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Biochemical Properties of MAL and MAL2 that Confer Specificity to Apical Delivery Pathways

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Biochemical Properties of MAL and MAL2 That Confer Specificity To Apical Delivery Pathways

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My focus is to identify regulators of apical delivery in polarized epithelial cells. Each plasma membrane (PM) domain of a polarized cell performs specific functions and has a unique distribution of proteins and lipids. In simple epithelial cells, newly synthesized apical proteins take a direct route to the apical plasma membrane from the trans-Golgi network. In contrast, apical proteins in hepatocytes take an indirect transcytotic route via the basolateral membrane. Myelin and lymphocyte protein (MAL) and MAL2 have been proposed to function in direct and indirect apical targeting, respectively. Hepatocytes lack endogenous MAL consistent with the absence of direct apical targeting. Does MAL expression reroute hepatic apical residents into the direct pathway? We found that MAL expression in WIF-B cells induced the formation of cholesterol and glycosphingolipid-enriched Golgi domains that contained glycosylphosphotidyl- inositol (GPI)-anchored and single transmembrane domain (TMD) apical proteins; polymeric IgA receptor (pIgA-R), polytopic apical, and basolateral resident distributions were excluded. Basolateral delivery of newly synthesized apical residents was decreased in MAL-expressing cells concomitant with increased apical delivery; pIgA-R and basolateral resident delivery was unchanged. These data suggest that MAL rerouted selected hepatic apical proteins into the direct pathway. Recently, it was proposed that lipid-raft microdomains are too small and transient to host apically destined cargo, and that lipid-associated proteins might serve to stabilize raft-sorting platforms. Do MAL and MAL2 promote raft-stablilization and clustering? Examination of lipid-association properties revealed that MAL is raft-

associated, while MAL2 is not. Does MAL and MAL2 overexpression promote lipid-association of apical proteins? MAL overexpression promoted lipid-association of both single TMD and GPI-anchored proteins whereas, MAL2 did not. Do MAL and MAL2 oligomerize to promote raft-coalescence? MAL is an oligomer and its overexpression altered the oligomeric states of single TMD and GPI-anchored apical proteins. MAL2 is monomeric and upon pIgA-R overexpression, shifted to high molecular weight fractions in velocity gradients indicating complex formation. Together, these results suggest that MAL oligomerization and lipid association may promote raft clustering and stabilization at the TGN, whereas the mechanism by which MAL2 regulates transcytosis and basolateral delivery of pIgA-R remains elusive.

This dissertation by Saiprasad Ramnarayanan fulfills the dissertation requirement for the doctoral degree in Cell and Microbial Biology approved by Dr. Pamela L. Tuma, PhD, as Director, and by Dr. J. Michael Mullins, Ph.D and Dr. James. J. Greene, Ph.D as Readers.

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Dr. Pamela L. Tuma, Ph.D., Director

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Dr. James J. Greene, Ph.D., Reader

DEDICATION

I dedicate this dissertation to my late grandfather, Mr. Siva Subrahmanyan, and my grandmother, Mrs. Sumana Devi, for giving me the freedom of thought to evolve as a sensitive secular human being.

….to my aunts, Vijayalakshmi, Sheila, Jyothi, Rajalakshmi, Swarna and Lakshmi, and my uncles, Nagaraj, Venkat and Satheesh, for all their love and affection and teaching me the importance of family.

….my parents, Mr. Ramnarayanan, and Mrs. Mangala, for giving my grandparents, aunts and uncles the freedom to raise me.

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I am also grateful to Dr. Maria Bastaki from Evergreen State College, Olympia, Washington for providing me with the MAL, DPP IV, HA and pIgA-R viruses

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LIST OF ABBREVIATIONS

5'NT, 5'nucleotidase

APN, aminopeptidase N

BC, bile canaliculus

BEE, basolateral early endosomes

BFA, Brefeldin A

CHAPS- 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CHX, cycloheximide

DPP IV, dipeptidyl peptidase IV

EDC, 1-ethyl-3- [3-dimethylaminopropyl] carbodiimide hydrochloride

EVH1, Ena-VASP homology 1; VAMP, vesicle associated membrane protein; Ena-VASP, Drosophila enabled/ vasodilator-stimulated phosphoprotein

GPI, glycophosphatidylinositol

HA, hemagglutinin

mβCD, methyl-β-cyclodextrin

MAL, myelin and lymphocyte

MRP2, multidrug resistance associated protein 2

pIgA-R, polymeric IgA receptor

PM, plasma membrane

SAC, subapical compartment

SNAP, soluble N-ethyl maleimide-sensitive factor attachment protein

SNARE, soluble N-ethyl maleimide-sensitive factor attachment protein receptor

Sulfo-NHS, N-hydroxysulfosuccinimide sodium salt

TMD; transmembrane domain

TPD52, tumor protein D52

INTRODUCTION

Liver function

The liver is the second largest organ in the human body, after the skin, and the largest internal organ. The liver performs a number of vital functions. It is the site for metabolism, detoxification, and inactivation of steroids, other hormones, drugs and toxins. The liver is also the major site for filtration of foreign particulate matter that includes bacteria, endotoxins, parasites, and aging red blood cells. Various hormones and vitamins are converted into their active forms in the liver, and, various enzymes convert lipophilic substances into more polar, water-soluble metabolites that are secreted in the bile. In addition, the liver is the major site of bile synthesis, which is essential for the digestion, and absorption of lipids from the intestine as well as the removal of bilirubin and cholesterol (Boron and Boulpaep, 2005). It is the primary site of synthesis of amino acids, glucose, glycogen, cholesterol, coagulation factors, growth factors and bile. The liver also secretes large amounts of albumin, which is required to maintain the osmolarity of blood. Thus, the numerous functions performed by the liver are major factors in the proper functioning of the human body.

Hepatocytes, cholangiocytes, sinusoidal endothelial cells, macrophages, lymphocytes, dendritic cells and stellate cells make up the cellular architecture of the liver. Together these cells are involved in carrying out the liver's many different functions. The human liver is composed of four lobes that have a rich supply of afferent and efferent blood vessels. The functional unit of the liver is the hepatic lobule. Each hepatic lobule consists of a hexagonal arrangement of plates of hepatocytes radiating outward from a central vein (Arias, 1988). The vertex of each lobule is a triad of

channels - artery, vein and bile duct. The hepatocytes are the epithelial cells of the liver, and form the bile canaliculi. The hepatic epithelium is one cell thick and forms a functional barrier between the canalicular lumen, containing bile, and the sinusoid, containing blood. While the majority of cells in the liver are composed of hepatocytes, about 6% of the liver is composed of other types of cells (Boron and Boulpaep, 2005). Cholangiocytes are epithelial cells that form the bile duct and help in bile secretion by secreting water and bicarbonates. Sinusoidal endothelial cells line the blood sinusoids forming a fenestrated structure. The Kupffer cells are present within the sinusoidal space and are macrophages that participate in the removal of particulate matter from the blood circulation. Hepatic stellate cells are found in a small region called the space of Disse between the sinusoid and the hepatocytes (Boron and Boulpaep, 2005). These cells store vitamin A and they can transform into proliferative, fibrogenic and contractile myofibroblasts. These cells are usually quiescent and are activated upon liver injury, and participate in fibrogenesis by secreting type I collagen, which can lead to cirrhosis. Thus all the cells of the liver have to perform in conjunction with each other to maintain the orderly functioning of the liver.

Hepatocyte structure and function

Hepatocytes are polarized epithelial cells. The formation of tight junctions between neighboring hepatocytes leads to the partitioning of the hepatocyte plasma membrane into the apical and the basolateral plasma membrane domains. Apical plasma membranes of neighboring hepatocytes come together to form the bile canaliculus, whereas the basolateral plasma membranes face the blood sinusoids. The establishment

and maintenance of polarity in hepatocytes is vital for the proper organization and functioning of the liver. Each plasma membrane domain of the hepatocyte performs specific functions. Functions at the apical surface include transport of bile, detoxification products, lipids, and the delivery of secretory IgA for mucosal immunity. Functions at the basolateral surface include transport of amino acids, glucose and bile acids, secretion of plasma and lipoproteins, and the absorption of macromolecules from the blood. Therefore, to understand how an epithelial cell establishes and maintains polarity and domain specific activities, it is important to understand polarized membrane transport. How are the apical and basolateral proteins transported to the correct destinations and how are their distributions maintained? The answer to this question, in part, lies in understanding polarized membrane transport. My focus is to understand the mechanisms regulating the trafficking of newly synthesized apical proteins in polarized epithelial cells.

The apical sorting pathways

There are two different pathways by which apical proteins are delivered to the apical plasma membrane in polarized epithelial cells: direct and indirect. In simple epithelial cells, like the Madine Darby Canine Kidney (MDCK) cells, newly synthesized apical proteins are ***directly*** transported from the Golgi compartment to the apical plasma membrane (Ikonen and Simons, 1998; Rodriguez-Boulan *et al.*, 2005) (Fig. 1). In hepatocytes, newly synthesized single transmembrane domain proteins and the glycosylphosphatidylinositol (GPI)-anchored proteins take a circuitous, ***indirect*** route to the apical plasma membrane (Bartles *et al.*, 1987; Bartles and Hubbard, 1988; Schell *et*

al., 1992a). The apical proteins are first transported to the basolateral plasma membrane where they are selectively internalized to basolateral, early endosomes and transcytosed to the sub apical compartment (SAC). The exceptions in this case are the polytopic

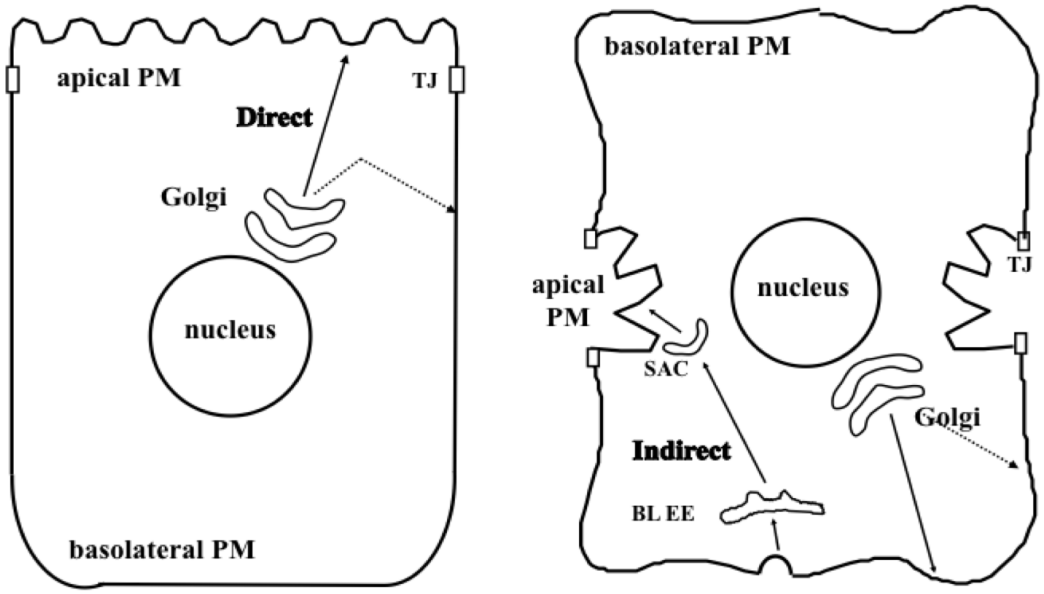

Fig. 1. Apical delivery routes. The panel on the left shows the direct pathway used by MDCK cells to deliver newly-synthesized apical proteins. The panel on the right shows the indirect apical delivery pathway used by hepatocytes. BL, basolateral; EE, early endosome; SAC, sub-apical compartment; TJ, tight junction

membrane proteins, such as multiple drug resistance protein 1 and multiple drug resistant protein 2 (MDR1 and MDR2), which are directly transported to the apical surface of hepatocytes from the Golgi and SPGP (sister of P-glycoprotein bile acids) which passes through an intracellular compartment before reaching the apical surface (Kipp and Arias, 2000).

Protein sorting signals

Twenty years ago it was thought that all exocytic trafficking from the Golgi apparatus was through bulk flow and that proteins transported from the Golgi did not

require specific signals (Pfeffer and Rothman, 1987). However, since the late 1980's it has become clearer that post-Golgi sorting of proteins requires specific signals whether the proteins are transported to the apical or the basolateral plasma membranes (Mostov *et al.*, 1986). In general, the cytoplasmic tails of basolateral proteins contain short, peptide signal motifs that bind to specific adaptor proteins to regulate basolateral sorting (Mostov *et al.*, 1986). The first demonstration that signals are present in the cytoplasmic tail of these proteins was by Mostov and coworkers in 1986 (Mostov *et al.*, 1986). They showed that deletion of the cytoplasmic tail of polymeric immunoglobulin A receptor (pIgA-R) to its direct apical sorting in MDCK cells. The pIgA-R basolateral sorting signal was later identified to be a 14-amino acid stretch within the 103 amino acid long cytoplasmic tail (Casanova *et al.*, 1991). The 14-amino acid stretch when fused to the cytoplasmic tail of an apical protein (PLAP) rendered that protein basolateral (Casanova *et al.*, 1991). This demonstrated that basolateral sorting signals could confer specific sorting when fused to the cytoplasmic tail of a different protein. Since then it has been shown that basolateral targeting signals are di-leucine (LL) (Hunziker and Fumey, 1994) or tyrosine (NPXY) (Nabi *et al.*, 1991) based signals, which in turn enable clathrin-mediated sorting of these proteins from the Golgi to the basolateral surface. Recently, it has been shown that a single leucine can also serve as a basolateral sorting signal (Wehrle-Haller and Imhof, 2001). Certain adapter proteins such as AP1B (Fölsch *et al.*, 1999), AP3 and AP4 (Simmen *et al.*, 2002) confer specificity to basolateral sorting.

In contrast, single transmembrane domain apical proteins do not have any known signals for apical sorting on their very short cytoplasmic tails (6-8 amino acids) and GPI-anchored proteins lack cytoplasmic tails. Only the polytopic membrane proteins such as

multiple drug resistance protein 2 (MRP2) have been shown to have an apical sorting signal in their C-terminal cytoplasmic tail. The last three amino acids, TKF (PDZ binding motif), in the C-terminal tail of MRP2 are required for apical transport (Harris *et al.*, 2001; Nies *et al.*, 2002). There are various proposed signals for apical targeting of single transmembrane domain and GPI-anchored apical proteins: 1. N-glycans that face the luminal surface on apical proteins can bind to specific adaptor proteins that enable their trafficking to the apical plasma membrane (Scheiffele *et al.*, 1995); 2. O-glycans that are usually present adjacent to the membranes of transmembrane proteins enabling proper positioning of the proteins (Yeaman *et al.*, 1997; Alfalah *et al.*, 1999); and 3. The transmembrane domains of apical resident proteins interact with "lipid-rafts" domains in the plasma membrane. My research is focused on the raft-hypothesis of apical transport.

Raft hypothesis for apical transport

The apical membrane domains of polarized epithelial cells are enriched with cholesterol and glycosphingolipids that promote the assembly of tightly packed regions in the plasma membrane called "lipid rafts" (Lisanti *et al.*, 1988; Harder and Simons, 1997; Brown and London, 1998). Certain apical proteins, like the GPI-anchored proteins, are recruited specifically to these lipid-raft regions in the plasma membrane because of their intrinsic, biophysical properties (Lisanti *et al.*, 1988). According to the raft-hypothesis, apical proteins encounter lipid rafts in the biosynthetic pathway, where they are packaged into specific vesicles and transported to the apical plasma membrane. Recently, it was proposed that these domains are too small and transient to host apically destined cargo, and that lipid-associated proteins might serve to stabilize raft-sorting platforms (Hancock,

2006). Therefore, the "new-raft hypothesis" invokes the requirement for a protein regulator that would enable coalescence and/or clustering of lipids into stable sorting platforms.

Both the direct transport of apical proteins from the Golgi to the apical plasma membrane in MDCK cells (Prydz and Simons, 2001), and indirect trafficking apical proteins from the basolateral early endosomes to the SAC in hepatocytes (Prydz and Simons, 2001; Nyasae *et al.*, 2003) of ***all*** types of apical proteins (raft or non-raft associated) are impaired on the depletion of cholesterol and glycosphingolipids; suggesting a general regulator of sorting whose function is lipid-dependent. Recently, using isopycnic centrifugation, Paladino and coworkers showed that apically destined GPI-anchored proteins were oligomeric, whereas basolaterally targeted GPI-anchored proteins were monomeric in MDCK cells (Paladino *et al.*, 2004). They also showed that the oligomerization of GPI-anchored proteins was important for its stabilization into lipid domains, leading to its incorporation into apically destined, lipid-raft vesicles and direct sorting to the apical plasma membrane (Paladino *et al.*, 2004). In addition, they proposed a putative interacting protein that would help in oligomerization of GPI-anchored proteins which might also lead to coalescence of stabilized rafts into bigger sorting platforms (Paladino *et al.*, 2004). I propose that the lipid-associated Myelin and lymphocyte (MAL) family of proteins are good candidates for promoting coalescence and stabilization of lipid-domains into stable, sorting platforms (Frank, 2000; Rodriguez-Boulan and Müsch, 2005; Weisz and Rodriguez-Boulan, 2009).

The Myelin and Lymphocyte proteins

The Myelin and Lymphocyte (MAL) proteins were so named because of their association with the myelin sheath and in T-cell differentiation (Alonso and Weissman, 1987a). These proteins are a family of highly hydrophobic, lipid-associated, ~20 kDa tetraspanning membrane proteins. They have four transmembrane helices and N and C terminal cytoplasmic tails (Fig. 2). All the MAL family proteins (MAL, MAL2, BENE and plasmolipin) share low sequence identity (29-37%) but have a signature-conserved motif (Q/YGWVMF/YV) between their first extracellular loop and the second transmembrane domains (Magyar *et al.*, 1997) (Fig. 2). Furthermore, the fact that the same lipophilic solvents that were used to extract lipids could extract these proteins led to them being called ***proteolipids*** (Rancaño *et al.*, 1994).

