A Bipartite Signal Regulates the Faithful Delivery of Apical Domain Marker Podocalyxin/Gp135[□]

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Podocalyxin/Gp135 was recently demonstrated to participate in the formation of a preapical complex to set up initial polarity in MDCK cells, a function presumably depending on the apical targeting of Gp135. We show that correct apical sorting of Gp135 depends on a bipartite signal composed of an extracellular O-glycosylation-rich region and the intracellular PDZ domain-binding motif. The function of this PDZ-binding motif could be substituted with a fusion construct of Gp135 with Ezrin-binding phosphoprotein 50 (EBP50). In accordance with this observation, EBP50 binds to newly synthesized Gp135 at the Golgi apparatus and facilitates oligomerization and sorting of Gp135 into a clustering complex. A defective connection between Gp135 and EBP50 or EBP50 knockdown results in a delayed exit from the detergent-resistant microdomain, failure of oligomerization, and basolateral missorting of Gp135. Furthermore, the basolaterally missorted EBP50-binding defective mutant of Gp135 was rapidly retrieved via a PKC-dependent mechanism. According to these findings, we propose a model by which a highly negative charged transmembrane protein could be packed into an apical sorting platform with the aid of its cytoplasmic partner EBP50.

INTRODUCTION

Multicellular organisms depend on polarized epithelia to carry out key physiological functions, such as vectorial flows of individual solutes across the renal tubular mucosa cells. To perform these vital functions, the epithelial cells maintain an asymmetrical distribution of lipid and protein components at the plasma membranes in the presence of a myriad of constant endocytic and secretory activities. This protein and lipid polarity was initially demonstrated to be achieved in the *trans*-Golgi network (TGN) complex where biomembrane components are sorted into different vesicles for apical or basolateral delivery (Griffiths and Simons, 1986), but accumulated evidences support a shifted paradigm that sorting may occur practically at every step of the biosynthetic pathway, either proximally or distally to the TGN (Rodriguez-Boulant and Musch, 2005). Although various signals have been linked to the targeting codes for protein delivery, a great deal of the mechanistic details about the sorting of membranous proteins are still not clear. The basolateral sorting signals described to date reside in the cytoplasmic domains, and in several cases the signals bear similarity to the tyrosine or di-leucine motif found in the endocytic proteins (Hunziker and Fumey, 1994; Matter *et al.*, 1994). In contrast to the more conserved nature of basolateral sorting

signals, apical signals appear to be rather diverse. These include N- and O-glycans found in many apical resident and secretory proteins (Scheiffele *et al.*, 1995; Yeaman *et al.*, 1997) and the small glycolipid moiety found in glycosphosphatidylinositol (GPI)-anchored proteins (GPI-APs; Brown *et al.*, 1989; Lisanti *et al.*, 1989). These signal codes, often found on the luminal side of the transported proteins, have been postulated to provide proteins a partition access to the sphingolipid-cholesterol rafts whose destiny is the apical domain. Recently, it has been recognized that lipid raft association might be required, but not sufficient for apical sorting. Protein oligomerization, for example, is demonstrated to be an essential secondary step for GPI-APs to be sent to apical domain (Hannan *et al.*, 1993; Paladino *et al.*, 2004). However, similar scenarios have so far not yet been demonstrated in apically sorted proteins other than GPI-APs.

Podocalyxin (PC) is a member of the CD34/endoglycan/PC sialomucin family proteins. It is expressed at the luminal surface of the highly specialized renal glomerular podocytes and is supposed to provide a charge barrier function to govern the filtration of plasma solutes into urinary flow (Kerjaschki *et al.*, 1984). This electric repulsive nature presumably results from the addition of the highly negatively charged sialic acid onto the complex glycosylation in PC (Kerjaschki *et al.*, 1985). We and others recently identified the canine homologue of PC as the apical domain marker protein Gp135 and demonstrated its expression on apical surface of canine renal tubular segments as well as in Madin-Darby canine kidney (MDCK) cells (Cheng *et al.*, 2005; Meder *et al.*, 2005). It has been demonstrated that Gp135 and sodium hydrogen exchanger regulatory factor 2 participate in the formation of a preapical complex (Meder *et al.*, 2005), a function presumably depending on the apical targeting of

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Gp135. Furthermore, knockdown of Gp135 expression in MDCK cells inhibits hepatocyte growth factor/scattering factor-induced tubulogenesis (Cheng *et al.*, 2005). Although Gp135/PC has been recognized as the hallmark of apical surface for decades, the apical sorting signal and the mechanism by which it is specifically delivered to the apical domain in polarized MDCK cells have never been characterized. In this article, we characterize the apical targeting signal of Gp135, and based on the results, we provide a model how both glycosylation and EBP50-mediated clustering contribute to apical delivery of Gp135.

MATERIALS AND METHODS

Cell Culture and Transfection

MDCK type II cells were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in a humidified incubator containing 5% CO₂. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All the Gp135-related constructs were transfected into wild-type MDCK cells. The transfected cells were selected in 700 µg/ml G-418 (Invitrogen), and all the trafficking assays were analyzed in at least three independently isolated cell clones.

