EXOCYST COMPLEX AND ENDOCYTIC TRAFFICKING IN POLARIZED EPITHELIAL CELLS

by

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EXOCYST COMPLEX AND MEMBRANE TRAFFICKING IN POLARIZED EPITHELIAL CELLS

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The octameric exocyst complex is associated with the junctional complex and recycling endosomes, and is proposed to selectively tether cargo vesicles directed toward the basolateral surface of polarized Madin-Darby canine kidney (MDCK) cells. I observed that the exocyst subunits Sec6, Sec8, and Exo70 were localized to early endosomes, transferrin-positive common recycling endosomes, and Rab11a-positive apical recycling endosomes of polarized MDCK cells. Consistent with its localization to multiple populations of endosomes, addition of function-blocking Sec8 antibodies to streptolysin-O permeabilized cells revealed exocyst requirements for several endocytic pathways including basolateral recycling, apical recycling, and basolateral-to-apical transcytosis. The latter was selectively dependent on interactions between the small GTPase Rab11a and Sec15A and was inhibited by the expression of the C-terminus of Sec15A or downregulation of Sec15A expression using shRNA. These results indicate that the exocyst complex may be a multi-purpose regulator of endocytic traffic directed toward both poles of polarized epithelial cells, and that transcytotic traffic is likely to require Rab11a-dependent recruitment and modulation of exocyst function, likely through interactions with Sec15A.

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PREFACE

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1.0 INTRODUCTION

Most cell types, such as epithelial cells and neurons, have the ability to form polarized membrane domains with different compositions and specialized functions (122). In polarized epithelial cells, proteins are delivered to different membrane domains to perform specific functions, and the disruption of the proper sorting of proteins (e.g. the low-density lipoprotein receptor [LDLR] or the epithelial sodium channel) can lead to heart disease and hypertension (e.g., familial hypercholesterolemia or Liddle's syndrome) (33, 146, 152); therefore, it is important to understand the sorting machinery involved in the generation and maintenance of epithelial polarity.

Several components of the polarized sorting machinery in epithelial cells have been identified and studied including sorting signals, protein complexes that recognize these signals, SNARE fusion complexes, as well as a growing list of GTPases (21). Although these components are crucial for the proper sorting of several proteins (137), recent studies indicate that the tethering complexes are also a part of polarized trafficking machinery. These protein complexes are a family of long coiled-coiled or multi-subunit protein complexes that are localized to specific compartments and form docking patches to which cargo vesicles are targeted (99, 193). One member of this family, the exocyst complex, has been suggested to regulate vesicle tethering in singlecell eukaryotes and metazoans (28, 66, 95, 118, 160). However, the role of the exocyst

complex in regulating specific trafficking pathways in polarized epithelial cells has not been explored in detail.

The overall purpose of my work was to better understand the protein machinery involved in polarized sorting in endocytic pathway; specifically, I focused on the possible role of the exocyst complex in regulating endocytic trafficking in polarized epithelial cells.

1.1 MEMBRANE TRAFFICKING PATHWAYS IN POLARIZED EPITHELIAL CELLS

Epithelial tissues line the internal and external body surfaces and serve as highly selective barriers between the outside and inside environments (157). Polarized epithelial cells are connected to each other with junctional complexes, and have two functionally and structurally different plasma membrane domains, an apical membrane facing the outside world and a basolateral membrane facing the underlying tissue (Figure 1.1A) (156). The functional integrity of epithelial tissue relies on the proper distribution of proteins at polarized membrane domains, which is mediated by the delivery of newly synthesized proteins to their specific membrane domains during biosynthetic trafficking and by the maintenance of this asymmetric distribution during endocytic trafficking (200).





Figure 1.1: Polarized epithelial cells

(A) A diagram of polarized epithelial cells. Apical and basolateral membrane domains are separated by tight junctions. (B) Transmission electron microscope image of polarized MDCK cells grown on permeable filter supports.

1.1.1 Biosynthetic trafficking in polarized epithelial cells

Polarized trafficking pathways in epithelial cells have been studied extensively by several groups using Madin-Darby canine kidney (MDCK) cells as a model system. When cultured on permeable filter systems, these cells form polarized monolayers (Figure 1.1B) that are selectively permeable to macromolecules, and serve as a model system to study polarized membrane traffic in epithelial cells (113, 114, 116, 128). In MDCK cells, 5% of the plasma membrane is newly synthesized each hour (116), and the delivery of biosynthetic cargo to specific membrane domains is regulated by different factors such as sorting signals as well as adaptor complexes that may function in multiple compartments (37, 138, 139).

Initial studies indicate that newly synthesized apical and basolateral proteins are trafficked to the *trans-Golgi* network (TGN) and then sorted into separate vesicles, which are delivered to the appropriate plasma membrane domain (138). However, recent studies show that biosynthetic pathway is more complicated then originally anticipated and protein sorting may start as early as in the endoplasmic reticulum (ER) (117, 138). Furthermore, instead of following a direct route to the plasma membrane, some newly synthesized basolateral or apical proteins transit through recycling endosomal compartments on their way to the plasma membrane (37, 138), indicating post-TGN sorting may take place during biosynthetic trafficking (Figure 1.2).

Sorting signals are a crucial part of protein trafficking during both biosynthetic and endocytic pathways. Basolateral sorting signals involved in delivery of newly synthesized proteins to the basolateral plasma membrane are localized to the cytoplasmic domains of cargo proteins (Figure 1.3) and generally fulfill two criteria: i)



Figure 1.2: Biosynthetic trafficking pathways in polarized MDCK cells

Biosynthetic trafficking of cargo destined to (A) basolateral and (B) apical domains. Newly synthesized proteins are sorted into vesicles destined to the plasma membrane at the TGN and delivered either (1) directly or through (2) recycling endosomes (RE) to the basolateral or apical surface as indicated.

deletion of the signal disturbs the basolateral targeting of the protein (115) and ii) transferring the signal to an apical protein mistargets it to the basolateral surface (23, 68, 105). Tyrosine based motifs such as NPXY or YXXØ are found in basolateral proteins like vesicular stomatitis virus G protein (VSV-G), whereas sorting signals with dileucine motifs are found in Fc receptors (138). Alternatively, some proteins such as polymeric immunoglobulin A and M (IgA and IgM) receptor, pIgR, contain unconventional basolateral sorting signals on their cytoplasmic tail that do not rely on tyrosine or leucine motifs (114, 125).

The basolateral trafficking information encoded by sorting signals is recognized by organelle-specific adaptor protein (AP) complexes (Figure 1.3). While AP2 is localized to the plasma membrane and regulates clathrin-dependent endocytosis (136), AP1, AP3, and AP4 are implicated in sorting of basolateral proteins (43, 155). AP4 is proposed to regulate basolateral sorting of the LDLR and TfR (155) and AP3 is associated with TGN as well as endosomes and involved in lysosomal targeting. In nonpolarized cells, AP3 is also implicated in regulating VSV-G exit from the TGN (123). Non-polarized cells have the ability to sort "apical" and "basolateral" cargo at the TGN (37), which suggests that sorting machinery may have common components in polarized and non-polarized cells. Therefore, AP3 may regulate basolateral trafficking of a subset of cargo in polarized cells. The AP1 complex has two forms; AP1A and AP1B, which share the same large (β 1, γ) and small subunits (σ 1) but differ in the medium (μ 1) subunit. AP1A medium subunit, µ1A, is ubiquitously expressed and this complex is involved in lysosomal targeting from the TGN. The µ1B subunit is 80% identical to the µ1A and expressed specifically in many, but not all, epithelial cells. In the polarized



Figure 1.3: Membrane trafficking steps and regulatory machinery

(1) Adaptor complexes recognize sorting signals and sort proteins into cargo vesicles. (2, 3, 4) Formation and budding of cargo requires small GTPases, coat proteins and possibly tethering complexes. (5) Cargo vesicles move from one membrane compartment to another via cytoskeletal rearrangements. (6, 7) Tethering of the cargo vesicle to the target membrane requires tethering complexes and small GTPases. (8, 9) Cargo vesicles fuse with the target membrane via SNAREs. kidney epithelial cell line LLC-PK1, that does not express µ1B, LDLR is mis-sorted to the apical surface (43, 44, 172). Other studies show that AP1B regulates delivery of a subset of cargo such as VSV-G to the basolateral surface during biosynthetic pathway while it is involved in sorting of LDLR and TfR during endocytic trafficking in polarized epithelial cells (22, 42, 50). These observations indicate that a subset of newly synthesized basolateral cargo is first delivered to the recycling endosomes and then sorted to the basolateral plasma membrane, therefore AP1B is involved in basolateral sorting of both biosynthetic and endocytic cargo at the recycling endosomes. Furthermore, these results place recycling endosomes at the crossroads of biosynthetic and endocytic trafficking in polarized epithelial cells and suggest that these compartments serve as a post-TGN sorting site in addition to their role in endocytic sorting.

Unlike basolateral sorting machinery, relatively less is known about apical sorting in the biosynthetic pathway. Apical signals can be localized to the cytoplasmic, lumenal and transmembrane domains of apical cargo. In addition to GPI-anchors and association with lipid rafts, N- and O-glycosylation of proteins act as apical sorting signals (37, 46, 130, 138, 150). Although several types of apical signals have been identified in different proteins, receptors that recognize these signals and regulate apical sorting of newly synthesized cargo at the TGN are not known. Interestingly, as described for basolateral trafficking, some apical cargo travel through intermediate endosomal compartments on their way to the apical surface from TGN and intriguingly, the apical sorting signals are involved in regulating which path the proteins follow (30). For instance, the non-lipid raft associated apical cargo endolyn is delivered to the apical

surface through the recycling endosomes whereas the lipid raft associated apical protein influenza hemagglutinin (HA) is delivered to the apical surface through a distinct endocytic compartment (30). This observation suggests that similar to biosynthetic trafficking of basolateral cargo, sorting of newly synthesized apical cargo can take place at a different population of recycling endosomes. In addition to sorting signals and adaptor complexes several other proteins such as GTPases, SNAREs, and tethering complexes (Figure 1.3) are involved in regulating biosynthetic trafficking towards apical and basolateral domains (79, 99, 101, 124, 162, 174, 190, 191) and the individual roles of these protein complexes are further discussed in sections 1.2-1.4.

1.1.2 Endocytic trafficking in polarized epithelial cells

In contrast to the relatively low rate of biosynthetic delivery to the plasma membrane, 50% of the cell surface area is endocytosed per hour in polarized MDCK cells (181). Internalized cargo can be trafficked through several endosomal compartments before reaching to its final destination and proper sorting during this pathway is central to maintain membrane polarity (181). Analyzing the protein machinery involved in these pathways is crucial to understanding how protein sorting is regulated during endocytic trafficking and how is it altered in disease states such as familial hypercholesterolemia.

Proteins endocytosed from either apical or basolateral domains can recycle, be delivered across the cell to the opposite domain in a process termed transcytosis, or be sent to lysosomes for degradation (Figure 1.4) (137, 142). The transcytotic pathway in polarized epithelial cells has been studied using MDCK cells transfected with plgR and following IgA transcytosis from the basolateral surface to the apical membrane (Figure



Figure 1.4: Endocytic trafficking in polarized MDCK cells

Proteins can be internalized from basolateral or apical poles of the polarized epithelial cells. Internalized proteins are either recycled back to the surface, transcytose to the other pole, or delivered to the lysosomes for degradation.

1.5) (6, 116). Polymeric IqA is synthesized and secreted by activated plasma cells in the lamina propria, internalized from the basolateral surface of epithelial cells by its receptor plgR, and then released to the mucosal surface where it acts to neutralize infectious pathogens (142). The first step of transcytosis is the internalization and delivery of plgR-lgA complex to the basolateral early endosomes (BEE) (Figure 1.5, 1a). While a small fraction of the receptor-ligand complex rapidly recycles back to the basolateral surface from this compartment (Figure 1.5, 1b), the majority of plgR-lgA complexes move to the common recycling endosome (CRE), which is a tubular endosomal compartment localized to the perinuclear region of the cell (Figure 1.5, 2a). In this compartment it is either recycled back to the basolateral surface (Figure 1.5, 2b) or sorted away from basolateral cargo such as Tf and delivered to the apical recycling endosome (ARE), which is a tubular vesicular compartment located underneath the apical pole of the cell (Figure 1.5, 3a). From the ARE, the IgA-plgR complex is finally carried to the apical membrane (Figure 1.5, 3b) and the extracellular domain of the plgR is cleaved by a protease, releasing the IgA associated with this domain in to the mucosal surface (8, 142).

In addition to the transcytotic pathway, polarized epithelial cells have recycling pathways that maintain the polarized distribution of internalized apical (e.g. aquaporin 2-water channel, plgR), and basolateral proteins (e.g. TfR, LDLR). The apical recycling pathway has been studied following IgA internalization from the apical surface in polarized MDCK cells (Figure 1.6). Upon binding to its ligand on the apical membrane,



Figure 1.5: Basolateral-to-apical transcytosis in polarized MDCK cells

(A) Polymeric IgA is internalized by binding to its receptor on the basolateral surface and delivered to the BEE (1a). A small fraction of the internalized cargo recycles back to the surface from this compartment (1b) while the rest is transported to CRE (2a). At CRE, while some of IgA-pIgR complex recycles back to the basolateral surface (2b), the rest is sorted away from the basolateral cargo and moved to ARE (3a). From ARE, the ligand is transported to the apical surface (3b) and released to the mucosal surface. plgR gets internalized and IgA-plgR complex is delivered to apical early endosomes (AEE, Figure 1.6, 1a). From this compartment some receptor-ligand complex rapidly recycles back to the apical domain (Figure 1.6, 1b) whereas the remaining population moves to the ARE (Figure 1.6, 2a) and then recycles back to the apical pole (Figure 1.6, 2b) (18, 90, 142).

The basolateral surface comprises a majority of the plasma membrane surface in a polarized epithelial cell and contains receptors such as TfR or LDLR that are involved in the homeostatic regulation of molecules like iron or cholesterol. Basolateral cargo, such as Tf, gets internalized from the basolateral surface by binding to its receptor, TfR, and the receptor-ligand complex is first delivered to BEE (Figure 1.7, 1a). In these endosomal compartments, some of the Tf-TfR complex recycles back to the basolateral surface (Figure 1.7, 1b), whereas the rest is transported to the CRE (Figure 1.7, 2a), and recycle back to the basolateral domain from this compartment (Figure 1.7, 2b) (5, 137).

Transcytosis of IgA from the basolateral surface to the apical membrane and secretion to the mucosal surface is crucial for the neutralization of pathogens that may enter to the body through mucosa (78, 142, 163). In addition, the apical recycling pathways are not only important for the cells to maintain apical polarity of certain proteins but also to regulate the number of channels and receptors on the apical surface, which are crucial for the regulation of reabsorption of certain ions or macromolecules (11, 152). As described previously, obstructing basolateral recycling of



Figure 1.6: Apical recycling in polarized MDCK cells

(A) An apical cargo such as IgA is internalized by binding to its receptor on the apical surface and is delivered to the AEE (1a). A small fraction of the internalized cargo recycles back to the surface from this compartment (1b), while the rest is transported to ARE (2a) before it is recycled back to the apical surface (2b).



Figure 1.7: Basolateral recycling in polarized MDCK cells

(A) A basolateral cargo such as Tf is internalized by binding to its receptor on the basolateral surface and is delivered to the BEE (1a). A small fraction of the internalized cargo recycles back to the surface from this compartment (1b), while the rest is transported to CRE (2a) and then recycles back to the basolateral surface (2b).

LDLR receptor may lead to impaired cholesterol uptake and hypercholesterolemia due to increased cholesterol levels in the blood (33, 81, 146, 152). Therefore, it is important to understand the protein machinery involved in regulating endocytic pathways.

Similar to polarized biosynthetic trafficking, sorting signals and adaptor complexes (Figure 1.3) are important factors for endocytic trafficking. Signals involved in endocytic trafficking of basolateral cargo, such as TfR and plgR, are different than the sorting signals that regulate biosynthetic trafficking of these receptors (114, 125). Furthermore, the adaptor complex AP1B, which is associated with recycling endosomes, regulates endocytic trafficking of internalized TfR in addition to its role in biosynthetic trafficking. Several factors are involved in basolateral-to-apical transcytosis of plgR such as phosphorylation of the receptor and binding of the ligand to plgR. However, adaptor molecules that recognize apical signals and regulate sorting of endocytosed proteins in apical endocytic pathways are not known. In addition to these players, GTPases, SNAREs and tethering complexes (Figure 1.3) are shown to be important regulators of endocytic pathways (79, 99, 101, 124, 162, 174, 190, 191) and will be analyzed further in the following sections.

1.2 SMALL GTPases INVOLVED IN REGULATING MEMBRANE TRAFFICKING PATHWAYS IN POLARIZED EPITHELIAL CELLS

The Ras superfamily of small GTPases are GDP/GTP-regulated switches with more then 150 members and based on sequence and functional similarities they can be divided into 5 groups; i) Ras sarcoma (Ras), ii) Ras homologous (Rho), iii) Ras-like

proteins in brain (Rab), iv) ADP-ribosylation factor (Arf) and v) Ras-like nuclear (Ran) protein. Several members of these groups are involved in multiple membrane trafficking steps including vesicle formation, budding, and fusion as well as vesicle motility possibly via cytoskeletal arrangements (Figure 1.3, steps 2-6) (191). In polarized epithelial cells, members of the Rho, Rab and Arf GTPase subfamilies are associated with specific compartments and regulate transit through these compartments (31, 89, 143, 144, 162, 186). The selectivity in distribution and function indicate that the small GTPases may influence the polarity of biosynthetic as well as endocytic trafficking pathways in epithelial cells.

1.2.1 Ras family

The Ras family of GTPases are extensively studied due to their role in oncogenesis. Members of this family include but are not limited to Ras, Rap and Ral small GTPases. They respond to a variety of extracellular and intracellular stimuli and activate downstream signaling cascades, which in turn regulate several cellular functions such as gene expression, cell proliferation and survival (191), as well as neoplastic transformation (20, 92). In the mammalian genome there are two Ral GTPases (RalA and RalB) with 80% homology and survival of cancer cells depend on a balance between RalA mediated proliferation and RalB mediated inhibition of apoptosis (20). In addition to their role in oncogenesis, RalA and RalB are also involved in membrane trafficking pathways. RalA is localized to the plasma membrane and synaptic vesicles in neurons (129) and dense granules on platelets (103). Constitutive expression of an

activated RalA mutant and down-regulation of RalA but not RalB expression by siRNA causes a non-polarized distribution of basolateral proteins but not apical ones (111).

1.2.2 Rho family

Rho GTPases have been studied in several systems including yeast and mammalian cells. Most of the family members such as RhoA, RhoB, and Cdc42 are shown to regulate cytoskeletal rearrangements and influence cell shape, cell-to-cell, and cell-to-matrix interactions (134). Furthermore, these small GTPases are involved in membrane trafficking pathways in different cells types (135). In polarized epithelial cells RhoA, RhoB and Cdc42 are shown to regulate endocytic trafficking (89, 143, 144). Another member, TC10, is associated with lipid rafts, regulates tethering and docking of GLUT-4 vesicles with the plasma membrane (25, 71, 148).

1.2.3 Arf family

Arf GTPase family members interact with coat proteins, influence phospholipid metabolism as well as actin cytoskeleton dynamics (31, 80). One member of this family, Arf6, is implicated in cell morphology and polarity (34). Furthermore, Arf6 is variably associated with the plasma membrane and recycling endosomes based on its nucleotide status. Dominant negative Arf6 is associated with the recycling endosomes and inhibits recycling to the plasma membrane from these compartments (26, 32). In addition to its role in recycling pathway, Arf6 is also implicated in regulated exocytosis in

PC12 cells (180). These observations indicate that Arf family of GTPases are important players in endocytic membrane trafficking pathways.

1.2.4 Rab family

Several members of the Rab GTPase family are involved in different stages of vesicular transport in biosynthetic and endocytic membrane trafficking pathways (52, 80, 124, 162), possibly through influencing other protein complexes that regulate different vesicle trafficking steps. Prenylation as well as C-terminal sequences of Rab proteins influence their distribution to different compartments (191). In yeast, Sec4p is associated with cargo vesicles and regulate delivery of vesicles from the TGN to plasma membrane (56). In mammalian cells, Rab1 is localized to ER as well as Golgi and regulates ER-to-Golgi trafficking while Rab5 is associated with early endosomes and regulate endosome fusion through its effector early endosome antigen 1 (EEA1) (52, 162, 191). These observations show that Rab GTPases are associated with specific compartments and regulate vesicle trafficking events in these compartments.