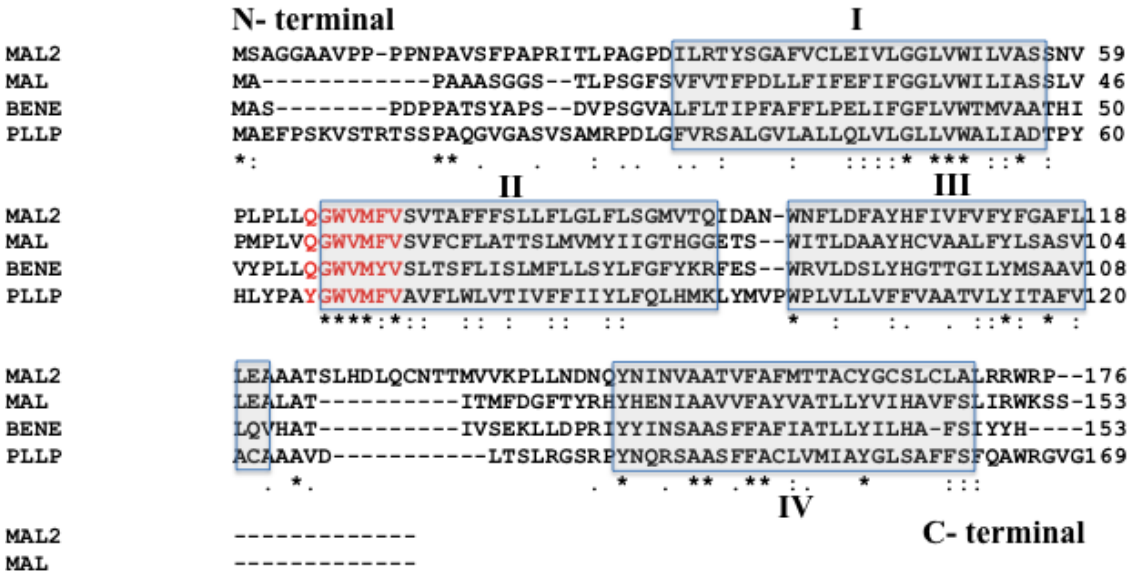

Fig. 2. The MAL family members. The panel above shows the sequence alignment of the human MAL family members. The transmembrane domains I through IV (MARVEL domains) are boxed and the MAL signature sequence conserved in all family members (Q/YGWVMF/YV) shown in red. They share very little identity (29-37%). * refers to conserved residues, : refers to conserved substitutions, .refers to semi conserved substitutions.

The four transmembrane helices of the MAL family share significant sequence similarity with the transmembrane helices of physin, gyrin and occludin family members (Sanchez-Pulido *et al.*, 2002). These transmembrane regions that share high similarity were named MAL and related proteins for vesicle trafficking and membrane link (MARVEL) domains (Sanchez-Pulido *et al.*, 2002). The physins and the gyrins have been found to be associated with small neurotransmitter containing vesicles in the neurons (Janz *et al.*, 1999) and in perinuclear vesicles of endothelial and epithelial cells and thereby implicating them in vesicle transport (Haass *et al.*, 1996). The occludin family members are integral membrane proteins that are found at tight junctions (Furuse *et al.*, 1993) where they provide a "fence" function (Saitou *et al.*, 2000; Tsukita *et al.*, 2001). It is, therefore, proposed that these MARVEL domains are important for lipid-association, stabilization and promoting membrane apposition events that could lead to apical vesicle formation (Sanchez-Pulido *et al.*, 2002).

MAL

MAL is a tetraspanning, raft-associated protein with a molecular weight of ~17 kDa. MAL was first identified as a hydrophobic protein required for T-cell differentiation in 1987 (Alonso and Weissman, 1987a). A year later, it was determined that MAL cDNA encoded a protein that shared a hydrophobicity pattern similar to proteolipid protein (PLP), the peripheral myelin protein-22, as well as myelin basic protein which are both required for wrapping of myelin sheaths around axons (Schaeren-Wiemers *et al.*, 1995).

MAL is expressed in a number of different tissues and cell types, including in tissues of the thyroid, thymus, kidney, brain, leukocytes, spleen and stomach (Martín-Belmonte *et al.*, 1998; Frank *et al.*, 2000). It is also endogenously expressed in cell types such as the Jurkat cells, MDCK, FRT and CaCo2 cells. At steady state, MAL is localized to the apical plasma membrane of polarized MDCK cells and FRT cells (Martín-Belmonte *et al.*, 2000; Puertollano *et al.*, 2001b). In non-polarized MDCK, Cos7, A498 and Jurkat cells MAL localizes to the plasma membrane and the Golgi compartment (Rancaño *et al.*, 1994; Puertollano and Alonso, 1998, 1999). Histopathology studies show that MAL is localized to the apical plasma membrane of distal tubules in the kidney, glandular stomach epithelium, enterocytes and the thyrocytes (Marazuela *et al.*, 2003).

MAL was first shown to be a component of the detergent insoluble membrane domains, or lipid-rafts, in T-lymphocytes (Millán *et al.*, 1997). Since then it has been consistently shown to be lipid-associated by means of low-density sucrose gradient flotations in various cell types including the MDCK cells, FRT cells and A498 cells (Martín-Belmonte *et al.*, 2000; Puertollano *et al.*, 2001b). MAL's C terminal LIRW juxtamembrane region is necessary for its association with lipids and the arginine residue within this sequence is of the most importance for this association (Puertollano and Alonso, 1998).

Evidence for MAL's regulation of direct apical transport

The earliest evidence of MAL's involvement in apical transport came when it was shown to be associated with apical transport vesicles in MDCK cells and was proposed to

be part of the apical transport machinery (Zacchetti *et al.*, 1995). Furthermore, MAL overexpression enhanced apical delivery and caused an expansion of apical surface domains in MDCK cells (Cheong *et al.*, 1999a). The experiments that confirmed MAL's involvement in apical transport were performed using antisense RNA in MDCK cells. First, knocking down MAL using antisense RNA in MDCK cells caused the Golgi accumulation of the apical, single transmembrane proteins influenza hemagglutinin (HA), gp114 and the missorting of a GPI-anchored apical protein, YFP-GL-GPI, to the basolateral plasma membrane (Cheong *et al.*, 1999a). Furthermore, it also led to the Golgi accumulation of an apically secreted protein, gp80 (Cheong *et al.*, 1999a). Experiments with antisense RNA against MAL in Fisher rat thyroid (FRT) cells led to the missorting of the single transmembrane proteins HA, P75NTR, and the GPI-anchored proteins, PLAP and GDI-DAF, to the basolateral plasma membrane. Another single transmembrane protein, DPP IV, accumulated in the Golgi on MAL knockdown (Martín-Belmonte *et al.*, 2000). All these experiments suggest that MAL knockdown perturbs apical trafficking of both raft-associated (HA, PLAP, GDI-DAF) and non-raft-associated (P75 NTR, DPP IV, gp80) proteins.

MAL is also itinerant between the TGN and the apical plasma membrane suggesting that it regulates trafficking between the Golgi and the apical plasma membrane (Puertollano and Alonso, 1999) (Fig. 4). In addition to all the reports of MAL's involvement in direct transport between the Golgi and the apical plasma membrane, MAL is also implicated in the regulation of apical endocytosis of pIgA-R in MDCK cells (Martín-Belmonte *et al.*, 2003). This suggests a dual role for MAL in both

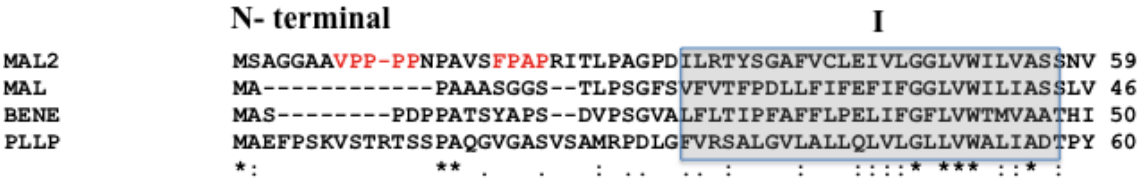

Fig. 3. Putative EVH1 binding sites in the MAL2 N-terminal tail. The panel above shows the sequence alignment of the N-terminal tail of human MAL family members. The N-terminal tail of MAL2 is longer and has the VPPP and FPAP sites similar to the EVH1 binding site F/LPPPP. * refers to conserved residues, : refers to conserved substitutions, .refers to semi conserved substitutions.

direct apical exocytosis from the Golgi and endocytosis from the apical plasma membrane.

In addition, MAL has also shown to form small homo-oligomers in SF-21 insect cells (Puertollano *et al.*, 1997) an interesting biochemical property that might enable MAL to coalesce and stabilize lipid-rafts into stable sorting platforms. Very few binding partners to MAL have been identified so far. MAL has been shown to interact with HA in MDCK cells (Tall *et al.*, 2003). Recently, mucin1 was shown to bind to MAL in breast cancer, yeast two-hybrid library screens (Fanayan *et al.*, 2009). The implications of these interactions are not yet fully understood.

MAL2

Another member of the MAL family of proteins, MAL2, is implicated in the regulation of the indirect apical trafficking pathway in hepatocytes. MAL2 was first discovered in a yeast two-hybrid screen of a human, breast carcinoma library as a binding partner to the tumor protein D52 (Wilson *et al.*, 2001a). Unlike MAL, MAL2 ‘s lipid-association properties are not consistent among different cell types. For example, MAL2 cannot be isolated in TritonX-100 insoluble low-buoyancy membranes in

oligodendrocytes (Bello-Morales *et al.*, 2009) whereas it is in low-buoyant density raft-fractions in HepG2 cells (de Marco *et al.*, 2002).

MAL2 is expressed in a number of tissues and cell types including the cells of the liver, kidney, brain, lungs, heart, placenta and the small intestine (Marazuela *et al.*, 2004). It is also expressed in MDCK, WIF-B, HepG2 and CaCo2 cells (de Marco *et al.*, 2002; In and Tuma, 2010). At steady state MAL2 is found in the apical plasma membrane of HepG2 and WIF-B cells (de Marco *et al.*, 2002; In and Tuma, 2010).

The C terminal region of MAL2 is very similar to that of MAL C terminal tail, which suggests that it might be important for lipid-association. The N-terminal cytoplasmic tail of MAL2 is 34 amino acids, longer than the 17 amino acid long MAL N-terminus (Fig. 3). The MAL2 N-terminal cytoplasmic tail is proline rich, which confers rigidity to the structure. It also contains FPPPP and FPAP sequences which are putative Ena-VASP homology 1 (EVH1) domain binding domains. The EVH1 domains are a 110 amino acid homologous region present on the Ena-VASP (Drosophila enabled/ vasodilator-stimulated phosphoprotein) family of proteins, which are key players in regulating actin filament assembly (Renfranz and Beckerle, 2002). The EVH1 domains have a high binding specificity to F/LPPPPP sites on proteins that are important for regulating a number of cellular processes ranging from cell signaling, and cytoskeletal remodeling to cellular trafficking (Renfranz and Beckerle, 2002). Thus, the N-terminal tail of MAL2 is divergent from other MAL family members (Fig. 3) and the presence of

the EVH1 binding sites suggest that MAL2 function might be regulated by actin dynamics.

Evidence for MAL2's regulation of the indirect pathway

The first reports on MAL2's involvement in trafficking showed that its knockdown impaired the indirect apical delivery of the professional transcytosing protein, pIgA-R, and the GPI-anchored CD59 in HepG2 human hepatoma cultures (de Marco *et al.*, 2002). MAL2 knockdown with antisense did not impair internalization at the basolateral plasma membrane of these proteins, but led to their accumulation at the basolateral early endosome. Therefore, MAL2 is proposed to be the regulator of indirect trafficking from the basolateral early endosome to the sub-apical compartment in hepatocytes (Fig. 4). Furthermore, live cell imaging studies showed that MAL2 regulated CD59 transcytosis to the apical plasma membrane (de Marco *et al.*, 2006). Recently using co-immunoprecipitations, antisense RNA and a morphological pulse chase assay, In and Tuma showed that MAL2, in addition to regulating the transcytosis of single transmembrane domain proteins and GPI-anchored proteins from the basolateral early endosome to the apical plasma membrane, also regulated the basolateral delivery of newly-synthesized pIgA-R from the Golgi (In and Tuma, 2010). Therefore, MAL2, like MAL, has a dual function in regulating protein transport: indirect delivery to the apical and basolateral targeting of pIgA-R (Fig. 4).

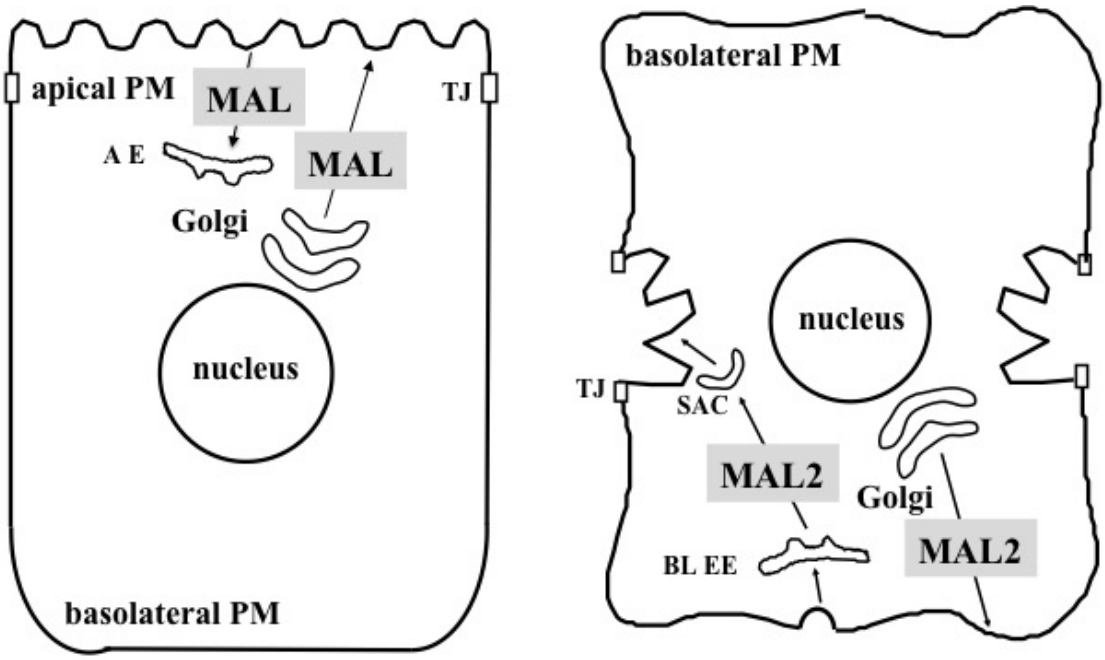

Fig. 4. Proposed sites of MAL and MAL2 function. The panel on the left shows that MAL in addition to regulating direct apical sorting also regulates the clathrin mediated apical internalization of pIgA-R in MDCK cells. The panel on the right shows that MAL2 in addition to regulating basolateral sorting of newly-synthesized pIgA-R, mediates delivery from the BL EE to the SAC in hepatocytes. Hepatocytes lack endogenous MAL. BL, basolateral; EE, early endosome; SAC, sub-apical compartment; TJ, tight junction; AE, Apical endosome

MAL2 binding partners

So far, MAL2 has been shown to interact with the TPD52-like proteins (Wilson *et al.*, 2001a) and Mucin1 (Fanayan *et al.*, 2009) in breast cancer cells. We showed that MAL2 also directly interacted with the pIgA-R in WIF-B cells (In and Tuma, 2010). MAL2's interactions with its binding partners rely on either its N-terminal cytoplasmic tail or its transmembrane domains. The N terminal cytoplasmic tail of MAL2 was shown to be required for its interaction with the TPD52-like family of proteins in human breast carcinoma yeast two-hybrid screens (Fanayan *et al.*, 2009). The transmembrane domains of MAL2 were shown to be important for its binding with mucin1 in yeast two-hybrid screens of human breast cancer libraries (Fanayan *et al.*, 2009). Interestingly, TPD52-

like proteins have been implicated in the calcium-dependent, secretory activity in pancreatic acinar cells (Thomas *et al.*, 2001). Mucin1 is expressed in the apical surface of many epithelial cells and is overexpressed in many cancers (Taylor-Papadimitriou *et al.*, 2002) . In addition, de Marco and coworkers have shown that the deletion of the N terminal cytoplasmic tail of MAL2 impaired the transcytosis of CD59 (de Marco *et al.*, 2002). The putative EVH1 binding domains at the N-terminal tail combined with all these other interactions suggest that MAL2 might interact with a number of different protein to regulate different steps of the transcytotic apical delivery as well as basolateral delivery pathways. Further experiments need to be performed to determine where MAL2 interacts with its different partners and what the functional consequences of these interactions are.

MALs and cancer

In addition to all the reports implicating MAL and MAL2 as regulators of polarized membrane trafficking, MAL and MAL2 have been shown to be important markers for cancer progression. MAL has been shown to be downregulated in human esophageal and cervical squamous cell cancers (Hatta *et al.*, 2004; (Mimori *et al.*, 2007). MAL expression in cancerous esophageal cells has been shown to suppress motility, invasion and tumorigenicity (Mimori *et al.*, 2003; Hatta *et al.*, 2004). MAL has also been used as a molecular marker for detection of primary, mediastinal large B-cell lymphomas (Copie-Bergman *et al.*, 1999; Copie-Bergman *et al.*, 2002). Most recently, MAL was shown to be overexpressed in ovarian epithelial cancers (Lee *et al.*, 2010). MAL's downregulation in colorectal and gastric cancers is accompanied by the hypermethylation

of its promoter region whereas its upregulation in ovarian cancer is accompanied by hypomethylation of its promoter (Buffart *et al.*, 2008; Lind *et al.*, 2008; Lee *et al.*, 2010). MAL2 is the binding partner to tumor protein D52-like family members and mucin1, which are frequently overexpressed in breast carcinomas. MAL2 and D52 overexpression in human breast carcinomas also correlate with an aggressive tumor phenotype indicating that any imbalance in the regulation of these molecules can cause tumor progression (Shehata *et al.*, 2008).

Therefore, it is clear that both MAL and MAL2 are important molecular markers for different types of tumors. However, how both of these molecules are involved in tumor progression is not clear. The answer to this question might lie in their regulation of protein sorting to the apical plasma membrane. Cancer cells are characterized by their loss or failure to achieve polarity. Therefore, any upregulation or downregulation in the regulators of polarized trafficking might be the cells attempt to suppress the cancer. All these data suggest that even a slight change in the expression levels of MAL and MAL2 and their binding partners, which regulate polarity, might cause mayhem to the trafficking pathways in the cell leading to or accompanying cancer progression.

Purpose of this research

Most columnar epithelial cells have both the direct and indirect pathways and express both MAL and MAL2. Interestingly, hepatocytes lack endogenous MAL and, correspondingly, rely on the indirect pathway for sorting GPI-anchored and single TMD apical proteins (Fig 4).

Will exogenous MAL expression reroute hepatic apical proteins into the direct pathway? I expressed MAL in WIF-B cells, a polarized hepatocyte model system lacking endogenous MAL. Because MAL normally encounters newly-synthesized apical proteins in cholesterol and GSL enriched domains in the TGN, and MAL2 encounters apical proteins in similar domains later in the pathway at early endosomes, I predicted that MAL expression would reroute single TMD and GPI-anchored apical residents through the direct pathway at the TGN in hepatocytes. I used both morphological and biochemical methods to investigate if MAL expression reroutes apical proteins into the direct pathway in WIF-B cells. I also investigated trafficking of single TMD, GPI-anchored, polytopic apical residents and the transcytosing polymeric immunoglobulin-A receptor.

