Exogenous MAL Reroutes Selected Hepatic Apical Proteins into the Direct Pathway in WIF-B Cells[□]

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Unlike simple epithelial cells that directly target newly synthesized glycosphosphatidylinositol (GPI)-anchored and single transmembrane domain (TMD) proteins from the *trans*-Golgi network to the apical membrane, hepatocytes use an indirect pathway: proteins are delivered to the basolateral domain and then selectively internalized and transcytosed to the apical plasma membrane. Myelin and lymphocyte protein (MAL) and MAL2 have been identified as regulators of direct and indirect apical delivery, 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 GPI-anchored and single 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.

INTRODUCTION

The plasma membrane (PM) of polarized epithelial cells is physically continuous but functionally and compositionally divided into two domains: the apical and basolateral. The sorting mechanisms and pathways by which newly synthesized PM proteins achieve their specific yet asymmetric distributions are actively being examined in many polarized epithelial cell types. Newly synthesized apical proteins are delivered to the apical PM by two pathways: direct or indirect. Proteins using the direct pathway are delivered directly from the *trans*-Golgi network (TGN) to the apical PM, whereas proteins using the indirect pathway take a more circuitous route. They are first delivered from the TGN to the basolateral domain where they are retrieved by endocytosis and transcytosed to the apical PM. The factors that specify and regulate which apical targeting pathway is used are not fully understood.

Unlike most simple epithelial cells, hepatocytes use the indirect pathway for apical delivery of newly synthesized

single transmembrane domain (TMD) and glycosphosphatidylinositol (GPI)-anchored proteins (Bartles *et al.*, 1987; Bartles and Hubbard, 1988; Schell *et al.*, 1992; Bastaki *et al.*, 2002; Tuma and Hubbard, 2003). We have previously shown that transcytotic sorting from the early endosome to the subapical compartment (SAC) in WIF-B cells requires cholesterol and glycosphingolipids (Nyasae *et al.*, 2003). In cells depleted of these lipids, efflux from early endosome of *all* apical residents examined and polymeric IgA receptor (pIgA-R) was significantly impaired irrespective of their detergent solubility properties. This indicated that apical residents do not require direct lipid-association for transcytotic sorting. Rather, the lipid-dependent sorting was likely conferred by a general regulator of transcytosis whose activity requires cholesterol and glycosphingolipids. Similarly, cholesterol depletion in Madin-Darby canine kidney (MDCK) cells impaired the direct apical transport of different classes of apical residents and secretory proteins irrespective of their solubility properties (Scheiffele *et al.*, 1997; Keller and Simons, 1998; Prydz and Simons, 2001). Thus, lipid-dependent sorting at the TGN is likely regulated by a general, lipid-associated molecule.

The myelin and lymphocyte protein (MAL) proteolipids are good candidates for mediating lipid-dependent apical sorting. These ~20-kDa tetraspanning membrane proteins are raft associated, and they 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.*, 1999; Puertollano *et al.*, 1999, 2001; Martin-Belmonte *et al.*, 2000, 2001). 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, we consider MAL a general

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Abbreviations used: 5'NT, 5' nucleotidase; APN, aminopeptidase N; BC, bile canaliculus; BFA, brefeldin A; CHX, cycloheximide; DPP IV, dipeptidyl peptidase IV; GPI, glycosphosphatidylinositol; HA, hemagglutinin; MAL, myelin and lymphocyte protein; mβCD, methyl-β-cyclodextrin; MRP2, multidrug resistance-associated protein 2; pIgA-R, polymeric IgA receptor; PM, plasma membrane; SAC, subapical compartment; TMD, transmembrane domain.

regulator of direct apical transport. Although MDCK cells express MAL2, its role in transcytosis remains unclear (Wilson *et al.*, 2001; 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, antisense 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). Interestingly, the block occurred between early endosomes and the SAC, reminiscent of the transcytosis defect we observed in lipid-depleted WIF-B cells (Nyasaie *et al.*, 2003). Thus, lipid depletion may prevent MAL2 from sorting transcytosing proteins at early endosomes and prevent MAL from sorting at the TGN.

Does MAL expression reroute newly synthesized apical proteins into the direct pathway in hepatocytes? To answer this question, we expressed MAL in polarized, hepatic WIF-B cells and examined the distributions of different classes of apical proteins. We 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.

MATERIALS AND METHODS

Reagents and Antibodies

Cycloheximide (CHX), brefeldin A (BFA), Triton X-100, F12 (Coon's modification) medium, and methyl- β -cyclodextrin (m β CD) were purchased from Sigma-Aldrich (St. Louis, MO). CHX was made fresh in 5% ethanol, and m β CD 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 GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom) and Pierce Chemical (Rockford, IL), 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). 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, Virus Production, and Infection

WIF-B cells were grown in a humidified 7% CO₂ 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 1.3×10^4 cells/cm² and grown for 8–12 d until they reached maximum density and polarity.