Rab11a, Rab11b, and Rab25 are closely related members of the Rab11 subfamily (58). In non-polarized cells, Rab11a is localized to the TGN and recycling endosomes (133, 173, 195) and is proposed to have a role in regulating Tf recycling (133, 173). However, in polarized cells, a large fraction of Rab11a is localized to the pericentriolar ARE (18, 24). In agreement with this distribution, inhibiting Rab11a function by expressing GTP binding deficient Rab11a blocks basolateral-to-apical transcytosis and apical recycling of IgA, but does not affect basolateral recycling of Tf (189). The specific distribution of Rab11a and its role in polarized epithelial cells makes

it an important regulator of apical pathways, but the details of Rab11 function and the roles of downstream effectors that are involved in this regulatory role are not completely understood. Although Rab11b has high sequence homology with Rab11a and is localized to apical pole of the cell, the colocalization between Rab11b and Rab11a is incomplete (83) and its regulatory role in polarized trafficking pathways is not known. Rab25 colocalizes with Rab11a at the ARE and is involved in regulating apical recycling and basolateral-to-apical transcytosis but not basolateral recycling pathways (24, 189).

Several proteins that contain a C-terminal Rab11 binding domain (RBD) have been identified and classified as the "family of Rab11a interacting proteins" (FIPs). According to additional structural motifs, FIPs are classified into three groups. Class I FIPs such as FIP2 and Rip11 contain a C2 domain, while class II FIPs have EF hand domains and class III FIPs have no homology to known proteins (58, 76). Mutations in an important Rab11a effector, FIP2, lead to inhibition of apical pathways (36, 58). FIP2 is also involved in association of actin motor protein myosin Vb to Rab11a and overexpression of myosin Vb tail which lacks the motor domain has a dominant negative effect by changing Rab11a distribution and blocking both basolateral-to-apical transcytosis and apical recycling (59, 84). Interestingly, several FIPs interact with Rab11b and Rab25 in addition to Rab11a (58) but the significance of these interactions is not well understood. These observations underline the importance of Rab11a in regulating polarized trafficking pathways. Although several effectors of Rab11a are identified, there are unknown questions about how it regulates different trafficking pathways.

Rab GTPases interact with Arf and Rho GTPases and their effectors (26, 70), and this crosstalk between different small GTPase families and their effectors may influence several membrane trafficking steps during biosynthetic and endocytic pathways. Furthermore, several studies show that Rab GTPases and their effectors interact with SNARE machinery and may influence their function (202). These observations suggest that small GTPases may act as "membrane organizers" to connect tethering complexes and SNAREs, and define their activity on different membrane domains.

1.3 SNARES

At the last stage of membrane trafficking, cargo vesicles fuse with the target membrane through the action of soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment receptor (SNARE) machinery (Figure 1.3, steps 8-9), which are originally classified as vesicle associated (v-SNARE) and target membrane associated (t-SNARE) complexes (159). This nomenclature is useful for asymmetric reactions such as cargo vesicle fusion with plasma membrane; however, it is not as definitive in homotypic fusion reactions where there is no asymmetry. Therefore, an alternative classification system, which defines SNARE complexes based on the presence of a key arginine (R-SNARE) or glutamine (Q-SNARE) residue has been developed (174).

T-SNAREs can be divided into either syntaxin types with a transmembrane domain or SNAP-25 types, which are anchored to the membrane through thioesterlinked acyl groups (174). Although the exact mechanism of SNARE mediated

membrane fusion is not well understood, it is suggested that SNARE complex assembly takes place when v- and t-SNAREs come together to form coiled-coiled helix bundles, which brings the vesicle and target membranes to close proximity to initiate membrane fusion (159, 174, 190). After the membrane fusion is complete, SNARE complexes disassemble by NSF and SNAP proteins to be recycled and used in additional rounds of fusion events. α -SNAP is suggested to recognize assembled SNARE complexes and recruit NSF, which provides the energy to initiate SNARE disassembly by hydrolyzing ATP (74).

In addition to NSF and SNAP proteins, several regulators of SNARE assembly and function have been identified. Munc18 is shown to regulate availability of syntaxins for SNARE complex assembly (205), while complexins bind to SNARE complexes and regulate exocytosis (159). Several members of Rab GTPase family are shown to be associated with fusion sites and regulate SNARE complexes. For instance, Rab5, which is associated with early endosomes, regulates membrane fusion in these compartments via the tethering protein EEA1, and t-SNAREs (syntaxin-13 and syntaxin-6) (106, 158, 202). This observation indicates the importance of small GTPases in regulating SNARE functions at different cellular compartments, possibly by recruiting their effectors to distinct compartments and catalyzing either the assembly or disassembly of SNARE complexes.

In polarized epithelial cells, TGN-to-basolateral delivery of newly synthesized proteins is blocked by antibodies against NSF and neurotoxins that cleave v-SNAREs. Furthermore, the t-SNARE syntaxin-4 is mainly localized to the basolateral domain (69). Although t-SNARE syntaxin-3 is associated with the apical membrane and required for

TGN-to-apical delivery, NSF may not be involved in this pathway (88, 98). This indicates that the original SNARE model that requires NSF-mediated disassembly may be different in apical trafficking. During endocytic trafficking, basolateral-to-apical transcytosis of plgR is independent of syntaxin-3 but requires NSF and SNAP25, however, the role of SNARE complexes in this pathway is not well understood (88, 98). Interestingly, the apical t-SNARE syntaxin-3 is involved in apical recycling pathway (98), which suggests that apical recycling and basolateral-to-apical transcytosis may be regulated through different mechanisms.

Both in yeast and mammalian cells, t-SNAREs are distributed ubiquitously on the membrane (193); however, the vesicle delivery is highly polarized toward certain parts of the membrane domains. For instance, during biosynthetic trafficking in polarized epithelial cells, vesicles carrying newly synthesized proteins from the TGN to the basolateral membrane are trafficked to the areas under the tight junctions, which are active exocytosis sites in polarized epithelial cells (51). Furthermore, different v- and t-SNAREs are shown to form promiscuous complexes *in vitro* (74) and interfering with SNARE complex function in squid synapses or flies increases the number of vesicles associated with target membranes (193). These observations question the ability of SNARE complexes to provide specificity to vesicle trafficking pathways. Furthermore, they suggest that SNAREs are involved in membrane fusion but there is an initial stage in which cargo vesicles are associated with the target membranes and this function is partially attributed to the family of tethering complexes (190, 193) (Figure 1.3, C).

1.4 TETHERING COMPLEXES

Although there is a growing understanding about the function of sorting signals, adaptor complexes, and SNAREs (21), significantly less is understood about tethering complexes, which are important components of the polarized sorting machinery. Tethering refers to a reversible physical interaction between a vesicle and target membrane preceding SNARE-mediated fusion (Figure 1.3, step 7) (118). Tethering complexes bring cargo vesicles in close proximity to their target membranes (168, 193). Furthermore, they provide specificity to vesicle delivery by targeting cargo vesicles to active exocytosis domains on plasma membranes. Although it was once thought that tethering complexes are assembled on the target membrane, tethering complexes can also be found on donor membranes and cargo vesicles (99, 101, 190, 193). Reflecting this expanded distribution, tethering complexes are also implicated in steps such as vesicle release (Figure 1.3, steps 2-4) (168, 193).

Tethering complexes are categorized broadly into two classes: long, coiled-coiled molecules that form dimers (e.g. EEA1) or multi-subunit complexes such as the exocyst (Figure 1.8 A&B) (99, 193). The coiled-coiled tethering proteins have been analyzed in yeast as well as mammalian cells. They have the ability to form homodimeric structures that have an extended confirmation, which may be longer then the diameter of a vesicle (193). EEA1 is an extensively studied coiled-coiled tethering protein that is associated with early endosomes and regulates fusion of endocytic vesicles with early endosomes (196). The C-terminus of EEA1 contains a FYVE domain, which binds to phosphatidylinositol 3-phosphate and is involved in recruitment of EEA1 to the early endosomal membrane (87). EEA1 also interacts with the small GTPase Rab5 via a



Figure 1.8: Tethering complexes

Two types of tethering complexes. (A) Coiled-coiled tethering proteins generally form dimers and attach vesicles to target membranes. They can recognize "receptor" proteins or lipids on cargo and target membrane domains. (B) Multi-subunit tethering complexes are composed of several subunits.
different domain (86). Both lipid and Rab5 interactions are necessary for the stable association of EEA1 with the early endosomes. Furthermore, EEA1 forms a complex with the t-SNAREs syntaxin-13 and syntaxin-6, which is involved in regulating endosome fusion (106, 158).

In contrast to coiled-coiled tethering proteins, multi-subunit tethering complexes are composed of several subunits; examples include the conserved oligomeric Golgi (COG), the transport protein particle (TRAPP), and the exocyst. These protein complexes are required for biosynthetic and endocytic traffic (127, 193). The COG complex contains 8 subunits (Figure 1.9A) and is mainly involved in retrograde protein trafficking within the Golgi complex (19, 132, 165). Although additional roles of the COG complex in other pathways are not completely understood, it is speculated to be involved in ER-to- Golgi, intra-Golgi trafficking and sorting at the endoplasmic reticulum (ER) (110, 177, 178). Several components of trafficking machinery such as coat proteins, SNAREs, and small GTPases interact with COG subunits (127), implicating this tethering complex in functions such as cargo sorting, vesicle formation, and delivery. Furthermore, COG subunits can be divided into two structurally distinct groups composed of COG1-4 and COG5-8, which form two subcomplexes associated with each other (Figure 1.9A) (127). The functional significance of these subcomplexes and whether they act independently of each other are not known.



Figure 1.9: COG and TRAPP tethering complexes

Subunits and assembly models of (A) COG and (B) TRAPP tethering complexes. (A) COG complex has 8 subunits that are assembled into 2 structurally distinct subcomplexes. (B) TRAPP complex contains 10 subunits and there are two structurally and functionally different TRAPP complexes. TRAPPI has 7 subunits whereas TRAPII contains all 10 subunits. Models are adapted from Oka and Krieger (127).

The TRAPP complex consists of 10 subunits (Figure 1.9B) and is associated with the Golgi complex (127). It is involved in ER-to-Golgi trafficking (127, 145), probably by regulating the association of ER-derived vesicles with the Golgi membrane. When resolved by gel filtration chromatography, two TRAPP complexes with different subunit composition have been identified (147). TRAPP-I contains 7 subunits (Figure 1.9B), binds to ER-derived COPII vesicles, and may be involved in ER-to-Golgi trafficking by regulating the uncoating of COPII vesicles (147). TRAPP-II contains all 10 subunits (Figure 1.9B), and is involved in regulating intra-Golgi trafficking (147). Interestingly, the presence of the 3 extra subunits on this complex prevents its association with COPII vesicles (147). Furthermore, each TRAPP complex may interact with a different set of GTPases to regulate distinct pathways (127), which indicates the importance of identifying small GTPases that can interact with these complexes and analyzing the functional significance of these interactions.

Another multi-subunit tethering factor is the exocyst complex and it is implicated in several cellular functions including mRNA splicing (9), protein synthesis at the ER (93, 94), cell division (39, 184, 185), cell mobility (164, 175, 206) as well as biosynthetic (51, 199) and endocytic traffic (131). Its role in regulating polarized trafficking pathways in epithelial cells is not completely understood and is the focus of my dissertation.

1.5 THE EXOCYST COMPLEX

The exocyst complex subunits were identified in *Saccharomyces cerevisiae* as a family of proteins involved in the secretory pathway. Both the *S. cerevisiae* and metazoan

exocyst are comprised of 8 subunits (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p), all of which are essential except Sec3p (169, 170). In the fission yeast *Schizosaccharomyces pombe*, the exocyst complex contains only 6 subunits (Sec6p, Sec8p, Sec10p, Sec15p and Exo70p) (184). The majority of *S. cerevisiae* exocyst subunits are associated with cargo vesicles while Sec3p is localized to the plasma membrane and Exo70p is found in both compartments (16). In higher organisms, exocyst subunit orthologs have been identified and are associated with the plasma membrane as well as intracellular compartments, where they regulate membrane trafficking steps that precede SNARE-mediated membrane fusion (Figure 1.3, step 7) (28, 42, 95).

The initial experiments that implicated the exocyst as a tethering complex came from analyzing mutant yeast strains that lacked exocyst subunits. These experiments showed that the deletion of any exocyst subunit with the exception of Sec3p caused an accumulation of secretory vesicles under the plasma membrane near the growing bud, in a step that preceded vesicle fusion (40, 41). In addition, as noted above, t-SNAREs are distributed ubiquitously on the yeast plasma membrane, however vesicle delivery is highly polarized toward the budding tip of the daughter cell, which is an active site of exocytosis during bud formation (66). Interestingly, the exocyst complex distribution on the plasma membrane is specifically restricted to active exocytic sites both in yeast and mammalian cells (66). These observations indicate that the exocyst acts as a tethering complex and regulates docking/tethering of cargo vesicles to specific sites at the plasma membrane.

As further evidence of exocyst involvement in late stages of vesicle trafficking, the exocyst associates with SNAREs, and SNARE associated proteins (e.g. Sec1p) (99, 168, 194). In accordance with this observation, overexpression of syntaxin (Sso1, Sso2) and SNAP25 (Sec9p) homologues of SNARE machinery rescues $\Delta sec15p$, while overexpression of Sso1 and Sso2 rescues $\Delta sec5p$ and overexpression of Sec9p rescues $\Delta sec8p$ phenotypes (171, 194). These observations suggest that the presence of excess SNAREs may bypass the requirement for certain exocyst subunits by stabilizing the association of the vesicle with the membrane to initiate membrane fusion, therefore implicating the exocyst complex in the vesicle tethering step that precedes SNARE-mediated fusion (Figure 1.3, step 7).

Although the sequence homology is limited between different exocyst subunits, recent structural analyses show that they fold into similar rod shaped structures composed of α -helices (118). Nearly full-length structures are available for the yeast and mouse Exo70. Both yeast (Figure 1.10A) and mouse (Figure 1.0B) Exo70 are similarly folded into long rod-like structures with α -helical bundles; however the orientation of the C-terminus is different between the two species (60, 109). The overall helical content of the Exo70 N-terminus is similar to the C-terminal domains of the yeast Sec6p, Exo84p, and *Drosophila* Sec15 (Figure 1.10C-E) (118). Furthermore, secondary structure predictions indicate that the remaining subunits (Sec3, Sec5, Sec8 and Sec10) may also fold into similar structures (118).

Based on these observations it is speculated that the rod-like exocyst subunits stack side-by-side to form the octomeric complex (Figure 1.11A) (118). This model is also consistent with the quick-freeze/deep-etch EM images of *in vitro* assembled



Figure 1.10: Exocyst subunit structures

Structures of complete *S.cerevisiae* (A) and mouse (B) Exo70. C-terminal domains of Sec6p, Exo84p and *Drosophila* Sec15 (C-E). N-terminals of the proteins are indicated. Colors represent predicted domains. Graphics were generated by PyMOL (http://pymol.sourceforge.net/).

exocyst complex (65), which resembles a mushroom with a rod-like structure and extended appendages. This assembly model requires several protein-protein interactions between different subunits, which have been identified by yeast-two-hybrid and pull-down assays (198), and are summarized in Figure 1.11B. Furthermore, based on this model, it is speculated that the exocyst complex may interact with other components of the trafficking machinery such as small GTPases or SNAREs via extended appendages (198). Although far from being complete, the structural analyses of individual exocyst subunits provide insight into the assembly mechanism of the exocyst complex.

1.5.1 Assembly and membrane recruitment of the exocyst complex

The function of the exocyst complex relies on the proper assembly of its subunits on the appropriate membrane domains; however, the details of the exocyst recruitment and assembly process are largely unknown. In yeast, Sec3p is proposed to act as a spatial landmark for exocyst assembly through its interaction with the small GTPase Rho1p (57). However, some exocyst subunits are correctly targeted to the plasma membrane in the absence of the Sec3p-Rho1p interaction (57), which suggests that other exocyst subunits or proteins may be involved in exocyst targeting. One possibility is Exo70, which was recently shown to bind phosphatidylinositol phosphate (PIP) species. In yeast, Exo70p interacts with phosphatidylinositol 4,5-bisphosphate (PI[4,5]P₂), and interfering with this interaction leads to mislocalization of other exocyst subunits (62).



Figure 1.11: Exocyst complex assembly model and subunit interactions

(A) Exocyst subunits assemble into rod-like structures. (B) Summary of multiple subunitsubunit and subunit-GTPase interactions in yeast and mammalian exocyst complexes. Interactions and model are adapted from Munson and Novick (118). The mammalian Sec3 homolog lacks a 30 kDa N-terminal domain that is involved in recruitment of Sec3p to the plasma membrane via Rho1p interaction (104). Similar to its yeast homologue, the mammalian Exo70 also interacts with PIP species via positively charged residues at its C-terminal domain, and therefore may regulate the recruitment of other exocyst subunits to the plasma membrane. In support of this argument, interfering with the Exo70-lipid interaction inhibits the recruitment of exocyst subunits to the plasma membrane and VSV-G exocytosis (97). In mammalian cells, phosphoinositides PI[3]P and PI[3,5]P₂ are associated with endosomal membranes whereas PI[4,5]P₂ is found on the plasma membrane (53). PI[3,4,5]P₃ is mainly associated with the basolateral membrane (47). It acts as a signaling ligand at the plasma membrane (29) and involved in cell proliferation and motility (72, 96). Interestingly, the mammalian Exo70 interacts with PI[4,5]P₂, PI[3,4,5]P₃, and to a lesser degree with PI[3,5]P₂ (97), which suggests that Exo70 has the potential to be associated with both the plasma membrane and the endosomal membrane domains.

Other subunits or protein complexes may be involved in the recruitment of the exocyst to different cellular compartments such as the ER or the TGN. For instance, in yeast strains lacking Exo84p, the polarized distribution of other exocyst subunits is impaired; however, deletions of subunits other then Sec5p do not interfere with Exo84p polarity (54). These observations suggest that Exo84 is capable of associating with the plasma membrane regardless of the presence of other subunits, and it may have a role in the recruitment of the remaining exocyst subunits to membrane domains. Alternatively, other protein complexes may be involved in the recruitment and assembly

of exocyst complex on different membrane domains. For instance, in MDCK cells the exocyst is found at the plasma membrane in a complex with E-cadherin and nectin- 2α during cell polarization (199). Based on these observations, it can be argued that the exocyst complex is recruited to specific membrane domains by multiple mechanisms including exocyst-lipid interactions, cell adhesion molecules, or small GTPases. The role of exocyst-GTPase interactions in regulating exocyst recruitment and function will be discussed further in the following sections.

Although studies analyzing the mechanisms involved in the membrane recruitment of the exocyst provide an insight into the hierarchy of exocyst subunit interactions, the process of exocyst assembly is not understood. In yeast, Sec15p and Sec10p form a subcomplex that is found in the cytosol as well as in the holocomplex. The interaction between Sec15p/Sec10p subcomplex with the rest of the subunits is mediated by Sec5p, which also interacts with Sec3p, Sec6p, Exo70p, and Exo84p (54). It is possible that after membrane binding, Sec3 and Exo70 recruit Sec5, which then recruits the other subunits, therefore acting as a core subunit. In accordance with these observations, the loss of Drosophila Sec5 impairs the membrane association of Sec6 and Sec8, while in the absence of Sec6, both Sec5 and Sec8 remain membrane associated (120). Other studies show that in mammalian cells, Sec15, Sec10, and Exo84 are associated with the secretory vesicles, and Sec5, via its interaction with Exo84 regulates assembly of the exocyst complex (112). Furthermore, the small GTPase RalA is also implicated in exocyst assembly by regulating Sec5/Exo84 assembly (112). These observations support the model, which relies on Sec5 as the core subunit that links membrane associated exocyst subunits with the rest of the

complex on the vesicles. Furthermore, small GTPases may facilitate exocyst assembly/disassembly by regulating the interactions between Sec5 and other subunits.