Does lipid-association and oligomerization of the MAL proteins promote lipid clustering into stable sorting platforms? MAL and MAL2 share significant sequence homology, but regulate different apical sorting pathways. Recently, it has been argued that rafts are too small and transient to sort apically-destined cargo. The 'new' raft hypotheses thus invoke raft-associated proteins that aid in stabilization of lipids into stable sorting platforms. Furthermore, raft-association and oligomerization of GPI-anchored proteins have been shown to be important for their direct apical sorting in MDCK cells. I investigated the lipid-association and oligomerization properties of the MAL proteolipids in WIF-B and Clone 9 cells. Although Clone 9 cells are non-polarized they are equipped with polarized delivery mechanisms despite lacking discrete membrane domains (Tuma *et al.*, 2002). They also lack endogenous MAL and MAL2 and thus are a good system for studying the intrinsic oligomerization and binding properties of MAL,

MAL2 and apical PM proteins. I used crosslinking, and velocity gradients to elucidate if MAL and MAL2 oligomerize and/or interact with other apical proteins.

METHODS

Reagents and Antibodies

Cycloheximide (CHX), brefeldin A (BFA), Triton X-100, F12 (Coon's modification) medium, and methyl-β-cyclodextrin (mβCD), 1-ethyl-3- [3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and, N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were purchased from Sigma-Aldrich (St. Louis, MO). CHX was made fresh in 5% ethanol, and 5 mM mβCD was made fresh in serum-free medium. BFA was stored at −20°C as a 10 mg/ml stock in dimethyl sulfoxide. Horseradish peroxidase (HRP)-conjugated secondary antibodies and Super Signal West Pico enhanced chemiluminescence (ECL) substrate were from Sigma-Aldrich and Perkin Elmer Life Sciences Inc. (Boston, MA), respectively. Alexa-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). Anti-myc epitope tag antibodies and anti-MAL polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-5′nucleotidase (5′NT) (monoclonal and affinity purified polyclonal), anti-hemagglutinin (HA), and anti-multidrug resistance-associated protein 2 (MRP2) antibodies were kindly provided by J. P. Luzio (Cambridge University, Cambridge, United Kingdom) M. Roth (University of Texas, Southwestern, Dallas, TX), and D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany), respectively. Antibodies against aminopeptidase N (APN), CE9, pIgA-R, dipeptidyl peptidase IV (DPP IV), HA321, and myc epitope tag (9E10) were all generously provided by A. Hubbard (Johns Hopkins University, School of Medicine, Baltimore, MD). Recombinant adenoviruses encoding V5/His6 epitope-tagged full-length DPP IV or pIgA-R and full-length HA were also all provided by A. Hubbard, and they have been described in detail previously (Bastaki et al., 2002 and Ramnarayanan et al, 2007). A

cDNA encoding full-length MAL was kindly provided by M. Alonso (Severo Ochoa Center for Molecular Biology, Universidad Autonoma de Madrid, Madrid, Spain).

Cell Culture

WIF-B cells were grown in a humidified 7% CO_2 incubator at 37°C as described previously (Shanks *et al.*, 1994). Briefly, cells were grown in F12 medium (Coon modification), pH 7.0, supplemented with 5% fetal bovine serum, 10 μM hypoxanthine, 40 nM aminopterin, and 1.6 μM thymidine. In general, cells were seeded onto glass coverslips at 1.3 x 10^4 cells/cm^2 and grown for 8–12 d until they reached maximum density and polarity. Clone 9 cells were grown in a humidified 5% CO_2 incubator at 37°C. Cells were grown in F12 medium (Coon modification), pH 7.0, supplemented with 10% fetal bovine serum. In general the cells were seeded onto glass coverslips at 2 x 10^5 cells/ well of a six well dish and grown for 2 days.

Virus Production and Infection

Recombinant MAL-myc, V5/ myc epitope tagged pIgA-R, V5/His6 epitope tagged DPP IV adenoviruses were generated using the Cre-Lox system as described previously (Bastaki *et al.*, 2002). WIF-B cells were infected singly or doubly with recombinant adenovirus particles (0.7–1.4 x 10^{10} virus particles/ml) for 60 min at 37°C as described previously (Bastaki *et al.*, 2002). The cells were washed with complete medium and incubated an additional 18–20 h to allow expression.

Immunofluorescence Microscopy

In general, cells were fixed on ice with chilled phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) for 1 min and permeabilized with ice-cold methanol for 10 min. To detect MRP2, cells were fixed and permeabilized at −20°C with methanol for 5 min. To detect MAL with anti-myc antibodies, cells were fixed for 30 min with 4% PFA at room temperature (RT) and permeabilized for 10 min at RT with 0.2% Triton X-100/PBS. Cells were processed for indirect immunofluorescence as described previously (Ihrke et al., 1993 blue right-pointing triangle). Alexa 488- or 568-conjugated secondary antibodies were used at 5 μg/ml. For some experiments, cells were treated for 1 h with 10 μg/ml BFA or up to 2 h with 50 μg/ml CHX at 37°C. To deplete cholesterol, cells were treated for 1 h with 1 or 5 mM mβCD in serum-free medium.

Labeled cells were visualized by epifluorescence on an Olympus BX60 fluorescence microscope (Opelco, Dulles, VA). Images were taken using an HQ2 digital camera (Photometrics, Tucson, AZ) and IPLabs image analysis software (Biovision, Exton, PA) or by using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) and SPOT Advanced software, version 3.5.8 (Diagnostic Instruments). Adobe Photoshop (Adobe Systems, Mountain View, CA) was used to compile figures.

Cells expressing HA, pIgA-R, or DPP IV alone or with MAL were treated for increasing times with 50 μg/ml CHX. Then, cells were fixed, permeabilized, and stained. Random fields were visualized by epifluorescence and digitized. From micrographs, the average pixel intensity of selected regions of interest (ROI) placed at the apical or basolateral PM of the same WIF-B cell were measured using the Measure ROI tool of the ImageJ imaging software (National Institutes of Health, Bethesda, MD). In general,

multiple ROI were collected in the same cell to verify that representative intensities were measured. The averaged background pixel intensity was subtracted from each value, and the ratio of apical-to-basolateral PM fluorescence intensity was determined. Approximately 100–300 cells were measured for each condition from at least three independent experiments. Values are expressed as the mean ± SEM.

Solubility in Triton X-100

WIF-B cells were rinsed in ice-cold PBS and extracted for 10 min on ice in 0.15 ml of ice-cold lysis buffer (1% [vol/vol] Triton X-100, 25 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.4) containing 1 μg/ml each of aprotinin, antipain, leupeptin, benzamidine, and phenylmethylsulfonyl fluoride (PMSF). The samples were centrifuged at 120,000 x g for 30 min at 4°C. The supernatant was recovered, SDS-PAGE buffer was added, and the sample was boiled for 3 min. The pellets were resuspended to volume with SDS-PAGE buffer and boiled for 3 min. When immunoblotting for MAL2, samples were not boiled, but incubated at RT for 30 min. The supernatants and pellets were immunoblotted with the indicated antibodies and percent insolubility was determined by densitometric analysis of the immunoreactive species.

Low Buoyant Density Flotations

Isolation of low buoyancy membrane fractions was performed as described previously (Brown and Rose, 1992). Control or MAL-infected WIF-B cells were rinsed in cold PBS and lysed on ice for 30 min with pre-chilled lysis buffer containing 1 μg/ml each of antipain, leupeptin, benzamidine, and PMSF. The lysates were diluted with an

equal volume of lysis buffer containing 80% sucrose and placed at the bottom of a 5–30% linear sucrose gradient. Samples were centrifuged in a swinging bucket rotor at 192,000 x *g* for 16 h at 4°C. 1.0 ml fractions were collected from the bottom of the gradient and immunoblotted with the indicated antibodies. HRP-conjugated secondary antibodies were used and immunoreactivity was detected with ECL. The relative distributions of the different proteins were determined by densitometric comparison of immunoreactive bands.

Antibody Labeling of Live Cells

Cells were cooled on ice for 5 min at 4°C. Selected PM proteins were surface labeled with specific antibodies for 20 min at 4°C. Because tight junctions restricted access of the antibodies to the apical PM, only antigens at the basolateral surface were labeled. For transcytosis assays, cells were washed two times for 2 min on ice and reincubated with prewarmed complete medium. Antibodies with bound antigens were allowed to chase for the indicated times at 37°C, and cells were fixed and stained.

For basolateral surface-labeling experiments, after antibody labeling on ice, cells were lysed by addition of SDS-polyacrylamide gel electrophoresis sample buffer. Lysates were immunoblotted with the indicated primary antibodies to detect the entire population of the selected PM protein. On a parallel immunoblot, lysates were probed directly with secondary antibodies to detect only the surface bound primary antibodies. The relative levels of immunoreactive species were determined by densitometry. The amount of surface-bound antibodies in control and MAL-infected cells was normalized to the amount of total antigen present. In all cases, control ratios were set to 100%.

Internalization Assays

Total IgG from serum (DPP IV) or ascites (5′NT) was purified (EZ-Sep; Pharmacia AB, Uppsala, Sweden) and biotinylated (EZ-Link sulfo-NHS-biotin; Pierce Chemical) according to the manufacturers' instructions. Internalization assays were performed as described previously (Tuma et al., 2002). Briefly, WIF-B cells were continuously labeled with biotinylated antibodies for the indicated times at 37°C. The remaining surface-associated antibodies were eluted with isoglycine (200 mM glycine and 150 mM NaCl, pH 2.5) for 5 min at RT, and the cells lysed in isoglycine containing 20 mM octylglucoside and 0.5% Triton X-100 for 30 min on ice. Aliquots of the eluate and lysate were incubated in streptavidin-coated 96-well plates (Pierce Chemical). Bound antibodies were detected with HRP-conjugated secondary antibodies followed by colorimetric detection with an HRP substrate detection kit (Pierce Chemical).

Velocity gradients

Velocity gradients were performed as described (Paladino et al, 2004). In brief, WIF-B cells (~ 5 x 10^6 cells) were lysed at 4°C for 30 min in 20 mM Tris, 100 mM NaCl, pH7.4 containing 0.4% SDS, 0.2% TritonX-100 and 1 μg/ml each of antipain, leupeptin, benzamidine, and PMSF. The lysate was loaded on top of a 5-30% linear sucrose gradient and the tubes were centrifuged at 192,000 x *g* for 18 h at 4°C. 1 ml fractions were collected from the bottom of the gradient and immunoblotted with the indicated antibodies. The relative distributions of various proteins were determined by densitometric comparison of immunoreactive species. Carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 KDa), a-amylase (250 kDa)

and apoferretin (443 kDa) were purchased from Sigma- Aldrich and used as molecular weight standards. To deplete cholesterol, cells were incubated in serum free medium containing 5 mM mβCD for 30 min at 37°C. To stop protein synthesis, cells were treated with 50 μg/ml cycloheximide for 60 or 120 min before lysis and centrifugation.

EDC crosslinking of non-nuclear membrane preparations

Membranes were crosslinked with EDC and sulfo NHS as described (Tuma and Collins, 1995). To prepare total membranes without nuclei, cells were detached with trypsin (137 mM NaCl, 0.058% $NaHCO_3$, 0.5% trypsin, pH 7.4) for 2 min at 37°C and then pelleted by centrifugation at 500 x g. The pellet was resuspended in 5 ml of ice-cold hypotonic buffer (1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) and incubated at 4°C for 5 min. The swollen cells were pelleted by centrifugation at 500 x g for 5 min. The cells were immediately dounce homogenized after resuspension in 0.5 ml 250 mM sucrose in PBS containing 1 μg/ml each of antipain, leupeptin, benzamidine, and PMSF. The samples were centrifuged at 1000 x g at 4°C for 5 min and total membranes were prepared by centrifugation of the post nuclear supernatant at 150,000 x g for 60 min. The membranes were resuspended in 0.5 ml PBS containing 1 μg/ml each of antipain, leupeptin, benzamidine, and PMSF with 20 strokes of the dounce homogenizer. The resuspended membranes were crosslinked with 10 mM EDC and 2.5 mM sulfo-NHS for 0, 15, 30 and 60 min. The reactions were stopped by addition of SDS-PAGE buffer. The samples were boiled for 3 min or kept at RT for 30 min (to detect MAL2). The samples were immunoblotted and the extent of crosslinking was determined by measuring the decrease in monomer levels by densitometric analysis of the immunoreactive bands. To

prevent post-Golgi trafficking, cells were preincubated for 2 h at 20°C before harvesting. For cholesterol depletion, cells were treated with 5mM mβCD in serum-free medium for 30 min before harvesting.

RESULTS: PART I

Exogenous MAL Reroutes Selected Hepatic Apical Proteins into the Direct Pathway

The MAL proteolipids are good candidates for mediating lipid-dependent apical sorting. These ~20 kDa, tetraspanning membrane proteins are raft-associated and have been implicated as important regulators of apical delivery in both the direct and indirect pathways. In MDCK cells lacking MAL, direct apical delivery was decreased; the ectopic expression of MAL rescued the defect (Cheong *et al.*, 1999b; Puertollano *et al.*, 1999; Martin-Belmonte *et al.*, 2000; Martin-Belmonte *et al.*, 2001; Puertollano *et al.*, 2001a). Because apical secretion of different classes of secretory proteins (thyroglobulin and gp80) and apical delivery of different classes of apical residents (single TMD and GPI-anchored) were both impaired in MAL-depleted cells, I consider MAL a general regulator of direct apical transport. Although MDCK cells express MAL2, its role in transcytosis remains unclear (Wilson *et al.*, 2001b; De Marco *et al.*, 2002) (see Discussion).

In contrast, hepatocytes express only MAL2 (Alonso and Weissman, 1987; Wilson *et al.*, 2001; De Marco *et al.*, 2002), consistent with the absence of direct apical delivery of single-TMD and GPI-anchored residents. In HepG2 cells, anti-sense MAL2 oligonucleotides impaired transcytosis of two classes of apical proteins: pIgA via its single TMD receptor and CD59, a GPI-anchored protein (De Marco *et al.*, 2002; In and Tuma, 2010). Interestingly, the block occurred between early endosomes and the SAC, reminiscent of the transcytosis defect observed in lipid-depleted WIF-B cells in previous

studies (Nyasae *et al.*, 2003). Thus, lipid depletion may prevent MAL2 from sorting transcytosing proteins at early endosomes and prevent MAL from sorting at the TGN.

Because hepatocytes lack MAL expression and the direct targeting pathway, we asked whether MAL expression reroutes newly-synthesized apical proteins into the direct pathway in hepatocytes. To answer this question, I expressed MAL in polarized, hepatic WIF-B cells and examined the distributions of different classes of apical proteins. I found that MAL expression induced the formation cholesterol and glycosphingolipid-enriched Golgi domains that contained GPI-anchored and single TMD apical residents; polytopic apical proteins, basolateral residents and pIgA-R were excluded. Basolateral surface-labeling revealed decreased basolateral delivery of GPI-anchored and single TMD apical residents in MAL-expressing cells whereas basolateral amounts of pIgA-R and basolateral residents were unchanged. By using a quantitative morphological assay, we determined that MAL was rerouting apical proteins into a direct pathway.

MAL induces intracellular populations of selected apical proteins

To determine whether MAL expression altered apical delivery in WIF-B cells, I examined the steady state distributions of different classes of apical proteins (single TMD, GPI-anchored and multispanning) and the transcytosing protein, pIgA-R. Importantly, adenovirus infection efficiencies were high; >95% of cells expressed MAL (data not shown). In control cells, the single TMD protein, HA, was present mainly at the apical PM, but sub-apical puncta and basolateral staining of transcytosing proteins were also apparent (Figure 5A). In MAL-expressing cells, numerous tubular structures and diffuse puncta were also observed that contained HA (Figure 5A, b). ~90% of cells expressing MAL contained these intracellular structures (Table 1).

Table 1. MAL-expressing cells contain intracellular populations of selected apical proteins.

Apical Protein	**% Cells**	**Treatment**	**% Cells**
HA	92.1 ± 2.5	--	87.5 ± 4.7
DPP IV	89.3 ± 3.6	BFA	87.0 ± 8.1
5'NT	69.2 ± 5.7	CHX	42.3 ± 7.1
pIgA-R	15.2 ± 0.2	CHX + mbCD	82.9 ± 4.5
MRP2	0		

For the experiments summarized in the left-hand side of the table, MAL-expressing cells were scored for the presence of intracellular (IC) tubules and puncta positive for the indicated apical protein. Values are expressed as the mean ± SEM. Measurements were performed on at least three independent experiments except for MRP2, where only one experiment was quantitated. On the right-hand side, WIF-B cells expressing MAL and HA were treated for 1 h with 10 mg/ml BFA or for 2 h with 50 mg/ml CHX alone or in the presence of 1 mM mbCD. Cells were fixed, stained and scored for HA^+ structures. Values are expressed as the mean ± SEM. Measurements were performed on at least three independent experiments.

