarily from studies of yeast and a few mammalian cell lines. It is unclear whether these generalizations carry over to well differentiated cells in tissues. For example, we find high levels of expression of PtdIns(3,4)P_2 in parotid secretory granule membranes.

Discrete and dynamic localization of PtdIns-kinases (PI-kinases) and PI-phosphatases regulate the production of PtdInsPs. Of these enzyme families, PI4-kinases have the dominant roles in Golgi function and secretion, although PI3-kinases and PI5-kinases also have roles in secretion (Wang et al. 2003; Roth 2004; Balla and Balla 2006). Several PI4-kinases are localized to the Golgi, TGN, or endosomes, and form complexes with several coat proteins including GGA and FAPP1/2 (D'Angelo et al. 2008). Careful immunofluorescence co-localization studies with MDCK cells have found PI4KIIα in the TGN and PI4KIIIβ in the cis/medial Golgi (Weixel et al. 2005). This is consistent with PtdIns4P being the most abundant PtdInsP in the Golgi. PI4KIIIβ is recruited by the coat protein Arf1 to the Golgi, and in turn PI4KIIIβ recruits Rab11 and the PtdIns4P-binding protein FAPP1 (Godi et al. 2004). Knock-down of PI4KIIα by RNAi has little effect on intra-Golgi transit, but inhibits TGN export of vesicles (Wang et al. 2003). These studies are interpreted as showing that PI4-kinases produce PtdIns4P on the cytosolic leaflet of the TGN and vesicles, which anchors essential adaptor proteins (Santiago-Tirado and Bretscher 2011). Importantly, class I PI3-kinases are also present on Golgi membranes, and are essential for tumor necrosis factor (TNF) secretion by macrophages (Low et al. 2010). Similarly, PI3-kinase was localized to secretory granules of PC12 cells by both immunofluorescence and cell fractionation (Meunier et al. 2005). Transfection of a PtdIns3P-binding domain (FYVE), or a catalytically-inactive PI3-kinase, blocked regulated secretion, possibly by interfering with fusion of the granule at the plasmalemma (Meunier et al. 2005). In summary, certain PI-kinases decorate the Golgi and TGN, interact with coat proteins, and produce PtdInsPs. These PtdInsPs are important for vesicle trafficking, however, the identities and roles of PI-kinases have not been investigated in cells that are highly specialized for bulk exocrine secretion, such as the parotid.

3. Selective trafficking for secretion

The presence of multiple different trafficking pathways for secretion has been described in many cell types (Dikeakos et al. 2007; De Matteis and Luini 2008; Park et al. 2008; Folsch et al. 2009; Perez et al. 2010; Stanley and Lacy 2010; Lacy and Stow 2011; Santiago-Tirado and Bretscher 2011). The best characterized of these pathways have a specific cargo protein that is a unique marker of that route, which is essential for molecular characterization (Lara-Lemus et al. 2006). For example, the cytolytic protein perforin of natural killer cells undergoes polarized secretion into the immune synapse, whereas the same cells secrete TNF in a non-polarized pattern (Reefman et al. 2010) thereby marking a different pathway. Also, some pituitary gonadotropes segregate luteinizing hormone into separate granules than follicle-stimulating hormone for different regulated secretion dynamics (Nicol et al. 2004). Alternatively, pathways can be marked by the use of different coat proteins. For example, the coat protein FAPP1 is essential for secretion by the basolateral pathway, whereas FAPP2

directs vesicles to the apical pathway (Godi et al. 2004; Vieira et al. 2005). Importantly, mutation of proteins involved with sorting between trafficking pathways can cause disease. This is also seen with non-genetic diseases, such as pancreatitis during which inappropriate basolateral (endocrine) secretion of cargo proteins occurs. Hence, pancreatic amylase or lipase in the serum are standard clinical markers of this disease. Given the presence of multiple different pathways for secretion, the key issue is to understand the molecular interactions of proteins destined for each pathway, which cause sorting to the correct immature granule or tubule as it forms, or which cause retention of the protein in the granule as it matures.

The best characterized model for specific sorting of soluble cargo proteins involves a transmembrane sorting receptor protein (Fig. 2). The receptor protein is present in the TGN membrane and is able to interact with coat proteins on the cytosolic side of the vesicle bud, and can also bind luminal cargo proteins. The transmembrane receptor is localized to the vesicle bud by the appropriate coat proteins, and in turn, selects the correct cargo. Hence, a single protein serves to coordinate the identity of the vesicle with the luminal contents. This model is exemplified by mannose-6-phosphate sorting receptors (MPRs), which are type I transmembrane receptors present in the TGN (Ghosh et al. 2003). Both the cation-dependent (MPR300) and the cation-independent (MPR46) MPRs deliver lysosomal enzymes from the TGN to endosomes for subsequent transfer to lysosomes. The cytosolic tails of MPRs have specific binding sites for multiple adaptor proteins, including AP1, AP2, GGAs and PACs1 (reviewed in (Ghosh et al. 2003)). In addition, the portion of MPR in the lumen of the vesicle binds to mannose 6-phosphate tags on cargo proteins. The mannose 6-phosphate is a specific posttranslational modification on the N-glycans of over 60 of acid hydrolases which need to be transported to lysosomes. Failure of this sorting causes lysosomal sorting disease, Mucolipidosis type II alpha/beta (I-cell disease) (Ghosh et al. 2003). A similar sorting mechanism has been described for stabilin-1 which binds GGAs and serves as a sorting receptor for a chitinase-like enzyme.

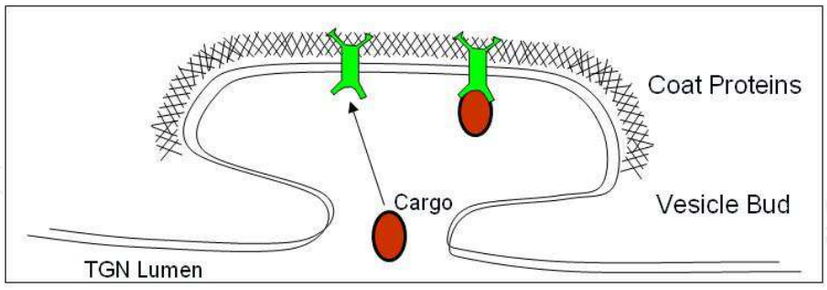

Fig. 2. Transmembrane sorting receptor model. The coat proteins localize a transmembrane sorting receptor, which collects the appropriate luminal cargo.

Various mechanisms have been suggested for sorting of secreted proteins into the regulated secretory pathway, as opposed to the constitutive secretory pathway or trafficking to intracellular targets (Dikeakos and Reudelhuber 2007; Park et al. 2008). Some proteins are secreted by the model discussed above. Phogrin (Ptprn2) is a type I transmembrane receptor present in the TGN and secretory granules of endocrine and neuroendocrine cells. Phogrin contains tyrosine and leucine motifs in the C-terminal (cytosolic) tail, which are important for localization to secretory granules, likely by interaction with coat proteins such as AP1

(Saito et al. 2011). Within the lumen, phogrin can bind to carboxypeptidase E (CPE), and contributes to sorting of the complex (Saito et al. 2011). Hence, information about the identity of the vesicle encoded by the coat proteins, is related through phogrin to determine the luminal cargo. Phogrin also has PI-phosphatase activity which may be important for regulating glucose-stimulated insulin secretion (Caromile et al. 2010).