1.5.2 The exocyst and subcomplexes

Another important aspect of the exocyst assembly is whether the subunits are always assembled together to form an octameric complex or whether there are subcomplexes with different subunit compositions. Functional studies from *S. cerevisiae* show that lack of most exocyst subunits leads to the same phenotype characterized by secretory deficiency (169, 170), which support presence of a single octomeric complex with multiple functions. In contrast, biochemical studies show that yeast Sec15p and Sec10p form a subcomplex that is separate from the octomeric complex (56). This observation indicates presence of exocyst subcomplexes separate from the octomeric complex. Alternatively, Sec15/Sec10 subcomplex may act as a bridge between the vesicle and plasma membrane associated exocyst subunits to regulate vesicle docking, therefore may not have a separate function. Further biochemical support for the presence of multiple exocyst subcomplexes comes from mammalian studies showing Sec10/Exo84, and possibly Sec15, form a subcomplex that is distinct from another subcomplex that contains Sec5 and Sec6 (112). However the functional significance of these subcomplexes are not known.

In addition, morphological studies in *Drosophila* show that the expression patterns of Sec5, Sec6, and Sec8 during development are different and their localizations are not identical in different tissues (13, 107). Furthermore, functional studies indicate that lack of *Drosophila* Sec5, Sec6, or Sec8 subunits is lethal, however

Sec15 mutant flies survive with deficient synapse formation (107). These observations further suggest presence of subcomplexes composed of separate subunits in a higher organism. In mammalian system, Sec8 knock-out mice are not viable (45), however a specific mouse strain (hbd) that lacks an isoform of Sec15 (Sec15I1/Sec15A) is normal aside from a hemoglobin deficiency (91, 203). While this difference can be explained by the presence of separate exocyst subcomplexes, an alternative explanation comes from the presence of different mammalian exocyst isoforms and splice variants. Although the functional significance of these alternative forms of exocyst subunits is not known, the milder phenotype of Sec15A-lacking hemoglobin deficient mice could be explained by the presence of Sec15B.

If there are multiple exocyst subcomplexes composed of different subunits, what is the mechanism that may regulate their function? One possibility is the interactions between different exocyst subunits and small GTPases. As described previously, small GTPases are associated with specific compartments and regulate distinct cellular events. Therefore, exocyst-GTPase interactions may provide specificity to the different exocyst subcomplexes.

1.5.3 The exocyst and GTPases

Several small GTPases that belong to the Rho, Ras, Arf, and Rab families interact with the various exocyst subunits (Figure 1.9B) and are proposed to regulate exocyst function (95). The mechanism of GTPase regulation of the exocyst function is not known. One possible model is the recruitment of the exocyst complex or different subcomplexes to specific membrane domains by the activation of small GTPases. For

example, Rho1p recruits Sec3p to the plasma membrane, and interfering with the Rho1p-Sec3p interaction by deleting the N-terminus of Sec3p interferes with the recruitment of Sec3p to the yeast plasma membrane (57).

However, not all exocyst-GTPase interactions are responsible for the recruitment of the exocyst complex to the membrane. For example, the small GTPase Sec4p, which is associated with the cargo vesicles, interacts with Sec15p subunit of the yeast exocyst complex. Furthermore, overexpression of Sec4p partially rescues the $\triangle sec15p$ phenotype (171, 194), which indicates that Sec4p is an upstream regulator of the exocyst complex, and may by-pass the Sec15p and interact with non-Sec15p components poorly when over expressed. The Sec4p-exocyst interaction is involved in cargo vesicle docking to the plasma membrane (56), however, Sec4p may not be involved in Sec15p recruitment to the plasma membrane, as Sec15p co-migrates with a vesicle marker Snc2p in Sec4p mutant yeast strains (56). Therefore, there may be additional factors involved in the recruitment of the exocyst complex to the specific membrane domains. The Exo70p subunit interacts with different lipid species (62, 97) and may be involved in GTPase-independent association of the exocyst complex with different compartments. In this model the activation of the small GTPase would recruit its downstream effectors to a specific membrane domain and the exocyst complex or subcomplexes, which are already associated with this membrane domain, may interact with these effectors and thereby regulate different membrane trafficking steps such as vesicle tethering or vesicle formation and budding (Figure 1.3, steps 2-7). Curiously, the exocyst complex can bind to both the cargo sorting machinery (AP1B) and the vesicle fusion machinery (SNAREs). Therefore, small GTPases may regulate the exocyst

function at the donor membrane by incorporating it into vesicle formation/budding machinery, and at the target membrane by combining the exocyst mediated tethering with SNARE mediated fusion to facilitate vesicle fusion steps.

In higher organisms, small GTPases that interact with the exocyst complex have a wide variety of functions. For example, Sec5 and Exo84 subunits compete to interact with RalA (111, 112), which is involved in oncogenic transformation, endocytosis, filopodia formation, and actin-cytoskeleton dynamics (38, 75, 121, 126, 192). Blocking the RalA-Sec5 interaction in permeabilized PC12 cells with the RalA binding domain of Sec5 inhibits GTP dependent exocytosis (186). In polarized MDCK and Caco2 cells, constitutive expression of an activated RalA mutant and down-regulation of RalA expression by siRNA causes a non-polarized distribution of basolateral proteins but not apical ones (111), which implicates RalA as a regulator of basolateral trafficking in polarized epithelial cells. However, whether the RalA-exocyst interaction is involved in regulating trafficking pathways in polarized epithelial cells is not known. It is possible that RalA may recruit some exocyst subunits to recycling endosomes and regulate the delivery of basolateral cargo from these compartments to the basolateral plasma membrane.

Arf6 is another small GTPase that interacts with the exocyst complex (131). Arf6 is localized to the plasma membrane as well as recycling endosomes, and is proposed to regulate membrane trafficking and cell spreading (26, 31). Expression of dominant active Arf6 displaces Sec10 from the perinuclear region, however dominant negative Arf6 does not affect the distribution of Sec10 (131). Since most GTPases recruit their effectors to specific membrane domains while they are activated, this observation

suggests that the Sec10 recruitment may require additional factors (e.g. exocyst-lipid interactions). One explanation is that the exocyst is associated with the Arf6 positive compartment regardless of the activation state of the small GTPase; however, other effectors are recruited to the same compartment only after Arf6 is bound to GTP. Therefore, Arf6 acts as a bridge between the exocyst complex and other Arf6 effectors. The interaction between these effectors and the exocyst may then regulate the trafficking events. Intriguingly, overexpression of a dominant negative Sec10 construct, which replaces this subunit form the exocyst complex and possibly blocks its interaction with Arf6, inhibits basolateral Tf recycling in non-polarized cells (131). Therefore, the exocyst complex, either by its interaction with Arf6 or RalA, has the potential to regulate basolateral trafficking pathways; however, neither of these GTPase interactions nor the functional significance of their interactions with the exocyst complex have been analyzed in polarized epithelial cells.

Rab11a is another small GTPase that interacts with the *Drosophila* and the mammalian exocyst complex via the Sec15 subunit (197, 204). Rab11a is involved in apical trafficking pathways in polarized epithelial cells (24, 183), and it has several effectors that share a common Rab11 binding domain, which is not found in Sec15 (204). This suggests that Sec15 may interact with Rab11a through an alternative domain and not compete with other effectors for Rab11a binding. In *Drosophila*, the binding motif involved in the Rab11-Sec15 interaction is located in the C-terminal domain of Sec15, which folds into α -helical bundles that are conserved among different species (197); however, the Rab11 binding domain of mammalian Sec15 is not known.

The interaction between the exocyst and Rab11a implicates the exocyst complex in regulation of endocytic trafficking directed towards the apical pole of the polarized epithelial cells, however this hypothesis has not been analyzed and the functional significance of the Sec15-Rab11a interaction in endocytic trafficking is not known. In Chapter 3, I define a potential role for Rab11a in the regulation of exocyst function during endocytic pathways in polarized MDCK cells. In addition to the small GTPases that are discussed here in detail, several others also bind to the exocyst complex (summarized in Figure 1.9B) (95).

As noted above, the exocyst complex may act as a single octomeric complex with different functions or as multiple subcomplexes with distinct functions. In either model, the exocyst-small GTPase interactions regulate the function of the exocyst complex (or subcomplexes) either by recruiting the exocyst to specific compartments or by facilitating its interaction with other components of the vesicle trafficking machinery. Currently, the mechanistic details of the exocyst-GTPase interactions are unknown. However as summarized in this section, the exocyst complex interacts with small GTPases such as RalA, Arf6 and Rab11a, which are involved in several polarized trafficking pathways, and these interactions may have important implications for polarized trafficking.

1.5.4 The exocyst and polarized traffic

As noted above, the exocyst complex is associated with polarized membrane growth sites in yeast (55), in *Drosophila* (107, 119), and mammalian nervous systems (61, 67), as well as polarized epithelial cells (51, 199, 201). In cultured hippocampal neurons,

interfering with exocyst function blocks neurite outgrowth (61), while in *Drosophila*, neurons lacking Sec5 have impaired delivery of membrane-associated proteins to the axons, whereas neurotransmitter release is not affected (119). These observations indicate that the exocyst complex is involved in polarized membrane trafficking during cell growth as well as delivery of membrane-associated proteins to specific domains; however, it is not required for all trafficking events. Intriguingly, the exocyst complex is also implicated in cytoskeletal arrangements, which may explain one of the mechanisms regulating polarized membrane growth. Specifically, the Exo70 subunit is implicated in microtubule and actin rearrangements, and it is also involved in the induction of membrane protrusions in migrating cells by regulating the Arp2/3 complex (188, 206). Furthermore, the Sec5-RalA interaction is involved in filopodia formation (164). These observations suggest that the exocyst may regulate polarized membrane trafficking to active exocytosis sites on the plasma membrane via cytoskeletal arrangements.

Other studies implicate the exocyst in regulating polarized protein trafficking at earlier stages such as the ER or TGN (120, 131). In polarized MDCK cells, a subpopulation of Sec6 is associated with the TGN and regulates the exit of newly synthesized basolateral cargo from this compartment (201). This observation is consistent with studies showing that in *Drosophila* larvae, lack of Sec6 interferes with protein expression, possibly at the TGN level (120). Furthermore, in polarized MDCK cells, overexpression of Sec10 selectively increases synthesis and delivery of basolateral and apical secretory proteins and basolateral but not apical, membrane proteins by stimulating ER translocation (93, 94).

The combination of these findings with previously described GTPase interactions suggests that the exocyst complex regulates various membrane trafficking pathways; however, its role in regulating endocytic trafficking pathways in polarized epithelial cells is largely unknown.

1.5.4.1 The exocyst and basolateral trafficking

The general dogma at present is that in polarized epithelial cells, the exocyst is involved in delivery of basolateral cargo from the TGN and endosomes to the plasma membrane, to a location near the tight junctions. This conclusion is based on studies done in polarized MDCK cells that show that the exocyst subunits Sec6 and Sec8 are variably localized to the TGN and tight junctions (51, 199, 201), and that delivery of newly synthesized LDLR to the basolateral membrane is inhibited by using function blocking antibodies in cells permeabilized with streptolysin-O (SLO), which is a bacterial toxin that binds to cholesterol in plasma membrane of the cells (51, 201). These observations indicate that the mammalian exocyst complex, similar to its yeast homologue, forms docking patches at active exocytosis sites and regulates the delivery of biosynthetic cargo to the basolateral plasma membrane in polarized epithelial cells. In Drosophila epithelial cells, the exocyst complex also regulates trafficking of DE-Cadherin from the TGN to the basolateral surface (82). However, in these cells, the exocyst subunits are associated with the recycling endosomes, and knocking down their expression leads to an accumulation of DE-Cadherin in these compartments (82). Based on the accumulation of cargo in recycling endosomes instead of vesicle accumulation next to the plasma membrane, it can be argued that in *Drosophila* epithelial cells, the exocyst complex regulates release of the cargo from the recycling endosomes.

These studies implicate the exocyst complex in basolateral delivery of newly synthesized proteins, and suggest that the site of exocyst action is both the recycling endosomes and the plasma membrane. In accordance with these observations, in nonpolarized mammalian cells, exocyst subunits are associated with the AP1B and Tfpositive endosomes (44). As mentioned in previous sections, AP1B is involved in regulating basolateral sorting of both biosynthetic and endocytic cargo at the recycling endosomes (22, 42, 50). The exocyst complex interacts with AP1B, and consistent with this observation, interfering with the exocyst function either by expressing dominant negative constructs or siRNA silencing leads to an accumulation of Tf in recycling endosomes (131). These observations implicate the exocyst complex in regulating basolateral trafficking from the recycling endosomes to the plasma membrane during both biosynthetic and endocytic trafficking in nonpolarized cells. However, whether the exocyst complex is involved in endocytic trafficking has not been analyzed in polarized epithelial cells. In order to test the involvement of the exocyst complex in the regulation of basolateral endocytic trafficking, I first analyzed the association of the exocyst subunits with recycling endosomal compartments that are involved in basolateral trafficking in polarized epithelial cells. Then I performed functional studies to test the role of the exocyst complex in regulating basolateral trafficking. The results of these studies are discussed in detail in Chapter 2.

1.5.4.2 The exocyst and apical trafficking

Although there are several clues that support the involvement of the exocyst in basolateral trafficking, its role in apical trafficking remains an open question. In SLO-permeabilized polarized epithelial cells, interfering with the exocyst function does not

affect delivery of p75 from the TGN to the apical surface (51). This study suggests that the exocyst is not involved in biosynthetic trafficking of apical cargo in polarized epithelial cells, however its role in regulating apical recycling or basolateral-to-apical transcytosis of endocytosed cargo is not known. Furthermore, in Drosophila photoreceptor cells, the exocyst regulates the delivery of rhodopsin from the TGN-torhabdomere, a densely packed apical compartment (13). In addition, overexpression of Sec10 increases synthesis and delivery of apical secretory proteins in polarized MDCK cells (93). Other evidence that implicates exocyst in apical trafficking is the association of Sec8 with Ca⁺² signaling molecules at the apical pole of the pancreatic acini cells. In these cells, antibodies against Sec8 inhibit Ca⁺² signaling (153), which suggests that Sec8 has a role in at least one type of apical trafficking. Further evidence that implicates the exocyst complex in apical pathways comes from Drosophila epithelial cells. In this system, lack of several exocyst subunits interferes with the "transcytosis" of DE-Cadherin from the lateral surface to the apical adherens junctions, which are localized above the tight junctions of *Drosophila* epithelial cells (82).

These observations challenge the model that the exocyst is simply a "basolateral tethering complex" and suggests it may have a broader role in polarized trafficking pathways, which has not been investigated. To date, the most important connection between the exocyst complex and apical trafficking comes from its interaction with Rab11a (197, 204). As described previously, Rab11a is localized to the ARE and regulates apical, but not basolateral, trafficking in polarized epithelial cells (24, 189). However, the localization of exocyst subunits to Rab11a-positive ARE has not been analyzed in polarized epithelial cells. Furthermore, the association of exocyst complex

with other compartments involved in apical recycling or basolateral-to-apical transcytosis is unknown. In summary, whether the exocyst acts as an apical tethering complex and whether the Rab11a-exocyst interaction has any functional significance in regulating this function have not been investigated in polarized epithelial cells. I analyzed the role of the exocyst complex in apical trafficking pathways and the functional significance of the Rab11a-exocyst interaction in Chapters 2 and 3.

1.6 GOALS OF THIS DISSERTATION

As summarized in the previous sections, several components of the protein machinery such as the adaptor complexes that recognize specific sorting signals, SNAREs and GTPases that regulate endocytic trafficking pathways in polarized epithelial cells have been extensively studied. However, other components of the trafficking machinery such as the tethering complexes are shown to be involved in regulating the delivery of endocytosed cargo vesicles to specific membrane domains in polarized epithelial cells. Initial studies suggested a model in which the exocyst complex is the missing link that regulates delivery of basolateral cargo vesicles to their specific membrane domain. This model may not be complete, and the exocyst complex may regulate additional trafficking pathways. My dissertation research seeks to better understand the role of the exocyst complex in regulating multiple endocytic trafficking pathways directed towards apical as well as basolateral surface of the polarized MDCK cells. Based on the information summarized above, I hypothesize that the exocyst complex regulates multiple endocytic pathways, and that the pathway specific role is dictated by its

interaction with different small GTPases. In Chapter 2.0, I characterize the general function of the exocyst complex in regulating endocytic trafficking. First by analyzing the distribution of the exocyst complex in fully polarized epithelial cells, I show that there is subpopulation of exocyst subunits associated with recycling а endosomal compartments. Then by blocking the function of the exocyst complex, I analyze its involvement in regulating different post-endocytic trafficking pathways. These studies show that the exocyst regulates basolateral-to-apical transcytosis as well as basolateral and apical recycling. Furthermore, in addition to its role as a tethering complex, I show that the exocyst is involved in vesicle budding from the ARE during basolateral-to-apical transcytosis. In Chapter 3.0, I present evidence that the Rab11a-exocyst interaction regulates basolateral-to-apical transcytosis. This observation supports the second part of my hypothesis, which argues that the interaction between exocyst and small GTPases provides specificity to the exocyst functions. The evidence provided in this thesis is important to our understanding of the regulatory components of polarized trafficking events in epithelial tissues. This information is valuable to the study of the molecular mechanisms underlying several pathological conditions that may be related to the mis-sorting and delivery of crucial proteins to incorrect plasma membrane domains as well as to our understanding of how certain pathogens might be hijacking the trafficking pathways to bypass epithelial barriers.

2.0 EXOCYST REQUIREMENT FOR ENDOCYTIC TRAFFIC DIRECTED TOWARD THE APICAL AND BASOLATERAL POLES OF POLARIZED MDCK CELLS*

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2.1 INTRODUCTION

The generation and maintenance of epithelial polarity, which is indispensable for the functional integrity of epithelial tissues, requires sorting, transport, and delivery of newly synthesized and endocytosed proteins to the correct apical or basolateral plasma membrane domain (200). The exocyst complex is required for tethering exocytic carriers to target membranes in eukaryotic cells (118, 187). In epithelial cells the exocyst is variably localized to the Golgi apparatus, TGN, recycling endosomes, and the junctional complex, and is proposed to promote the targeting and fusion of biosynthetic and endocytic recycling cargo carriers with the basolateral plasma membrane domain, possibly at sites near the tight junction (44, 131, 199, 201). Although, exocyst is implicated in basolateral biosynthetic pathway, association of exocyst subunits with

endocytic compartments and its role in regulating endocytic trafficking pathways in polarized epithelial cells in unknown.

Initial studies of exocyst subunit distribution in MDCK cells showed that Sec6 and Sec8 were localized at or near the tight junctions of cells after initiation of cell-to-cell contact or tubulogenesis (51, 93, 140). Other studies showed that the exocyst was associated with intracellular compartments including the Golgi, the TGN of MDCK cells expressing a kinase-inactive mutant of protein kinase D, and the recycling endosomes of wild-type MDCK cells (3, 131, 201). The discrepancy in exocyst localization may reflect, in part, the degree of cellular polarization, the growth conditions of the cells, the observation that some monoclonal antibodies differentially recognize pools of junctional versus intracellular populations of exocyst subunits (199, 201), and the possibility that the exocyst complex may exist in different conformational states (extended or closed) depending on its localization in the cell (118).

Exocyst complex was proposed to promote the targeting and fusion of biosynthetic and endocytic recycling cargo carriers with the basolateral plasma membrane domain, possibly at sites near the tight junction (44, 131, 199, 201). Consistent with this model, addition of function-blocking antibodies to Sec8 inhibits delivery of newly synthesized proteins from the TGN to the basolateral, but not apical, surface of polarized MDCK cells (51). Overexpression of Sec10 stimulates the synthesis and delivery of basolateral, but not apical membrane proteins (93), while mutations in Sec5 or Sec6 inhibit trafficking of DE-cadherin from recycling endosomes to the basolateral domain of *Drosophila* epithelial cells (82). Furthermore, the RaIA GTPase, which interacts with Exo84 and Sec5, is required for basolateral but not apical trafficking

in polarized MDCK cells (154), and AP1B, a basolateral-selective epithelial cargo adaptor, recruits the exocyst complex to recycling endosomes (4, 44). Although it has not been experimentally shown that basolateral recycling is exocyst dependent, interfering with Sec10 function or decreasing Sec5 expression interferes with recycling endosome morphology and Tf recycling in non-polarized cells (131).