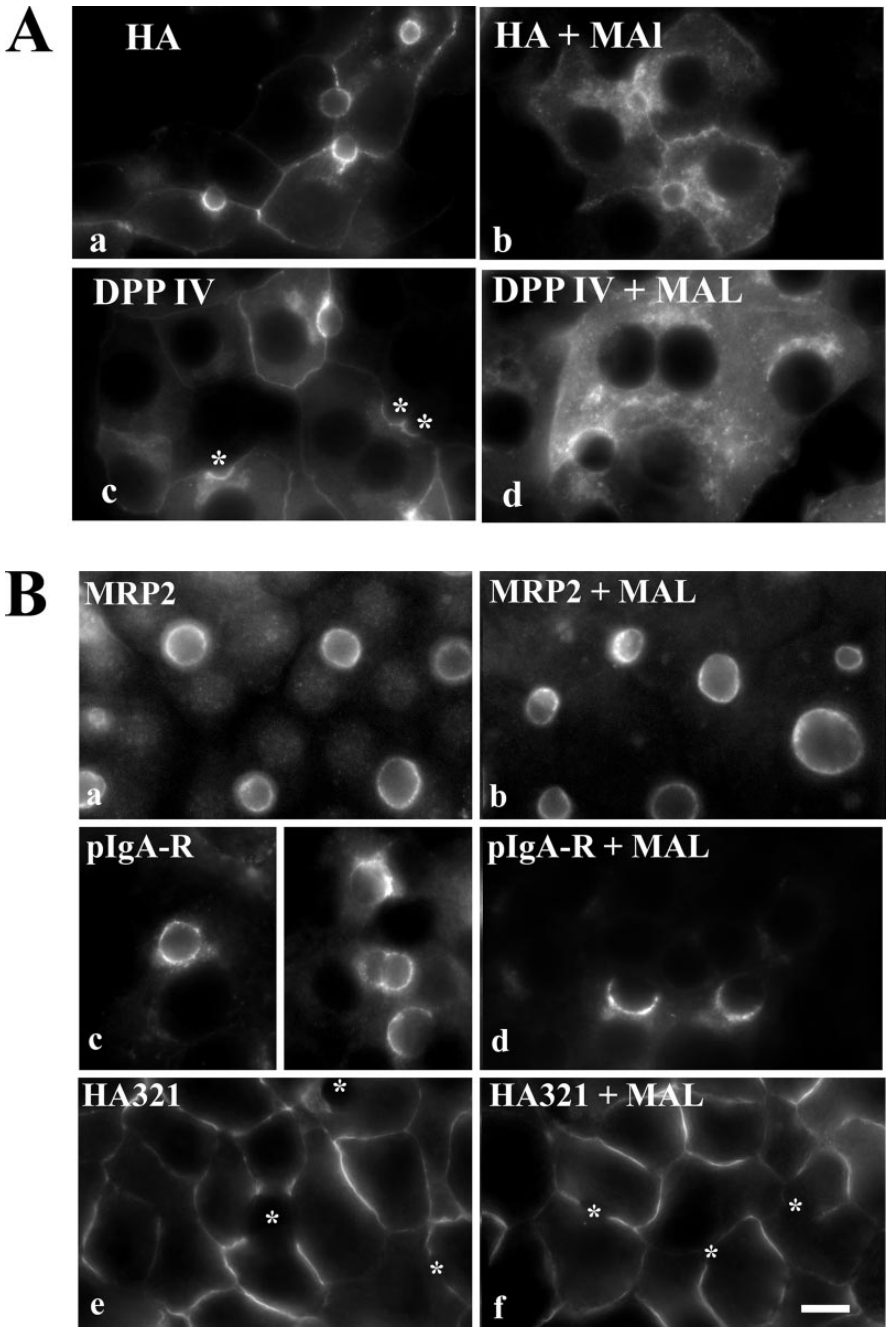

Figure 5. Single TMD and GPI-anchored apical proteins, but not polytopic apical residents, basolateral proteins or pIgA-R, redistribute in MAL-expressing cells. A, Cells were infected with recombinant adenoviruses expressing MAL (b and d), HA (a and b) or DPP IV (c and d). The intracellular populations of HA (b) and DPP IV (d) are apparent in MAL-infected cells (d). B, Cells were infected with recombinant adenoviruses expressing MAL (b, d and f) and/or pIgA-R (c and d). After 20 h, the steady state distributions of MRP2 (a and b), pIgA-R (c and d) or HA321 (e and f) were determined. Asterisks are marking selected BCs. Bar = 10 mm

Similar redistribution was observed for exogenous DPP IV (Figure 5A, d and Table 1), another single TMD apical protein, and 5'nucleotidase (5'NT), an endogenous GPI-anchored apical resident. Fewer MAL-infected cells contained 5'NT^{+} intracellular structures (~70%; Table 1), which likely reflects lower rates of endogenous protein synthesis. In contrast, the distributions of MRP2, a polytopic apical resident, were not changed in MAL-expressing cells. In both cases, MRP2 was exclusively localized to the apical PM; no intracellular staining was observed (Figure 5B and Table 1). Similarly, the distributions of exogenously expressed pIgA-R were not changed by MAL expression (Figure 5B, c and d). In both control and MAL-expressing cells, pIgA-R was present mainly at the apical PM with a small population in sub-apical puncta. Only 15% of MAL-expressing cells contained increased amounts of intracellular pIgA-R (Table 1). MAL expression also did not alter the distributions of the basolateral residents, HA321 (Figure 5B, f) or CE9 (data not shown), indicating that MAL selectively altered the distributions of single TMD and GPI-anchored apical proteins.

The structures are cholesterol and glycosphingolipid-enriched Golgi domains

According to the "raft hypothesis" for protein sorting, cholesterol and glycosphingolipid-enriched domains form in the biosynthetic pathway where they recruit apically-destined proteins, then the rafts and their recruited cargo are transported in vesicles directly to the apical domain (Simons, 1997). Because MAL has been shown to be raft-associated and to function at the TGN, I tested whether MAL expression induced the formation of biosynthetic, cholesterol and glycosphingolipid-enriched Golgi domains in WIF-B cells.

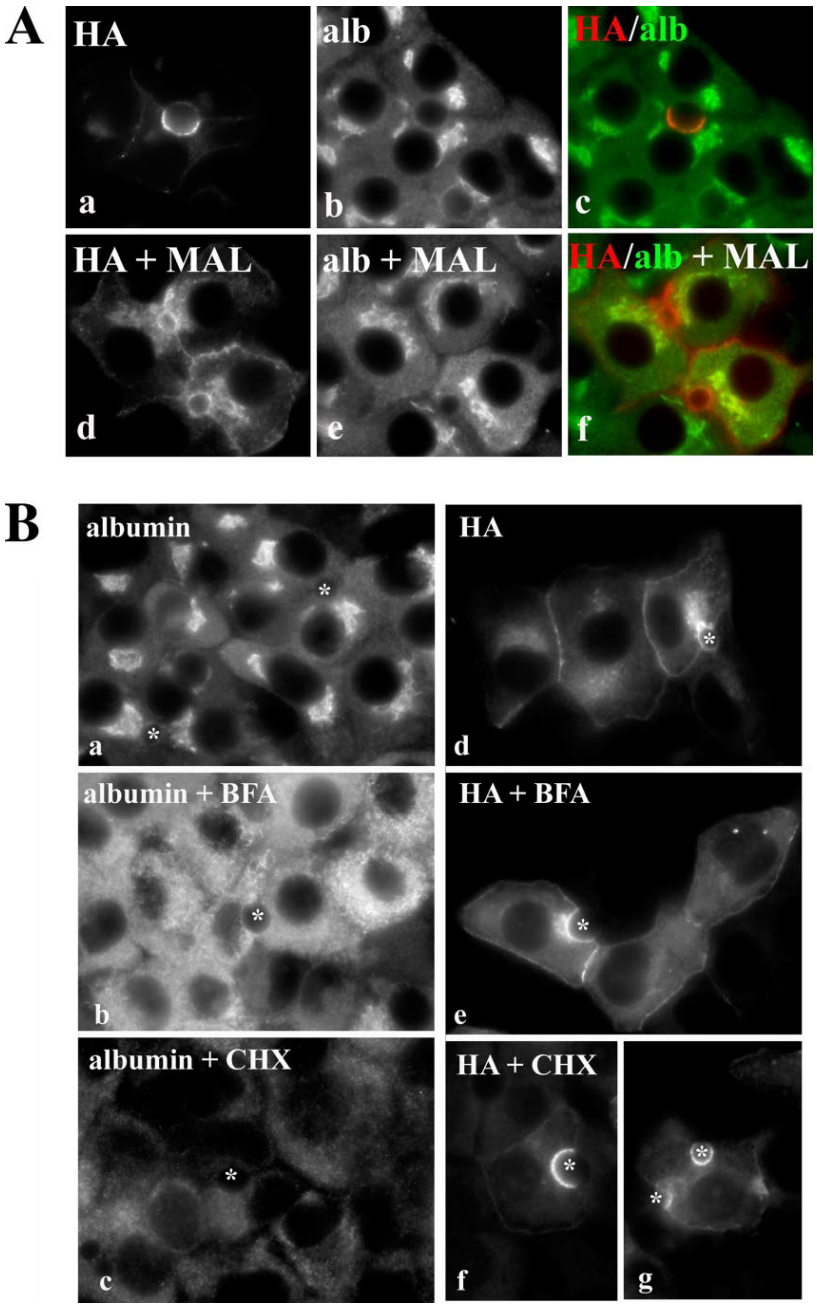

Figure 6. The MAL-induced intracellular structures are Golgi-derived, biosynthetic intermediates. **A,** Cells were infected with recombinant adenoviruses expressing MAL (d–f) and HA (a-f). After 20 h, the steady state distributions of HA and albumin were determined as indicated. Merged images are shown in c. The intracellular staining of HA in MAL-infected cells significantly overlapped with the Golgi marker, albumin (f). **B,** WIF-B cells were co-infected with MAL and HA adenoviruses for 20 h (d-g). Cells were incubated in the absence or presence of 10 mg/ml BFA for 1 h (b and e) or 50 mg/ml CHX for 2 h at 37^0C (c, f and g). Cells were stained for albumin (a-c) or HA (d-g). Asterisks are marking selected BCs. Bar = 10 mm

I first examined whether HA in MAL-expressing cells was present in the Golgi. In control cells, HA was primarily localized to the apical PM showing no overlap with the Golgi marker, albumin (Figure 6A, a-c). In contrast, the intracellular HA in MAL overexpressing cells largely colocalized with albumin (Figure 6A, c-f) indicating its presence in the Golgi.

I next examined the distributions of HA in MAL-expressing cells treated with either BFA or CHX. To first determine the effectiveness of the drugs, we monitored albumin distributions in control and treated cells. As predicted, albumin fully redistributed to the ER in the presence of BFA (Figure 6B, b). In CHX-treated cells, albumin staining was nearly absent (Figure 6B, c) indicating that protein synthesis was inhibited and the biosynthetic pipeline was emptied. The intracellular HA population in MAL-expressing cells was reduced by BFA treatment and ER staining was observed (Figure 6B, e), confirming the Golgi localization. Because cells were scored as "negative" or "positive", no change was observed in the percent cells positive for intracellular HA (Table 1). In contrast, there was much less (Figure 6B, g) to no (Figure 2B, f) intracellular HA in CHX-treated cells. Only 42.5% of treated cells contained HA^+ structures (Table 1) indicating that they were biosynthetic, i.e., the apical cargo was delivered during treatment.

If MAL expression induced biosynthetic raft formation, one prediction was that cholesterol depletion should impair accumulation of the apical proteins in the Golgi domains. To test this, I treated cells with mβCD for 60 min, conditions that deplete 80% of cholesterol in WIF-B cells (Nyasae *et al.*, 2003). However, HA remained in the Golgi in treated cells (Figure 7A, b).

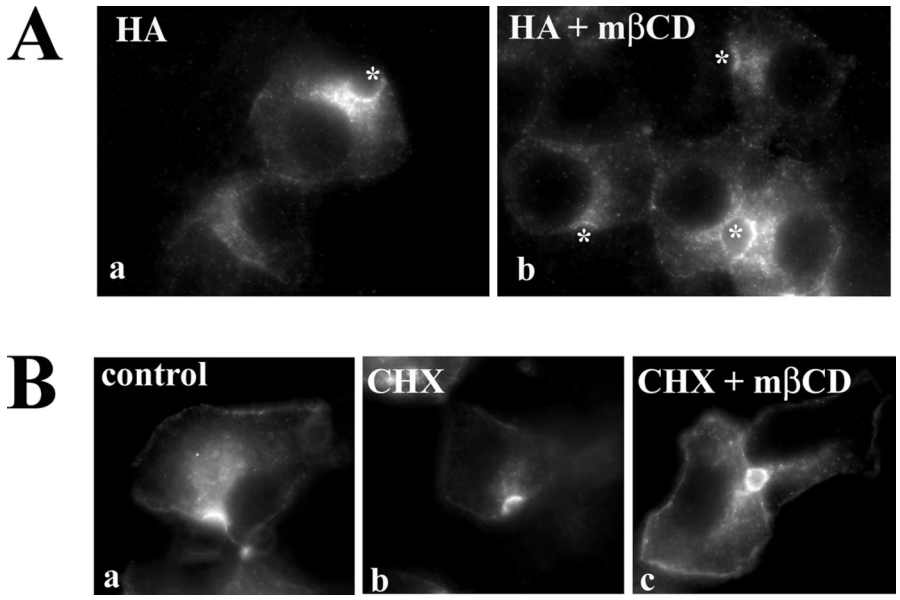

Figure 7. Cholesterol depletion impairs the apical delivery of apical residents from the MAL-induced compartment. **A,** Cells expressing both HA and MAL were treated in the absence (a) or presence (b) of 5 mM mβCD for 1 h. The intracellular pool of HA remained in cholesterol depleted cells. **B,** Cells expressing both MAL and HA (a-c) were treated with 50 mg/ml CHX alone (b) or in the presence CHX and 1 mM mβCD (c) for 2 h at 37^0C. The distributions of HA are shown. Asterisks are marking selected BCs.

Because direct targeting requires cholesterol (Scheiffele *et al.*, 1997; Keller, 1998; Prydz and Simons, 2001), another possibility was that apical staining should persist in cholesterol-depleted cells reflecting impaired apical delivery. To test this prediction, we incubated CHX-treated cells with mβCD, conditions whereby "chase" from the compartment could be monitored. As shown above, CHX treatment decreased intracellular HA staining indicating that apical delivery occurred (Figure 7B, b). However, in cells also treated with mβCD, the intracellular HA staining remained (Figure 7B, c and Table 1) indicating that apical delivery was impaired, and thus cholesterol-dependent.

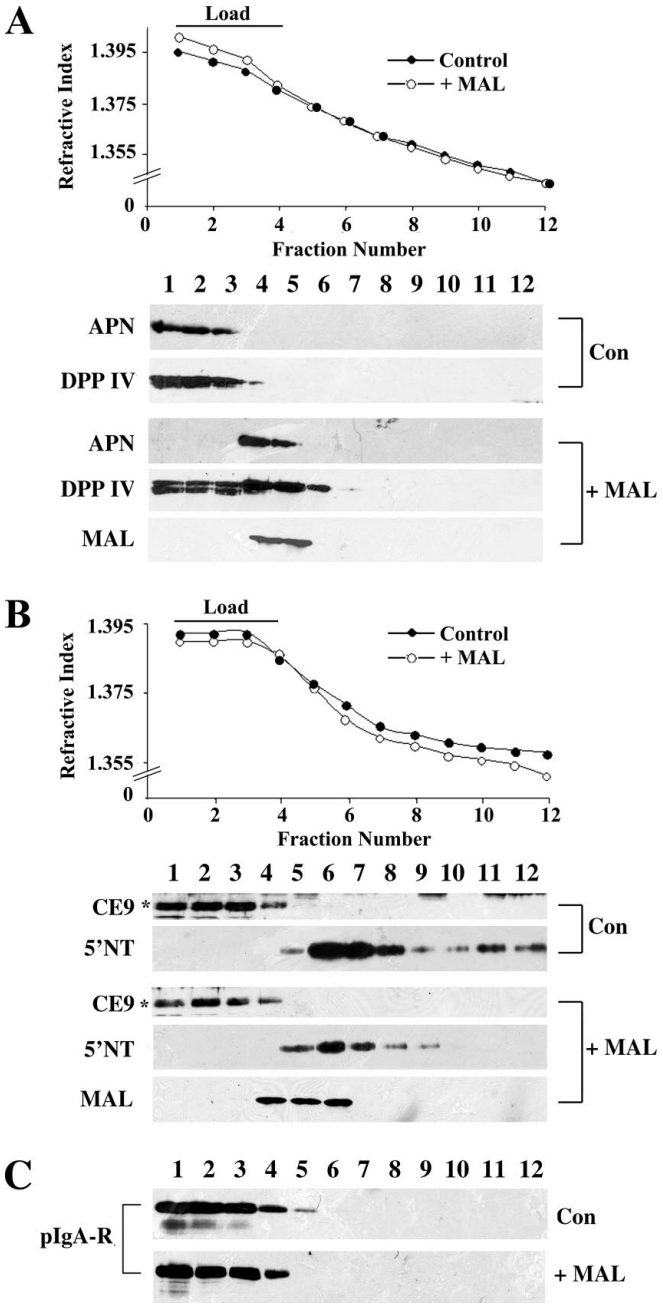

Figure 8. MAL expression alters the density of selected apical proteins in lipid flotations. Control or MAL-expressing cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 and subjected to low-density flotation. Fractions were collected from the bottom with the first four fractions corresponding to the load as indicated. **A**, the refractive index of each fraction from control and MAL-expressing gradients was plotted. Fractions were immunoblotted for APN, DPP IV or MAL as indicated. In **B**, the gradient fractions were immunoblotted for CE9 (marked with an asterisk), 5'NT or MAL, and in **C**, flotations from control or MAL-expressing cells were immunoblotted for pIgA-R.

If MAL induced raft formation, another prediction was that incorporation of the apical residents into raft domains should increase in expressing cells. To test this, I assayed the distributions of apical proteins in control and MAL-expressing cells using low-density flotations. In control cells, none of APN or DPP IV (single TMD proteins) was found in floated fractions (Figure 8A). Similarly, the basolateral protein, CE9, was detected only in the load fractions (Figure 8B). In contrast, all of the GPI-anchored protein, 5'NT floated, peaking in fractions 6-8 and at the top of the gradient in fractions 11-12 (Figure 8B). In infected cells, MAL floated just above the load in fractions 4-6 (Figures 8A and B) that contained 22-26% sucrose.

This is in agreement with preparations from other epithelial cells where MAL floated in fractions containing 24-25% sucrose (Martin-Belmonte *et al.*, 1998; Martin-Belmonte *et al.*, 2000; Tall *et al.*, 2003). As predicted, the raft-association of the apical residents was altered by MAL expression. All of APN and 46% of exogenously expressed DPP IV were found in low density fractions (Figure 9A). Only the high molecular weight, mature forms of DPP IV were found in the floated fractions consistent with the identification of the MAL-induced structures as Golgi-derived. 5'NT distributions also changed in cells expressing MAL, and no longer distributed to the top of the gradient (Figure 8B). Although 5'NT levels peaked in fractions 5-7 that were less dense than the peak of MAL in fractions 4-6, the two molecules significantly overlapped. The shift down in density for APN and DPP IV, and the shift up for 5'NT into fractions containing MAL suggest that MAL was enhancing raft association of the apical residents. For comparison, the basolateral resident, CE9, remained in the load fraction in preparations from MAL-expressing cells (Figure 10B). Similarly, pIgA-R remained in

the load fractions in control and MAL-expressing cells (>97% and 100%, respectively) consistent with the lack of pIgA-R redistribution.

MAL expression reroutes apical proteins into the direct pathway

My original question asked whether exogenous MAL rerouted hepatic apical proteins into the direct pathway. If so, decreased basolateral delivery is predicted. To test this, I measured the basolateral populations of the apical residents in control and MAL-expressing cells by surface-labeling with specific antibodies. Because tight junctions restricted antibody access to the apical PM, only antigens at the basolateral PM were labeled. The labeled cells were lysed and immunoblotted directly with secondary antibodies to detect the surface-bound primary antibodies. On a parallel immunoblot, lysates were probed with the corresponding primary antibody to detect the entire population. The amount of surface-bound antibodies in control or MAL-infected cells was normalized to the amount of total antigen present and control ratios were set to 100%. Importantly, there were no changes in apical or basolateral protein levels in MAL-expressing cells (data not shown).

As predicted, the basolateral populations of HA, DPP IV and APN were significantly decreased in MAL-expressing cells (Figure 9A). HA and APN basolateral labeling was decreased by 80% whereas DPP IV labeling was reduced by ~50%. The basolateral population of 5'NT was decreased to a lesser extent (~70% of control) which is consistent with the fewer number of MAL-expressing cells with intracellular 5'NT staining (Table 1). In contrast, no significant decrease in labeling was observed for basolateral residents (HA321 and CE9) or for pIgA-R (Figure 9A).