Other sorting receptors have been reported in neural and endocrine cells. CPE itself binds the granule membrane. The C-terminus of CPE can span the membrane, although only 5 amino acid residues are cytosolic (Dhanvantari et al. 2002). Recycling of CPE from the plasmalemma requires ARF6 apparently due to direct binding to the coat protein. CPE acts as a sorting receptor for proopiomelanocortin (POMC) and proBDNF trafficking into the regulated secretion pathway (reviewed in (Park and Loh 2008)). Secretogranin III (SgIII) can also serve as a sorting receptor. Despite the absence of a transmembrane domain, SgIII binds to granule membranes or to cholesterol-rich liposomes, and anchors chromograninA (CgA) to the membrane (Hosaka et al. 2004). SgIII also interacts with CPE, POMC, and adrenomedullin (Hosaka et al. 2005; Han et al. 2008). It is unclear how SgIII is targeted to specific granules, but this may occur due to selective interactions with membrane rafts, or due to interaction with CPE and, indirectly, phogrin.

Dikeakos et al. (Dikeakos et al. 2007; Dikeakos and Reudelhuber 2007) have shown that a hydrophobic patch in short amphipathic alpha helices is sufficient to sort cargo proteins. Helical domains are implicated in sorting of somatostatin, CPE, prohormone convertase enzymes (PC1/3, PC2), and chromogranin A (CgA). The proposed mechanism for this sorting is that the hydrophobic patch of the helix embeds into the membrane of the forming granule (Dikeakos and Reudelhuber 2007). One of the sorting-competent helical domains (Hels13-5) bound liposomes composed of phosphatidylcholine (PC) and phosphatidylglycerol (PG) with a Kd=9.7 μM (Kitamura et al. 1999). This Hels13-5 peptide integrated into the non-polar layer to a variable extent depending on the pH of the liposome. No cholesterol was necessary for this interaction. In addition, the helical domain of PC1/3 was shown to directly interact with CHAPS detergent micelles (Dikeakos et al. 2009). Characterization of both natural and artificial helices which confer sorting of a cargo protein at the TGN is an important step forward, however, at this point it is unclear how the identity of the vesicle (determined by the cytosolic coat proteins) directs selective sorting of such cargo into the correct pathway.

For many years, aggregation of secreted proteins has been seen as critical to sorting into the regulated secretory pathway in neuroendocrine cells (Gorr et al. 2005). Dense-core secretory granules of the regulated pathway contain large aggregates comprising chromogranins secretogranins and other secreted proteins, and which are not present in the constitutive secretion pathway. Aggregation of granins and many hormones can be demonstrated *in vitro* in a Ca^{++} and pH-dependent fashion. The pH of the TGN is approximately 6.2. In AtT20 cells the pH decreases further as the granule matures, reaching pH 5.5 in mature secretory granules (Wu et al. 2001). The acidic pH and high Ca^{++} present in the regulated secretory pathway is essential for aggregation. This relatively non-specific interaction may allow trafficking of large aggregates of proteins even where only a few specific interactions with transmembrane sorting receptors are present. Nonetheless, it must be recognized that aggregation is a fairly ill-defined concept, and it will be necessary to characterize these interactions in order to determine how some cargo is excluded from the aggregate to be sorted into the constitutive pathway.

4. Sorting for regulated secretion in the parotid gland

The parotid salivary gland provides an excellent model for the study of regulated secretion of proteins. This gland has evolved to secrete copious amounts of specific proteins into the saliva. It secretes salivary proteins including amylase, Parotid Secretory Protein (PSP), a family of acidic (aPRP) and basic (bPRP) Proline-Rich Proteins, and less abundant proteins such as histatin and statherin (Helmerhorst and Oppenheim 2007). Secreted PSP has anti-bacterial activity which contributes to protection of the oral cavity (Gorr et al. 2011). Human PSP (SPLUNC2, BPIFA2) has been shown to be expressed in saliva as several isoforms due to alternative splicing of the mRNA (Bingle et al. 2009; Bingle et al. 2011). Another abundant salivary protein, amylase, initiates digestion of starch, and also adheres to oral bacteria and enamel. PRPs contribute to secretion of other cargo proteins (Venkatesh and Gorr 2002; Venkatesh et al. 2007), are part of the acquired dental pellicle, and also bind bacteria. These three proteins are the most abundant luminal cargo proteins within the secretory granule. In addition, hundreds of other proteins are secreted into saliva by the parotid gland, and have been cataloged by proteomic approaches (Denny et al. 2008). As these proteins move through the trans-Golgi network, they are each presumably sorted into the correct pathway for secretion. We previously reviewed the pathways of sorting and secretion in the parotid gland (Gorr et al. 2005). Secretion in the parotid, as with other exocrine cells, includes the major regulated pathway, a minor regulated pathway, apical and basolateral constitutive secretory pathways, and a constitutive-like secretory pathway (Perez et al. 2010). Similarly, endocrine cells have both regulated secretory and constitutive secretory pathways, in addition to pathways within the cell (Kim et al. 2006; Park and Loh 2008). Of the major salivary proteins, PSP is an excellent marker for the regulated secretory pathway. Western blot analysis of rat serum demonstrates that some fraction of salivary amylase is normally present in serum, however, PSP was undetectable (Venkatesh et al. 2007). This indicates that under normal conditions PSP is tightly sorted into the apical regulated secretory pathway, whereas a portion of amylase enters a basolateral pathway *in vivo*.

Salivary glands are being studied for their potential to produce and secrete therapeutic proteins from transgenes introduced to patients (Perez et al. 2010). Towards this goal, it is important to understand the molecular mechanisms that control parotid sorting and secretion, in order to regulate whether the transgenic protein is secreted by an apical regulated (exocrine) or basolateral (endocrine) pathway (Perez et al. 2010). Progress has been made in defining molecular interactions that affect sorting in some cell types; however, many of these mechanisms do not appear to be present in parotid acinar cells. As described above, the pH of the secretory granules of neuroendocrine and endocrine cells decreases during maturation from about 6.2 at the TGN to 5.5 – 5.0 in the mature granule (Wu et al. 2001; Kim et al. 2006). This acidic environment is important for sorting in PC12 and AtT20 cells, and is essential for protein aggregation (reviewed in (Kim et al. 2006)). However, in the parotid gland, the pH of the acinar cell granule increases from about pH 6.2 at the TGN to 6.8 or higher after maturation (Arvan and Castle 1986). Furthermore, granule cargo proteins from parotid acinar cells (amylase and PSP) are unable to aggregate even in the presence of Ca^{++} and low pH, whereas pancreatic exocrine granule proteins (used as a control) aggregate in a fashion similar to endocrine cells (Venkatesh et al. 2004). This indicates that sorting of amylase and PSP in the parotid gland have at least some important differences from the mechanisms described for neuro/endocrine cells. Therefore, we have investigated

the molecular interactions of PSP in the secretory granule in an attempt to understand how sorting may be controlled (Venkatesh et al. 2011).