In contrast to the basolateral pathway, significantly less is understood about how targeted fusion is accomplished at the apical pole of epithelial cells, although numerous indirect data suggest that the exocyst may play some role in these events. The exocyst is localized to the primary cilium (141); it regulates Ca²⁺ signaling at the apical domain of pancreatic acinar cells (153); it is required for targeting secretory vesicles to the rhabdomere (a densely packed tuft of microvilli located at the apical pole of insect photoreceptor cells)(13); it regulates exocytosis of apical secretory proteins in MDCK cells (93); it is associated with aquaporin-2 containing vesicles (which recycle at the apical pole of collecting duct principle cells) (11), and it may be involved in apical trafficking of Spdo/Notch/Delta (73). Intriguingly, the exocyst may also modulate a form of "transcytosis" whereby DE-cadherin is delivered from the lateral membranes to the adherens junctions localized above the septate junction of insect epithelial cells (82). In mammalian epithelial cells, the adherens junction is localized below the tight junctions and transcytosis refers to the transfer of endocytosed membrane and solutes between the apical and basolateral poles of the cell; however, it is unknown whether the exocyst directs basolateral-to-apical transcytosis or other forms of apically directed endocytic traffic in these cells.

2.2 RESULTS

2.2.1 The intracellular pool of exocyst subunits is associated in part with EEA1-, Tf-, and Rab11a-positive endosomes but not the TGN of polarized MDCK cells

In order to understand the localization of exocyst complex in polarized epithelial cells, I analyzed the distribution of Sec6, Sec8, and Exo70 in polarized, filter-grown MDCK cells. I initially tested a number of fixation conditions to optimize staining conditions and to determine whether the distribution was fixation or antibody sensitive. Variables included permeabilization with saponin prior to fixation (a treatment that removes the cytoplasmic pool of exocyst) (201), and fixation with either methanol, 4% paraformaldehyde in sodium cacodylate buffer (pH 7.4), or 4% paraformaldehyde at varying pHs (the pH-shift protocol) (8, 10). I initially examined the distribution of Sec6 and Sec8 using commercially available rSec6 and rSec8 monoclonal antibodies used in previous studies (51, 67, 93). Similar to these earlier studies I observed that Sec6 and Sec8 co-localized with the tight junction marker ZO-1 in cells fixed with either methanol or the pH-shift protocol (Figure 2.1A-B and D-E). Some tubulo-vesicular staining was noted, particularly in cells fixed using the pH-shift protocol and stained with the rSec8 antibody (Figure 2.1E). Samples fixed with 4% paraformaldehyde in cacodylate buffer gave a staining pattern similar to that observed for the pH-shift protocol.

In contrast, when cells were permeabilized with saponin prior to fixation with the pH-shift protocol, the distribution of Sec6 using the rSec6 antibody shifted from one associated with tight junctions to a tubulo-vesicular staining pattern (Figure 2.1C). Similarly, permeabilization with saponin enhanced detection of the tubulo-vesicular pool

of Sec8 (Figure 2.1F); however, Sec8 localization to tight junctions was still apparent under these conditions (Figure 2.1F). The Sec6-positive tubulo-vesicular elements (detected in saponin permeabilized cells using the rSec6 antibody) were observed in close proximity to the lateral cell surfaces and in the apical cytoplasm, both at the level of the tight junctions as well as in structures near the apical pole of the cell (Figure 2.2A). A similar distribution was observed for the tubulo-vesicular pool of Sec8 detected using the rSec8 antibody (data not shown).

In contrast to the rSec8 antibody, three Sec8 specific monoclonal antibodies (10C2, 5C3, or 2E12) (51) showed only the tubulo-vesicular elements, and no tight junction localization was observed under any fixation conditions tested (representative images using the 10C2 antibody are shown in Figure 2.2B). Sec8 was localized to tubulo-vesicular structures that were found along the lateral surface and in the apical cytoplasm of the cell. The tubulo-vesicular staining pattern was most apparent when the cells were permeabilized with saponin prior to fixation, but a similar staining pattern was discernable irrespective of fixation method, even in cells fixed without prior saponin treatment (data not shown). The specificities of these Sec8 antibodies were confirmed by western blot analysis, which yielded a single band of the expected ~100kD size (data not shown). Finally, I examined the distribution of Exo70 using previously described anti-Exo70 monoclonal antibody 13F3 (179). I observed a tubulo-vesicular staining pattern, regardless of fixation condition, that was similar to that observed for Sec6 or Sec8 (Figure 2.2C).

The intracellular pool was also observed in pre-extracted, semi-polarized MDCK cells grown on glass coverslips (data not shown). By removing soluble proteins, the



Figure 2.1: Effect of fixation conditions on the distribution of exocyst subunits in polarized MDCK cells

Cell were fixed using methanol (A&D), fixed using a pH-shift protocol (B&E), or permeabilized with saponin for 5 min at 4°C prior to fixation using the pH-shift protocol (C&F). (A-C) Distribution of Sec6 (detected using the rSec6 monoclonal antibody; green) and ZO-1 (red). (D-F) Distribution of Sec8 (detected using the rSec8 monoclonal antibody; green) and ZO-1 (red). (A-F) XZ sections through the monolayer are shown at the top of each set and single merged optical sections from the apical pole of the cell or at the level of the tight junctions are shown below. Scale bar = 10 μ m.

extraction procedure may cause exocyst-interacting proteins to dissociate from the complex, thus allowing antibody binding. Alternatively, the extraction procedure may alter the conformation of the exocyst, revealing the intracellular pool of subunits. Importantly, the intracellular pool of Exo70 (and of Sec8 using the 10C2, 5C3, and 2E12 antibodies) was observed even in cells not permeabilized with saponin prior to fixation (see for example Figures 2.3 and 2.4), confirming that the intracellular pool of exocyst is not simply an artifact of the saponin pre-treatment.

Next I assessed whether there was co-localization between Sec8 (using mAb 10C2) or Exo70 (using mAb 13F3) and the TGN marker furin. The tight junction protein ZO-1 was labeled to mark the position of the apico-lateral junction. Furin was localized to a ribbon-like structure that resided in a supranuclear position in the cell (Figure 2.3A&2.3C). I observed occasional regions where the furin-labeled TGN and Sec8 were in close proximity, but generally there was little co-localization between furin and Sec8 (Figure 2.3A). This lack of co-localization was also apparent for two other Sec8 antibodies (5C3 or 2E12) and Sec6 (data not shown). Exo70 was occasionally found associated with the ends of the TGN ribbons (see boxed region, Figure 2.3C), but there was generally not much overlap between Exo70 and furin (Figure 2.3C). Next, I analyzed if any of the exocyst-associated tubulo-vesicular elements were associated with EEA1-positive apical and basolateral early endosomes (AEE and BEE, respectively) (90). Although there was no co-localization between EEA1 and Sec8 (Figure 2.3B), or EEA1 and Sec6 (data not shown), there appeared to be some localization of Exo70 to EEA1-positive endosomes (Figure 2.3D). In this case, Exo70



Figure 2.2: Distribution of exocyst subunits in MDCK cells

(A) Cells were permeabilized with saponin for 5 min, fixed using a pH shift protocol, and then stained with the rSec6 monoclonal antibody (green) and an antibody to ZO-1 (red). The top two panels are identical to those shown in Supplementary Figure 1C. (B) Cells were permeabilized with saponin for 5 min, fixed using a pH-shift protocol, and then stained with the Sec8 monoclonal antibody 10C2 (green) and an antibody to ZO-1 (red). (C) Cells were fixed using a pH-shift protocol and then stained with the 3F3 antibody (green) and an antibody to ZO-1 (red). (A-C) XZ sections are shown at the top of each column, and individual merged optical sections at the designated levels are shown below. Scale bar = 10 μ m.

appeared to concentrate at the periphery of the EEA1-positive endosomal elements (Figure 2.3D, inset).

I next examined whether Sec6, Sec8, or Exo70 were associated with Tf-positive BEE or the common recycling endosomes (CRE) of polarized MDCK cells. BEE are found closely apposed to the basal and lateral surfaces of the cell, while CRE are found in a peri- and supranuclear distribution (151, 183). Following basolateral uptake of Tf for 45 min, the cells were fixed and double labeled with antibodies specific for canine Tf, Sec8, or Exo70. I observed some co-localization between the peripherally localized Tf-labeled BEE and the exocyst subunits (lower panels, Figure 2.4A-B). However, co-localization was more apparent for the Exo70 subunit. Co-localization was also readily observed in the supranuclear CRE (upper panels, Figure 2.4A-B), and Sec6 showed a similar degree of co-localization with Tf as that observed for Sec8 (data not shown). As further confirmation of our localization studies, I immunoisolated Sec8-positive endosome-enriched compartments and observed that the Tf receptor was associated with these membranes (Figure 2.4C). As a control I used non-specific mouse IgGs, which failed to capture the Tf receptor.

The recent reports that Sec15 interacts with Rab11 (197, 204) prompted me to explore whether the exocyst was associated with the Rab11-positive ARE of polarized MDCK cells. The ARE, is morphologically distinct from the AEE, BEE, and CRE, is located at the apical pole of polarized MDCK cells, and is a site of regulation of apical recycling and basolateral-to-apical transcytosis of the plgR (8, 18, 24, 183). The plgR normally transports basolaterally-internalized IgA from BEE, to CRE, to the ARE, and



Figure 2.3: Localization of exocyst subunits in polarized MDCK cells

(A-B) Cells were treated with saponin and then fixed using a pH-shift protocol. (A) Distribution of Sec8 (green), furin (red), and ZO-1 (blue). (B) Distribution of Sec8 (green), EEA1 (red), and ZO-1 (blue). (C-D) Cells were fixed using the pH-shift protocol. (C) Distribution of Exo70 (green), furin (red), and ZO-1 (blue). (D) Distribution of Exo70 (green), EEA1 (red), ZO-1 (blue). (A-D) An XZ section is shown in the top of each column and single optical sections at the designated position of the cell are shown below. The bottom-most panels show the distribution pattern of exocyst subunits (green) and cellular markers (red) within the regions marked with the white boxes. Scale bar = 10 μ m.

then to the apical surface where the receptor is cleaved, releasing it along with bound IgA into secretions (8, 18). However, a significant fraction of pIgR escapes proteolysis and is then endocytosed and recycled through the ARE *en route* to the apical cell surface. In our experiments, IgA was either internalized basolaterally for 10 min and chased for 20 min, or internalized from the apical pole of the cell for 10 min, to accumulate ligand in the ARE. Following apical uptake surface-bound ligand was removed by proteolysis, the cells were then fixed and labeled with antibodies specific for exocyst subunits, Rab11a, and IgA. I observed co-localization between Sec8 or Exo70 and Rab11a (Figure 2.5A&C) or Sec8 or Exo70 with basolaterally internalized IgA (Figure 2.5B&D). Triple label experiments confirmed that Sec8/Exo70, Rab11a, and apically internalized IgA were co-distributed in a subset of ARE (Figure 2.5E-F).

Additional experiments confirmed the association of exocyst subunits with Rab11-positive endosomes and directly with dominant active Rab11aSV. An endosomal-enriched fraction was incubated with Dynabeads coated with an antibody that recognizes both a and b isoforms of Rab11 (the Rab11a specific antibodies I used for immunofluorescence were not functional in this protocol). The immunoisolated fractions were resolved by SDS-PAGE and western blots were probed with plgR, Rab11, and Sec8 specific antibodies (Figure 2.5G). Consistent with the immunofluorescence analysis, a fraction of Sec8 and the plgR was associated with Rab11-positive endosomes. Little plgR, rab11, or Sec8 association was observed in control reactions incubated with non-specific rabbit IgGs (Figure 2.5G). The Golgi marker GM130 was not observed in the Rab11a-positive endosome fraction (data not shown), confirming that.



Figure 2.4: Association of exocyst subunits with Tf-positive endosomes

(A) Distribution of Sec8 (green), Tf (red), and ZO-1 (blue). (B) Distribution of Exo70 (green), Tf (red), and ZO-1 (blue). An XZ section is shown in the top panel, the middle panel is a 3D reconstruction of optical sections taken from the apical to supranuclear level of the cells, and the bottom panel is a 3D reconstruction of optical sections taken along the lateral surfaces of the cells. Examples of co-localization between exocyst subunits and Tf-positive endosomes are marked by arrows. In XZ sections, the scale bar is equal to 10 μ m. In 3-D reconstructions each length of the grid is equivalent to 3.8 μ m. (C) Endosomal-enriched fractions were incubated with a pool of Sec8-specific antibodies (10C2, 5C3, 2E12) or IgG and recovered using Dynabeads coated with goat-anti-mouse secondary antibodies. The lane at the left is a loading control showing that Sec8 and the Tf receptor were present in the starting PNS. The other lanes show the immunoisolated fraction bound to Sec8-specific antibodies or non-specific rabbit IgG antibodies that were resolved by SDS-PAGE and then sequentially probed with antibodies to Sec8 or the Tf receptor.



Figure 2.5: Localization of exocyst subunits to Rab11a- and IgA-positive recycling endosomes

(A-D) Distribution of Sec8/Exo70 (green) and Rab11a (A&C) or IgA (B&D). The distribution of ZO-1 was also examined but is not apparent in all of the panels. (B&D) IgA was internalized from the basolateral pole of the cell for 10 min and chased 20 min at 37°C. (E&F) IgA was internalized from apical pole of the cell for 10 min. (E) The cells were fixed and stained with antibodies to Sec8 (green), Rab11a (red), and IgA (blue). (F) Cells were stained with antibodies to Exo70 (green), Rab11a (red), and IgA (blue). In B and E cells were treated with saponin prior to fixation whereas in A, C, D and F cells were fixed prior to permeabilization. Arrows indicate areas of co-localization between the three markers. Scale bar = 10 μ m. (G) Association of Sec8 and pIgR with immunoisolated Rab11-positive endosomes. The lane at the left shows that Sec8, pIgR, and Rab11 were present in the starting PNS, and the other lanes show the immunoisolated fraction bound to Rab11-specific antibodies or non-specific rabbit IgG antibodies to Sec8, pIgR, or Rab11.

the immunoisolated sample represents an endosome-enriched fraction and does not pull-down other non Rab11a-associated membrane bound compartments.

Likely reflecting the transient nature of the Rab11a/exocyst interaction, I was unable to co-immunoprecipitate endogenous Rab11a with antibodies to Sec8, Sec15A, or Exo70. However, when I infected MDCK cells with an adenovirus encoding a GTPase deficient mutant of Rab11a fused to GFP and containing an HA tag (GFP/HA-Rab11aSV), I observed that Exo70 was associated with GFP-Rab11aSV-positive endosomes (Figure 2.6A). Furthermore, when I performed co-immunoprecipitation using Sec8 antibodies I observed that multiple exocyst subunits (Sec6/Sec8/Sec15A/Exo70) as well as GFP/HA-Rab11aSV were found in a complex (Figure 2.6B). Exo70 did not colocalize with a dominant negative mutant of Rab11a (GFP/HA-Rab11aSN), which appeared to be primarily cytosolic (data not shown).

Taken together, the above data indicate that exocyst subunits are localized to multiple endocytic compartments including early endosomes, Tf-positive recycling endosomes, and the Rab11a-positive ARE, but not to a significant degree with the TGN of polarized MDCK cells.

2.2.2 Basolateral recycling, apical recycling, and basolateral-to-apical transcytosis are exocyst-dependent trafficking pathways

Next, I examined whether there was a functional role for the exocyst in postendocytic trafficking pathways. For this analysis I used SLO-permeabilized cell assays that I


Figure 2.6: Association of exocyst subunits with GFP/HA-Rab11aSV

(A) MDCK cells were infected with adenovirus encoding GFP/HA-Rab11aSV and then fixed and processed for immunofluorescence. The distribution of GFP/HA-Rab11aSV (green) and Exo70 (red) are shown. A merged image is shown at the right. Scale bar = 10 μ m. (B) Cells were infected with virus encoding GFP/HA-Rab11aSV, lysed, and Sec8 and associated proteins were immunoprecipitated using Sec8-specific monoclonal antibody 10C2 or protein G alone. The cell lysate (1/20th of the total) or the immunoprecipitated proteins were resolved by SDS-PAGE and a western blot was sequentially probed with antibodies to Sec6, Sec8, Sec15, Exo70, and Rab11a.

previously developed to measure transcytosis and recycling (7, 88). Important advantages of this technique include the ability to examine exocyst function after the cells have already polarized, the ability to test the acute effects of inhibiting exocyst function on defined trafficking events, and the ability to uniformly permeabilize the entire monolayer, ensuring equal delivery of the reagents to each cell.

I first examined whether Tf recycling in polarized MDCK cells (Figure 2.7A) was dependent on the exocyst. ¹²⁵I-Tf was internalized from the basolateral surface of filtergrown MDCK cells, the cells were permeabilized with SLO, and following cytosol washout, vesicle trafficking was reconstituted at 37° C in the presence of exogenous cytosol and an ATP regenerating system. A pool of function-blocking Sec8 mAbs (10C2, 5C3, 2E12) (51), was included in the washout step and the reconstitution reaction. As a control I substituted the Myc 9E10 mAb for the Sec8 antibodies in the reaction. At the end of the reconstitution reaction, the percentage of ligand that was recycled was calculated. The ATP-dependent values were normalized to control reactions that contained an ATP regenerating system and cytosol, but no antibody. The addition of Sec8 antibodies significantly inhibited basolateral recycling of Tf by ~45%; however, no effect was observed upon addition of the non-specific Myc antibody (Figure 2.7B).

In the next experiment, I examined whether exocyst is involved in regulating IgA transcytosis (Figure 2.8A). Cells were basolaterally pulsed for 10 min with ¹²⁵I-IgA, washed, and then chased in the absence of ligand for 5 min. Cell permeabilization and reconstitution of vesicle trafficking was performed as described for Tf recycling. IgA transcytosis to the apical surface was measured in the presence and absence of



Figure 2.7: Exocyst requirement for basolateral recycling in SLO-permeabilized MDCK cells

(A) Basolateral transferrin recycling pathways in polarized epithelial cells. (B) Basolateral recycling of ¹²⁵I-Tf in SLO-permeabilized MDCK cells incubated in the presence of an ATP-regenerating system (ATP), cytosol, and either Myc antibodies (Myc) or a pool of Sec8 antibodies (10C2, 5C3, 2E12). The ATP-independent fraction was ~12% and values for control reactions performed in the presence of an ATP-regenerating system and cytosol were ~46%. Data is mean ± S.E.M. (n = 3; performed in triplicate). *Statistically significant difference between reactions performed in the presence of Myc or Sec8 antibodies (p < 0.05).

exocyst antibodies. Function blocking antibodies inhibited IgA transcytosis by ~60%, while Myc antibodies did not have a significant effect (Figure 2.8B). As described in earlier chapters, IgA transcytosis includes several compartments including BEE, CRE and ARE. In order to test if exocyst is specifically involved in trafficking at ARE, I analyzed if transit of ¹²⁵I-IgA from the ARE and release from the apical pole of the cell was exocyst-dependent (Figure 2.9A). ¹²⁵I-IgA was internalized for 10 min at 37° C, washed, and then chased for 20 min to accumulate IgA in the Rab11a-positive elements of the ARE. Immunofluorescence confirmed that under these internalization conditions, IgA was present at the apical pole of the cell in tubulovesicular structures that colocalized with Rab11a, but not with Tf receptor (Figure 2.9B). The cells were then permeabilized with SLO and ¹²⁵I-IgA release from the ARE was measured in the presence or absence of exocyst antibodies. Blocking antibodies significantly inhibited IgA trafficking from the ARE by ~40% (Figure 2.9B), while Myc antibodies had no effect.