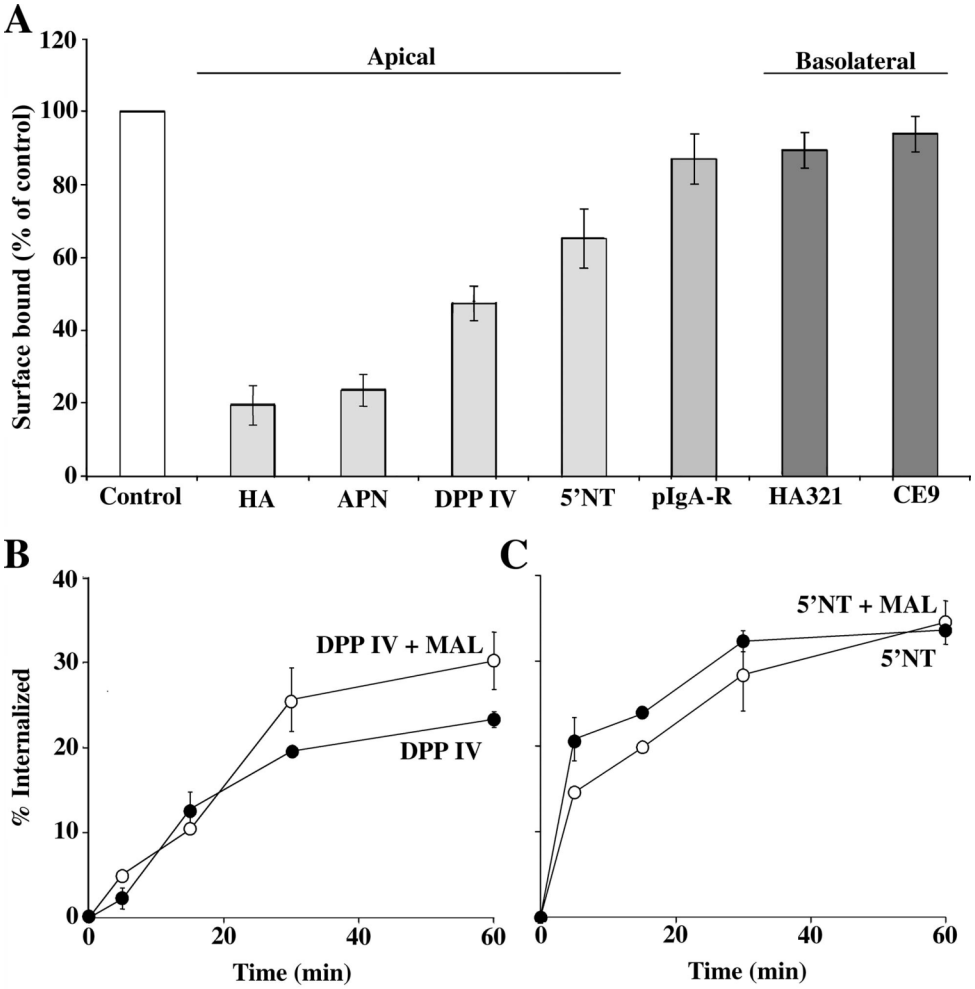

Figure 9. MAL expression decreases basolateral delivery of newly-synthesized apical residents, but does not enhance basolateral internalization. **A,** Control or MAL-expressing WIF-B cells were surface labeled with the indicated antibodies for 30 min at 4^0C. Cells were immediately lysed and the lysates immunoblotted with primary antibodies to detect the entire population of the selected PM protein. On a parallel immunoblot, lysates were probed directly with secondary antibodies to detect only the surface bound primary antibodies. The amount of surface bound antibodies was normalized to the total antigen amount. In all cases, control ratios were set to 100%. Values are expressed as the mean ± SEM. Measurements were done on at least three independent experiments. **B** and **C,** Control and MAL-expressing cells were continuously labeled with biotinylated anti-DPP IV (B) or anti-5'NT antibodies (C) for the indicated times at 37^0C. The remaining PM-associated antibodies were eluted and the cells lysed. Aliquots of the eluate and lysate (the internalized population) were assayed for amounts of biotinylated antibodies using streptavidin-coated 96-well plates and colorimetric detection of HRP-conjugated secondary antibodies. Values are expressed as the mean ± SEM. Measurements were done on at least three experiments each performed in duplicate.

Greater than 90% of control amounts were detected at the basolateral PM in MAL-infected cells. These results suggest that the basolateral delivery of single TMD and GPI-anchored apical residents was reduced in MAL-expressing cells, while delivery of basolateral residents and pIgA-R was not changed. I next measured basolateral internalization in MAL-expressing cells to determine whether decreased basolateral labeling could be explained by increased internalization. Although the extent of surface labeling was decreased, the percent DPP IV internalized was minimally enhanced (<10%) in MAL-expressing cells (Figure 9B). This minor increase does not account for the >50% decrease in basolateral labeling observed. Likewise, virtually no change in 5'NT internalization was observed (Figure 9C). Thus, increased basolateral internalization was not responsible for the decreased basolateral labeling observed in MAL-expressing cells.

To rule out that any of the intracellular HA in MAL-infected cells was present on transcytotic intermediates, we performed two sets of experiments. First, I co-labeled steady state HA with transcytosing APN. Although less APN was present at the basolateral surface, sufficient labeling was achieved to monitor its apical delivery. After 45 min of chase, APN was present at the apical PM indicating that transcytosis was not impaired in MAL-expressing cells (Figures 10A, b). Trafficked APN was also detected in small puncta (Figure 10A, b) in MAL-expressing cells, but these puncta did not overlap with the intracellular HA (Figure 10A, c) indicating that HA was not in transcytotic intermediates, confirming its Golgi localization.

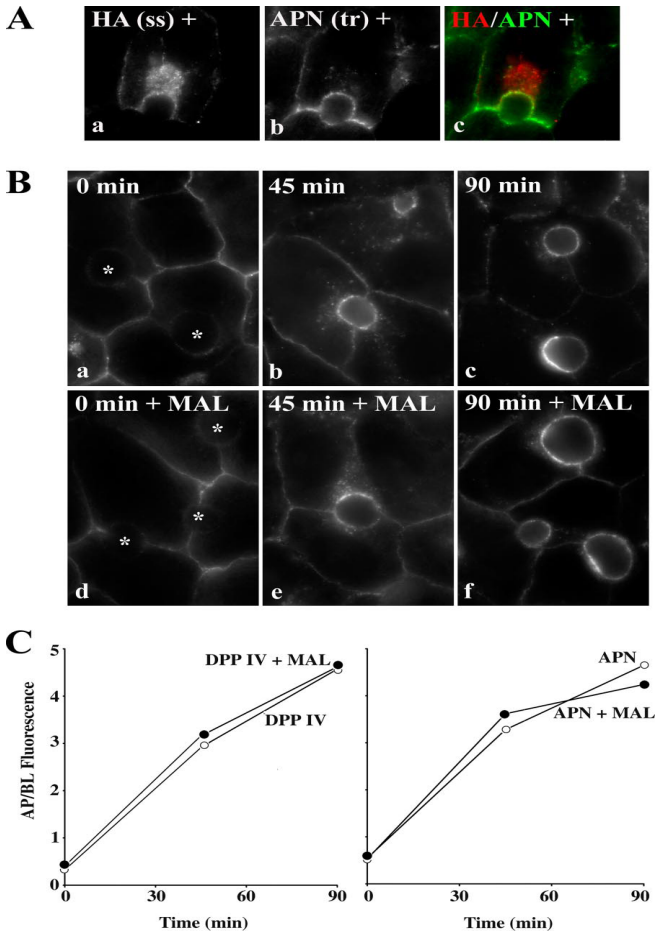

Figure 10. MAL expression does not alter transcytosis. **A,** Cells were infected with recombinant adenovirus expressing MAL and HA (a-c). Basolateral populations of APN were labeled with specific antibodies and chased for 45 min at 37^0C. Cells were fixed and labeled for steady state (ss) distributions of HA (a) and transcytosed (tr) APN (b). Merged images are shown in c revealing little colocalization between the intracellular HA and transcytosing APN in MAL-expressing cells. Asterisks are marking selected BCs. **B,** Basolateral populations of APN were labeled in control (a-c) or MAL-expressing cells (d-f) for 15 min at 4^0C then chased for 0 (a and d), 45 (b and e) or 90 (c and f) min at 37^0C. Cells were fixed and labeled for the transcytosing APN. No accumulated transcytosing populations of APN were observed in MAL-expressing cells. **C,** Basolateral populations of DPP IV or APN were labeled in MAL-expressing cells, and their transcytosis monitored as described in A. Random fields were visualized by epifluorescence and digitized. From micrographs, the average pixel intensity of each marker at selected regions of interest placed at the apical or basolateral PM of the same WIF-B cell were measured. The averaged background pixel intensity was subtracted from each value and the ratio of apical PM to basolateral PM fluorescence intensity was determined. For both DPP IV and APN, no changes in transcytosis were observed in MAL-expressing cells.

In my second set of experiments, we monitored transcytosis in control and MAL-expressing cells at different times of chase. Virtually no differences were observed in the transcytosis of APN in control or infected cells (Figure 10B). After 90 min, most of the APN was chased to the PM and no increased intracellular staining was observed in MAL-expressing cells (Figures 10B, e and f). I quantified this by measuring the relative fluorescence intensities of DPP IV or APN at the apical vs. basolateral PM after 0, 45 or 90 min of chase. This somewhat unconventional analysis was used because the morphology of polarized WIF-B cell precludes more standard methods for measuring apical delivery. The apical PM, located between adjacent cells, is sequestered from the extracellular milieu preventing direct access, and thus, direct measurement of apical delivery. In both control and MAL-expressing cells, the ratios of apical to basolateral fluorescence increased with increased time of chase indicating successful apical delivery. The values were nearly identical in control and infected cells indicating that MAL expression was not interfering with transcytosis and that the intracellular structures were not transcytotic intermediates.

To discriminate between retention vs. redirection of apical proteins, I developed a morphological "pulse-chase" analysis where I measured relative fluorescence intensities of a cohort of HA, DPP IV or pIgA-R at the apical or basolateral PM in CHX-treated cells. We first examined the transcytotic delivery of HA, DPP IV and pIgA-R in control cells to determine the feasibility of our assay. As shown in Figure 11, the ratio of apical/basolateral fluorescence decreased for all markers after 15 min of CHX treatment (75% of 0 min for HA and DPP IV and 85% for pIgA-R) indicating that each cohort was being delivered to the basolateral PM (a decreased ratio reflects increased basolateral

delivery). For DPP IV, peak basolateral delivery was seen after 30 min indicating it was delivered more slowly. After 60 min, the ratios for all three proteins increased beyond that observed at 0 min, signifying that the proteins had traversed the basolateral PM. The ratios for pIgA-R increased much more rapidly (150% vs. 95%) indicating the receptor was more rapidly internalized and transcytosed. By 120 min, all three apical proteins had achieved ratios greater than 100% indicating successful apical delivery confirming that the assay was monitoring transcytosis.

In MAL-expressing cells, the kinetics of pIgA-R apical delivery were not changed indicating that the receptor was delivered via the indirect pathway (Figure 11C) consistent with the lack of intracellular pIgA-R and unaltered basolateral delivery. In contrast, the ratios for HA and DPP IV apical/basolateral fluorescence in MAL-expressing cells did not drop below that seen at 0 min indicating decreased basolateral delivery (Figures 11A and C). The ratios steadily increased to ~170% indicating increased apical delivery. Thus, we conclude that HA and DPP IV were directly delivered to the apical PM from the Golgi.

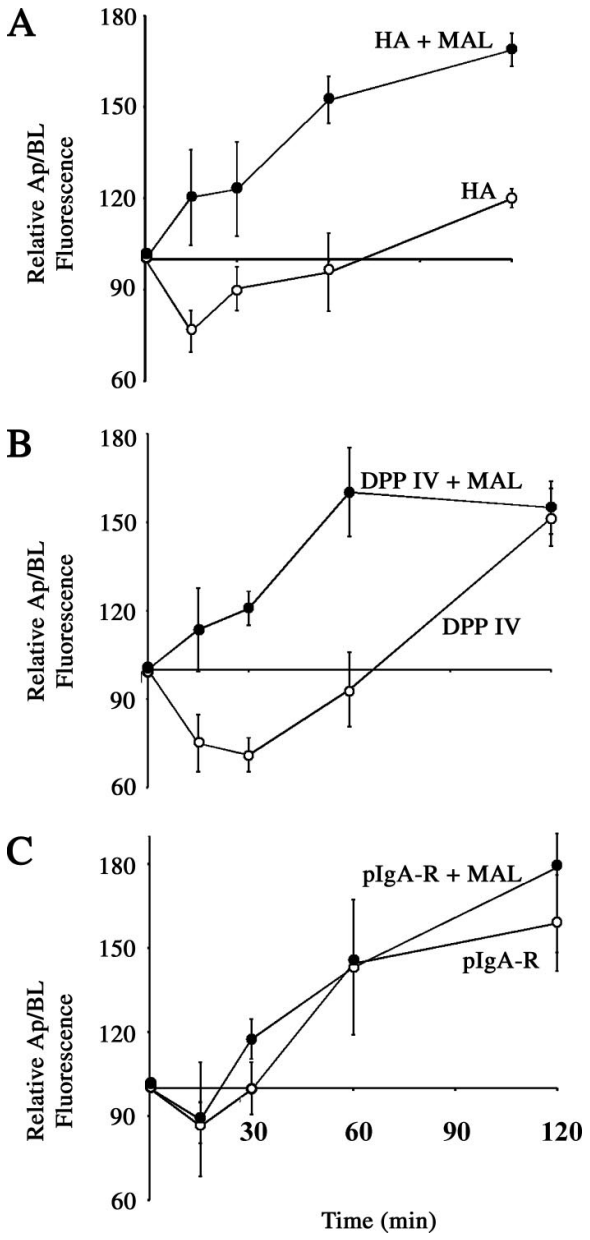

Figure 11. MAL expression reroutes apical proteins into the direct pathway. Cells expressing HA (A), DPP IV (B) or pIgA-R (C) alone or with MAL were treated for the indicated times with 50 mg/ml CHX. The cells were fixed, permeabilized and labeled. Random fields were visualized by epifluorescence and digitized. From micrographs, the average pixel intensity of each marker at selected regions of interest placed at the apical or basolateral PM of the same WIF-B cell were measured. The averaged background pixel intensity was subtracted from each value and the ratio of apical PM to basolateral PM fluorescence intensity was determined. In all cases, control ratios were set to 100% from which the percent of 0 min values were determined. Values are expressed as the mean ± SEM. Measurements were done on at least three independent experiments.

This study was initiated by a simple observation and a question. The observation: MAL regulates direct apical delivery in polarized epithelial cells, and in hepatocytes that lack MAL, apical delivery of GPI-anchored and single TMD proteins is via an indirect route. The question: does exogenous MAL reroute hepatic apical proteins into the direct pathway? I believe the answer is yes. In MAL-expressing cells, single TMD and GPI-anchored proteins were found in intracellular structures; polytopic apical proteins, basolateral residents and pIgA-R were excluded. The structures were Golgi-derived, biosynthetic intermediates, and their apical delivery was impaired by cholesterol depletion. Furthermore, basolateral delivery of single TMD and GPI anchored proteins (but *not* delivery of basolateral residents or pIgA-R) was impaired in MAL-expressing cells implying decreased transcytosis. By using a morphological "pulse-chase" assay, I determined that MAL selectively rerouted apical proteins into a direct route, but did not alter pIgA-R transcytosis. These results not only more clearly define the role of MAL in regulating apical membrane delivery, but also may explain, in part, the long-standing puzzle as to why hepatic cells display transcytotic apical sorting for single TMD and GPI-anchored proteins; they lack MAL expression.

RESULTS: PART II

MAL and MAL2 oligomerization properties in polarized hepatic cells: implications for differential apical protein sorting

Using isopycnic centrifugation, Paladino and coworkers showed that apically destined GPI-anchored proteins Ire oligomeric whereas basolaterally targeted GPI-anchored proteins Ire monomeric in MDCK cells (Paladino *et al.*, 2004). They also showed that oligomerization of GPI-anchored proteins was important for its stabilization into rafts leading to its incorporation into apically destined lipid-raft vesicles and direct sorting to the apical plasma membrane (Paladino *et al.*, 2004). They proposed a putative interactive protein that would aid in GPI-anchored protein oligomerization and coalesce rafts into bigger sorting platforms. MAL and MAL2 are raft-associated and are good candidates for promoting lipid coalescence into stable sorting platforms. In this chapter, I have investigated the raft-association and oligomerization properties of MAL and MAL2 to determine if these proteins function as proposed in raft coalescence.

MAL, but not MAL2, associates with low-buoyant density membranes and alters apical protein buoyant density

If MAL and MAL2 function in lipid coalescence in WIF-B cells, one prediction is that they are lipid-associated. I first tested this by measuring their solubility in 1% TritonX-100 at 4°C. As shown in Fig. 12A, both MAL (41.8 ± 1.3%) and MAL2 (68.0 ± 5.8%) were partially insoluble in TritonX-100. Furthermore, MAL expression did not change MAL2 solubility (Fig. 12A) (and see below). As expected, cholesterol depletion with 5 mM mβCD for 1 h rendered the majority of MAL2 soluble; only 26 ± 11%

remained in the pellet (Fig. 12B). However, MAL's solubility did not change upon cholesterol depletion; 41 ± 2.08% vs.40 ± 0.88% of MAL was soluble in control vs., treated cells, respectively (Fig. 12B). A smear of higher molecular Weight is also visible in the MAL2 blot, which corresponds, to a proposed glycosylated form of MAL2 (Fig 12 A and B) (De Marco et al, 2002). Interestingly, the solubility of the glycosylated forms mirrors that of the unglycosylated MAL2 (Fig. 12B) in all conditions.

I next assayed for MAL and MAL2 lipid association in WIF-B cells using low-density flotations. As predicted, MAL floated just above the load in fractions 4–6 (Fig. 12C) that contained 22–26% sucrose. This is in agreement with preparations from other epithelial cells where MAL floated in fractions containing 24–25% sucrose (Martin-Belmonte *et al.*, 1998, 2000; Tall *et al.*, 2003). In contrast and somewhat surprisingly, MAL2 was detected only in the load fractions (fractions 1-4) (Fig. 12C) indicating that it is not associated with detergent resistant membranes in WIF-B cells. Because MAL has been shown to be oligomeric (Puertollano *et al.*, 1997; Magal *et al.*, 2009) and because MAL induces the raft association of different classes of apical residents (Ramnarayanan *et al.*, 2007), I examined MAL2 buoyant density in MAL overexpressing cells. As in control cells, MAL2 distributed entirely to the load fractions indicating that MAL and MAL2 do not hetero-oligomerize and that the lipid domains induced by MAL do not contain MAL2.

Previously I determined that MAL expression induced raft formation and altered the buoyant properties of single TMD and GPI-anchored proteins, but not pIgA-R (Ramnarayanan *et al.*, 2007). To determine whether cholesterol depletion reversed these associations, I treated MAL-expressing cells with 5 mM mβCD for 30 min, conditions

known to deplete ~60% of cholesterol in WIF-B cells (Nyasae *et al.*, 2003). As shown previously, in cells without MAL expression, APN (a single TMD apical resident) and CE9 (a basolateral resident) Ire detected only in the load fractions (Fig. 13A) whereas all of the GPI-anchored protein 5'NT floated (fraction 6 – 12). As also shown previously, in cells expressing MAL, a subpopulation of APN was detected in more buoyant density fractions while 5'NT distributions shifted to higher density fractions, both overlapping with MAL in fraction 8 (Fig. 13B). In contrast, the basolateral resident, CE9 remained in the load (Figure 13B). The shift down in density for APN and the shift up for 5'NT into fractions containing MAL, suggest that MAL was enhancing raft association of the apical residents. In cholesterol-depleted cells, the majority of MAL no longer floated and was detected in the load (Fig. 13C). Similarly, all of APN was detected in fractions 1-4 (Fig. 13C). Although a substantial pool of 5'NT remained in the buoyant fractions, they Ire of higher density than in untreated MAL-expressing cells indicating decreased association with detergent resistant membranes.