4.1 Parotid secretory protein (PSP) binds to secretory granule membranes

We analyzed rat parotid granule membranes by mass spectrometry with the goal of identifying integral membrane proteins that may be candidate sorting receptors in the parotid gland. Parotid gland homogenates were fractionated on sucrose gradients to isolate the secretory granules, which were osmotically lysed. Membranes were washed and further enriched by an additional sucrose gradient. Sucrose gradient-purified granule membranes were electrophoresed on polyacrylamide gels, and trypsinized peptides identified by MS/MS as described (Uriarte et al. 2008). Numerous integral or membrane-bound proteins were identified, including several involved in vesicle trafficking and cytoskeletal proteins, as expected. However, potential sorting receptors such as SgIII (Han et al. 2008) or carboxypeptidase E (Dhanvantari et al. 2002) were not identified in parotid membranes by this method. Nonetheless, one salivary cargo protein, PSP, was identified. Other abundant soluble cargo proteins such as amylase and acidic Proline-Rich Protein (PRP) were not detected. To confirm the binding of PSP to granule membranes, western blot analysis was done with independent preparations of purified and extensively washed granule membranes. This confirmed that PSP is selectively bound, whereas amylase and PRP are absent from purified membranes (Fig. 3) (Venkatesh et al. 2011). While this approach failed to identify candidate sorting receptor proteins, it did demonstrate that PSP is a good marker for interactions with the membrane. In other cell types, putative sorting proteins such as secretogranin III (Hosaka et al. 2004), carboxypeptidase E (Dhanvantari et al. 2002), PC1/3, and PC2 (Jutras et al. 2000) are also associated with isolated secretory granule membranes.

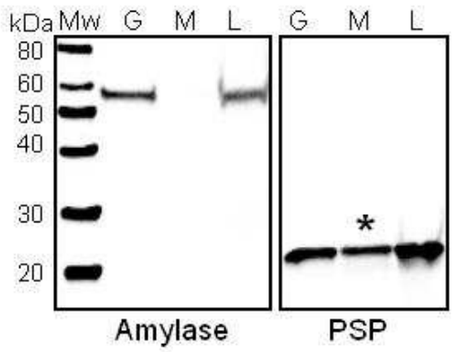

Fig. 3. Western blots of purified secretory granule membranes, probed with either anti-amylase or anti-PSP. Lanes contain either intact granules (G), purified granule membranes (M), or soluble cargo protein lysate (L). Equal proportions (0.5%) of each fraction was analyzed. The star indicates PSP in the purified membrane fraction. Mw: molecular size standards.

Given the existing models for sorting receptors, we tested whether PSP was bound to a sorting receptor protein in the secretory granule membrane. Numerous experiments were done attempting to crosslink PSP to a membrane protein; however, PSP never crosslinked

into a specific membrane-dependent higher molecular weight band. Taking the opposite approach, we extensively digested parotid granule membranes with either trypsin or pronase to destroy all membrane-associated proteins, and subsequently found that exogenous PSP still binds quite effectively. These results indicated that PSP does not require a protein receptor for binding to the membrane. In contrast, exogenous amylase did not bind the trypsinized membranes, and was present in the unbound fraction only, emphasizing the specificity of the binding of PSP.

4.2 PSP binds specifically to phosphatidylinositol 3,4-bisphosphate

The binding of a cargo protein to the vesicle membrane is of great interest in defining the mechanisms of sorting. However, the ability of PSP to bind granule membranes in the absence of any sorting receptor protein ruled out the most common model for sorting. Several secreted proteins have been shown to interact with lipid microdomains (e.g., CPE, SgIII, PC1/3), presumably by relatively non-specific hydrophobic interactions (Park and Loh 2008; Dikeakos et al. 2007), whereas other classes of protein bind to a highly specific lipid headgroup (Di Paolo and De Camilli 2006). Therefore, we tested the ability of PSP to bind specific lipids, and to bind liposomes. Parotid secretory granules were isolated, and the lysate supernatant containing soluble cargo proteins was used in lipid-overlay assays to determine whether PSP or other salivary proteins (amylase or PRP) bind specific lipids (Dowler et al. 2000). Importantly, none of the cargo proteins bound directly to the most abundant membrane lipids (phosphatidylcholine, phosphatidylethanolamine, cholesterol, or sphingomyelin). Similarly, acidic PRP never bound any lipid spots, and amylase showed little or no binding. In contrast, PSP bound with remarkable selectivity to phosphatidylinositol phosphates (PtdInsPs), but did not bind to unphosphorylated PtdIns (Fig. 4). We observed decreased PSP binding to PtdInsPs at more acidic conditions, but clear binding was still present at pH 6.0. Hence, this interaction could contribute to sorting of PSP in the TGN where the pH is approximately 6.2 (Arvan and Castle 1986), and may also contribute to retention as the pH increases during granule maturation.

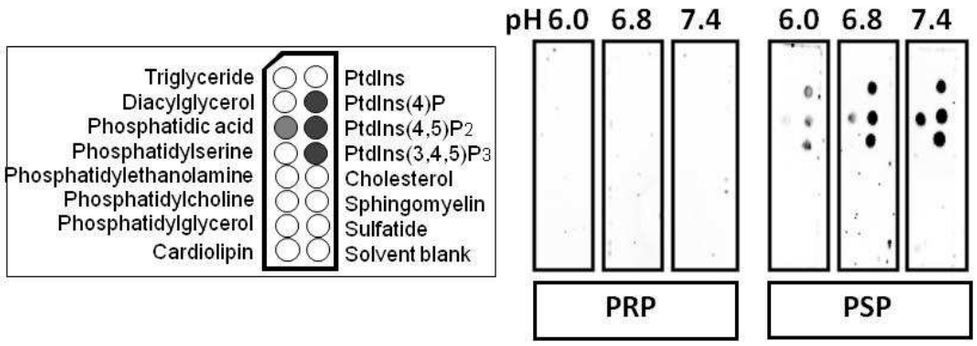

Fig. 4. PSP binds to phosphatidylinositol phosphates. Lipid strips (Echelon Biosciences) were incubated with parotid granule soluble lysate at 2 µg/ml (Venkatesh et al. 2011). Bound protein was detected with antibodies to PSP or acidic PRP. A schematic of the lipid strips is shown on the left (filled circles represent PSP binding).

The inability of PSP to bind unphosphorylated PtdIns suggested that specific interactions with the headgroup were required. Therefore, we compared the binding of PSP to a dilution

series of each of the seven different phosphorylated forms of PtdInsPs. We found that native PSP binds 3- to 5-fold more to PtdIns(3,4)P_2 compared to PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3, and 10-fold greater than PtdIns(3,5)P_2 or PtdIns(4)P (Fig. 5). PSP does not bind PtdIns(3)P, PtdIns(5)P, or PtdIns. Half-maximal binding of PSP was with approximately 35 pmoles PtdIns(3,4)P_2. Parallel blots failed to detect any bound amylase or acidic PRP, both of which are abundant in the granule lysates. This high degree of specificity indicates that PSP binds the head group of PtdInsPs, analogous to known PtdInsP-binding proteins (Di Paolo and De Camilli 2006). For example, PSP is more selective than the PH-domain protein DAPP1 which binds PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 with similar avidity (Dowler et al. 2000).

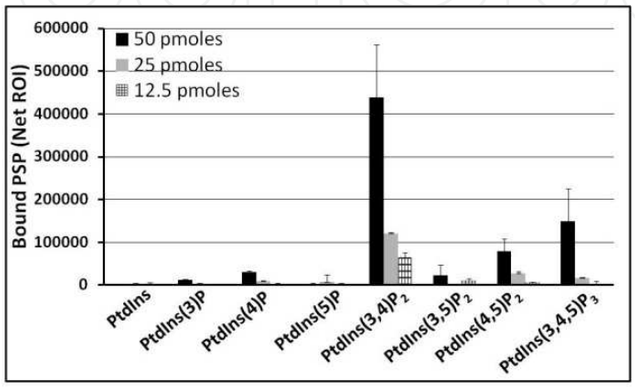

Fig. 5. Binding of PSP to phosphatidylinositol phosphates. PtdInsP array membranes spotted with serially diluted lipids were used to define the binding of native PSP. Membranes were blocked and probed with anti-PSP as described (Venkatesh et al. 2011).