Recombinant MAL-myc adenoviruses were generated using the Cre-Lox system as described previously (Bastaki *et al.*, 2002). WIF-B cells were infected with recombinant adenovirus particles ($0.7\text{--}1.4 \times 10^{10}$ virus particles/ml) encoding myc-epitope tagged MAL, green fluorescent protein (GFP), or V5/His6 epitope-tagged DPP IV or pIgA-R or untagged HA 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-MAL 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). 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 m β CD 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 $\pm$ SEM.

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 ice-cold lysis buffer (1% [vol/vol] Triton X-100, 150 mM NaCl, and 5 mM EDTA, pH 7.4) containing 1 μg /ml each of antipain, leupeptin, benzamide, and phenylmethylsulfonyl fluoride. The lysates were diluted with an equal volume of lysis buffer containing 80% sucrose and placed in the bottom of a 12-ml centrifuge tube. A 5–30% linear sucrose gradient was poured on top of the extracts, and the tubes were centrifuged in a swinging bucket rotor at $192,000 \times g$ for 16 h at 4°C. One-milliliter fractions were collected from the bottom 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).

RESULTS

MAL Induces Intracellular Populations of Selected Apical Proteins

To determine whether MAL expression altered apical delivery in WIF-B cells, we examined the steady-state distributions of different classes of apical proteins (single TMD, GPI anchored, and multispinning) 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 subapical puncta and basolateral staining of transcytosing proteins were also apparent (Figure 1A). In MAL-expressing cells, numerous tubular structures and diffuse puncta were also observed that contained HA (Figure 1Ab). Approximately 90% of cells expressing MAL contained these intracellular structures (Table 1). Similar redistribution was observed for exogenous DPP IV (Figure 1Ad and Table 1), another single TMD apical protein, and 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.

Figure 1. 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 mark selected BCs. Bar, 10 μ m.

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

Apical protein	% Cells	Treatment	% Cells
HA	92.1 $\pm$ 2.5		87.5 $\pm$ 4.7
DPP IV	89.3 $\pm$ 3.6	BFA	87.0 $\pm$ 8.1
5'NT	69.2 $\pm$ 5.7	CHX	42.3 $\pm$ 7.1
pIgA-R	15.2 $\pm$ 0.2	CHX + m β CD	82.9 $\pm$ 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 tubules and puncta positive for the indicated apical protein. Values are expressed as the mean $\pm$ 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 μ g/ml BFA or for 2 h with 50 μ g/ml CHX alone or in the presence of 1 mM m β CD. Cells were fixed, stained, and scored for HA⁺ structures. Values are expressed as the mean $\pm$ SEM. Measurements were performed on at least three independent experiments.

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 1B and Table 1). Similarly, the distributions of exogenously expressed pIgA-R were not changed by MAL expression (Figure 1B, c and d). In both control and MAL-expressing cells, pIgA-R was present mainly at the apical PM with a small population in subapical 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 1Bf) 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, and then the rafts and their recruited cargo are transported in vesicles directly to the apical domain (Simons and Ikonen, 1997). Because MAL has been shown to be raft associated and to function at the TGN, we tested whether MAL expression induced the formation of biosynthetic, cholesterol- and glycosphingolipid-enriched Golgi domains in WIF-B cells. We 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 2A, a–c). In contrast, the intracellular HA in MAL-overexpressing cells largely colocalized with albumin (Figure 2A, c–f), indicating its presence in the Golgi.

We 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 endoplasmic reticulum (ER) in the presence of BFA (Figure 2Bb). In CHX-treated cells, albumin staining was nearly absent (Figure 2Bc), 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

Figure 2. The MAL-induced intracellular structures are Golgi-derived, biosynthetic intermediates. (A) Cells were infected with recombinant adenoviruses expressing MAL (d–f) and HA (a–c). 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 coinfecting with MAL and HA adenoviruses for 20 h (d–g). Cells were incubated in the absence or presence of 10 μ g/ml BFA for 1 h (b and e) or 50 μ g/ml CHX for 2 h at 37°C (c, f, and g). Cells were stained for albumin (a–c) or HA (d–g). Asterisks mark selected BCs. Bar, 10 μ m.