Together these results indicate that MAL expression induces the production of detergent resistant membranes that promote the lipid association of single TMD and GPI-anchored proteins and that this association requires cholesterol. In and Tuma recently showed that pIgA-R overexpression leads to the dramatic redistribution of MAL2 from the apical membrane to all the transcytotic intermediates occupied by the receptor (In and Tuma, 2010), I examined MAL2 distributions on flotations in pIgA-R expressing cells. As shown in Fig. 13D, both MAL2 and pIgA-R remained in the load indicating these proteins are not present in detergent resistant complexes.

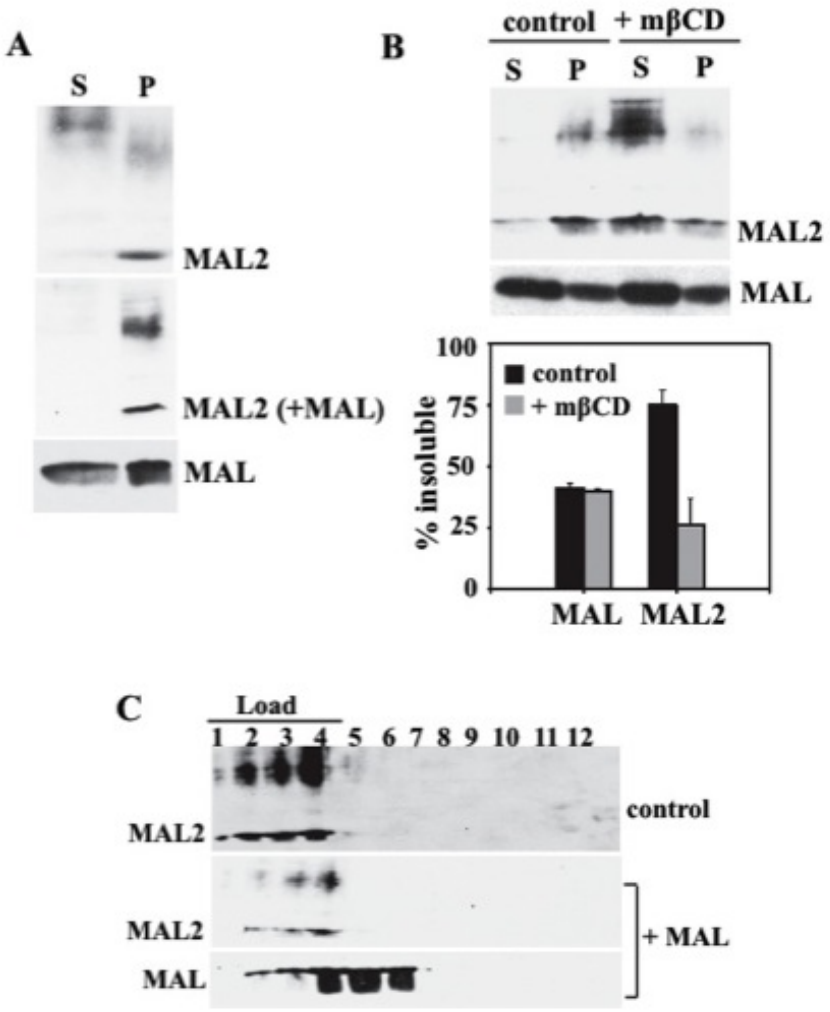

Figure 12. Both MAL and MAL2 are insoluble in Triton X 100, but only MAL is associated with low-buoyant density membranes in WIF-B cells. Control and MAL-myc expressing cells Ire extracted in ice-cold lysis buffer containing 1% Triton X-100 and centrifuged for 30 min at 120,000g. Both MAL and MAL2 are found in the Triton X-100 insoluble fractions (A and B). After cholesterol depletion with 5 mM mβCD for 30 min, MAL2 becomes almost completely soluble in ice-cold Triton X-100 whereas MAL does not (B). Measurements were done on three independent experiments. Values are expressed as the mean ± SEM. Control or MAL expressing cells Ire lysed in ice-cold lysis buffer containing 1% Triton X-100 and subjected to low-density flotation. Fractions Ire collected from the bottom with the first four fractions corresponding to the load as indicated (C). MAL2 does not float in control or MAL expressing cells whereas MAL floats in the low-density fractions (C).

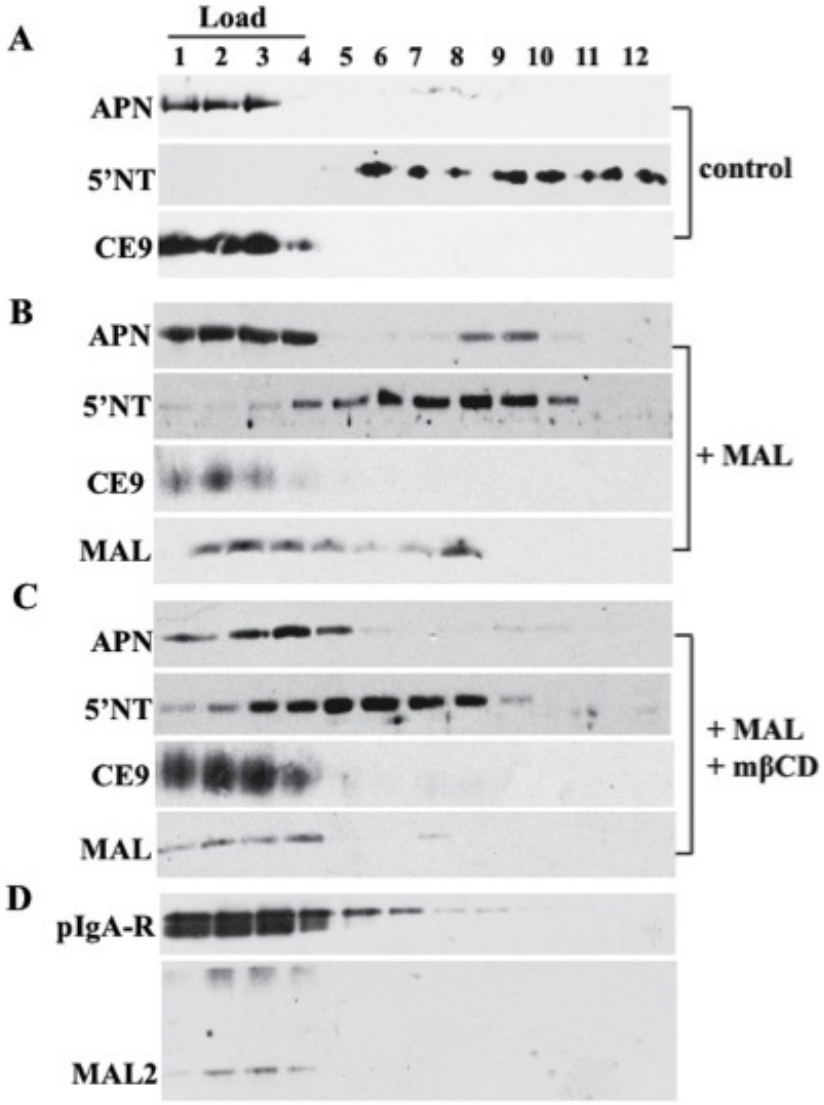

Figure 13. MAL expression alters density of single TMD and GPI-anchored apical proteins. Control or MAL expressing cells Ire lysed in ice-cold lysis buffer containing 1% Triton X-100 and subjected to low-density flotation. Fractions Ire collected from the bottom with the first four fractions corresponding to the load as indicated. APN does not float in control gradients whereas 5'NT has a biphasic distribution in the floated fractions (A). APN shifts to floated fractions in MAL expressing cells while 5'NT shifts to MAL containing "raft" fractions (B). MAL expressing cells Ire depleted of cholesterol with 5 mM mβCD for 30 min and lysed in ice-cold lysis buffer containing 1% Triton X-100 and subjected to low-density flotations (C). Both APN and MAL remain in the load fractions after cholesterol depletion (C). 5'NT shifts to lower density fractions on cholesterol depletion (C). MAL2 is found in the load fractions along with pIgA-R in low-density gradients performed in cells expressing pIgA-R (D).

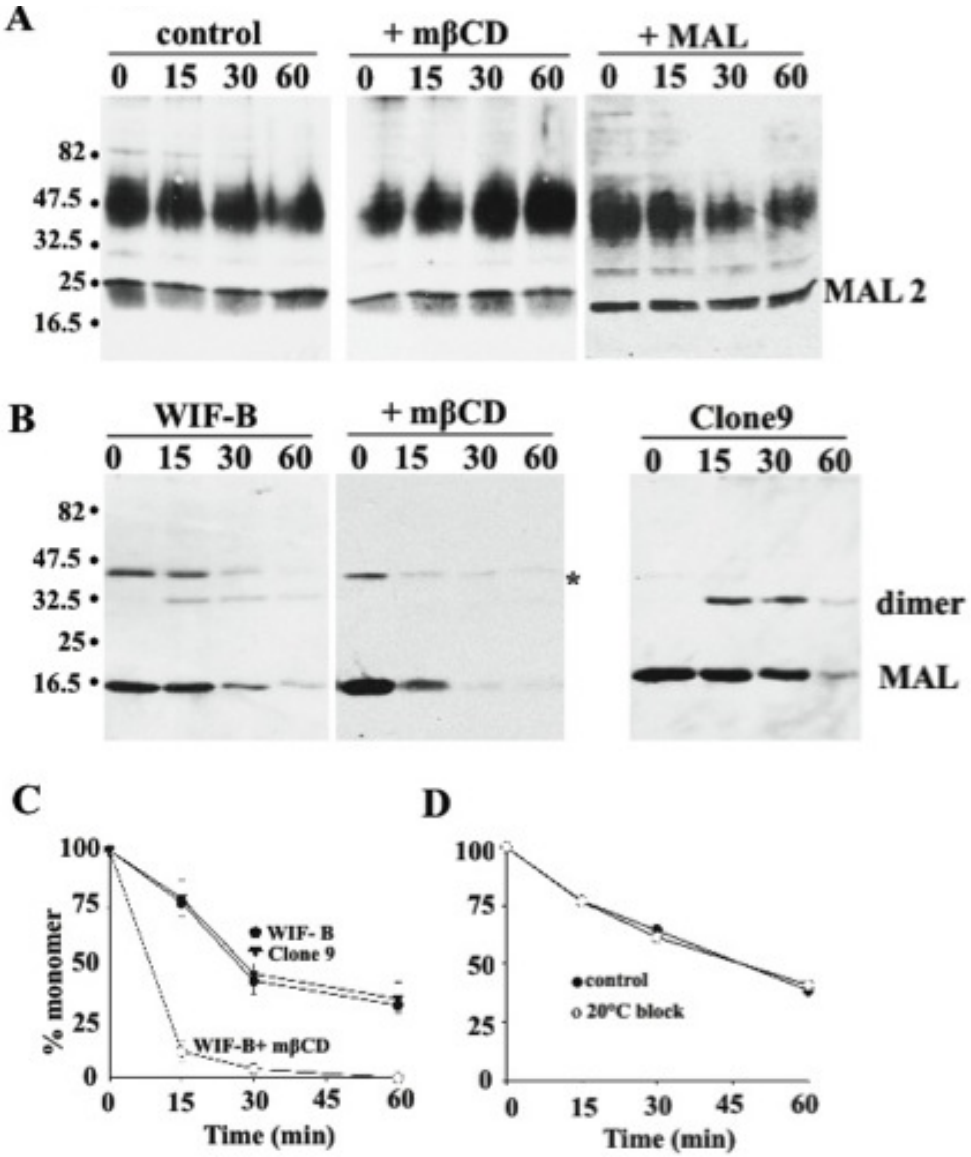

Figure 14. MAL is a dimer whereas MAL2 is monomeric. Post-nuclear membranes of control cells or expressing adenoviral MAL-myc Ire isolated and crosslinked with 10 mM EDC and 2.5 mM sulfo-NHS for 0, 15, 30 and 60 min (A and B). MAL2 is monomeric after EDC crosslinking and does not hetero-oligomerize with MAL (A). MAL is at least a dimer in both WIF-B and Clone 9 cells (B). MAL follows identical crosslinking kinetics in both WIF-B and Clone 9 cells and cholesterol depletion with 5 mM mβCD enhances the kinetics of MAL crosslinking with EDC (C). Measurements Ire made on three independent experiments. Values are expressed as ± SEM. MAL expressing cells Ire subjected to a 20°C temperature block for 2 hours before crosslinking (D). MAL crosslinking does not change on blocking post-Golgi trafficking with a 20°C temperature block (D). Asterisk indicates a cross-reactive species associated with anti-myc antibodies used to detect exogenous MAL in WIF-B cells.

MAL is an oligomer whereas MAL2 is monomeric

If MAL and MAL2 are required for raft coalescence, another prediction is that they form stable complexes with themselves or with apical resident proteins. To test for direct interactions, I crosslinked MAL and MAL2 containing vesicles with the zero length crosslinker, EDC. In non-crosslinked samples, MAL was detected only as a 17 kDa monomer. The ~40 kDa band marked with an asterisk is an unidentified cross-reactive species in WIF-B cells (no such band is detected in Clone 9 cells, see Fig. 14B). However, after 15 min in EDC, an additional band at ~32.5 kDa was observed with a concomitant loss of monomer reflecting MAL dimer formation (Fig. 14B). By 60 min, only ~30% of the monomeric and traces of the dimeric forms Ire detected indicating that MAL is present in higher order oligomers that are too large to be resolved in the gel. I next crosslinked MAL when overexpressed in Clone 9 cells. Importantly, these cells lack endogenous MAL and apical protein expression thereby allowing us to examine intrinsic MAL oligomeric properties. As shown in Fig. 14B, a similar loss in the MAL monomeric species and increase in the dimeric form was observed. In fact, when loss of monomer was plotted for MAL expressed in WIF-B or Clone 9 cells, nearly overlapping lines Ire obtained (Fig. 14C). After 15 min of crosslinking, 77 ± 1.5% and 78.84 ± 8% of monomer Ire detected in WIF-B and Clone 9 cells, respectively, and by 60 min, only 32 ± 3.51% and 34.75 ± 7.32% of the monomer remained.

To our surprise, cholesterol depletion of WIF-B cells enhanced MAL crosslinking (Fig. 14B and 14C). After only 15 min in EDC, only 15% of the monomer was detected and by 60 min, no monomer remained. No intermediate dimer was detected in cholesterol-depleted cells suggesting that MAL was readily crosslinked into large

complexes. Because the prediction is that MAL would encounter and recruit newly synthesized apical residents in the Golgi, I also investigated MAL crosslinking after 2 h at 20^{o}C. However, no changes in MAL crosslinking Ire observed (Fig. 14D) suggesting that the interaction between MAL and apical proteins is indirect and mediated through lipid domains.

Unlike for MAL, no MAL2 higher order oligomers Ire observed after prolonged incubations with EDC (Fig. 14A). Even after 60 min, no changes in the levels of the 19 kDa monomeric protein Ire observed (Fig. 14A) which was confirmed using densitometry (Figure 14B). Also interestingly, the levels of the 35 kDa diffuse MAL2-positive species did not change after incubation with EDC suggesting this species is also monomeric (Fig. 14C). Furthermore, over-expression of MAL did not alter MAL2 oligomerization state (Fig. 3A; + MAL) nor did cholesterol depletion with mβCD (Fig. 14A; + mβCD). Thus I conclude that MAL2 is present mainly as a monomer in WIF-B cells.

If MAL and MAL2 serve to coalesce lipid domains, another prediction is that overexpression of apical proteins provokes complex formation that would be revealed with EDC crosslinking. However, DPP IV (a single TMD apical resident) overexpression did not alter MAL oligomer detection. In both cases, small levels of dimer Ire detected with a gradual loss of monomer in the presence of EDC (Fig. 15 B and C). Furthermore, DPP IV crosslinking was not changed by MAL overexpression (Fig. 15C). In both control and MAL expressing cells, loss of monomer detection was nearly identical (Fig. 15C). Similarly, pIgA-R overexpression did not alter MAL2 EDC crosslinking (Fig. 15A).

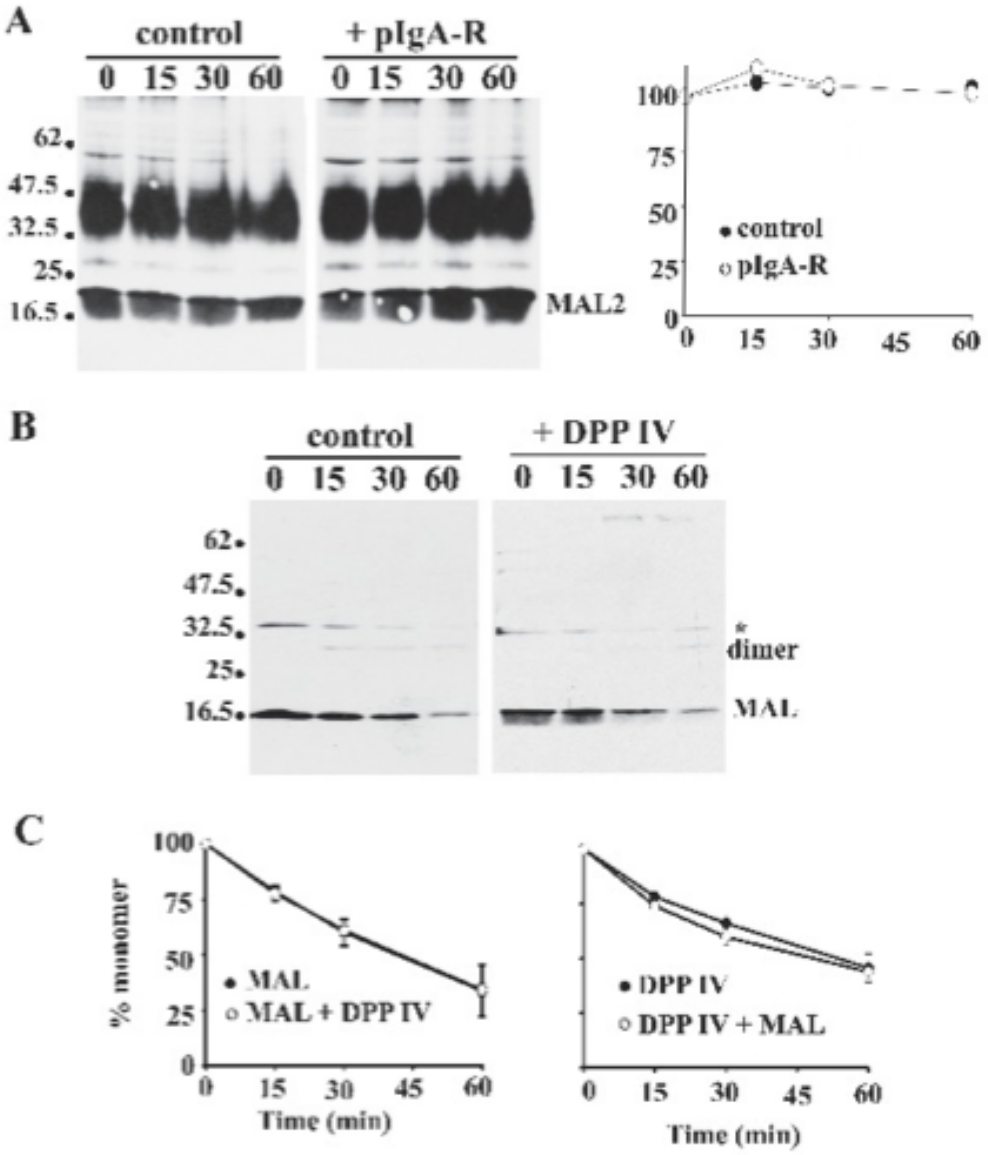

Figure 15. Apical protein expression does not alter MAL2 or MAL oligomerization state. Post-nuclear membranes of control cells or cells expressing adenoviral pIgA-R or control Ire isolated and crosslinked with 10 mM EDC and 2.5 mM sulfo-NHS for 0, 15, 30 and 60 min (A). MAL2 oligomerization state does not change on pIgA-R expression (A). Post-nuclear membranes of cells expressing adenoviral MAL and/or adenoviral DPP IV Ire isolated and crosslinked with 10 mM EDC and 2.5 mM sulfo-NHS for 0,15, 30 and 60 min (B) and (C). The kinetics of MAL crosslinking do not change on DPP IV expression and MAL expression does not change DPP IV crosslinking kinetics (B and C). Asterisk indicates a cross-reactive band associated with anti-myc antibodies used to detect exogenous MAL in WIF-B cells. Measurements were done on three independent experiments. Values are expressed as mean ± SEM.