The experiments described above use a secretory granule lysate as the source of PSP. This leaves open the possibility that PSP binds indirectly to PtdInsP_2. Therefore, PSP was expressed *in vitro* in rabbit reticulocyte lysates, and also was expressed in bacteria. Chloramphenicol acetyltransferase (CAT) was used as a negative control since CAT and rPSP are similar in size and also have similar acidic pI values. Rat PSP, and human PSP (human Splunc2, BPIFA2), each with a V5 tag, were translated *in vitro* and bound selectively to PtdIns(3,4)P_2 and PtdIns(4,5)P_2 demonstrating that this activity is conserved between species. CAT-V5 was unable to bind any of the lipids. Similarly, both human and rat PSP proteins were expressed in bacteria as glutathione-S-transferase (GST) fusion proteins having V5 tags. Again, GST-rPSP-V5 and GST-hPSP-V5 each bound strongly to PtdIns(3,4)P_2 and did not bind PtdIns, whereas GST-V5 had no binding activity (Venkatesh et al. 2011). Bacterially expressed and GST-affinity purified rPSP-V5 also preferentially binds PtdIns(3,4)P_2 (Fig. 6). The binding of *in vitro* synthesized, and bacterially expressed, rPSP and human PSP to PtdIns(3,4)P_2 demonstrates that the interaction is independent of other parotid granule proteins.

Pleckstrin homology domain proteins bind phosphoinositides with a moderate to high affinity (Vicinanza et al. 2008). We used bacterially expressed rPSP-V5 to determine the affinity of PSP for PtdIns(3,4)P_2. Bacterially expressed affinity-purified rPSP-V5 was incubated at 0.1 to 3.5 µg/ml with membranes spotted with 50 pmoles of PtdIns(3,4)P_2. Bound protein was detected with anti-V5 antibody, and the intensity of the signal used to calculate the amount of free and bound protein from a PSP-V5 standard curve, as described

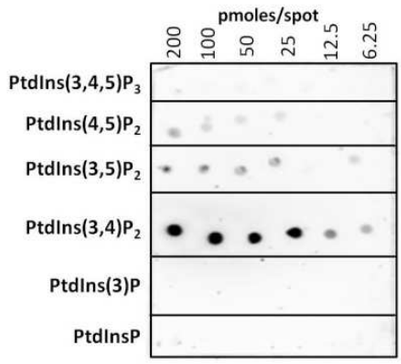

Fig. 6. Bacterially expressed rPSP-V5 binds to PtdIns(3,4)P_2. GST-PSP-V5 was affinity purified, and the rPSP-V5 was isolated separate from GST using PreScission protease. Protein overlay assays were performed using the purified rPSP-V5 (1 µg/ml) on nitrocelluose membranes spotted with lipids (200-6.25 pmoles/spot). Bound protein was detected with anti-V5 antibody. Bacterially expressed rPSP shows a similar pattern of specificity as native PSP.

(Venkatesh et al. 2011). The affinity was derived from the binding curve (Fig. 7). In three independent experiments, the binding affinity of PSP ranged from K_d= 1.85 x 10^{-10} to 3.9 x 10^{-11} M demonstrating a high affinity interaction. This is similar to the affinity of 5 x 10^{-9} M for TAPP1 binding PtdIns(3,4)P_2 measured by a similar method (Dowler et al. 2000). The affinity of p47phox for PtdIns(3,4)P_2 is reported as 3.8 x 10^{-8} (Karathanassis et al. 2002). In a direct comparison of bacterially expressed p47phox-V5 and rPSP-V5 we confirmed that PSP binds PtdIns(3,4)P_2 more strongly than p47phox.

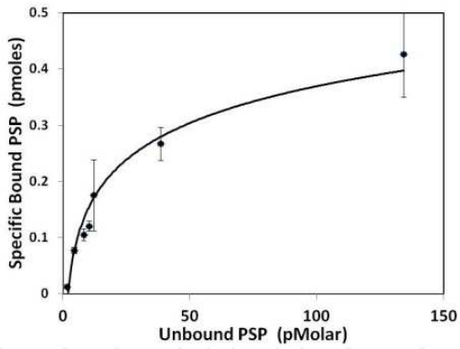

Fig. 7. Binding curve of PSP to PtdIns(3,4)P_2. Bacterially expressed affinity-purified rPSP-V5 was incubated at different concentrations to define binding to PtdIns(3,4)P_2 as described (Venkatesh et al. 2011).

We studied the interaction of PSP with lipid bilayers in liposomes for three reasons. Primarily, we wanted to determine if PSP binds specifically to PtdInsPs in intact membranes. In addition, it was important to test whether PSP can bind non-specifically to membranes due to hydrophobic interactions similar to helical peptides (Kitamura et al. 1999; Dikeakos et al. 2007) and to compare these two types of interaction. Synthetic liposomes were made by standard methods to contain phosphatidylcholine (PC), phosphatidylethanolamine (PE), and PtdIns (or PtdInsP) at a molar ratio of 77:20:3. We did

not detect any interaction of PSP with liposomes comprising PC, PE, and unphosphorylated PtdIns (77:20:3), nor did it bind to liposomes with PtdIns(3,5)P_2 (Fig. 8). This suggests that PSP does not interact with membranes through the relatively non-specific hydrophobic interactions observed for amphipathic alpha helices, and shown to be important for sorting of certain cargo proteins (Kitamura et al. 1999; Dikeakos et al. 2007). Conversely, native rat PSP, in granule lysates, bound repeatably to lysosomes spiked with 3% PtdIns(3,4)P_2 or PtdIns(4,5)P_2 (Fig. 8), consistent with the previous results. Amylase present in the same granule lysates did not bind to any of these liposomes. PSP also bound to stabilized PIPosomes (from Echelon Biosciences Inc.) containing 5% PtdIns(3,4)P_2. These results show that native PSP binds PtdIns(3,4)P_2 in an intact lipid membrane. This binding is not detectably due to hydrophobic interactions of an alpha helical domain, does not require a different protein to act as a membrane tether or a transmembrane sorting receptor, nor does it require cholesterol-rich domains.

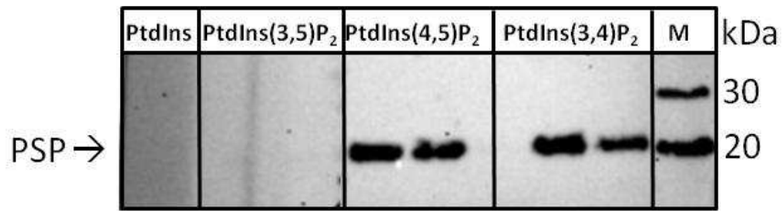

Fig. 8. PSP binds to PtdIns(3,4)P_2 and PtdIns(4,5)P_2 in lipid bilayers. Parotid granule extract was incubated with liposomes spiked with 3% either PtdIns, PtdIns(3,4)P_2, PtdIns(4,5)P_2 or PtdIns(3,5)P_2. After binding, liposomes were extensively washed and analyzed by SDS-PAGE and western blotting with anti-PSP. Each lane is a separate incubation. PSP bound to PtdIns(3,4)P_2 and PtdIns(4,5)P_2 only. M: molecular size markers.