As further confirmation that the exocyst modulated apically directed traffic, I also explored apical recycling of IgA in SLO-permeabilized cells (Figure2.10A). Filter-grown cells were pulsed with ¹²⁵I-IgA from the apical domain for 10 min, the cells were washed and chased in the absence of ligand for a total of 5 min, and then membrane bound IgA was stripped from the surface with trypsin at 4°C. The cells were permeabilized with SLO, and apical IgA release was reconstituted in the presence of Sec8 or Myc antibodies as described above. In the presence of the Sec8 antibodies, the pool of IgA that recycled apically and was dependent upon ATP and cytosol was significantly inhibited by ~80% relative to control (Figure 2.10B). It is worth noting that I observed a relatively large ATP independent pool of recycling IgA in these assays (~30%),



Figure 2.8: Exocyst requirement for apical transcytosis in SLOpermeabilized MDCK cells

(A) IgA transcytosis in polarized epithelial cells. (B) IgA was internalized basolaterally for 10 min at 37°C, washed, and chased in the absence of ligand for 5 min. Apical release of ¹²⁵I-IgA was quantified in SLO-permeabilized cells. ATP independent fraction was ~5%) Data is mean \pm S.E.M. (n = 3; performed in triplicate). *Statistically significant difference between reactions performed in the presence of Myc or Sec8 antibodies (p < 0.05).



Figure 2.9: Exocyst requirement for apical transcytosis from ARE in SLOpermeabilized MDCK cells

(A) IgA transcytosis from ARE in polarized epithelial cells. (B) IgA was internalized basolaterally for 10 min at 37°C and the cells were incubated in the absence of IgA for 20 min. The cells were fixed and stained with antibodies to IgA (blue), ZO-1 (blue), Tf receptor (green), and Rab11a (red). 3-D reconstructions are shown. Each length of the grid = 3.8 μ m. (C) Ligand was internalized as described in panel B, and apical release of ¹²⁵I-IgA was quantified in SLO-permeabilized cells. The ATP independent fraction was ~8% and values for control reactions were ~42%. Data is mean ± S.E.M. (n = 2; performed in triplicate). *Statistically significant difference between reactions performed in the presence of Myc or Sec8 antibodies (p < 0.05).

indicating that either reconstitution was inefficient or that apical recycling had little requirement for ATP. The ATP-independent pool of recycling was insensitive to the addition of Sec8 antibodies (data not shown).

It was previously shown that apical delivery of newly synthesized p75 neurotrophin receptor was independent of exocyst function (51). Consistent with this previous analysis, I found that addition of function blocking Sec8 antibodies to SLO permeabilized MDCK cells had no significant effect on apical delivery of this protein (data not shown). This latter observation confirms that only a subset of trafficking events are exocyst dependent in SLO permeabilized cells.

While the exocyst is generally thought to be involved in promoting transit between intracellular compartments and the plasma membrane, some studies indicate that it may also play a role in modulating cargo exit from TGN or endosomes (13, 82, 201). To explore this possibility, I reconstituted vesicle budding from ARE in mechanically perforated cells (Figure 2.11A). ¹²⁵I-IgA was internalized basolaterally for 20 min at 18° C and then chased for 20 min to accumulate IgA in the ARE. The apical membrane was then mechanically perforated with nitrocellulose (15) and release of ¹²⁵I-IgA in transport vesicles was measured in the presence of Sec8 or Myc antibodies, cytosol, and an ATP regenerating system. Addition of antibodies against Sec8, but not Myc, resulted in a significant inhibition of ¹²⁵I-IgA release from labeled ARE (Figure 2.11B). Taken together the above results indicate that the exocyst modulates a broad spectrum of endocytic trafficking events in polarized cells including those directed toward the apical and basolateral pole of the cell. Furthermore, the exocyst may modulate the exit of IgA-pIgR cargo from the ARE.



Figure 2.10: Exocyst requirement for apical recycling in SLO-permeabilized MDCK cells

(A) Apical recycling pathways in polarized epithelial cells. (B) Apical ¹²⁵I-IgA recycling in SLO-permeabilized cells. The ATP independent fraction was ~30% and values for control reactions were ~47%. Data are mean \pm S.E.M. (n = 2; performed in triplicate). *Statistically significant difference between reactions performed in the presence of Myc or Sec8 antibodies (p < 0.05).





(A) Vesicle release assay in perforated cells. (B) Release of ¹²⁵I-IgA-labeled cargo vesicles from mechanically perforated MDCK cells. The ATP independent fraction was ~8% and values for control reactions were ~30%. Data is mean \pm S.E.M. (n = 3; performed in duplicate). *Statistically significant difference between reactions performed in the presence of Myc or Sec8 antibodies (p < 0.05).

2.3 DISCUSSION

2.3.1 Localization of exocyst to multiple endocytic compartments in polarized MDCK cells

I examined the distribution of Sec6, Sec8, and Exo70 in polarized MDCK cells using antibodies and fixation conditions that revealed a tubulo-vesicular pool of these proteins. In contrast to previous reports showing exocyst subunit localization to the Golgi or TGN of NRK and sub-confluent MDCK cells (131, 201), I generally did not observe co-localization between exocyst subunits and the TGN marker furin. This may reflect cell type differences or the use of different antibodies and fixation conditions. Consistent with previous studies (44, 131), I observed that Sec6, Sec8, and Exo70 colocalized with basolaterally internalized Tf in what appeared to be BEE and the CRE. While Sec6 and Sec8 showed little association with EEA1, Exo70 appeared to distribute to the periphery of these early endosomal structures. Work in *Drosophila* has previously established that the distribution of individual exocyst components is not always identical (13, 120). The function of Exo70 in early endosomes is unknown but the absence of Sec6 or Sec8 may indicate that Exo70 is acting independently or as part of a subcomplex. An alternative possibility is that Exo70 is marking the site of assembly of the octameric exocyst complex, a function proposed for Exo70p in yeast (16).

The recent finding that Rab11 interacts with Sec15 (197, 204) prompted me to also explore whether exocyst subunits associated with the Rab11a-positive ARE, an endosome that regulates apically directed endocytic traffic in MDCK cells (24, 189). Indeed, I observed a fraction of exocyst subunits that colocalized with Rab11a in the apical region of polarized MDCK cells. There was a pool of exocyst subunits that did not colocalize with any of the markers I employed and may represent exocyst association with other organelles such as the endoplasmic reticulum (94). Taken together, the data indicate that the exocyst localizes to multiple endocytic compartments, including those involved in traffic directed toward both the basolateral and apical poles of the polarized MDCK cell.

2.3.2 Requirement for exocyst in both basolateral- and apical-directed endocytic transport

While not all membrane trafficking steps are dependent on the exocyst (28, 51, 119), the association of the exocyst with multiple endocytic compartments indicated that it may be involved in a broader range of trafficking events than originally proposed. To examine this possibility, I reconstituted endocytic trafficking events in SLO-permeabilized cells. Consistent with the localization of exocyst subunits to Tf-positive endosomes, I observed that a cocktail of function-blocking Sec8 antibodies significantly inhibited basolateral recycling of Tf. The inhibition of Tf recycling was not complete, perhaps indicating that the blocking antibodies had access to only a subset of exocyst complexes involved in recycling or that Tf recycling is occurring by more than one mechanism. While previous studies showed that Tf recycling is exocyst dependent in

non-polarized cells and that the exocyst is localized to recycling endosomes in MDCK cells (44, 131), it was unknown whether basolateral recycling was exocyst dependent in polarized epithelial cells.

The localization of exocyst subunits to the Rab11a-positive ARE prompted me to explore further whether the exocyst was involved in apical recycling. I observed that the apical recycling of IgA was inhibited by function-blocking Sec8 antibodies. Like Tf, the inhibition was not complete and this may again reflect issues of antibody access or of a multiplicity of mechanisms and/or pathways. Our observation that the exocyst is important for apically directed traffic is consistent with observations that the exocyst is important for delivery of cargo to the apical membrane-associated rhabdomeres of epithelial photoreceptor cells in *Drosophila* (13).

By staging IgA in the ARE prior to reconstitution, I further showed that a late step in the basolateral-to-apical transcytotic pathway, namely movement from ARE to the apical plasma membrane, was sensitive to function-blocking Sec8 antibodies. This observation is consistent with current models in which the exocyst promotes tethering at the plasma membrane. However, my finding that vesicle budding also showed some dependence on the exocyst indicates that the exocyst may also play some role in steps that precede vesicle fusion, possibly including cargo selection or vesicle transport. It was previously reported that function-blocking antibodies against Sec6/Sec8 inhibit cargo exit from the TGN (51, 201), and expression of mutant alleles of Sec5, Sec6, or Sec15 result in accumulation of DE-cadherin in Rab11-positive endosomes (82), and not, for example, in vesicles near the plasma membrane. It is interesting to note that the octameric conserved oligomeric Golgi (COG) complex, which regulates a variety of

functions (cargo sorting, vesicle trafficking/tethering) in different intracellular compartments (ER, Golgi, and endosomes), shares structural and sequence similarities to the exocyst (127). Thus, the exocyst may also regulate multiple membrane traffic steps in the endocytic/biosynthetic systems, including ones I did not examine in this study.

3.0 RAB11-EXOCYST INTERACTION REGULATES BASOLATERAL-TO-APICAL TRANSCYTOSIS IN POLARIZED EPITHELIAL CELLS

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3.1 INTRODUCTION

Studies discussed so far show that exocyst is involved in multiple endocytic trafficking pathways directed towards both apical and basolateral poles of the polarized epithelial cells. However, the factors that regulate specificity of the exocyst function are not known. Exocyst subunits interact with multiple small GTPases, which are associated with different compartments and regulate specific trafficking pathways. Therefore it is possible that the interaction between small GTPases and the exocyst complex regulates the specificity of exocyst function. A potentially important link between endocytic traffic and the exocyst is the association between Sec15 and Rab11, a GTPase that regulates both biosynthetic and endocytic traffic (3, 189). Structural analysis has defined the site of *Drosophila* Rab11 interaction to a single helix in the C-terminal region of Sec15 (197).

The functional significance of the Sec15/Rab11 interaction is not well characterized, but overexpression of Sec15A-GFP in COS cells slows the egress of Tf from recycling endosomes (204); while in *Drosophila*, mutations in Sec5, Sec6, or Sec15 result in the accumulation of cargo in enlarged Rab11-positive endosomes (13, 82).

Rab11 is localized to the TGN and recycling endosomes of non-polarized cells (133, 173, 195). However, in polarized MDCK cells the majority of the Rab11 "a" isoform (Rab11a) appears to be localized to pericentriolar-localized apical recycling endosomes (ARE)(18, 24, 90), where it regulates apical, but not basolateral, recycling and basolateral-to-apical transcytosis (189). Thus far, there is little information available regarding whether Sec15 and Rab11a interact in polarized mammalian epithelial cells or whether this interaction is of functional significance.

3.2 RESULTS

3.2.1 The C-terminus of Sec15A binds to Rab11a

The potential requirement for the exocyst in basolateral-to-apical transcytosis and apical recycling prompted me to further explore the molecular requirements for this dependence. I initially focused on the previously described interaction between Sec15 and Rab11 (197, 204). Although mapping of Sec15-Rab11 interactions was recently described using *Drosophila* proteins (197), I confirmed these interactions with their mammalian orthologs (Figure 3.1). Using a two-hybrid approach and a quantitative β -galactosidase assay, I observed that full-length rat Sec15A interacted with wild-type

Rab11a as well as with GTPase-deficient Rab11a-SV. However, no interaction was observed with the dominant negative mutant Rab11aS25N (Rab11a-SN), Lamin C, or empty vector (Figure 3.1B). Next, I broadly examined the region of Sec15A that was involved in these interactions. The Sec15A N-terminus (Sec15NT; amino acids 1–390) showed no interactions with Rab11a (Figure 3.1C). However, the Sec15A C-terminus (Sec15CT; amino acids 391-822) interacted, like the intact protein, with wild type Rab11a and Rab11a-SV, but not with Rab11a-SN (Figure 3.1D). It was previously reported that a point mutation that converted Asn₆₅₉ to an alanine residue in *Drosophila* Sec15CT blocked its interaction with Rab11 (197). I observed that the analogous mutation in mammalian Sec15CT, in which Asn₇₀₉ was converted to an alanine residue (Sec15CT(NA)), prevented the interaction of Rab11a with the C-terminus of Sec15A (Figure 3.1E).

To confirm that Sec15CT can interact with Rab11a *in vivo*, I generated stable cell lines expressing GFP-Sec15CT or GFP-Sec15CT(NA), which were subsequently infected with GFP/HA-Rab11aSV adenovirus. The cells were crosslinked with the reversible cross-linker DSP, lysed, and GFP/HA-Rab11aSV was immunoprecipitated using an anti-HA antibody. Western blots of the immunprecipitates were probed with the 15S2G6 antibody (which recognizes the C-terminus of Sec15A) or with antibodies against Rab11a. Consistent with our two-hybrid analysis, anti-HA antibodies co-immunoprecipitated a complex between GFP/HA-Rab11aSV and GFP-Sec15CT, but little interaction was observed between GFP/HA-Rab11aSV and GFP-Sec15(NA) (Figure 3.1F).



Figure 3.1: Interaction between Rab11a and the C-terminus of Sec15A

(A) Sec15A constructs used to identify the Sec15A-Rab11a interaction domain. (B-E) Results of CPRG assay between the Sec15A constructs shown in panel A and either wild-type Rab11a (Rab11a), dominant active Rab11a-SV, dominant negative Rab11a-SN, lamin C (LamC), or empty vector (EV). These assays were repeated two times. Data from one determination are shown. Mean \pm SD (n=3). (F) Untransfected MDCK cells or stable MDCK cells lines expressing GFP-Sec15CT or GFP-Sec15CT(NA) were infected with adenovirus encoding GFP/HA-Rab11aSV. The cells were cross-linked, lysed, and GFP/HA-Rab11aSV was immunoprecipitated with anti-HA antibodies. The immunoprecipitates were resolved by SDS-PAGE and western blots were sequentially probed with antibodies that recognize Rab11a or Sec15A.

3.2.2 Expression of Sec15CT or downregulation of Sec15A impairs basolateralto-apical transcytosis of plgR-lgA complexes

I next examined whether Sec15CT expression affected the distribution of Rab11a, potentially by impairing interactions between endogenous Sec15 and Rab11a. I transiently transfected polarized filter-grown MDCK cells with GFP-tagged Sec15CT (GFP-Sec15CT), and ~24 h post-transfection the cells were fixed and labeled with Rab11a-specific antibodies (Figure 3.2A). I estimate that ~20-30% of the cells expressed GFP-Sec15CT following transfection. I observed that GFP-Sec15CT was localized to small vesicular structures at the apical pole of the cell as well as very large "vesicular" structures in the medial cytoplasm. Rab11a co-localized with both pools of GFP-Sec15CT (Figure 3.2B), as did the plgR (see Figure 3.3B). However, the large medial GFP-Sec15CT-positive elements did not co-localize with or alter the distribution of Sec8 nor did they co-localize with basolaterally internalized IgA (data not shown). When examined by live-cell imaging, GFP-positive vesicular elements were observed to enter and exit the large vesicular structures (data not shown), demonstrating that these structures are dynamic and unlikely to be cytoplasmic accumulations of GFP-Sec15CT in aggresomes. The mutant version of GFP-tagged Sec15CT (GFP-Sec15CT(NA)) showed some puncta at the apical pole of the cells that were positive for Rab11a (Figure 3.2C), indicating that the mutant may have bound to this compartment in a Rab11a-independent fashion. However the mutant did not induce the formation of large vesicular structures in the medial cytoplasm and appeared to be predominantly cytoplasmic.



Figure 3.2: Expression of GFP-Sec15CT and GFP-Sec15CT(NA) in polarized MDCK cells

(A) Filter transfection protocol. (B) Distribution of GFP-Sec15 (green), Rab11a (red) and the nucleus (blue). (C) Distribution of GFP-Sec15(NA) (green), Rab11a (red) and the nucleus (blue). Individual optical sections from the apical or medial regions of the cell are shown. A merged image (overlay) is shown in the right-hand panels. Examples of co-localization between exocyst subunits and Rab11a are marked by arrows.

Next, I examined whether expression of GFP-Sec15CT altered basolateral-toapical transcytosis of IgA using a morphological assay that scored the delivery of basolaterally-internalized IgA to the apical surface of polarized MDCK cells (Figure 3.3A). IgA was internalized from the basolateral surface of the cells for 20 min at 18° C to trap a cohort of IgA in BEE (161). Following this pulse, I confirmed that basolaterally internalized IgA was found in BEE subjacent to the basolateral surface of the cells expressing either GFP-Sec15CT or GFP-Sec15CT(NA) (see medial projections, Figure 3.3B). The cells were also labeled with an anti-plgR mAb (SC166) to confirm plgR expression. To initiate transcytosis, the basolateral surfaces of the cell were washed and the cells were incubated in the absence of ligand for 20 min at 37° C. A Cy3-labeled secondary antibody specific for IgA was included in the apical medium during the 37° C chase to label IgA-bound pIgR complexes as they appeared at the apical cell surface (Figure 3.3A). As described above, a significant fraction of the pIgR escapes cleavage and recycles at the apical pole of the cell. After the chase in the presence of anti-IgA antibody, the cells were washed, fixed, and stained. I observed that in cells expressing GFP-Sec15CT, there was little uptake of Cy3-labeled secondary antibody (Figure 3.3C, blue), whereas in untransfected cells or those transfected with GFP-Sec15CT(NA), significant uptake of the anti-IgA antibody was detected at the apical pole of the cells (Figure 3.3C, blue), which co-localized with the plgR. These results indicate that the expression of GFP-Sec15CT inhibited IgA delivery to the apical surface of the cell whereas Sec15CT(NA), which binds inefficiently to Rab11a, had less of an effect. I also examined the effect of expressing GFP fused to full-length Sec15A; however, our



Figure 3.3: IgA transcytosis in polarized MDCK cells expressing GFP-Sec15CT or GFP-Sec15CT(NA)

(A) Protocol for detecting basolaterally internalized IgA at the apical cell surface. (B) Distribution of GFP-Sec15CT or GFP-Sec15CT(NA) (green), the pIgR (red), and IgA (blue) endocytosed from the basolateral pole of the cell for 20 min at 18°C. An optical section from the apical pole of the cell and a projection of sections along the lateral region of the cell are shown; (C) IgA was internalized from the basolateral pole of the cell for 20 min at 37°C. CY3-labeled anti-IgA antibodies were included in the apical medium during the incubation at 37°C. The distribution of GFP-Sec15CT or GFP-Sec15CT(NA) (green), pIgR (red), and anti-IgA (blue) is shown in projected optical sections taken from the apical pole of the cell.

analysis was thwarted by preliminary studies that showed expression of this construct resulted in the rapid formation of apoptotic cells characterized by nuclear fragmentation, and they had very low plgR expression.

As further evidence of the effects of Sec15CT on transcytotic traffic, I used the stable MDCK cells lines expressing GFP-Sec15CT or GFP-Sec15CT(NA) described above. The level of expression of these two constructs was approximately equivalent to that of the endogenous protein (Figure 3.4A), which is likely to be an underestimate as only ~ 40-50% of the cells expressed these constructs, and was less than that observed using the transient transfection protocol described above. The vesicular structures were present at the medial levels of these cells, but were somewhat smaller in dimension, likely reflecting the lower levels of GFP-Sec15CT expression in these cell lines. To measure transcytosis, the cells were first infected with an adenovirus encoding the plgR. After 24 h to allow for receptor expression, ¹²⁵I-IgA was internalized from the basolateral surface of the cell for 10 min at 37°C, the cells were washed, and the percentage of internalized ¹²⁵I-IgA released into the basolateral medium (recycled) or apical medium (transcytosed) was measured during a 2-h incubation at 37°C. Consistent with the morphological assay, I observed that IgA transcytosis was significantly impaired by expression of GFP-Sec15CT. The effect was kinetic with an \sim 50% inhibition observed at 15 min and \sim 20% at the 2-h time point (Figure 3.4B). There was little effect on basolateral recycling or degradation, but there was a compensatory increase in the amount of cell-associated ligand after the 2-h chase (data not shown). In contrast, there was no effect on apical recycling of IgA (Figure 3.4D) or basolateral recycling of Tf (Figure 3.4F). Expression of GFP-Sec15CT(NA) resulted in a small but



Figure 3.4: Effect of expressing GFP-Sec15CT and GFP-Sec15CT(NA) on the postendocytic fate of IgA and Tf in polarized MDCK cells infected with adenovirus encoding the plgR

(A) Lysates of cells expressing GFP-Sec15CT or GFP-Sec15CT(NA) were resolved by SDS-PAGE and western blots were probed with an antibody against Sec15A. (B-C) Fate of basolaterally internalized ¹²⁵I-IgA in control MDCK cells or those expressing GFP-Sec15CT or GFP-Sec15CT(NA) (panels B and C, respectively). (D-E) Fate of apically internalized ¹²⁵I-IgA in control MDCK cells or those expressing GFP-Sec15CT or GFP-Sec15CT(NA) (panels D and E, respectively). (F-G) Fate of basolaterally internalized ¹²⁵I-Tf in control MDCK cells or those expressing GFP-Sec15CT or GFP-Sec15CT (NA) (panels D and E, respectively). (F-G) Fate of basolaterally internalized ¹²⁵I-Tf in control MDCK cells or those expressing GFP-Sec15CT or GFP-Sec15CT(NA) (panels F and G, respectively). In each panel, the fraction of ligand released from the apical or basal pole of the cell is shown. Data are mean \pm S.E.M. (n \geq 2; performed in triplicate). *Values are significantly different (p < 0.05) from those observed in control MDCK cells.

significant stimulation of basolateral-to-apical transcytosis of IgA (Figure 3.4C), but had no effect on apical recycling of ¹²⁵I-IgA (Figure 3.4E), or basolateral recycling of ¹²⁵I-Tf (Figure 3.4G).