This suggests that both MAL and MAL2 do not interact directly with apical proteins and that their oligomerization states are independent of apical protein.

MAL alters the oligomerization states of single TMD and GPI anchored proteins, whereas, MAL2 oligomerization state is altered with the pIgA-R expression

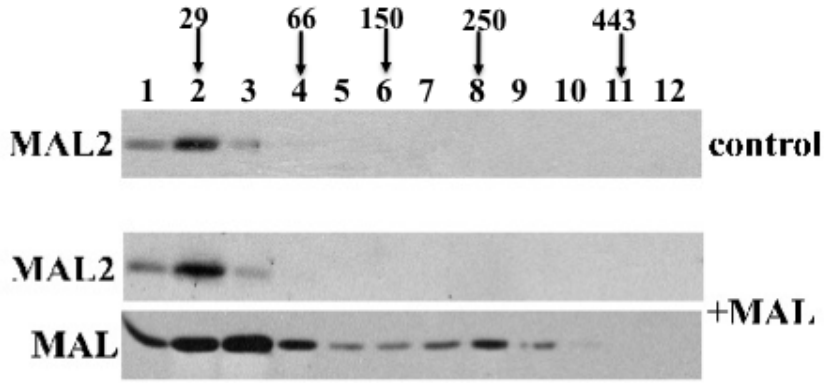

Figure 16. Velocity gradient centrifugation shows that MAL is an oligomer whereas MAL2 is a monomer. Control cells or cells expressing adenoviral MAL-myc or control Ire lysed in ice-cold lysis buffer containing 0.4% SDS and 0.2% Triton X-100 and subjected to velocity gradient centrifugation. MAL2 is found at the top of the gradient in low-density fractions indicating that it is monomeric whereas MAL is found to peak in fraction 8 which corresponds to ~250 kDa.

To confirm our crosslinking results, I next examined MAL and MAL2 complex formation using velocity sucrose gradient centrifugation. Post-nuclear detergent lysates Ire loaded on a 5-30% sucrose gradient and centrifuged at high speed for 18 hrs. As shown in Fig. 16, MAL (17 kDa) was found in higher density fractions (fractions 7, 8 and 9) corresponding to a molecular weight of around 250 kDa. In contrast, MAL2 (19 kDa) remained on top of the gradient in fractions corresponding to molecular weight less than 29 kDa indicating that MAL2 is monomeric (Fig. 16). Consistent with results in Fig 14A, MAL overexpression had no effect on MAL2 oligomerization state (Fig. 16). I next

investigated the oligomerization properties of the single TMD apical protein, APN, and GPI-anchored apical protein, 5'NT, in control and MAL expressing cells using velocity gradients. I predicted that the oligomerization properties of apical proteins would change on MAL expression. In control cells, APN (125 kDa) distribution is uniform in fractions number 7-14 (Fig. 17A) corresponding with dimeric or tetrameric APN complexes (250 kDa to 500 kDa). In MAL expressing cells, APN peaks in fraction 7 (Fig. 17B) along with MAL suggesting that MAL expression causes APN to shift to a predominantly dimeric state (~250 kDa). The GPI anchored apical protein 5'NT (62 KDa) is found in fractions 5-14 in control cells (Fig. 17A) suggesting that it can form higher order oligomers consistent with previous observations that GPI-anchored proteins oligomerize (Paladino *et al.*, 2004). In cells expressing MAL, 5'NT, like APN peaks in fraction 7 along with MAL (Fig. 17B) suggesting that MAL expression causes 5'NT to shift to a predominantly tetrameric state. Thus MAL expression alters the oligomerization properties of both single TMD and GPI-anchored proteins.

Previously I determined that MAL overexpression causes the intracellular accumulation of single TMD and GPI-anchored proteins in WIF-B cells at the Golgi (Ramnarayanan *et al.*, 2007). This alteration in oligomerization states of single TMD and GPI-anchored proteins implies that MAL induces the formation of transport intermediates, which contain single TMD and GPI-anchored apical proteins. Since MAL expression enhances the raft-association properties of single TMD and GPI-anchored apical proteins I wanted to verify if the alteration in oligomerization of single TMD and GPI-anchored proteins was lipid-dependent. To test this, I depleted cells of cholesterol with 5 mM mβCD for 30 minutes. As shown in Fig 17C, APN, 5'NT and MAL no

longer peaked in fraction number 7 in cholesterol depleted cells. Instead their distributions appeared as in control without MAL expression (Fig. 17A).

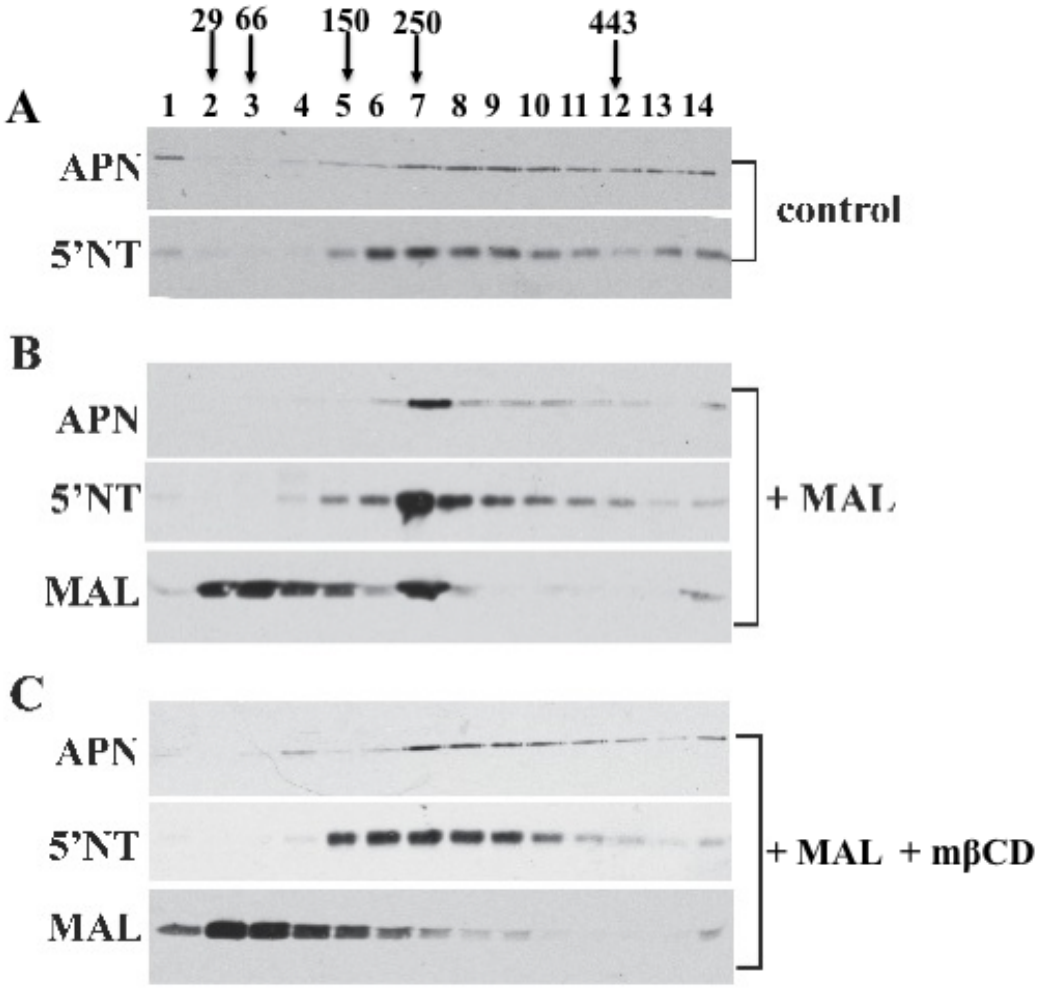

Figure 17. MAL expression alters the oligomerization states of single TMD and GPI-anchored apical proteins. Cells expressing adenoviral MAL-myc or control Ire lysed in ice-cold lysis buffer containing 0.4% SDS and 0.2% Triton X-100 and subjected to velocity gradient centrifugation. APN and 5'NT are dimers or oligomers in control gradients (A). After MAL expression, both APN and 5'NT peak in fraction 7 corresponding to ~250 kDa indicating that their oligomerization states are altered because of MAL expression (B). After cholesterol depletion with 5 mM mβCD for 30 min, both APN and 5'NT revert to control distributions (C).

This experiment suggests that the MAL induced alteration in oligomerization state of single TMD and GPI-anchored apical proteins is lipid-raft dependent. I have

previously shown that cholesterol depletion impaired the apical delivery from the MAL induced transport intermediates to the apical plasma membrane (Ramnarayanan *et al.*, 2007). Depletion of cholesterol thus likely prevents MAL oligomerization, which in turn perturbs lipid-stabilization and incorporation of apical proteins into lipid-domains.

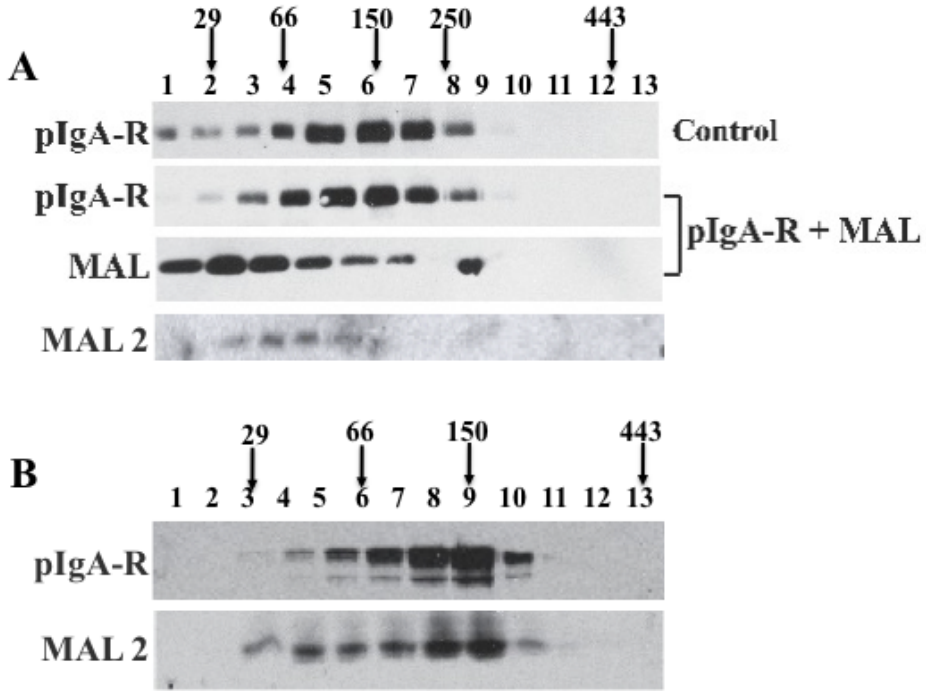

Figure 18. pIgA-R expression alters MAL2's oligomerization state, but not MAL's in WIF-B cells. Control cells or cells co-expressing adenoviral MAL-myc and pIgA-R, or pIgA-R alone, Ire lysed in ice-cold lysis buffer containing 0.4% SDS and 0.2% Triton X-100 and subjected to velocity gradient centrifugation. pIgA-R peaks in fraction number 5, 6 and 7 in both MAL expressing and control cells (A). MAL peaks at fraction number 8 (A). After pIgA-R expression, MAL2 shifts to pIgA-R containing fractions 2,3,4 and 5 (A). MAL2 and pIgA-R peak in the same fractions (7,8 and 9) of velocity gradient in the liver where pIgA-R is endogenously expressed.

As a negative control, I also investigated the oligomerization state of pIgA-R a professional transcytosing protein whose trafficking and lipid-association is independent of MAL expression in WIF-B cells (Ramnarayanan *et al.*, 2007). I found that pIgA-R

(120 KDa) peaked in fractions 5, 6 and 7 in both control and MAL expressing cells, not in fraction 7 with MAL like single TMD or GPI-anchored proteins (Fig. 18A). Thus as predicted, pIgA-R's basolateral sorting is independent of the sorting provided by MAL at the TGN (Ramnarayanan *et al.*, 2007).

Recently In and Tuma from our laboratory showed that MAL2 selectively regulated newly-synthesized pIgA-R's trafficking to the basolateral surface in WIF-B cells (In and Tuma, 2010). They also showed an interaction between pIgA-R and MAL2 using co-immunoprecipitations. I used velocity gradients to determine whether pIgA-R expression would alter MAL2's localization in the gradient. As predicted, I found that pIgA-R expression shifted MAL2 to higher molecular weight fractions containing pIgA-R (Fig 18A). MAL2 shifts to a molecular weight of ~150 kDa suggesting that a dimer of MAL2 (~38 kDa) likely interacts with one molecule of pIgA-R (120 kDa). I further verified this by performing velocity gradient centrifugations on rat liver fractions which endogenously express pIgA-R. I found that both MAL2 and pIgA-R distributed in fractions 3-9 and both peaked in the same fraction in velocity gradients performed in liver fractions (Fig. 18B). This also corresponds to ~150 kDa suggesting that one MAL2 dimer likely interacts with a pIgA-R monomer. The complete overlap in the liver might be because pIgA-R is endogenously expressed whereas the shift is subtler in WIF-B cells because of high levels of exogenous pIgA-R expression. Much of the exogenous pIgA-R is present in the ER where MAL2 is not present thus much of the pIgA-R population is unable to bind MAL2. All these results put together suggest that MAL through oligomerization and lipid-association drives the formation of apical vesicles, which enables direct sorting of proteins when these proteins are expressed in hepatocytes

whereas MAL2 directly interacts with newly-synthesized pIgA-R to selectively regulate its basolateral delivery. Therefore, based on all the biochemical properties examined, MAL functions as the proposed lipid-coalescing protein at the TGN, enabling the formation of stable sorting platforms.

DISCUSSION

MAL redirects single TMD and GPI-anchored apical proteins into the direct pathway

The first part of this study was initiated by a simple observation and a question. The observation: MAL regulates direct apical delivery in polarized epithelial cells, and in hepatocytes that lack MAL, apical delivery of GPI-anchored and single TMD proteins is via an indirect route. The question: Does exogenous MAL reroute hepatic apical proteins into the direct pathway? I think the answer is yes. In MAL-expressing cells, single TMD and GPI-anchored proteins were found in intracellular structures; polytopic apical proteins, basolateral residents, and pIgA-R were excluded. The structures were Golgi derived, biosynthetic intermediates, and their apical delivery was impaired by cholesterol depletion. Furthermore, basolateral delivery of single TMD and GPI-anchored proteins (but not delivery of basolateral residents or pIgA-R) was impaired in MAL-expressing cells, implying decreased transcytosis. By using a morphological pulse-chase assay, we determined that MAL selectively rerouted apical proteins into a direct route, but they did not alter pIgA-R transcytosis. These results not only more clearly define the role of MAL in regulating apical membrane delivery but also they may explain, in part, the long-standing puzzle as to why hepatic cells display transcytotic apical sorting for single TMD and GPI-anchored proteins; they lack MAL expression.

My Working Model

MAL is mainly expressed at the apical PM, yet its sorting activity is thought to occur at the TGN, whereas MAL2 is found at the SAC in hepatic cells, yet it is thought to sort transcytosing residents from early endosomes. Thus, the MAL proteolipids must be

itinerant proteins, a conclusion consistent with findings from Cos7 cells and live cell imaging (Puertollano and Alonso, 1999; de Marco et al., 2006). In our model, which is based on and similar to those previously proposed (e.g., Martin-Belmonte et al., 2001; Puertollano et al., 2001; de Marco et al., 2006), MAL normally encounters single TMD and GPI-anchored apical residents in cholesterol and glycosphingolipid-enriched domains in the TGN, whereas MAL2 encounters apical single TMD and GPI-anchored proteins in similar domains at early endosomes. The proteolipids and newly synthesized apical proteins associate; are packaged into vesicles; and the divergent, cytoplasmic N-terminal domains of each MAL isoform recruit specific (yet unidentified) regulators that target vesicles to either the apical PM or SAC. Because hepatocytes lack MAL, apical sorting at the TGN does not occur, and apical proteins are routed instead into the transcytotic pathway where MAL2-mediated sorting occurs at the basolateral early endosome. Thus, in MAL-expressing hepatic cells, we propose that the newly synthesized apical proteins encountered MAL first at the TGN, and this association rerouted them directly to the apical PM, bypassing interactions with MAL2 and the transcytotic pathway.

This hypothesis is consistent with studies from MDCK cells where apical residents were missorted to the basolateral PM when MAL expression was knocked down (Cheong et al., 1999; Puertollano et al., 1999, 2001; Martin-Belmonte et al., 2000, 2001). Curiously, despite the endogenous expression of MAL2 in MDCK cells, the missorted apical proteins were not transcytosed to the apical PM. One possibility is that MAL2 was not expressed at high enough levels to handle the large load of missorted proteins. Alternatively, MAL2 may regulate transcytotic sorting of different cargo. Thus, it remains to be determined what the specific role of MAL2 is in MDCK cells. Also in

MDCK cells, MAL has been implicated in regulating apical endocytosis (Puertollano et al., 2001; Martin-Belmonte et al., 2003). Whether MAL functioned similarly in WIF-B cells remains to be determined.