4.3 The PSP family of BPI-fold proteins

The results described above suggest that PSP binds directly to the headgroup of PtdIns(3,4)P_2 in a membrane. It is of interest to compare this activity to related proteins. Following the newly developed nomenclature (Bingle et al. 2011), rat PSP/BPIFA2E is a member of the BPI-fold superfamily. This diverse superfamily includes bactericidal/permeability-increasing protein (BPI), LPS-binding protein (LBP), cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP). In addition, the superfamily includes the BPIFA subfamily (previously referred to as the SPLUNC family, containing PSP), and the BPIFB subfamily (previously termed LPLUNCs). The history of cloning the PSP/Splunc/BPIFA family, and changes in the nomenclature have recently been described in depth (Bingle et al. 2011). An important observation is that proteins in this superfamily have quite divergent amino acid sequences. For example, the optimal possible alignment of rat PSP to rat BPI gives a sequence identity of only 19%. However, there is strong conservation of secondary structure and predicted tertiary structures across the superfamily. The crystal structure of BPI shows a hollow boomerang-shaped structure (Beamer et al. 1997). The two halves (domains) of the boomerang show clear similarity to each other at both the primary sequence level, and at the structural level. The Long PLUNC (BPIFB) subfamily maps across both the two domains, whereas PSP and the rest of the Short PLUNC (BPIFA) subfamily consists of only one domain. Many of these proteins have important roles that involve binding to lipids. For example, both BPI and LBP bind to

lipopolysaccaride (LPS) by the lipid A region containing multiple acyl chains. The crystal structure of BPI shows the presence of two bound phosphatidylcholine molecules, each with the acyl chains deeply embedded in the hollow protein tube (Beamer et al. 1997). A similar model is found for CETP, and PLTP (Huuskonen et al. 1999; Qiu et al. 2007). This is distinctly different from PSP binding to lipids observed in our results. Using two different assays, PSP does not bind to phosphatidylcholine. Further, PSP requires critical interactions with the headgroup of the lipid, which are not apparent in BPI binding to phosphatidylcholine. Based on these differences, it does not appear that PSP binding to PtdIns(3,4)P_2 uses the same mechanism as the well-characterized binding of BPI to lipids. As an initial hypothesis, we predict that separate binding sites on PSP will be identified for binding PtdIns(3,4)P_2 and lipopolysaccaride. PSP could bind PtdIns(3,4)P_2 in membranes during trafficking, and subsequently use a different interaction to bind LPS in the saliva.

4.4 Phosphoinositides in parotid granule membranes

The observed binding of PSP to PtdIns(3,4)P_2 could support sorting of PSP; and in addition PSP could act as a membrane tether (or chaperone) to aid in sorting of other cargo. However, PtdIns(3,4)P_2 binding can mediate selective trafficking into granules only if the presence of PtdInsP_2 is somehow linked to the identity of the forming granule. The idea that a specific type of rare lipid may direct the sorting of cargo proteins is an entirely novel suggestion. However, it is just an extrapolation of the role of PtdInsPs on the other side of the membrane. In the following sections we address two key questions. Is PtdIns(3,4)P_2 present on parotid secretory granule membranes? Can PtdIns(3,4)P_2 cross to the luminal side of the granule membrane?

PtdInsPs have highly specific intracellular distributions, anchoring critical proteins to specific membranes (Graham and Burd 2011). PtdIns(3,4)P_2 could reasonably be present in the TGN since both PI3-kinase and PI4-kinase are bound at the TGN, and both PI-kinase activities are required for regulated secretion (Meunier et al. 2005; Low et al. 2010). However, PtdIns(3,4)P_2 has tended to be neglected in studies of PtdInsP distribution, so little information is available. Nonetheless, immunofluorescence of a transfected PtdIns(3,4)P_2-binding protein (TAPP1) showed clear localization to a Golgi-like structure adjacent to the nucleus (Hogan et al. 2004). We isolated parotid gland secretory granules, and methanolic extracts of purified granule membranes were spotted on PVDF and probed with antibodies to specific phosphoinositides. As expected, we detected PtdIns(4)P which is reported to be on vesicles and TGN of several cell types. In addition, strong immunoreactivity was observed for PtdIns(3,4)P_2 (Venkatesh et al. 2011). Standard curves of PtdIns(4)P and PtdIns(3,4)P_2 were used to calculate the amounts of each lipid. PtdIns(4)P is abundant in the TGN, however, we find that PtdIns(3,4)P_2 is present at a slightly higher amount than PtdIns(4)P in parotid granule membranes.

As a separate approach, we used immunofluorescence to localize PtdIns(3,4)P_2 within the parotid acinar cell. Anti-PSP labels the secretory granules, which collect near the center of the acinus, in the apical end of each cell, but did not label the basal ends. Anti-PtdIns(3,4)P_2 also labeled the apical end of parotid acinar cells, giving a similar pattern. Superimposing the images shows that PSP and PtdIns(3,4)P_2 co-localize to the secretory granules (Fig. 9; yellow and orange). Therefore, using either biochemical or histological methods, we consistently find that PtdIns(3,4)P_2 is present in parotid granule membranes.

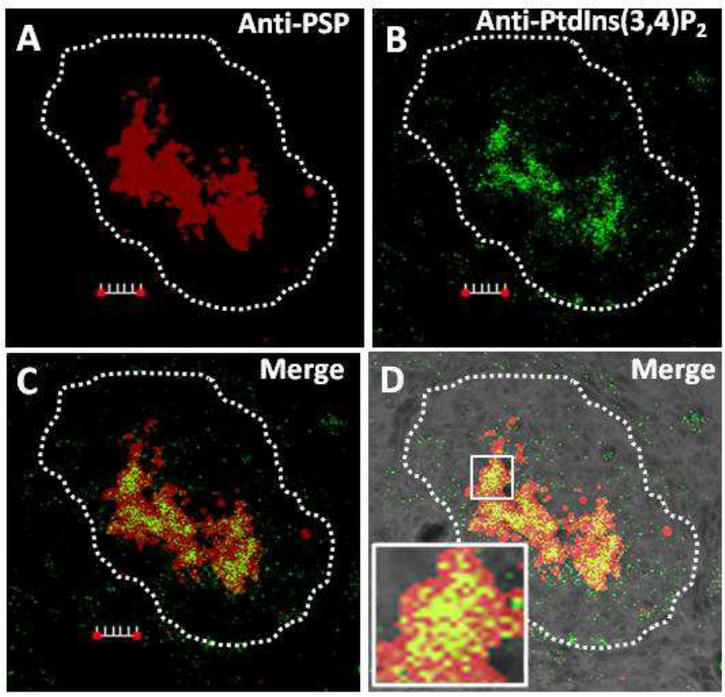

Fig. 9. PtdIns(3,4)P_2 co-localizes to secretory granules of parotid acinar cells. Rat parotid tissue sections were probed with anti-rPSP (red) and monoclonal anti-PtdIns(3,4)P_2 (green). A merge of the two images shows co-localization (C). The dotted line marks the boundary of the acinus. The boxed area of the Nomarski image (D) is enlarged in the inset. The scale bar is 5 μm.

4.5 Lipid translocases in parotid granule membranes

The observation that PtdIns(3,4)P_2 is present in parotid granule membranes supports our model that PSP binds the membrane by interacting with this lipid. However, the PI-kinases that produce PtdInsPs are located on the cytosolic leaflet of the TGN and granule membranes, yet PSP is present only inside the secretory granule.