staining was observed (Figure 2Be), confirming the Golgi localization. Because cells were scored as “negative” or “positive,” no change was observed in the percentage of cells positive for intracellular HA (Table 1). In contrast, there was much less (Figure 2Bg) to no (Figure 2Bf) 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, we treated cells with m β CD for 60 min, conditions that deplete 80% of cholesterol in WIF-B cells (Nyasa *et al.*, 2003). However, in ~85% of cells expressing MAL, HA remained in the Golgi in treated cells (Figure 3Ab). Because direct targeting requires cholesterol (Scheiffele *et al.*, 1997; Keller and Simons, 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

Figure 3. 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 m β CD 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 μ g/ml of CHX alone (b) or in the presence CHX and 1 mM m β CD (c) for 2 h at 37°C. The distributions of HA are shown. Asterisks mark selected BCs.

m β CD, conditions whereby “chase” from the compartment could be monitored. As shown above, CHX treatment decreased intracellular HA staining, indicating that apical delivery occurred (Figure 3Bb). However, in cells also treated with m β CD, the intracellular HA staining remained (Figure 3Bc and Table 1), indicating that apical delivery was impaired and thus cholesterol dependent.

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, we assayed the distributions of apical proteins in control and MAL-expressing cells by using low-density flotations. In control cells, none of APN or DPP IV (single TMD proteins) was found in floated fractions (Figure 4A). Similarly, the basolateral protein CE9 was detected only in the load fractions (Figure 4B). 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 4B). In infected cells, MAL floated just above the load in fractions 4–6 (Figure 4, A 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, 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 4A). 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 4B). 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 4B). 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 (Figure 4C). Also importantly, MAL2 distributions were not changed in MAL-infected cells (unpublished data).

Figure 4. 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. (B) The gradient fractions were immunoblotted for CE9 (marked with an asterisk), 5'NT, or MAL. (C) Flotations from control or MAL-expressing cells were immunoblotted for pIgA-R.

MAL Expression Reroutes Apical Proteins into the Direct Pathway

Our original question asked whether exogenous MAL rerouted hepatic apical proteins into the direct pathway. If so, decreased basolateral delivery is predicted. To test this, we 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

Figure 5. MAL expression decreases basolateral delivery of newly synthesized apical residents, but it 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°C. 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 $\pm$ 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°C. The remaining PM-associated antibodies were eluted, and the cells were 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 $\pm$ SEM. Measurements were done on at least three experiments each performed in duplicate.

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 5A). 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 5A). 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.

We 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 percentage of DPP IV internalized was minimally enhanced (<10%) in MAL-expressing cells (Figure 5B). 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 5C). 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, we colabeled 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 (Figure 6Ab). Trafficked APN was also detected in small puncta (Figure 6Ab) in MAL-expressing cells, but these puncta did not overlap with the intracellular HA (Figure 6Ac), indicating that HA was not in transcytotic intermediates, confirming its Golgi localization.

In our 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 6B). After 90 min, most of the APN was chased to the PM, and no increased intracellular staining was observed in MAL-expressing cells (Figures 6B, e and f). We quantified this by measuring the relative fluorescence intensities of DPP IV or APN at the apical versus 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 versus redirection of apical proteins, we developed a morphological "pulse-chase" analysis where we 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 7, the ratio of apical-to-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

Figure 6. 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°C. 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 mark selected BCs. (B) Basolateral populations of APN were labeled in control (a–c) or MAL-expressing cells (d–f) for 15 min at 4°C, and then chased for 0 (a and d), 45 (b and e) or 90 (c and f) min at 37°C. 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 to basolateral PM fluorescence intensity was determined. For both DPP IV and APN, no changes in transcytosis were observed in MAL-expressing cells.

ratios >100%, indicating successful apical delivery confirming our 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 7C) consistent with the lack of intracellular pIgA-R and unaltered basolateral delivery. In contrast, the ratios for HA and DPP IV

Figure 7. 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 μ g/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 percentage of 0-min values was determined. Values are expressed as the mean $\pm$ SEM. Measurements were done on at least three independent experiments.

apical-to-basolateral fluorescence in MAL-expressing cells did not drop below that seen at 0 min, indicating decreased basolateral delivery (Figure 7, A and C). The ratios steadily increased to $\sim$ 170%, indicating increased apical delivery. Thus, we conclude that HA and DPP IV were directly delivered to the apical PM from the Golgi.

DISCUSSION

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? We 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.

Our 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 apical residents in cholesterol and glycosphingolipid-enriched domains in the TGN, whereas MAL2 encounters apical 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, exoge-

nous 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). We 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-Boulant *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 multispreading proteins may be sorted to the apical PM via these motifs (Fanning and Anderson, 1999). We 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, we suggest that sorting is not directly conferred via cytosolic targeting proteins. Rather, we 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; Tall *et al.*, 2003). 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).

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 pro-

motes raft formation or clustering that is required for apical sorting, a hypothesis we are actively examining.

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