Intestinal cells express both MAL and MAL2 (De Marco et al., 2002; Marazuela et al., 2003, 2004; Marazuela and Alonso, 2004). If our model is correct, the prediction is that MAL should directly target proteins to the apical membrane. However, intestinal cells rely on both the indirect and the direct pathways for delivery of newly synthesized apical proteins (Tuma and Hubbard, 2003). In our studies, exogenous MAL expression did not fully reroute the apical proteins (Figure 5A), suggesting that relative levels of MAL and the newly synthesized apical proteins are important, i.e., high levels of MAL expression are required for efficient direct targeting. Studies are needed to first confirm the roles of MAL and MAL2 in intestinal apical delivery and second to examine the rates of apical protein synthesis and delivery routes with respect to MAL and MAL2 expression levels.

MAL Reroutes a Subset of Resident Apical Proteins

Exogenous MAL altered the apical delivery of single TMD and GPI-anchored apical proteins, whereas trafficking of pIgA-R, basolateral residents, and the polytopic apical resident MRP2 was not affected. How did the latter three classes of proteins elude MAL-mediated sorting? The cytoplasmic tails of pIgA-R and basolateral residents encode targeting signals that mediate their delivery to the basolateral PM (Casanova et al., 1991; Keller and Simons, 1997). I propose that these targeting signals are dominant or independent to the sorting provided by MAL. This explains why pIgA-R takes the

indirect route in MDCK cells where most other single span proteins take the direct pathway. This conclusion is also consistent with the finding that many apical targeting signals can function only in the absence of basolateral targeting signals (Matter and Mellman, 1994; Keller and Simons, 1997; Rodriguez-Boulan et al., 2005). The apical sorting signal for MRP2 has been mapped to its cytoplasmic, C-terminal domain that contains a PDZ-binding motif (Harris et al., 2001; Nies et al., 2002). Similarly, other apical multispanning proteins may be sorted to the apical PM via these motifs (Fanning and Anderson, 1999). I suggest, that like the basolateral targeting signals, these motifs are dominant to the sorting conferred by MAL.

In contrast, DPP IV, APN, and HA encode short, cytoplasmic tails (6, 8, and 12 amino acids, respectively) that contain no known targeting information. Because of the shortness of these cytoplasmic domains and the lack of cytoplasmic regions of GPI-anchored proteins, I suggest that sorting is not directly conferred via cytosolic targeting proteins. Rather, I favor the possibility that exogenous MAL redirected these (and other?) apical proteins via interactions that occurred within the bilayer. Consistent with this hypothesis is the finding that the 10 amino acids of the TMD of HA that span the bilayer outer leaflet were important for apical targeting, raft association, and MAL binding (Lin et al., 1998 blue right-pointing triangle; Tall et al., 2003 blue right-pointing triangle). Within this 10 amino acids, glycine 520 and serine 521 have been shown to mediate apical delivery (Scheiffele et al., 1997; Lin et al., 1998) which are not in the TMDs of APN or DPP IV. In fact, there is virtually no sequence conservation between the HA TMD and those of DPP IV or APN. This lack of sequence conservation suggests that the interactions between MAL and the apical proteins are indirect or weak. This is

consistent with our inability to coimmunoprecipitate MAL with any apical protein (data not shown). Similarly, only 0.7–2% of HA was coimmunoprecipitated with MAL in MDCK cells (Tall et al., 2003).

Do MAL and MAL2 stabilize lipid-rafts and apical proteins into stable sorting platforms?

According to the raft-hypothesis, apical proteins encounter lipid rafts in the biosynthetic pathway, where they are packaged into specific vesicles and transported to the apical plasma membrane. Recently, it was proposed that these domains are too small and transient to host apically destined cargo, and that lipid-associated proteins might serve to stabilize raft-sorting platforms (Hancock, 2006). Therefore, the "new-raft hypothesis" invokes the requirement for a protein regulator that would enable coalescence and/or clustering of lipids into stable sorting platforms. Interestingly, each of the TMD sequences of the MAL family members share significant sequence identities (Sanchez-Pulido et al., 2002), suggesting that these domains regulate specific interactions with proteins or lipids that might be required for sorting. Recently, it was proposed that oligomerization of GPI-anchored proteins promotes raft association that then mediates apical delivery (Paladino et al., 2004). The authors suggest that the association is driven either by oligomerization and subsequent stabilization into rafts or by coalescence of rafts that then promote oligomerization. One possibility is that MAL promotes raft formation or clustering that is required for apical sorting, a hypothesis that I examined in the second part of this dissertation.

Using biochemical techniques, we investigated whether MAL and MAL2 are the proteins required to coalesce and stabilize rafts into stable sorting platforms. Since such proteins are predicted to reside in lipid domains, we examined their lipid-association in WIF-B cells. Interestingly, while both MAL and MAL2 are highly insoluble in Triton X-100, they show a marked difference in their low-density buoyant density flotation properties. MAL floats to low-buoyant density fractions in sucrose gradient flotations whereas MAL2 is present in the high-density "load" fractions. This indicates that MAL is indeed raft-associated, while MAL2 is not. This suggests that there is a difference in the membrane environments of the proteins. While the results for MAL raft-association are consistent with previous results, our results for MAL2 flotation are not with results in HepG2 cells where MA2 was found in low-buoyant density fractions (de Marco *et al.*, 2002). However, our results are consistent with other reports on MAL2 flotations performed in oligodendrocytes and breast cancer cell lines (Bello-Morales *et al.*, 2009) suggesting that MAL2's lipid-association is cell type dependent.

If MAL or MAL2 function to coalesce lipid domains and apical proteins into stable sorting platforms, another prediction is that their overexpression should promote association of apical proteins in buoyant membrane fractions. Only MAL showed this property. MAL expression enhanced flotation of the single TMD apical protein APN and shifted GPI-anchored apical protein 5'NT to MAL containing floated fractions. Furthermore, cholesterol depletion completely redistributed both APN and MAL to the high-density "load" fractions and caused a partial shift of the GPI-anchored protein 5'NT to the "load" fractions further indicating that the MAL induces the formation of cholesterol-enriched apical sorting platforms in WIF-B cells.

MAL is an oligomer whereas MAL2 is monomeric

Another prediction if MAL and MAL2 function in raft-coalescence is that they oligomerize. Using a zero-length crosslinker we showed that MAL is oligomeric as predicted and consistent with report from Puertollano and coworkers (Puertollano et al, 1997) whereas MAL2 is only monomeric. Velocity gradient centrifugation confirmed these results and further indicated that MAL expression altered the oligomerization states of single TMD and GPI-anchored apical proteins. These results suggest that MAL oligomerization causes clustering and stabilization of lipid-rafts at the TGN that recruit apical proteins, which may enable direct sorting of single TMD, and GPI-anchored apical proteins.

Model for MAL's regulation of sorting from the TGN to the apical plasma membrane

MAL expression alters the raft-association properties of the single TMD and GPI-anchored apical proteins and induces the formation of glycolipid and cholesterol enriched post Golgi vesicles with these proteins. These vesicles are then trafficked directly to the apical plasma membrane bypassing the indirect route. We have also shown that, pIgA-R, a professional transcytosing protein, with its own basolateral targeting signal, escapes MAL's regulation at the TGN and interacts directly with MAL2 (In and Tuma, 2010). Our studies confirm those reports and also suggest a mechanism by which rafts are stabilized into stable sorting platforms (Fig 19). When MAL is expressed in WIF-B cells it forms homo-oligomers in the biosynthetic pathway, which lead to the clustering of the lipid microdomains at the TGN. The clustering of these microdomains causes a shift in the lipid-association properties of the single TMD and GPI-anchored proteins. Single

TMD proteins, which were previously in non-raft domains, now shift into the lipid-raft clusters containing MAL and the GPI-anchored proteins. The MARVEL domains in the TMD regions of MAL then enable apical raft-vesicle formation at the TGN. The apical raft-vesicles with MAL and the single TMD and GPI-anchored apical proteins are then delivered to the apical plasma membrane (Fig 19).

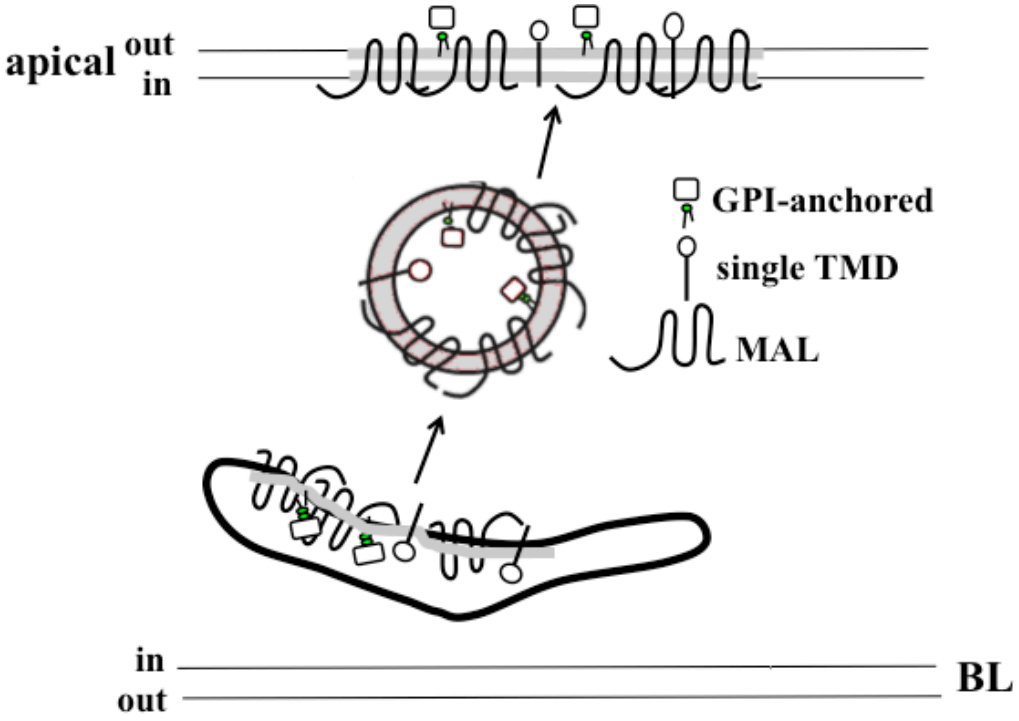

Fig. 19. Model for MAL's regulation of apical transport. MAL oligomerizes and causes clustering of rafts at the Golgi. The apical proteins are incorporated into MAL clustered raft-platforms. MAL and apical proteins are packaged into raft-vesicles and delivered to the apical plasma membrane.

There are a few questions that still remain about the mechanisms involved in the direct trafficking of newly synthesized apical proteins to the apical plasma membrane. How are these proteins specifically directed to the apical plasma membrane and what are the molecules that mediate docking at the apical plasma membrane? There are a few Rab proteins that have been implicated in regulating trafficking between various

compartments in MDCK cells. Rab 13 is proposed to mediate traffick between the TGN and the common recycling endosome (Nokes *et al.*, 2008) whereas; Rab 14 is proposed to mediate the transport of influenza hemagglutinin (HA) to the apical surface of MDCK cells (Kitt *et al.*, 2008). There has been various soluble- vesicle NSF (N-ethylmaleimide sensitive factor) attachment protein receptors (SNARES) implicated in the docking and fusion of apical proteins. The ablation of function of syntaxin-3 impairs the apical delivery of both raft-associated and non-raft associated apical proteins in MDCK cells (Low *et al.*, 1998; Lafont *et al.*, 1999). Syntaxin-2 distributes equally between the apical and basolateral membranes and its importance in apical transport is not yet known. Two vesicle associated membrane proteins (VAMPS)- VAMP7 and VAMP 8 have been shown to associate with apical SNARES to initiate membrane docking and fusion events in MDCK cells (Steegmaier *et al.*, 2000). Recent studies have shown that there are specific microtubule motors involved in transporting apical proteins. KIFC3, a minus end kinesin is involved in the targeting of influenza HA and annexin XIII B to the apical surface in MDCK cells (Noda *et al.*, 2001). Surprisingly, KIF5B, a plus end kinesin is involved in the transport of p75 neurotropin receptor a single pass membrane protein, to the apical surface in MDCK cells (Jaulin *et al.*, 2007). How MAL interacts with these molecules remains an open-ended question that has to be addressed in future research and incorporated in future models of MAL induced apical transport.

Model for MAL2's regulation of transcytosis

Unlike MAL, MAL2 did not display many of the features we predicted that a protein involved in raft-coalescence should possess. For example, MAL2 did not

distribute to low-density fractions, it does not oligomerize nor did it change the lipid-association of apical proteins. These data suggest that unlike MAL, MAL2 does not cause raft-coalescence or clustering. However, In and Tuma have previously shown that MAL2's subcellular localization is dependent on cholesterol and on cholesterol depletion, MAL2 redistributes to the basolateral plasma membrane (In and Tuma, 2010). Furthermore, upon cholesterol depletion, MAL2 becomes completely soluble in Triton X-100 consistent with change in distribution suggesting that MAL2 is associated with cholesterol-enriched regions. In addition, there is also strong evidence from earlier studies performed in WIF-B cells that transcytotic trafficking is dependent on cholesterol and glycosphingolipids (Nyasae et al, 2003).

How does MAL2 regulate the indirect delivery pathway when it does not share the buoyant density properties with MAL in Triton X 100 gradients? The answer may lie in studies performed in oligodendrocytes where MAL2, despite being completely soluble in buoyant density gradients performed with Triton X-100, floats to detergent insoluble fractions in CHAPS gradients. The differences in MAL2's flotation properties in different detergents suggest that the composition of MAL2's lipid microdomains is different from that of MAL's. Furthermore, MAL2's N-terminal tail has EVH1 domain, which likely facilitates interactions with the actin cytoskeleton (see Fig. 3 in introduction) and contributes to its high levels of insolubility in Triton X-100 detergent extracts.

Therefore, according to my model, MAL2 likely interacts with single TMD and GPI anchored proteins in CHAPS microdomains at the basolateral early endosomes (Fig. 20). It has been shown that the N-terminal region of MAL2 interacts with TPD52- family of proteins and the transmembrane domains interact with mucin I in breast cancer library

screens (Fanyan et al, 2009). Whether MAL2 regulates the transcytotic delivery of apical proteins through these interactions is not known. Recent yeast two-hybrid screens performed using liver library in our lab (In and Tuma, unpublished data) suggests that MAL2 also interacts with Rab17, suggesting that this Rab might be involved in

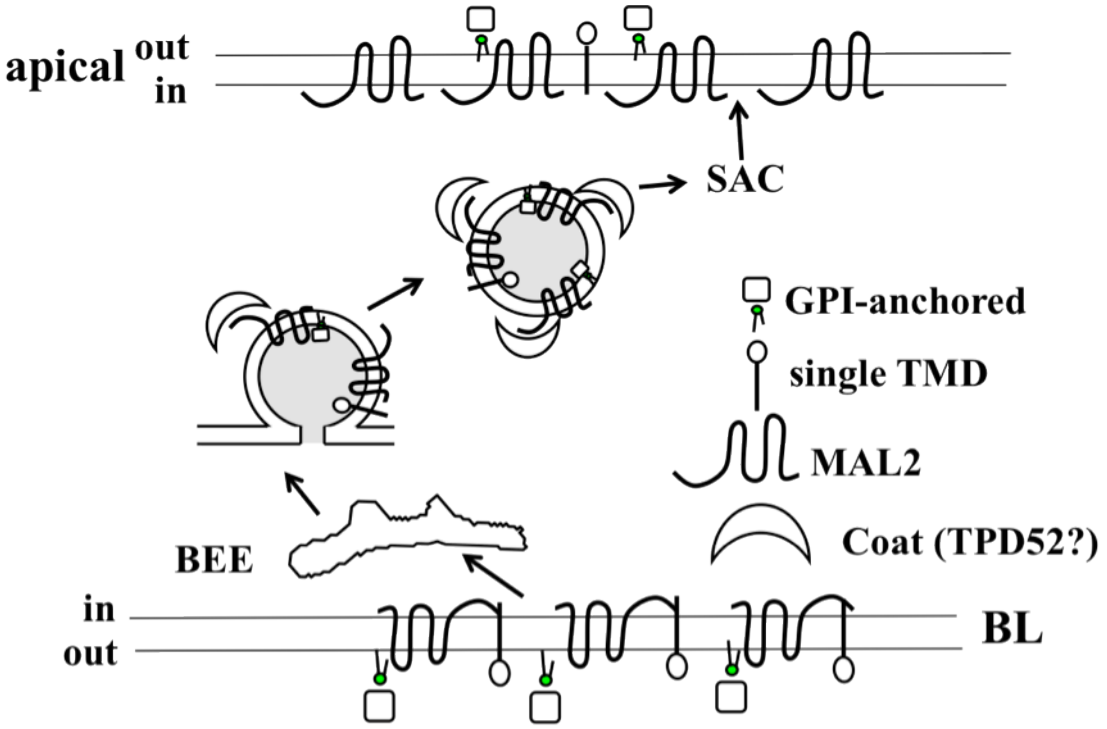

Fig. 20. Model for MAL2's regulation of transcytosis. Single TMD and GPI-anchored proteins associate with MAL2 in cholesterol-enriched domains at the basolateral PM. They are internalized to the basolateral early endosome (BEE). Certain coat proteins and accessory factors are recruited and vesicles formed are delivered to the sub-apical compartment (SAC).

regulating transcytotic delivery with MAL2. Therefore according to our model, MAL interacts with apical proteins in specialized lipid domains at the basolateral early endosomes and with the help of other interacting partners regulates the transcytotic transport of these proteins from the basolateral early endosome (Fig. 20).

Interestingly, In and Tuma have recently shown that MAL2 interacts with pIgA-R which, I confirmed using velocity gradients in this dissertation (In and Tuma, 2010). MAL2 in addition, also specifically regulates the basolateral delivery of pIgA-R (In and Tuma, 2010). Further research needs to be done to determine which region of MAL2 interacts with pIgA-R. MAL2 is thus involved in regulating multiple transport pathways, and its possible involvement in direct transport of polytopic membrane proteins is being actively investigated upon in our laboratory.

The consequences of mis-regulation of apical and basolateral targeting pathways

The transport of newly synthesized proteins is tightly regulated in a normal polarized cell. Cancerous cells are typically characterized by their failure to achieve or maintain polarity. Both MAL and MAL2 regulate multiple trafficking pathways in the cell. Interestingly, both these proteins have been linked with cancer. MAL has been linked with esophageal cancer, gastric cancer, colo-rectal cancer, B cell lymphoma and epithelial ovarian cancer (Copie-Bergman *et al.*, 1999; Xu *et al.*, 1999; Lind *et al.*, 2007; Buffart *et al.*, 2008; Lee *et al.*, 2010). MAL2 interacts with human tumor protein 52- like family members (Shehata *et al,* 2008) and Mucin1 in human breast carcinomas (Wilson *et al.*, 2001; Fanayan *et al.*, 2009). Any imbalance, in the expression of these proteins may lend to defects in protein sorting that can have major deleterious effects on cell function. Therefore, studying how these proteins regulate trafficking might provide insights into the mechanisms contributing to the development of cancer.

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