The coat protein complex on budding vesicles of the TGN includes translocases which flip phospholipids to maintain lipid asymmetry, and can contribute to bending of membranes (van Meer and Sprong 2004; Natarajan et al. 2009; Contreras et al. 2010). Translocases (flippases) are reported on the TGN and post-Golgi vesicles, and are linked to vesicle budding (Muthusamy et al. 2009). Translocases on yeast TGN are involved in a clathrin-dependent pathway, vesicle bud formation, and membrane trafficking (Graham 2004; Daleke 2007; Natarajan et al. 2009). Translocase activity is also present on pig gastric parietal cell secretory vesicles (Suzuki et al. 1997), and adrenal chromaffin granules (Zachowski et al. 1989). Three types of lipid translocases have been described at the TGN or on post-Golgi vesicles. P-type ATPase translocases are present on the TGN and secretory granules (Suzuki et al. 1997), and are important for secretion (Muthusamy et al. 2009). Alternatively, the ATP-binding cassette (ABC) superfamily of transporters includes lipid translocases which can be found on the TGN, lysosomes, and secretory vesicles of lung type II cells (Stahlman et al. 2007). Similarly, the phospholipid scramblase family mediates bidirectional flipping of

phospholipid across the membrane, and PLSCR1 is present on neutrophil secretory vesicle membranes (Frasch et al. 2004). Un-phosphorylated PtdIns can be translocated by a flippase, however, none of the translocase families have been tested for the ability to flip PtdInsPs.

Our data suggest that PtdIns(3,4)P_2 may be present on the luminal face of the parotid granule membrane as a binding site for PSP, however, it is unclear how it would get there. Therefore, intact rat parotid secretory granules were incubated with fluorescent NBD-tagged PtdInsP to measure flipping, according to (Natarajan and Graham 2006; Natarajan et al. 2009). In this method, added NBD-lipids integrate rapidly into the outer leaflet of the granule membrane on ice. After incubation at 37 ^{o}C to allow flipping, label remaining in the outer leaflet is quantitatively destroyed by addition of BSA and dithionite, however, NBD-lipids which had translocated to the luminal leaflet are protected by the membrane. We found that incubation for 1 hour at 37 ^{o}C allowed 15% of integrated NBD-PtdIns(3,4)P_2 to translocate to the protected inner leaflet (Fig. 10). Less than 2% of the PSP leaked from the granules after incubation at 37 ^{o}C for 1 h, indicating that the granules remained sealed during the assay.

Unphosphorylated PtdIns is translocated by a flippase at a rate similar to phosphatidylcholine (Vishwakarma et al. 2005). This provides us with a benchmark for comparison with the extent of flipping of PtdInsPs. In our experiments, approximately 10% of PtdIns was flipped to the inner leaflet of parotid granules, whereas 15-18% of PtdIns(4)P, PtdIns(3,4)P_2, or PtdIns(3,5)P_2 was translocated (Fig. 10). Hence, the flippase activity is relatively non-selective, and the phosphate groups do not inhibit translocation. Parotid granule membranes support flipping of phosphorylated forms to a greater extent than PtdIns. Taken together, our results demonstrate that PtdIns(3,4)P_2 is present in the granule membrane, and can flip to the inner face of the membrane. Further, the presence of PtdInsPs on the luminal leaflet of membranes raises the possibility that other intra-organelle proteins may be localized by PtdInsP anchors.

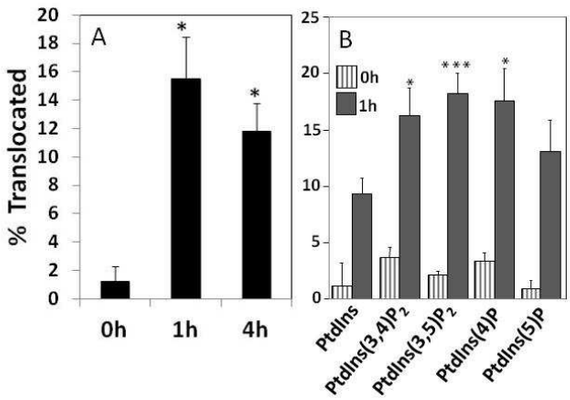

Fig. 10. A. Parotid secretory granules translocate PtdIns(3,4)P_2. Purified intact parotid secretory granules were incubated with fluorescent NBD-labeled PtdIns(3,4)P_2 to measure translocation, as described (Natarajan and Graham 2006; Venkatesh et al. 2011). Data show the amount of lipid flipped to the inner leaflet. Data are Mean±SE of 3 experiments in triplicate. *$p<0.01$ compared with 0h. B. Several PtdInsPs translocate parotid granule membranes. Data are the amount of flipped lipid at 0 or 1 hour. Data are Mean ± SE of 3-6 experiments in triplicate. *$p<0.05$, ***$p<0.01$ compared to PtdIns.

5. Conclusions: Lipids as sorting receptors

As discussed above, a central aspect of selective trafficking is ensuring that the vesicle targeting information encoded by the granule coat matrix directs the choice of which cargo proteins fill the lumen of the granule. Current models for selective sorting all rely on interactions with a transmembrane protein to convey that information, directly or indirectly. Our results with parotid secretory granules suggest the possibility of a variant of this model. Rather than a transmembrane sorting receptor, translocation (flipping) of a rare lipid, PtdIns(3,4)P_2, may convey the character of the coat matrix (Fig. 11).

Both PI3-kinase and PI4-kinase are present on the TGN or vesicles in several cell types, and create specific PtdInsPs in the outer leaflet of the membrane. In addition, lipid translocases, or flippase activity, has been reported on mammalian secretory granules. Our results demonstrate that PtdIns(3,4)P_2 is present in parotid secretory granule membranes. We observe that PtdIns(3,4)P_2 can translocate to the luminal bilayer of the granule membrane. Further, we find that PSP binds strongly to PtdIns(3,4)P_2 in the membrane. Since the translocase likely is recruited and localized by the coat proteins, we suggest that the translocase may create a local region of higher concentration of PtdIns(3,4)P_2 in the luminal leaflet within the forming vesicle bud, compared to other areas of the TGN. This may serve to localize PSP within the budding vesicle, thereby sorting it for secretion. The membrane-bound PSP may in turn act as a sorting chaperone for other cargo proteins. This hypothetical model has the advantage that it suggests specific interactions which can be tested for a role in sorting for secretion.

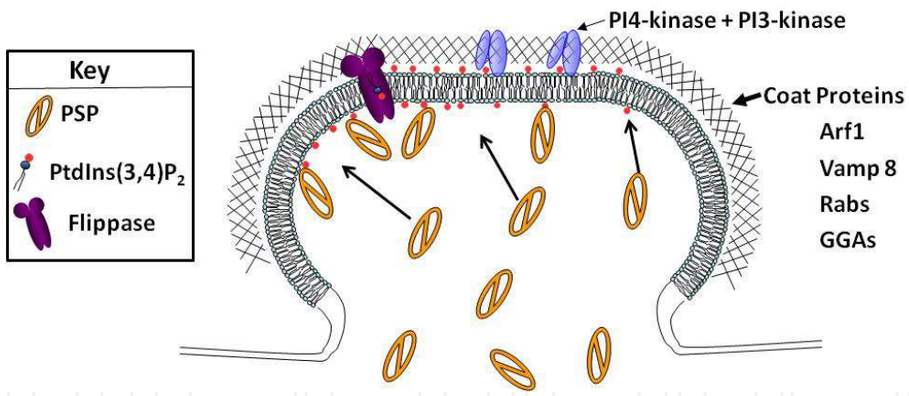

Fig. 11. A hypothetical model for how PtdIns(3,4)P_2 could mediate sorting of PSP.