As a final experiment I downregulated expression of Sec15A by transiently expressing a plasmid (pSuper-Sec15A) that expresses a Sec15A specific shRNA. Compared to cells expressing vector alone (not shown) or a control construct (pSupercontrol), expression of pSuper-Sec15A resulted in a decrease in both Sec15A mRNA and protein expression as assessed by RT-PCR analysis or by western blotting (Figure 3.5A). I estimated that mRNA expression was decreased by \sim 80 % and that protein expression was decreased by ~ 50 %. Because the 15S2G6 antibody made against Sec15A may also bind to Sec15B, I used RT-PCR to confirm that pSuper-Sec15A had little effect on mRNA expression for Sec15B (Figure 3.5; decrease of ~ 10-20 %); however, the lack of isoform specific antibodies prevented me from examining the protein levels of Sec15B in the cells. There was no effect of silencing Sec15A expression on levels of Sec8 (Figure 3.5A). Expression of pSuper-Sec15A shRNA, but not pSuper-control, had a similar phenotype to expression of GST-Sec15CT: basolateral-to-apical transcytosis was significantly inhibited at all time points (Figure 3.5B), but there was no effect on apical or basolateral recycling (Figure 3.5C-D). Taken together, the above results indicate that Sec15A, possibly acting through Rab11a, modulates basolateral-to-apical transcytosis in polarized MDCK cells.



Figure 3.5: Dependence of postendocytic traffic on expression of Sec15A

(A) The effect of expressing pSuper-Sec15A or pSuper-control on Sec15A or Sec15B mRNA expression is shown in the upper panel. The lower panel shows a western blot sequentially probed with antibodies to Sec15A and then Sec8. (B) Basolateral-to-apical transcytosis of ¹²⁵I-IgA in cells expressing pSuper-Sec15A or pSuper-control. Shown is the amount of ligand released in the apical or basal chamber of the Transwell. (C) Apical recycling of ¹²⁵I-IgA in cells expressing pSuper-Sec15A or pSuper-control. (D) Basolateral recycling of ¹²⁵I-Tf in cells expressing pSuper-Sec15A or pSuper-control. Data are mean ± S.E.M. (n ≥ 3; performed in triplicate). *Values are significantly different (p < 0.05) from those observed in cells expressing pSuper-control.

3.3 DISCUSSION

The association of the exocyst with multiple intracellular compartments and the dependence of several trafficking steps on exocyst function indicate that there must be a mechanism(s) to affect exocyst localization and exploit its functions in a pathway-specific manner. Likely players in this regard include a growing list of Ras family GTPases that interact with various exocyst subunits in metazoans: Arf6 with Sec10; RalA/RalB with Sec5 and Exo84; Tc10 with Exo70; and Rab11 with Sec15 (52, 118). Small GTPases exhibit a highly specialized distribution in the cell and are involved in recruiting effector proteins to intracellular compartments where they regulate specific membrane trafficking events.

Confirming previous reports (197, 204), I observed that Rab11a interacts with the C-terminus of Sec15A. My analysis further demonstrated that the GFP-tagged Sec15A C-terminus was associated with Rab11a-positive endosomes, that it caused the accumulation of Rab11a in large vesicular structures, and that it acted as a dominant-negative inhibitor of basolateral-to-apical transcytosis. In contrast, this mutant had no effect on the basolateral recycling pathway, which was previously shown to be independent of Rab11a (189). Inhibition of transcytosis was not observed when I expressed the GFP-Sec15CT(NA) mutant, which blocks interactions between the Sec15A C-terminus and Rab11a. The *Drosophila* Sec15 C-terminus also binds to Rab3, Rab8, and Rab27 in two-hybrid assays (197). As such, I cannot exclude the possibility

that GFP-Sec15CT expression may cause inhibition as a result of its interactions with other GTPases. However, there is no evidence as yet that the exocyst interacts with these GTPases *in vivo*, nor is it known whether they bind to Sec15CT at the same site as Rab11a. Furthermore, while there is some evidence that Rab3b regulates transcytosis (176), Rab8 is generally thought to regulate basolateral events (3), and there is no evidence that Rab27a/b are expressed in MDCK cells. While our data are consistent with a model whereby Rab11a recruits the exocyst via Sec15A to modulate transcytotic traffic, I cannot exclude a role for the closely related Rab11b isoform that shares many of the same effectors as Rab11a. However, Rab11b localization is distinct from that of Rab11a and Rab11b does not co-localize with Tf or the plgR in polarized MDCK cells (83).

Consistent with the observations described above, I found that downregulation of Sec15A, using shRNA, inhibited basolateral-to-apical transcytosis, but did not affect receptor recycling pathways. The inhibition of transcytosis most likely reflected defects in the movement into or out of the Rab11a-positive elements of the ARE. However, it is possible that earlier trafficking steps were also affected. The inhibition of transcytosis was modest, possibly reflecting my inability to achieve complete Sec15A knockdown or the possibility that there may be alternative mechanisms/pathways for transcytosis. An additional possibility is that Sec15B may compensate for the loss of Sec15A (however, see discussion below for an alternative possibility). Unfortunately, there are few reagents to study Sec15B at present, so the role of this isoform in endocytic traffic is left to future studies.

The lack of effect of expressing GFP-Sec15CT or pSuper-Sec15A on apical IgA recycling was surprising as both apical recycling and basolateral-to-apical transcytosis are regulated by Rab11a (197), and I confirmed that expression of GFP/HA-Rab11aSN inhibited both of these pathways (Oztan and Apodaca, unpublished observations). A likely possibility is that Rab11a regulates these two pathways through recruitment of a distinct subset of effectors or their functions. Consistent with this possibility I recently observed that the SARG and Δ C mutants of Rab11-interacting protein 2 affect basolateral-to-apical transcytosis, but have no impact on apical recycling (35), and other studies have noted differences between apical IgA recycling and basolateral-to-apical transcytotic pathway. However, because I did not achieve complete knockdown of Sec15A expression, I cannot rule out that further downregulation of Sec15A may impact other trafficking pathways including receptor recycling.

There are additional explanations for why Sec15A may selectively regulate basolateral-to-apical transcytosis and not other exocyst-dependent endocytic trafficking pathways. One possibility is that there are distinct exocyst holocomplexes containing either Sec15A or Sec15B subunits and the complex containing Sec15A is selectively involved in regulating the basolateral-to-apical transcytotic pathway. An alternative possibility is that there are "physically distinct subcomplexes" comprised of a subset of exocyst subunits, which would function independent of the holocomplex and would act downstream of GTPases such as Rab11a to regulate basolateral-to-apical transcytosis. In yeast, Sec15p and Sec10p form a subcomplex found in the cytosol as well as part of

the holocomplex (56), and in mammalian cells Sec10 and Exo84 (and presumably Sec15) may form a similar subcomplex (112). However, it is unknown whether the Sec10/Sec15/Exo84 subcomplex is functionally active, or if it modulates distinct trafficking events. Our finding that multiple exocyst subunits are found on the ARE and CRE suggest an additional possibility: the exocyst may form "functionally distinct subcomplexes." In this case all subunits are present in the octameric complex; however, the functionally active ones depend on their cellular localization and association with regulatory GTPases. Thus Rab11a could recruit the exocyst holocomplex via Sec15A and then modulate Sec15A function to promote basolateral-to-apical transcytosis. While these models are not mutually exclusive they suggest ways that the exocyst subunits, either as part of the holocomplex or part of physically distinct subcomplexes, may act downstream of compartment-specific GTPases such as Rab11a to modulate specific cellular events such as basolateral-to-apical transcytosis.

4.0 CONCLUSION

4.1 INTRODUCTION

Biosynthetic and endocytic trafficking pathways are involved in the establishment and maintenance of polarity in epithelial cells. Several components of the polarized trafficking machinery that regulates biosynthetic and endocytic trafficking have been identified (113, 116). During membrane trafficking, cargo molecules are first recruited into separate vesicles that are destined to specific membrane domains by sorting signals and adaptor complexes. Interfering with the function of these components can lead to protein mistargeting (137, 139). Furthermore, small GTPases are involved in recruitment of proteins to both the donor and target membrane domains. At the last stages of membrane trafficking, SNAREs and tethering complexes regulate the association of cargo vesicles with the target membrane domain and ultimately fusion between the two compartments (108, 113, 137, 139). The octomeric exocyst complex was originally proposed to act as a tethering factor that directs vectorial delivery of newly synthesized basolateral cargo to the junctional complex, which is an active site of exocytosis in polarized epithelial cells (42). Further studies showed that the exocyst complex is also associated with recycling endosomes and may regulate basolateral trafficking through these compartments (42, 131); therefore, the model was further

expanded to include a role for the exocyst in endosomal traffic. Most recently, the basolateral "specificity" of the exocyst has been challenged by studies in *Drosophila* photoreceptor cells that show the exocyst is involved in trafficking of apical cargo from the TGN to the rhabdomere (13).

In this dissertation, I analyzed the role of the exocyst complex in regulating apically and basolaterally directed endocytic pathways in polarized epithelial cells, and contrary to existing models, I found that the exocyst complex regulates endocytic trafficking directed to both poles of the epithelial cells. This prompted me to examine how the exocyst was differentially engaged to orchestrate traffic through multiple endocytic pathways. In the second part of my dissertation, I have tested the involvement of small GTPases in regulating exocyst function and show that through its interaction with Rab11a, the exocyst complex regulates basolateral-to-apical transcytosis, whereas this interaction is not involved in basolateral or apical recycling pathways. My data and that of others indicate that the association of the exocyst with different GTPases may modulate its function so that it can regulate distinct events such as insulin secretion, cell division, and membrane trafficking (95).

The results I have obtained in this study lead to an important question: Is there a single exocyst complex with multiple functions (Figure 4.1A), or multiple exocyst subcomplexes with individual roles (Figure 4.1B)? Initial models suggest that all exocyst subunits assemble at the target domain and act as a single complex, whereas some of my results and those from other studies support an alternative view in which different subcomplexes with variable subunit compositions may have different functions.



Figure 4.1: Exocyst models

(A) Functionally distinct subcomplexes. In this model all subunits are present in the octameric complex; however, the functionally active ones depend on their cellular localization and association with regulatory GTPases. (B) Physically distinct subcomplexes. In this model there are distinct exocyst subcomplexes comprised of different subset of exocyst subunits, which would function independent of the holocomplex and would act downstream of GTPases to regulate specific pathways.

4.2 THE EXOCYST: A SINGLE COMPLEX WITH MULTIPLE ROLES OR MULTIPLE SUBCOMPLEXES WITH DIFFERENT FUNCTIONS

The original model for the exocyst is based on studies that showed that all eight exocyst subunits assemble to form possibly a single functional holocomplex. If this model is true, then how can one exocyst complex regulate multiple trafficking pathways? As discussed in section 3.3, one way this can be achieved is by having "functionally" distinct octomeric exocyst complexes in various compartments (Figure 4.1A). According to this model, all subunits are present in the holocomplex, however only some are active and this can be achieved by interaction with specific small GTPases. For instance, the interaction between Rab11a and the exocyst (via Sec15) may alter the confirmation of the whole complex to create a functionally distinct exocyst, which regulates trafficking, in this case, at the ARE. In a similar fashion RalA may affect exocyst at the CRE. An alternative way for the small GTPases to influence the function of the holocomplex may be through their effectors. By bringing the exocyst and other effectors together at specific compartments, the small GTPases may influence the function of the exocyst holocomplex and regulate different trafficking pathways.

Although initial studies favored the presence of a single octomeric exocyst complex with multiple functions, the differences in expression profiles of the *Drosophila* exocyst subunits Sec5, Sec6, and Sec8 (13) as well as the different effects of mammalian Exo70 and Sec8 truncation mutants on AMPA receptor delivery (48), suggest that different subunits may form separate subcomplexes with distinct functions. Furthermore, my morphological analysis shows that although exocyst subunits have similar distribution, Exo70 has a slightly different intracellular distribution then Sec8 and

Sec6 in polarized MDCK cells. In addition, by interfering with exocyst function using antibodies against Sec8 subunits, I observe a broader functional effect whereas targeting Sec15 subunit inhibits a specific trafficking pathway (i.e. basolateral-to-apical transcytosis). These observations support the presence of the "physically distinct" exocyst subcomplexes (Figure 4.1B), which are comprised of a subset of exocyst subunits and would function independent of the holocomplex to regulate different trafficking pathways. In addition, some of these subcomplexes may also be involved in regulating other cellular events that are attributed to the exocyst complex such as protein synthesis, cell division or mRNA synthesis.

Small GTPases may play a pivotial role in regulating the function of different subcomplexes by influencing the recruitment of the exocyst subunits and assembly of the subcomplexes on correct cellular compartments. Upon activation, a small GTPase may recruit a specific exocyst subunit to the target membrane compartment and induce assembly of the subcomplex on this site. Alternatively, some exocyst subunits may already be associated with different compartments due to their affinity to different lipids or due to interaction with other resident proteins such as SNAREs, coats or other tethering family proteins. Under these circumstances, recruitment of the missing subunit via small GTPase may induce the assembly of a subcomplex on a specific compartment. In addition, GTPases may not be involved in recruitment of the subunits but regulate the assembly of the subcomplexes on specific compartments through their other effectors.

Although there are several clues, which indicate the presence of different exocyst subcomplexes and that the small GTPases are involved in regulating the exocyst

function, presence of these subcomplexes as well as the details of how GTPases influence the exocyst function have not been analyzed in polarized epithelial cells. In the following sections I will describe different approaches that could be used to test if there is a single holocomplex with different functions or if there are multiple exocyst subcomplexes.

4.2.1 Comparative analysis of exocyst subunit distribution in polarized epithelial cells

In order to answer if the exocyst acts as a single complex or as multiple subcomplexes, it is crucial to analyze the co-localization of exocyst subunits in polarized epithelial cells. Although I have analyzed the distribution patterns of Sec6, Sec8, and Exo70, the localization of other subunits has not been examined in polarized epithelial cells. It is not known which subunits are found in the various compartments of polarized cells. If there is a single exocyst complex responsible for regulating multiple pathways, it is expected that all subunits would be found in any one compartment. However, if there are subcomplexes, some of the subunits are expected to be associated with a subset of compartments, whereas others would be enriched in different compartments. Because some subunits may be part of a core complex that is required for several functions these subunits may be observed in multiple compartments together.

Cellular fractionation and differential centrifugation approaches may be useful to analyze presence of different exocyst complexes with variable subunit compositions. Alternatively, immunoisolation of different compartments and quantification of the presence of different exocyst subunits in these compartments would provide additional

supporting evidence. In Chapter 2, I analyzed the association of Sec8 with Rab11positive endosomes and TfR association with Sec8-positive compartments. This approach can be further optimized either i.) to isolate different compartments and compare the presence of different subunits or ii.) to isolate exocyst-positive compartments using antibodies specific towards different subunits and analyze the presence of other subunits. One problem with this approach is the possibility of dissociation of some subunits from the endosomal compartments during homogenization or immunoisolation stages. In mammalian cells, a subpopulation of Sec8 was shown to fractionate independent of the other subunits (51). Furthermore, in yeast, a pool of Exo84 subunit was shown to be independent of the other subunits (54). These observations may either suggest that some subunits dissociate from the complex during experimental procedures or that there are exocyst subcomplexes with different subunits. Therefore the presence of subunits on a compartment would be useful information, whereas the absence of a subunit may not be as conclusive. One way to address this problem is to use a reversible crosslinker such as DSP as I have done in Chapter 3 to stabilize the protein interactions prior to homogenization and immunoisolation. Although this approach may solve the dissociation problem, it may also lead to nonspecific crosslinking between exocyst subunits that are in close proximity but not necessarily in the same complex. To demonstrate the presence of different subcomplexes, it is important to show that these subunits interact with each other. One possible approach to analyze this is to use co-immunoprecipitation of endosomal fractions to show that the exocyst subunits are in a complex. However, as mentioned previously, dissociation of subunits during homogenization or
immunoisolation stages due to week subunit-subunit interactions is a concern and may interfere with the results of these studies. The combination of both morphological and biochemical assays will allow one to compare the distributions of different exocyst subunits, ultimately examining whether the subunits are all present together or if only a subset are involved.

4.2.2 Functional analysis of different exocyst subunits

An additional way to explore possible subcomplexes or the individual functions of exocyst subunits is to interfere with their roles in intact or permeabilized polarized MDCK cells. In vitro studies by Wang et.al. (188) indicate differentiable roles for exocyst subunits in cytoskeletal arrangements, whereas in Drosophila, silencing of Sec6 and Sec5 expression leads to different phenotypes (120). Therefore, in order to understand the mechanism of exocyst function in the regulation of polarized membrane trafficking pathways, it is important to study the individual roles of subunits. One direct approach is to knock down each subunit and analyze the functional effects on different pathways. In Chapter 3, I used shRNA constructs to knock-down Sec15A expression in polarized MDCK cells, and using the same technique other subunits can be targeted. Knocking down exocyst subunits may affect the ability of the cells to fully polarize; however, in this protocol transfected cells are directly placed on filters after transfection, giving the cells time to start polarizing prior to significant silencing of the protein expression. Under these circumstances, the functional role of all the subunits can be tested in polarized MDCK cells.

If there is a single exocyst holocomplex that is involved in regulating multiple pathways, it is expected that knocking down any of the subunits may lead to the same phenotype, whereas if there are multiple subcomplexes with different functions, knocking down one subunit might affect a single pathway but not others. For example, a subcomplex that contains the Sec5 subunit but not Sec15 may regulate the basolateral recycling pathways and silencing Sec5 may inhibit basolateral but not apical trafficking. Furthermore, assembly of a specific subcomplex may be rate limiting for the assembly of another subcomplex that regulates a different pathway due to availability of common subunits between the two subcomplexes. Accordingly, while silencing the expression of a subunit that is specific for a subcomplex may inhibit a pathway, it may induce the assembly of an alternative subcomplex and have a gain of function effect for a different pathway. While in both yeast and Drosophila there are single copies of exocyst subunits, in mammalian systems, isoforms and splice variants have been identified. These variations of exocyst subunits may have functional significance in different exocyst-regulated pathways, and isoform specific knock-down studies can be useful to understand the role of these exocyst variants in regulating endocytic trafficking in polarized epithelial cells. It is possible that silencing some isoforms may be more lethal then the others but, examining the cells shortly after may overcome this problem.

One problem that may be encountered is that if there is a block in earlier trafficking stages, it may be impossible to analyze the role of downstream trafficking steps. For instance, Exo70 is associated with both early and recycling endosomes and by knocking down Exo70, trafficking through early endosomes may be blocked and therefore the role of Exo70 in recycling endosomes cannot be analyzed. As an

alternative approach, SLO-permeabilized cells can be used to stage ligands in different compartments and to analyze the role of individual or combination of exocyst subunits in regulating trafficking pathways passing through that specific compartment. Initially, the exocyst complex can be depleted from rat or rabbit cytosols by using antibodies against all the subunits. Then this exocyst-depleted cytosol can be supplemented with individual or combinations of bacterially expressed recombinant exocyst subunits. Incomplete depletion of the exocyst complex from the cytosol as well as inefficient expression of exocyst subunits in bacterial system may be possible challenges in this approach. These problems can be addressed by using exocyst antibodies instead of depleted cytosol to block the function of different subunits. If there is a single exocyst complex with multiple functions, all the subunits will be necessary to reconstitute the trafficking pathways. However, if there are subcomplexes or subunits with individual roles, combinations of different subunits will rescue some pathways. Results obtained from these studies will not only advance our understanding of the role of exocyst complex subunits individually or as different subcomplexes but also suggest how the tethering complexes may function in general.