6. Acknowledgments

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7. References

Arvan, P. and Castle, J. (1986) Isolated secretion granules from parotid glands of chronically stimulated rats possess an alkaline internal pH and inward-directed H+ pump activity. J. Cell Biol. 103(4): 1257-1267.

Balla, A. and Balla, T. (2006) Phosphatidylinositol 4-kinases: old enzymes with emerging functions. Trends Cell Biol 16(7): 351-361.

Beamer, L.J., Carroll, S.F., et al. (1997) Crystal Structure of Human BPI and Two Bound Phospholipids at 2.4 Angstrom Resolution. Science 276(5320): 1861-1864.

Bingle, C.D., Seal, R.L., et al. (2011) Systematic nomenclature for the PLUNC/PSP/BSP30/SMGB proteins as a subfamily of the BPI fold-containing superfamily. Biochem Soc Trans 39(4): 977-983.

Bingle, L., Barnes, F.A., et al. (2009) Characterisation and expression of SPLUNC2, the human orthologue of rodent parotid secretory protein. Histochem Cell Biol 132(3): 339-349.

Caromile, L.A., Oganesian, A., et al. (2010) The Neurosecretory Vesicle Protein Phogrin Functions as a Phosphatidylinositol Phosphatase to Regulate Insulin Secretion. J. Biol. Chem. 285(14): 10487-10496.

Contreras, F., Sanchez-Magraner, L., et al. (2010) Transbilayer (flip-flop) lipid motion and lipid scrambling in membranes. FEBS Lett 584(9): 1779-1786.

Cullen, P.J. (2011) Phosphoinositides and the regulation of tubular-based endosomal sorting. Biochem Soc Trans 39(4): 839-850.

D'Angelo, G., Vicinanza, M., et al. (2008) The multiple roles of PtdIns(4)P - not just the precursor of PtdIns(4,5)P2. J. Cell Sci. 121(12): 1955-1963.

Daleke, D.L. (2007) Phospholipid flippases. J Biol Chem 282(2): 821-825.

De Matteis, M. and Luini, A. (2011) Mendelian disorders of membrane trafficking. N Engl J Med 365(10): 927-938.

De Matteis, M.A., Di Campli, A., et al. (2005) The role of the phosphoinositides at the Golgi complex. Biochim Biophys Acta 1744(3): 396-405.

De Matteis, M.A. and Luini, A. (2008) Exiting the Golgi complex. Nat Rev Mol Cell Biol 9(4): 273-284.

Denny, P., Hagen, F.K., et al. (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. J Proteome Res 7(5): 1994-2006.

Dhanvantari, S., Arnaoutova, I., et al. (2002) Carboxypeptidase E, a prohormone sorting receptor, is anchored to secretory granules via a C-terminal transmembrane insertion. Biochemistry 41(1): 52-60.

Di Paolo, G. and De Camilli, P. (2006) Phosphoinositides in cell regulation and membrane dynamics. Nature 443(7112): 651-657.

Dikeakos, J.D., Di Lello, P., et al. (2009) Functional and structural characterization of a dense core secretory granule sorting domain from the PC1/3 protease. Proc Natl Acad Sci U S A 106(18): 7408-7413.

Dikeakos, J.D., Lacombe, M.J., et al. (2007) A hydrophobic patch in a charged alpha-helix is sufficient to target proteins to dense core secretory granules. J Biol Chem 282(2): 1136-1143.

Dikeakos, J.D. and Reudelhuber, T.L. (2007) Sending proteins to dense core secretory granules: still a lot to sort out. J Cell Biol 177(2): 191-196.

Dowler, S., Currie , R., et al. (2000) Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. Biochem J 351(Pt 1): 19-31.

Folsch, H., Mattila, P.E., et al. (2009) Taking the scenic route: biosynthetic traffic to the plasma membrane in polarized epithelial cells. Traffic 10(8): 972-981.

Frasch, S.C., Henson, P.M., et al. (2004) Phospholipid flip-flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils. J Biol Chem 279(17): 17625-17633.

Fujita-Yoshigaki, J., Katsumata, O., et al. (2006) Difference in distribution of membrane proteins between low- and high-density secretory granules in parotid acinar cells. Biochem Biophys Res Commun 344(1): 283-292.

Ghosh, P., Dahms, N.M., et al. (2003) Mannose 6-phosphate receptors: new twists in the tale. Nat Rev Mol Cell Biol 4(3): 202-212.

Godi, A., Di Campli, A., et al. (2004) FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat Cell Biol 6(5): 393-404.

Gorr, S.-U., Venkatesh, S.G., et al. (2005) Parotid Secretory Granules: Crossroads of Secretory Pathways and Protein Storage. J Dent Res 84(6): 500-509.

Gorr, S.U., Abdolhosseini, M., et al. (2011) Dual host-defence functions of SPLUNC2/PSP and synthetic peptides derived from the protein. Biochem Soc Trans 39(4): 1028-1032.

Graham, T.R. (2004) Flippases and vesicle-mediated protein transport. Trends Cell Biol 14(12): 670-677.

Graham, T.R. and Burd, C.G. (2011) Coordination of Golgi functions by phosphatidylinositol 4-kinases. Trends Cell Biol 21(2): 113-121.

Guerriero, C.J., Lai, Y., et al. (2008) Differential sorting and Golgi export requirements for raft-associated and raft-independent apical proteins along the biosynthetic pathway. J Biol Chem 283(26): 18040-18047.

Han, L., Suda, M., et al. (2008) A large form of secretogranin III functions as a sorting receptor for chromogranin A aggregates in PC12 cells. Mol Endocrinol 22(8): 1935-1949.

Helmerhorst, E.J. and Oppenheim, F.G. (2007) Saliva: a Dynamic Proteome. J Dent Res 86(8): 680-693.

Hogan, A., Yakubchyk, Y., et al. (2004) The phosphoinositol 3,4-bisphosphate-binding protein TAPP1 interacts with syntrophins and regulates actin cytoskeletal organization. J Biol Chem 279(51): 53717-53724.

Hosaka, M., Suda, M., et al. (2004) Secretogranin III binds to cholesterol in the secretory granule membrane as an adapter for chromogranin A. J Biol Chem 279(5): 3627-3634.

Hosaka, M., Watanabe, T., et al. (2005) Interaction between secretogranin III and carboxypeptidase E facilitates prohormone sorting within secretory granules. J. Cell Sci. 118(20): 4785-4795.

Huuskonen, J., Wohlfahrt, G., et al. (1999) Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis. J. Lipid Res. 40(6): 1123-1130.

Jutras, I., Seidah, N.G., et al. (2000) A predicted alpha -helix mediates targeting of the proprotein convertase PC1 to the regulated secretory pathway. J Biol Chem 275(51): 40337-40343.

Karathanassis, D., Stahelin, R., et al. (2002) Binding of the PX domain of p47(phox) to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction. EMBO J 21(19): 5057-5068.

Kim, T., Gondre-Lewis, M.C., et al. (2006) Dense-core secretory granule biogenesis. Physiology (Bethesda) 21: 124-133.

Kitamura, A., Kiyota, T., et al. (1999) Morphological behavior of acidic and neutral liposomes induced by basic amphiphilic alpha-helical peptides with systematically varied hydrophobic-hydrophilic balance. Biophys J 76(3): 1457-1468.

Lacy, P. and Stow, J.L. (2011) Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood 118(1): 9-18.

Lang, T. (2007) SNARE proteins and 'membrane rafts'. J Physiol 585(Pt 3): 693-698.

Lara-Lemus, R., Liu, M., et al. (2006) Lumenal protein sorting to the constitutive secretory pathway of a regulated secretory cell. J. Cell Sci. 119(9): 1833-1842.

Low, P.C., Misaki, R., et al. (2010) Phosphoinositide 3-kinase delta regulates membrane fission of Golgi carriers for selective cytokine secretion. J Cell Biol 190(6): 1053-1065.

Meunier, F.A., Osborne, S.L., et al. (2005) Phosphatidylinositol 3-kinase C2alpha is essential for ATP-dependent priming of neurosecretory granule exocytosis. Mol Biol Cell 16(10): 4841-4851.

Muthusamy, B.P., Natarajan, P., et al. (2009) Linking phospholipid flippases to vesicle-mediated protein transport. Biochim Biophys Acta 1791(7): 612-619.

Natarajan, P. and Graham, T. (2006) Measuring translocation of fluorescent lipid derivatives across yeast Golgi membranes. Methods 39(2): 163-168.

Natarajan, P., Liu, K., et al. (2009) Regulation of a Golgi flippase by phosphoinositides and an ArfGEF. Nat Cell Biol 11(12): 1421-1426.

Nicol, L., McNeilly, JR, et al. (2004) Differential secretion of gonadotrophins: investigation of the role of secretogranin II and chromogranin A in the release of LH and FSH in LbetaT2 cells. J. Mol. Endocrinol. 32(2): 467-480.

Park, J.J., Cawley, N.X., et al. (2008) Carboxypeptidase E cytoplasmic tail-driven vesicle transport is key for activity-dependent secretion of peptide hormones. Mol Endocrinol 22(4): 989-1005.

Park, J.J., Gondre-Lewis, M.C., et al. (2011) A distinct trans-Golgi network subcompartment for sorting of synaptic and granule proteins in neurons and neuroendocrine cells. J. Cell Sci. 124(5): 735-744.

Park, J.J. and Loh, Y.P. (2008) How peptide hormone vesicles are transported to the secretion site for exocytosis. Mol Endocrinol 22(12): 2583-2595.

Perez, P., Rowzee, A.M., et al. (2010) Salivary epithelial cells: an unassuming target site for gene therapeutics. Int J Biochem Cell Biol 42(6): 773-777.

Puri, N. and Roche, P.A. (2006) Ternary SNARE complexes are enriched in lipid rafts during mast cell exocytosis. Traffic 7(11): 1482-1494.

Qiu, X., Mistry, A., et al. (2007) Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. Nat Struct Mol Biol 14(2): 106-113.

Reefman, E., Kay, J.G., et al. (2010) Cytokine secretion is distinct from secretion of cytotoxic granules in NK cells. J Immunol 184(9): 4852-4862.

Roth, M.G. (2004) Phosphoinositides in Constitutive Membrane Traffic. Physiol Rev 84(3): 699-730.

Saito, N., Takeuchi, T., et al. (2011) Luminal interaction of phogrin with carboxypeptidase E for effective targeting to secretory granules. Traffic 12(4): 499-506.

Santiago-Tirado, F.H. and Bretscher, A. (2011) Membrane-trafficking sorting hubs: cooperation between PI4P and small GTPases at the trans-Golgi network. Trends Cell Biol 21(9): 515-525.

Shilo, B.Z. and Schejter, E.D. (2011) Regulation of developmental intercellular signalling by intracellular trafficking. EMBO J 30(17): 3516-3526.

Simons, K. and Sampaio, J.L. (2011) Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol 3(10): a004697.

Stahlman, M.T., Besnard, V., et al. (2007) Expression of ABCA3 in developing lung and other tissues. J Histochem Cytochem 55(1): 71-83.

Stanley, A.C. and Lacy, P. (2010) Pathways for cytokine secretion. Physiology (Bethesda) 25(4): 218-229.

Suzuki, H., Kamakura, M., et al. (1997) The Phospholipid Flippase Activity of Gastric Vesicles. J. Biol. Chem. 272(16): 10429-10434.

Trucco, A., Polishchuk, R., et al. (2004) Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. Nat Cell Biol 6(11): 1071-1081.

Uriarte, S.M., Powell, D.W., et al. (2008) Comparison of Proteins Expressed on Secretory Vesicle Membranes and Plasma Membranes of Human Neutrophils. J. Immunol. 180(8): 5575-5581.

van Meer, G. and Sprong, H. (2004) Membrane lipids and vesicular traffic. Curr Opin Cell Biol 16(4): 373-378.

Venkatesh, S.G., Cowley, D.J., et al. (2004) Differential aggregation properties of secretory proteins that are stored in exocrine secretory granules of the pancreas and parotid glands. Am J Physiol Cell Physiol 286(2): C365-371.

Venkatesh, S.G. and Gorr, S.U. (2002) A sulfated proteoglycan is necessary for storage of exocrine secretory proteins in the rat parotid gland. Am J Physiol Cell Physiol 283(2): C438-445.

Venkatesh, S.G., Goyal, D., et al. (2011) Parotid secretory protein binds phosphatidylinositol (3,4) bisphosphate. J Dent Res 90(9): 1085-1090.

Venkatesh, S.G., Tan, J., et al. (2007) Isoproterenol increases sorting of parotid gland cargo proteins to the basolateral pathway. Am J Physiol Cell Physiol 293(2): C558-565.

Vicinanza, M., D'Angelo, G., et al. (2008) Function and dysfunction of the PI system in membrane trafficking. EMBO J 27(19): 2457-2470.

Vieira, O.V., Verkade, P., et al. (2005) FAPP2 is involved in the transport of apical cargo in polarized MDCK cells. J Cell Biol 170(4): 521-526.

Vishwakarma, R.A., Vehring, S., et al. (2005) New fluorescent probes reveal that flippase-mediated flip-flop of phosphatidylinositol across the endoplasmic reticulum membrane does not depend on the stereochemistry of the lipid. Org Biomol Chem 3(7): 1275-1283.

Wang, C.C., Shi, H., et al. (2007) VAMP8/endobrevin as a general vesicular SNARE for regulated exocytosis of the exocrine system. Mol Biol Cell 18(3): 1056-1063.

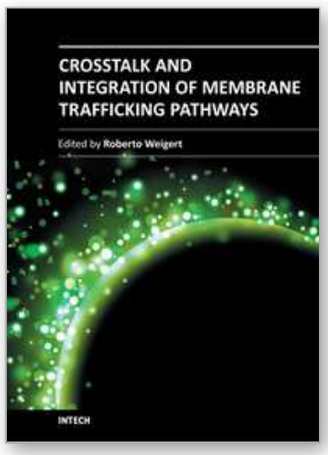
Wang, Y.J., Wang, J., et al. (2003) Phosphatidylinositol 4 Phosphate Regulates Targeting of Clathrin Adaptor AP-1 Complexes to the Golgi. Cell 114(3): 299-310.

Weixel, K.M., Blumental-Perry, A., et al. (2005) Distinct Golgi populations of phosphatidylinositol 4-phosphate regulated by phosphatidylinositol 4-kinases. J Biol Chem 280(11): 10501-10508.

Wilson, C., Venditti, R., et al. (2011) The Golgi apparatus: an organelle with multiple complex functions. Biochem J 433(1): 1-9.

Wu, M.M., Grabe, M., et al. (2001) Mechanisms of pH Regulation in the Regulated Secretory Pathway. J. Biol. Chem. 276(35): 33027-33035.

Zachowski, A., Henry, J., et al. (1989) Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. Nature 340(6228): 75-76.



Crosstalk and Integration of Membrane Trafficking Pathways
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Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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