4.3 SMALL GTPASES AS REGULATORS OF EXOCYST FUNCTION

In polarized epithelial cells, small GTPases are compartmentalized and regulate specific pathways (26, 162, 202). While the exocyst binding partner Rab11a is localized to ARE, where it regulates apical but not basolateral trafficking (24, 189), another binding partner RalA is variably associated with plasma membrane and recycling endosomes

and regulates basolateral trafficking between these two compartments (154). Therefore, interaction between small GTPases and exocyst subunits may regulate the exocyst complex and specify which pathway the exocyst is involved with in any one compartment. The mechanism of regulation of exocyst function by small GTPases is not known, however, they may be involved in the recruitment of the complex to specific membrane domains. Alternatively, the binding of an activated GTPase may change the structure of the exocyst complex to facilitate its role, or GTPases may act as a bridge between the exocyst complex and their other effectors. Furthermore, GTPases may bring different components of the vesicle trafficking machinery including the coat complexes, the exocyst and v-SNAREs together to facilitate vesicle formation and budding from the donor compartment. In addition, it can be speculated that the exocyst complex may regulate the GTPase activity of the small GTPases on specific compartments. However, to date there is no known incidence in which the exocyst acts as a GTP exchange factor or regulates the GTP hydrolysis.

Different exocyst models discussed in previous sections rely on the involvement of small GTPases as "regulators" of the exocyst complex. In Chapter 3, I analyzed the role of the Rab11a-exocyst interaction in regulating endocytic trafficking and showed that the Rab11a-exocyst interaction via Sec15 subunit regulates basolateral-to-apical transcytosis but not apical or basolateral recycling. An open area that remains to be explored is the identity of other small GTPases that are involved in endocytic trafficking by regulating exocyst activity. As noted in the previous sections, RalA is another GTPase that interacts with the exocyst complex. Due to the involvement of RalA in basolateral trafficking in polarized epithelial cells (154), it can be speculated that the

RalA-exocyst interaction regulates basolateral recycling pathways. This hypothesis can be tested by interfering with the RalA-exocyst interaction and observing the effect on basolateral recycling of the Tf in polarized MDCK cells. One approach to block the exocyst-RalA interaction is to use the RalA binding domain of Sec5 (1-120 aa) (186) as a GST fusion protein in SLO-permeabilized MDCK cells, and measure the effect on transferrin recycling as described in Chapter 2. As an alternative approach, antibodies against the RalA binding domain of Sec5 can be used to inhibit the interaction between the two proteins (164). If the RalA-Sec5 interaction is not involved in regulation of the post-endocytic pathways, the RalA-Exo84 interaction can also be investigated using the RalA binding domain of Exo84 (122-333 aa) in SLO-permeabilized cells as described above.

It is expected that interfering with the RalA-exocyst interaction will block basolateral trafficking of endocytosed cargo, but will not interfere with apical recycling, since RalA is not implicated in regulating this pathway. However, if the RalA-exocyst interaction regulates trafficking at the CRE compartment, interfering with this interaction may have an indirect effect on other cargo passing through this compartment such as IgA during basolateral-to-apical transcytosis. If there is an effect on the transcytotic pathway, further studies can be performed to analyze the consequences of inhibiting the RalA-exocyst interaction on CRE morphology and the possible accumulation of basolaterally internalized Tf and IgA in this compartment.

In addition to RalA, there are other GTPases involved in endocytic trafficking pathways and they may interact with the exocyst complex. For instance, Rab11b and Rab25, other members of the Rab11 family, are possible candidates. These two

GTPases share most of the Rab11a effectors, therefore it is expected that they also interact with the exocyst complex. As shown in Chapter 3, Rab11a-exocyst interactions regulate basolateral-to-apical transcytosis but not apical recycling. Therefore, it is intriguing to speculate that other Rab11 family members such as Rab25, which is involved in apical recycling of IgA, may interact with the exocyst complex. In addition, Rab11b has a similar but not identical apical distribution compared to Rab11a, and my morphological analysis shows that there exists a pool of exocyst subunits at the apical pole of the cell that does not co-localize with Rab11a positive endosomes. These compartments may be Rab11b positive, and exocyst may interact with Rab11b in addition to Rab11a. Yeast-two-hybrid assays can be used to test the interaction between these GTPases and different exocyst subunits. It is possible that the Sec15 subunit may interact with these additional Rab11 family members, however if not, other exocyst subunits should be tested. If the exocyst complex interacts with Rab11b or Rab25 via a different subunit then Sec15, this may fortify a subcomplex model in which different exocyst subcomplexes regulate separate pathways by interacting with different set of small GTPases.

In addition to analyzing the roles of the exocyst-small GTPase interactions in regulating different endocytic trafficking pathways, it is important to explore *how* these interactions regulate different pathways. As noted above, GTPases may change the conformation of the exocyst complex, recruit different exocyst subunits to specific membrane compartments, or serve as a bridge between their different effectors and the exocyst complex. Furthermore, overexpression of SNAREs homologues in yeast partially rescues exocyst mutant strains, which suggests direct or indirect interaction

between the tethering and SNARE complexes. How does the exocyst and SNAREs form a complex and regulate vesicle fusion is not well understood. It is possible that the small GTPases may also regulate the communication between the exocyst complex and SNAREs, which is an important area to explore. The exocyst complex may regulate several membrane trafficking steps in addition to vesicle tethering, and small GTPases, specifically Rab GTPases, are implicated in these steps. Therefore, understanding the mechanistic details of the exocyst-GTPase interactions will advance our understanding of membrane trafficking steps.

4.4 THE EXOCYST: REGULATOR OF MULTIPLE MEMBRANE TRAFFICKING STEPS

Although the exocyst complex regulates the association of cargo vesicles with the plasma membrane, my studies and others indicate that it may regulate additional steps during vesicle trafficking. For instance, inhibiting exocyst function in *Drosophila* epithelial cells leads to an accumulation of cargo in recycling endosomal compartments (82), rather then in secretory vesicles accumulated underneath the plasma membrane. These data suggest that the exocyst complex may be involved in the cargo release from endocytic compartments in addition to its role at the plasma membrane. Furthermore, in yeast, most of the exocyst subunits are shown to be associated with exocytic cargo vesicles (16), which may implicate the exocyst in vesicle formation, budding, or vesicle motility.

Currently it is not known if, like its yeast homologue, the mammalian exocyst complex is associated with the cargo vesicles. However, Exo70 is associated with the actin modifying proteins such as Arp2/3 complex and it implicated in cytoskeletal arrangements. These observations suggest that the exocyst may regulate cargo vesicle motility from the donor membranes (e.g. recycling endosomes) to target membranes (e.g. plasma membrane). In order to analyze this possibility, the association of different exocyst subunits with cargo vesicles derived from the ARE or CRE can be analyzed using immuno-gold labeling and transmission electron microscopy. In this assay, a cargo molecule such as IgA is first accumulated in the ARE, followed by perforation of the cells using nitrocellulose as described in Figure 2.11A. Then vesicle release is resumed by adding cytosol and ATP regenerating system. Cargo vesicles are collected from perforated cells and prepared for immuno-electron microscopy analysis. Since this preparation will contain different vesicle populations derived from different compartments, the ARE-derived vesicles can be specifically labeled with an antibody against IgA. Association of different exocyst subunits with IgA-positive vesicles can be analyzed by co-labeling samples with antibodies against different exocyst subunits. The same approach can also be used to test the association of exocyst subunits with CREderived cargo vesicles, by accumulating basolaterally internalized Tf in CRE and then analyzing the association of different exocyst subunits with Tf containing vesicles. The presence of different subunits on cargo vesicles can be analyzed biochemically, however enriching enough cargo vesicles to be able to measure the presence of exocyst subunits using biochemical detection methods might be challenging.

The association of a specific exocyst subunit with cargo vesicles does not mean that the subunit is involved in vesicle budding from a given compartment. In order to test this role, the vesicle budding assay I used in Figure 2.11 can be performed with minor modifications. As described previously, exocyst subunits can be immuno-depleted from the cytosols using antibodies against all the subunits. Then this exocyst-depleted cytosol can be supplemented with individual or combinations of bacterially expressed recombinant exocyst subunits. The presence of some subunits may regulate the budding of vesicles from a specific compartment such as the CRE, while other subunits may regulate vesicle release from the ARE. It is equally possible that a core "budding complex", which contains the same subset of exocyst subunits, may regulate vesicle budding in all the compartments. As an alternative approach, instead of using exocyst depleted cytosol and recombinant exocyst subunits, function-blocking antibodies against different exocyst subunits can be used to test their roles in vesicle budding from different compartments.

The interaction between the exocyst and AP1B adaptor complex (44) is another intriguing observation that hasn't been analyzed in detail. Why does a tethering complex interact with an adaptor complex involved in cargo sorting? One possible explanation is that some exocyst subunits may interact with adaptor complexes to combine cargo sorting and vesicle budding steps by acting as a bridge between the cargo sorting complexes, coat proteins, and the remaining vesicle budding machinery. In this model, Rab GTPases, which are implicated in cargo sorting as well as vesicle formation (26, 80, 162, 193), play a central role possibly by recruiting all of the necessary components of cargo sorting and vesicle formation machinery to a specific membrane compartment.

However, it is important to note that during delivery of ER derived vesicles to the Golgi complex, it is proposed that the interaction between TRAPPI tethering complex subunit Bet3 and COPII coat molecule Sec23 activates Ypt1/Rab1 GTPase, which is followed by the recruitment of the coiled tether Uso1/p115. According to this model, the coat-tethering complex interaction is upstream of the GTPase activity, however as discussed previously the role of GTPases in exocyst mediated pathways in not fully understood and it is possible that both models may be true for different pathways. To date, the only adaptor that is shown to interact with the exocyst is AP1B, which regulates a subset of basolateral trafficking pathways. The interaction of the exocyst complex with other adaptor complexes has not been analyzed, and may be an interesting area to explore further.

Accumulation of cargo molecules at intermediate compartments in exocyst deficient *Drosophila* and mammalian cells can also occur due to inhibition of the fusion step with downstream compartments. To test if the exocyst complex regulates the endosome-endosome fusion step of vesicle trafficking, an *in vitro* endosome fusion assay can be used. In this experiment, different populations of endosomes isolated from polarized cells are mixed in the presence of an ATP regenerating system and cytosol. As described previously for vesicle budding, a cytosol that lacks all exocyst subunits can be used by supplementing different subunits either individually or in combination. If endosome fusion is an exocyst-dependent process, this opens up another interesting research area to analyze the interaction of the exocyst with endosome-associated fusion complexes. For example, Rab5 and its effectors, which include EEA1, are associated with early endosomes and regulate endosome fusion (27, 196). Interestingly,

my morphological studies show that Exo70 is associated with EEA1 positive endosomes. Therefore, it would be intriguing to focus on Exo70 initially to analyze its association with the endosome fusion machinery and potential role in regulating endosome-endosome fusion. However, it is important to note that due to its association with different lipid species and the cytoskeleton, Exo70 may have an indirect role in endosome motility and fusion by associating with early endosomes and regulating the actin cytoskeleton rather then directly interacting with endosome fusion machinery.

As discussed previously, in yeast, the exocyst complex regulates the cargo vesicle tethering to the plasma membrane (55, 57, 169). In accordance with this observation, it is also implicated in delivery of cargo vesicles to the basolateral membrane in polarized epithelial cells (51). However, its role as an apical tethering factor in these cells has not been tested. Although there is evidence that suggests the exocyst complex is not involved in p75 trafficking from the TGN to the apical membrane (51), my data from Chapter 2 and Chapter 3 indicates the exocyst complex is involved in endocytic trafficking pathways directed towards the apical pole of the polarized MDCK cells. Furthermore, Sec8 is localized to the primary cilium at the apical membrane in these cells (141). Based on these observations, it can be hypothesized that, the exocyst complex may regulate apical trafficking not only at recycling endosomes but also as a tethering factor on the apical membrane. In order to test this hypothesis, a cell-free system that was originally described by Sztul et.al. could be used (167). In this assay, fusion between the transcytotic vesicles and the apical plasma membrane isolated from rat liver is reconstituted in the presence of ATP regenerating

system and cytosol. Involvement of the exocyst complex in this event can be tested by using exocyst-depleted cytosol as described previously.

The results obtained from these studies will be useful to understand the roles of individual exocyst subunits during a variety of trafficking stages such as vesicle budding, endosome-endosome fusion, and cargo vesicle-apical membrane fusion. As the simple tethering complex model indicates, these protein complexes regulate the very last stages of membrane trafficking steps preceding SNARE mediated fusion, and analyzing their possible roles in earlier stages will further increase our understanding of how different membrane trafficking players such as adaptor complexes, GTPases, SNAREs, and tethering molecules crosstalk to each other to regulate complicated membrane trafficking steps during various pathways.

4.5 THE EXOCYST: TRAFFICKING OF CHANNELS AND PATHOGENS

In this dissertation, I have analyzed the role of the exocyst complex in the regulation of endocytic trafficking in polarized epithelial cells. In addition, other studies indicate that the exocyst regulates trafficking in different systems such as the delivery of NMDA and AMPA receptors in neurons (48, 149). Exocyst subunits as well as Rab11 have also been identified in aquaporin 2-positive compartments (11), which may indicate that the exocyst complex regulates trafficking of this channel to the apical surface. Therefore, it can be speculated that the exocyst complex is involved in trafficking of other channels such as epithelial sodium channel (ENaC), which regulates sodium uptake, or the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates chloride

transport across the epithelial tissue. Although both of these channels play important roles in the pathogenesis of Liddle's syndrome or cystic fibrosis, respectively (2, 152), the protein complexes involved in regulating their trafficking are not well understood. Interestingly, ENaC is shown to be associated with Rab11-positive endosomes (100), and if this population of ENaC represents a "recycling" pool, the exocyst complex might be involved in regulating ENaC trafficking between Rab11-positive endosomes and the apical surface. Recently, Rab11 and its effector Myosin Vb were shown to be involved in CFTR trafficking (166); however, involvement of other Rab11a effectors such as the exocyst in regulating CFTR delivery is not known. The regulatory role of Rab11 in CFTR trafficking indicates that upon internalization, a population of CFTR may be delivered to an endosomal compartment and recycle back to the apical surface from this compartment.

In addition to channel trafficking in polarized cells, the exocyst might be involved in other trafficking pathways such as apical-to-basolateral transcytosis, in which ligands bind to their receptors on the apical surface and transcytose to the basolateral membrane through endocytic compartments (142). This pathway is an important route for several pathogens to pass mucosal epithelia and invade the host. For instance, mother-to-infant transfer of Hepatitis B virus (HBV) accounts for 30% of new infections (64), and transcytosis through placental epithelia is speculated to be a mechanism involved in this transmission. In tissue culture, HBV is shown to infect the polarized BeWo placental cell line and transcytose from the apical surface to the basolateral media (14), however the details of the internalization or transcytosis are not known. Another pathogen, Epstein-Barr virus (EBV), has also been studied intensively and

shown to infect epithelial cells. Interestingly, EBV interacts with IgA and uses pIgRmediated internalization of the IgA from the apical surface as its infection route (77). In addition to viral pathogens, bacteria such as *Streptococcus pneumoniae*, which is the most common cause of bacterial meningitis, are shown to directly bind to pIgR on the apical surface of cells and hijack the transcytotic machinery to pass the epithelial barrier and invade the host (77). The growing list of pathogens infecting or bypassing epithelial cells using the transcytotic pathway underlines the importance of understanding the protein machinery involved in regulating this pathway. It will be intriguing to analyze the role of the exocyst complex in apical-to-basolateral pathway in polarized epithelial cells since apically internalized cargo may go through the compartments that the exocyst is associated with, including the ARE and CRE.

So far the trafficking pathways I have discussed are closely related to the pathways I have analyzed in my dissertation. An unrelated but equally interesting pathway that the exocyst might be involved is the trafficking of odorant receptors (OR) to the cell surface in olfactory sensory neurons (OSN). Mammalian ORs are G-protein coupled seven transmembrane proteins that bind to odor molecules and activate downstream signaling pathways that carry the "odor information" to higher data processing centers in central nervous system (182). Although mammalian and *Caenorhabditis elegans* ORs are shown to have similar structures, recently it was shown that *Drosophila* ORs have different membrane topology and may not be G-protein coupled proteins (12). Furthermore, *Drosophila* ORs are shown to associate with another seven transmembrane protein, OR83b, and this cofactor is required for the trafficking of most ORs to the plasma membrane. In OSNs, OR-OR83b complex is

localized to the cilia in the dendrites (85); however, the protein complexes involved in the delivery of this complex to the cilia are not known. Furthermore, the fate of "activated" OR after binding to its specific ligand is not well understood. It is not known if desensitization to a specific odor is due to internalization of the receptors and degradation or delivery to a recycling compartment where they can recycle back to the surface after the stimulus is removed from the environment. Interestingly, the Sec8 subunit of the exocyst complex is associated with the cilia in polarized epithelial cells (141). Analyzing the association of Sec8 with the cilia in OSNs and colocalization of other exocyst subunits with ORs in these structures would be an intriguing project. It is possible that OR83b may act as a link between the cellular factors and ORs, and it would be interesting to analyze the interaction between its cytoplasmic domains and protein complexes involved in trafficking such as the exocyst. Based on the wide variety of pathways that the exocyst regulates, it wouldn't be surprising to find that exocyst subunits are also involved in OR trafficking in *Drosophila* OSNs.

4.6 CLOSING COMMENTS

In mammalian epithelial cells, the octameric exocyst complex is suggested to direct the fusion of cargo carriers with the basolateral membrane of the cell (51, 118). In contrast, I observed that i) the exocyst is associated with both apical and basolateral endocytic compartments, ii) the exocyst regulates endocytic traffic directed toward both the basolateral and apical poles of the cell, and iii) the pathway specificity of exocyst function may be dictated through its interaction with small GTPases. I propose a model

whereby having multiple subunits provide the exocyst complex the flexibility to adapt to different compartments and regulate different trafficking pathways. The observations I have made in this dissertation expands our knowledge about the role of the exocyst complex in polarized trafficking and raises several questions about the individual roles of the exocyst subunits as well as presence of different subcomplexes on different compartments. The sum total of current data paints a picture in which the exocyst complex regulates vesicle trafficking events at multiple levels, i.) cargo sorting and vesicle budding at the donor compartment, ii.) movement of the cargo vesicle to a specific membrane domain through rearrangement of cytoskelaton by Exo70 and iii.) tethering of the cargo vesicle to the target membrane domain through interaction between subunits that are present on the cargo vesicle and the membrane, which is followed by the SNARE-mediated fusion of the vesicle with the target membrane. These speculations can be further tested using knock-down studies as well as in vitro vesicle binding and fusion assays as described above. The list of regulatory functions associated with the exocyst complex will probably increase in the future and it will be implicated in regulating trafficking of several receptors and channels that have functional significance due to their role in different disease states or infections.

5.0 MATERIALS AND METHODS

5.1 ANTIBODIES

Ascites containing mouse mAbs against Sec8 (10C2, 5C3, or 2E12) were characterized previously (51), and used at 1:500 dilution for immunofluorescence labeling, 1:5000 dilution for western blotting, and 1:100 dilution for SLO assays. The rSec6 and rSec8 mAbs (Stressgen, Ann Arbor, MI) were used at 1:100 dilution for immunofluorescence labeling. Hybridoma supernatant containing the Sec15A mAb 15S2G6 was used undiluted for western blotting. Hybridoma supernatant from cells expressing mouse antibody Exo70 monoclonal 13F3 against was used 1:10 dilution for immunofluorescence labeling and western blotting (179). Hybridoma supernatant containing the Sec6 mAb 9H5 was used undiluted for western blotting (199). Ascites containing the anti-Myc 9E10 mAb (Dr. S.W. Whiteheart, University of Kentucky, Lexington, KY) was diluted 1:100 for use in SLO studies. Rat anti-ZO-1 hybridoma R40.76 culture supernatant (Dr. D.A. Goodenough, Harvard University, Cambridge, MA) was used at a dilution of 1:10. Serum containing anti-canine Tf antibodies (Apodaca et al., 1994) was used at a 1:250 dilution for immunofluorescence. Rabbit anti-EEA1 antibody (Dr. S. Corvera, University of Massachusetts Medical School, Worcester, MA) was used at a dilution of 1:500. An affinity-purified rabbit anti-Rab11 antibody specific

for the N-terminus of Rab11 (ab3612; Abcam, Cambridge, MA) was used for immunoisolation; Mouse monoclonal anti-Rab11 antibody (610656; BD Transduction Labs, San Jose, CA) was used at 1:1000 dilution for western blots; Anti-Rab11a-specific serum (Dr. J. Goldenring, Venderbilt University, TN) was used for immunofluorescence at a dilution of 1:500 for immunofluorescence and 1:2500 for western blotting. Rabbit anti-furin (Alexis Biochemicals, San Diego, CA) was used at a 1:500 dilution. Mouse monoclonal anti-Tf receptor antibody H68.4 (Invitrogen, Carlsbad, CA) was used 1:100 for IF and 1:2000 for western blotting. Mouse anti-HA antibody (Covance, Berkeley, CA) was used in 1:250 for immunoprecipitation reactions. Mouse monoclonal anti-polymeric immunoglobulin receptor (plgR) antibody Sc166 was used 1:100 for IF and 1:1000 for western blots. Human polymeric IgA was purchased from Dr. JP. Vaerman (Catholic University of Louvain, Belgium) and used at 0.2 mg/ml. All Cy5 and FITC conjugated affinity-purified and minimal cross reacting goat anti-mouse, rabbit, rat and human secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and used at 1:200 dilution. Cy3 conjugated secondary antibody (Jackson Immunoresearch Laboratories) was used at 1:1000 dilution. For western blotting, HRP conjugated goat anti-mouse and goat-anti rabbit secondary antibodies (Jackson Immunoresearch Laboratories) were used at 1:10,000 and 1:50,000 dilutions, respectively.

5.2 DNA CONSTRUCTS AND PRODUCTION OF ADENOVIRUSES

Full-length rat Sec15A, a 1170-bp fragment (1-1170) encoding the first 390 amino acids of the N-terminus of Sec15A, or a 1296-bp fragment (1171-2466) encoding the Cterminal 431 amino acids were cloned into the yeast two-hybrid vector pGADT7 (BD Biosciences) using BamHI and XhoI sites. For two-hybrid analysis Rab11a wild-type (Rab11a), dominant negative Rab11a-S25N (Rab11aSN), and dominant active Rab11a-S20V (Rab11aSV) were subcloned into the pBDGAL-Cam vector (Stratagene, La Jolla, CA) using standard DNA technologies. The GFP-Sec15CT chimera was generated by cloning the 1296 bp fragment of Sec15A into the pEGFP-C3 vector (Clontech, Palo Alto, CA) using XhoI and BamHI sites. The GFP-Sec15CT(NA) construct was generated by mutating amino acid Asn₇₀₉ to an alanine residue using the Quickchange Site-Directed Mutagenesis Kit (Stratagene). Adenovirus expressing GFP and triple HA tagged Rab11a (pAdTet-GFP/HA-Rab11a) was kindly provided by Robert Edinger (University of Pittsburgh). A recombinant adenovirus expressing GFP/HA-Rab11aSV was generated by mutating Ser₂₀ in pAdTet-GFP/HA-Rab11a to a valine residue using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) and the virus was produced as described previously (63).

5.3 CELL CULTURE, GENERATION OF STABLE CELL LINES, AND INFECTION WITH ADENOVIRUS

MDCK strain II cells expressing the wild-type rabbit pIgR (pWe) have been described (Breitfeld *et al.*, 1989a). Cells were maintained in MEM (Cellgro, Herndon, VA) supplemented with 10% (v/v) FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin in a 37° C incubator gassed with 5 % CO₂. Cells were cultured on 12-mm or 75-mm, 0.4 μ m Transwells (Costar, Cambridge, MA) as described (Breitfeld *et al.*, 1989a) and used 3–4 d after culture. Stable cell lines expressing GFP-Sec15CT or GFP-Sec15CT(NA) were created by transfecting MDCK-II cells with the appropriate vector using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected with 500 μ g/ml G418. Cells were infected with recombinant adenovirus encoding the pIgR as described previously (1). For GFP/HA-Rab11SV under the control of a tetracycline-regulated promoter and an additional adenovirus that expressed the tetracycline-repressible transactivator (AvTA) to induce expression in the absence of antibiotic (63).

5.4 IMMUNOFLUORESCENCE LABELING, CONFOCAL MICROSCOPY, AND IMAGE PROCESSING

When described, cells were permeabilized with 0.05% saponin in PIPES-KOH buffer (80 mM PIPES-KOH, pH 6.8, containing 2 mM MgCl₂, and 5 mM EGTA) for 5 min at

4°C. Cells were fixed with methanol for 10 min at – 20°C, with 4% (w/v) paraformaldehyde in 100 mM sodium cacodylate buffer (pH 7.4) for 10 min at room temperature, or with a previously described pH-shift protocol (8, 10). Following fixation with paraformaldehyde containing fixatives, unreacted paraformaldehyde was guenched with phosphate buffered saline (PBS) containing 20 mM glycine, pH 8.0 and 75 mM NH₄CI for 10 min at room temperature. Fixed cells were incubated with block buffer (0.025% [w/v] saponin, and 8.5 mg/ml of fish skin gelatin in PBS) containing 10% (v/v) goat serum for 10 min at room temperature. Cells were incubated with primary antibody for 1 h at room temperature, washed three times with block buffer for 5 min, and then incubated with fluorescent-labeled secondary antibodies for 1 h at room temperature. Following three additional 5-min washes with block buffer, the cells were rinsed with PBS, fixed with 4% paraformaldehyde in 100 mM sodium cacodylate, pH 7.4 for 5 min at room temperature, and then mounted as described previously (8). Imaging was performed using a TCS-SL confocal microscope (Leica, Dearfield, IL) equipped with argon, green helium-neon, and red helium-neon lasers. Images were acquired using a 100 x 1.4 NA oil objective. Photomultipliers were set to 600-900 V and zoom at 4 X. Images were collected every 0.25 μ m and averaged 3 times. The images (512 x 512 pixels) were saved in a TIFF format and compiled using Volocity software (Improvision, Lexington, MA).

5.5 IMMUNO-ISOLATION OF RAB11- AND SEC8-POSITIVE ENDOSOMES AND WESTERN BLOTTING

An "early" endosome fraction was enriched as described previously (49). Preliminary results confirmed that this fraction was also enriched in Rab11-positive endosomes. Briefly, filter grown cells (cultured on 75-mm Transwells) were washed with ice-cold PBS, gently recovered by scraping into PBS, and recovered by centrifugation. The cells were resuspended in 300 µl of homogenization buffer (3 mM imidazole, pH 7.4, 250 mM sucrose, 0.5 mM EDTA, and complete proteinase inhibitor cocktail from Roche). Cells were homogenized by 21 strokes of a tight fitting Dounce homogenizer and then centrifuged for 10 min at 3,000 rpm in a Heraeus Biofuge Fresco table-top centrifuge. The resulting post-nuclear supernatant (PNS) was reserved and the nuclear pellet was resuspended in an additional 300 µl of homogenization buffer and centrifuged again. The two PNS fractions were pooled, an aliquot was reserved for western blot analysis, and the PNS was adjusted to 40.6% (w/w) sucrose using 62% (w/w) sucrose. The diluted PNS was placed in 12-ml capacity Polyclear centrifuge tubes and overlayed with 6 ml of 35% (w/w) sucrose and 4 ml of 25% (w/w) sucrose. The tubes was topped off with homogenization buffer and centrifuged in a TH-641 rotor at 108,000 x g for 3 h at 4° C. The endosome-enriched fraction (~1 ml) at the 25%/35% sucrose interface was collected with a needle.

Sheep anti-rabbit magnetic Dynabeads (50 μ l; Invitrogen) were washed with 0.2% (w/v) BSA in PBS two times and incubated with 1 ml 5% (w/v) BSA in PBS overnight at 4° C. The following day the beads were recovered with a magnetic particle concentrator

(Dynal, Oslo, Norway) and resuspended in 1 ml 5% BSA in PBS containing 5 μ g of Rab11 polyclonal antibody (ab3612) or non-specific rabbit IgG and incubated overnight at 4° C. The beads were washed with 1% (w/v) BSA in PBS, resuspended in ~2 ml of 5 % BSA in PBS and incubated with ~1 ml of the endosome fraction 3 h at 4° C on a rotator. The Rab11-positive endosomes associated with the Dynabeads were collected using a magnetic plate, washed two times with 0.2% BSA in PBS and then one additional time with PBS. The endosome suspension was transferred to a new tube, magnetic beads were collected using a magnetic particle concentrator, and PBS was removed by aspiration. The endosomes bound to beads were boiled in Laemlli sample buffer and resolved on 15% SDS PAGE gel. Western blots were performed as described previously (102). In some cases the blot was stripped using Restore Plus western blot stripping buffer (Pierce, Rockford, IL) and then re-probed with different antibodies. For immunoisolation of Sec8-containing endosomal compartments, 5 ul of a 1:1:1 mixture of Sec8 antibodies (10C2, 5C3, and 2E12 ascites) was incubated with sheep anti-mouse magnetic Dynabeads. A nonspecific mouse IgG was used as a control. The immunoisolation was performed and samples were analyzed as described above.

5.6 CO-IMMUNOPRECIPITATION ANALYSIS

Filter-grown MDCK cells were washed with ice-cold Ringer's saline (10mM HEPES, pH 7.4, 154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl₂) twice, the filters were excised from their plastic holder and placed in Eppendorf tubes. A half ml of IP lysis buffer (50 mM Tris,

pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% NP-40, 1 mM PMSF, and 5 µg/ml of pepstatin, leupeptin, and antipain) was added and cells were lysed by vortex shaking for 10 min at 4 C. The supernatants were transferred into a new tube, a 1:500 dilution of anti-Sec8 antibody 10C2 was added and the samples were incubated overnight at 4° C on a rotator. Protein G sepharose (Roche, Mannheim, Germany) was washed with icecold PBS, and 50 ul of a 50 % slurry was added to each tube and the reaction was incubated at 4 C for 3 h on a rotator. Tubes were centrifuged 30 s at maximum speed in a 5414D microcentrifuge (Eppendorf, Westbury, NY) and the supernatants were aspirated. Beads were washed 3 times with IP lysis buffer, twice with low salt wash buffer (2mM EDTA, 10 mM Tris, pH 7.4), and then resuspended in 30 ul of 2 X Laemmli sample buffer. The samples were heated at 95 C for 3 min and then centrifuged for 2 min to pellet the protein G beads. The cell lysate was resolved by SDS-PAGE and western blots were probed with the indicated primary antibodies. For coimmunoprecipitation of Sec15CT with GFP/HA-Rab11SV, the cells were crosslinked with 0.2 ug/ul DSP (Pierce, Rockford, IL) at room temperature for 30 min prior to cell lysis. The reaction was stopped by incubating filters with 50 mM glycine at room temperature for 10 min and then the cells were lysed and processed as described above. A 1:250 dilution of mouse anti-HA antibody and 50 ul of protein G sepharose were added to the cell lysate and incubated overnight at 4 C on a rotator. Samples were centrifuged to pellet the beads and washed as described above. Prior to loading the samples on the gel, 10ul of 1M DTT was loaded into each well to ensure reversal of the DSP crosslinks.

5.7 SLO PERMEABILIZATION OF MDCK CELLS AND RECONSTITUTION OF MEMBRANE TRAFFICKING IN SEMI-PERMEABILIZED CELLS

Basolateral recycling of ¹²⁵I-Tf in SLO-permeabilized cells was performed as described previously (88). Delivery of IgA from the ARE to apical pole of the cell was performed as follows. ¹²⁵I-IgA was internalized from the basolateral surface of the cells for 10 min at 37°C, the cells were then washed three times with MEM/BSA (minimal essential medium containing 20 mM HEPES, pH 7.4, 0.35 g/l NaHCO₃, 0.6% (w/v) BSA, and penicillin/streptomycin (1:100, Gibco-Invitrogen)), and then chased in ligand free medium for 20 min at 37°C. The cells were permeabilized with SLO (Murex Diagnostics, Norcross, GA) and transcytosis reconstituted as described previously (7). To reconstitute apical recycling, cells were rinsed with warm MEM/BSA and ¹²⁵I-IgA was internalized from the apical pole of the cell for 10 min at 37° C. The cells were washed three times with warm MEM/BSA and then two times with ice-cold MEM/BSA. The apical surface of the cells was treated with 25 µg/ml TPCK-treated trypsin (in MEM/BSA) for 30 min at 4° C, rinsed with cold MEM/BSA, and then incubated with soybean trypsin inhibitor (125 µg/ml in MEM/BSA) for 20 min at 4° C. The cells were then permeabilized with SLO as described for the transcytosis assay. For each assay, control reactions were performed in the presence of cytosol, ATP, and an ATPregenerating system. ATP independence was assessed by performing reactions in the presence of 40 U/ml apyrase (Type VI; Sigma, St. Louis, MO) and cytosol, but lacking ATP and an ATP-regenerating system. For experimental samples, ascites containing function-blocking Sec8 mAbs (10C2, 5C3, and 2E12) or non-specific Myc antibodies were included during the cytosol washout and during the reconstitution step (performed

in the presence of cytosol, ATP, and an ATP-regenerating system). Experiments were performed 2-3 times in triplicate. ATP independent values were subtracted from control values and experimental values, and the resulting values were normalized to control reactions.

5.8 YEAST-TWO-HYBRID

For *yeast-two-hybrid* experiments the MATCHMAKER GAL4 Two-Hybrid System (Clontech) was used. Experiments were performed according to the directions supplied by the manufacturer.

5.9 VESICLE BUDDING

Reconstitution of vesicle-release from endosomes was performed as described previously (15) with the following modifications. ¹²⁵I-IgA was internalized from basolateral surface of cells grown on 75-mm Transwell filters for 20 min at 18.5° C. The cells were washed with ice-cold MEM/BSA three times quickly, and then three times 5 min on an orbital shaker at 4° C. The cells were then incubated at 37°C for 20 min to accumulate IgA in the ARE. After perforation with nitrocellulose, the cytosol was washed out in the presence of ascites containing anti-Sec8 mAbs (10C2, 5C3, 2E12) or anti-Myc mAb at 4° C for 45 min. Control reactions were reconstituted in the presence of an ATP-regenerating system and rat liver cytosol (2 mg/ml). ATP independence was

assessed in reactions containing apyrase and cytosol. Experimental samples contained an ATP regenerating system, cytosol, and either ascites containing the Sec8 or Myc mAbs. Data were analyzed as described above for the SLO assays.

5.10 TRANSFECTION OF POLARIZED FILTER-GROWN CELLS

Cells were plated on 12-mm Transwells and 2 d later the medium was aspirated and replaced with low calcium medium (DME-F12 medium containing 1.2 g/l NaHCO₃, 5 µM CaCl₂ and 10% [v/v] dialyzed FBS) and cultured for an additional 2 d. On the day of transfection, the low calcium medium was aspirated, and the cells were incubated with PBS containing 1 mM MgCl₂ for 10 min at room temperature. Plasmid DNA (2-6 μ g) and 4 µl of Lipofectamine 2000 (Invitrogen) were each diluted in 100 µl of Opti-MEM (Gibco-Invitrogen) and incubated for 5 min at room temperature. The DNA and Lipofectamine 2000 containing solutions were mixed and then incubated for 20 min at room temperature. Filters were placed on 40 µl drops of DNA/Lipofectamine complex (performed on Parafilm) and the remaining 160 µl of DNA/Lipofectamine complex was added to the apical well of the Transwell unit. Cells were incubated for 5-7 h at 37° C. An additional 900 μ l of complete MEM medium containing 10% (v/v) FBS and penicillin/streptomycin/fungizone was added to the apical chamber and 1.5 ml added to the well facing the basolateral surface of the cell. The cells were used 24 h posttransfection.

5.11 IF TRANSCYTOSIS ASSAY

Filter-grown cells were washed with MEM/BSA and incubated at 18° C for 15 min and pulsed with 200 µg/ml IgA from the basolateral surface of the cells for 20 min at 18° C. The cells were then placed on ice or incubated for 20 min at 37° C. During this chase, 25 µg/ml Cy3-labeled anti-human IgA was added to the apical media. The cells were then washed with ice-cold MEM/BSA and then PBS, and finally fixed with 4% (w/v) paraformaldehyde in 100 mM sodium cacodylate, pH 7.4 for 10 min at room temperature. Cells were labeled as described above.

5.12 POSTENDOCYTIC FATE OF ¹²⁵I-Tf AND ¹²⁵I-IgA

The postendocytic fate of a pre-internalized cohort of ¹²⁵I-IgA or Tf was performed as described previously (17, 102).

5.13 shRNA AND RT-PCR ANALYSIS IN POLARIZED MDCK CELLS

For shRNA studies, an algorithm from MIT (<u>http://jura.wi.mit-.edu</u>/bioc/siRNAext/home.php) was used to search for siRNA sequences that were predicted to target splice-variants of canine Sec15A (XM_534966, XM_844233), but not Sec15B (XM_540235, XM_861541, XM_861551). Three candidate sequences were selected and custom-synthesized sense and anti-sense DNA oligos (IDT, Coralville, IA) were

Early passage MDCK cells expressing the pIgR (pWe) were plated at low density to achieve 50-70% confluency at the time of transfection. The MDCK cells were trypsinized, resuspended in MEM culture medium, and recovered by centrifugation. Cells (0.5-1.0 x 10⁶) were resuspended in 100 ul of transfection buffer, which was prepared by mixing 10 ul of Solution I (362 mM disodium salt of ATP, 590 mM MgCl₂ • 6H₂O; stored at -80° C) with 1 ml of Solution II (88.2 mM KH₂PO4, 14.3 mM NaHCO₃, 2.2 mM glucose, pH 7.4; stored at -20° C). The appropriate DNA construct (6 ug) was added to the cell suspension and cell/DNA mixture was placed into an Amaxa (Gaithersburg, MD) electroporation cuvette. Cells were nucleofected using the T-20 program. Immediately after transfection, 800 ul of MEM culture medium was added to the cells were resuspended using a sterile glass Pasteur pipette 6-8 times,

and the cells were then plated in the apical chamber of collagen-coated 12-mm Transwell filters. MEM culture medium (1.5 ml) was added to the basolateral chamber and the culture medium was aspirated and replaced 24 h post-transfection. Experiments were performed 48 h post-transfection.

For RT-PCR studies, mRNA was isolated from filter grown cells using an RNAgueous RNA isolation kit according to the manufacturer's protocol (Ambion, Austin, TX). cDNA was synthesized using 1 ug total RNA, oligo(dT) primer, and the M-MLV reverse transcriptase. For RT-PCR reactions, 1/5th of the total cDNA reaction mixture was mixed with 5 mM dNTPs, 2 uM sense and anti-sense primers, and polymerase from the Expand High Fidelity PCR System (Roche). Canine Sec15A message was (GATGCTATTGGACAGTGAGT) amplified using sense and anti-sense (CTATGTATGCTGGGACATCCCAT) primers. То amplify canine Sec15B messagelused the following primers: sense (ACTTTTGCTGGAAGCTGAAG) and antisense (TCATGAGTGGTGGCTGCTGATGAGT). For RT-PCR the DNA was denatured at 94 C for 4 min followed by 25 cycles of amplification (94 C for 2 min, 55 C for 45 s, 72 C for 1 min) and finally 1 cycle of incubation for 5 min at 72 C. The complete PCR reaction was loaded on 1% agarose gel to observe amplified products. The gel pictures were scanned and quantified using QuantityOne software (BioRad, Hercules, CA). For Western Blot analysis of shRNA transfected cells, ECL reactions were exposed to film and the images were quantified by densitometry using QuantityOne software. Background was subtracted from each well, values were normalized to actin or Sec8 expression, and the percentage decrease was calculated.

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