Construction of Expression Vectors

Plasmids expressing various Myc-Gp135 constructs were generated by standard molecular biology techniques. All Myc-tagged Gp135-related constructs were derived from pcDNA3.1(+) (Invitrogen). The Flag-tagged EBP50 construct was derived from pCMV-2A (Stratagene, La Jolla, CA). The EGFP-Gp135 and EGFP-Gp135dC fusion constructs were derived from pcDNA3.1(+). The site-directed mutations of the N-glycosylation sites were made by using the QuickChange kit (Stratagene). All constructs had been verified by automatic nucleotide sequencing. The verified sequence information of all the constructs can be found at http://w3.mc.ntu.edu.tw/department/clinmed/new_page_3.htm.

EBP50 Knockdown by Small Interfering RNA Approach

Chemically modified RNA oligonucleotides (Stealth, Invitrogen) targeting at canine EBP50 (siE50-1, 5' GAGACUGAUGAGUUCUUAAGAAAAU 3') and Ezrin (siEzrin, 5' UGGCCUCCAGUAUGTGAUAAUAAA 3') were designed and generated, and transfected into MDCK cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Antibodies and Chemicals

Hybridoma cells secreting anti-Gp135 (3F2/D8) and tight junction marker ZO-1 (R40.76) monoclonal antibodies (mAb) were gifts from Dr. George Ojokian and Dr. Dan Goodenough, respectively. Mouse anti-CD7 antibody and rabbit anti-Myc antibody (A14) were from Santa Cruz Biotechnology, (Santa Cruz, CA). Mouse anti-FLAG mAb (M2), rabbit polyclonal anti-FLAG antibody, and mouse anti-Actin mAb were from Sigma-Aldrich (St. Louis, MO). Anti-Myc hybridoma cells (9E10) were from ATCC (Manassas, VA). Mouse anti-p230 *trans*-Golgi antibody was from BD-Transduction Laboratories (Lexington, KY). Rabbit anti-EBP50 polyclonal and mouse anti-Ezrin monoclonal antibodies were from Abcam (Cambridge, MA) and Lab Vision (Fremont, CA), respectively. PKC inhibitor Gö6976 and endoglycosidase-H were purchased from Calbiochem (La Jolla, CA). Cyclohexamide (CHX) was from Sigma.

Immunofluorescence, Immunoprecipitation, Western Blotting Analysis, and Image Acquisition

These procedures were performed as previously described (Cheng *et al.*, 2005).

Steady State Surface Biotinylation

MDCK cells stably expressing mutant proteins were cultured on Transwells filters (Costar, Cambridge, MA) for 4 d for polarization. Transepithelial electric resistance was measured each time before performing the experiments to confirm the tightness of the monolayer, and only samples having resistance larger than 250 Ω/cm² were used for the biotinylation study. Filters were washed three times with cold Ringer's buffer (10 mM HEPES, pH 7.4; 154 mM NaCl; 7.2 mM KCl; 1.8 mM CaCl₂). Sulfo-NHS-biotin (200 µg/ml; Pierce, Rockford, IL) in Ringer's buffer was applied to either apical (0.4 ml) or basolateral (0.8 ml) chambers. Filters were incubated on ice for 30 min on a rocker platform. Excess biotin was quenched by washing cells in five changes of Tris-saline (10 mM Tris-HCl, pH 7.4, 120 mM NaCl). The cells were then extracted in CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, and 0.5% [vol/vol] Triton X-100) for 10 min at 4°C and prepared

for immunoprecipitation and Western blotting. Blots were incubated with horse radish peroxidase (HRP)-conjugated streptavidin solution (1:200, R&D Systems, Minneapolis, MN) and processed for electrochemiluminescence. The fluorography signals were quantified using ImageGauge V3.41 (Fujifilm, Tokyo, Japan).

Metabolic Labeling and Protein-trafficking Assays

Arrival of Newly Synthesized Proteins at the Target Membrane. Polarized MDCK cells on 24-mm Transwell filters (Costar) were starved of Met/Cys for 16 h, then labeled with 150 µCi ³⁵S-Met/Cys (New England Nuclear, Boston, MA) for 30 min, and chased for the indicated time specified in the individual experiments. Domain-specific biotinylation was performed as described in the subsection *Steady State Surface Biotinylation*, except sulfo-NHS-S-S-biotin (Pierce) was used. Labeled cells were processed as described in the subsection *Immunoprecipitation*, and the bound proteins were released from beads by incubating with acidic elution buffer (0.2 M glycine, pH 2.6, 1% TX-100) for 25 min at room temperature. The eluted proteins were recaptured by immobilized avidin gel (Pierce) at 4°C for 16 h. The recaptured proteins were washed with PBS, denatured by boiling in SDS sample buffer, separated by SDS-PAGE, and processed for fluorography using a phosphorimager (BAS-1000; Fujifilm).

Plasma Membrane Stability. MDCK cells stably expressing Gp135 mutant proteins were prepared as those for "arrival of first wave proteins at the target membrane" experiment, except the metabolic labeling was extended for 2 h. After quenching free biotin, cells were incubated at 37°C to resume endocytosis. The monolayer was then extracted after indicated times, immunoprecipitated, and recaptured by avidin gel as described above.

Postsecretory Fate Study of the Basolaterally Missorted m135dC Mutant.

MDCK cells stably expressing the mutant protein were prepared as those for the steady state surface biotinylation experiments, except the cells were incubated at 37°C for 2 h with 2 µM Gö6976 to accumulate the mutant protein at the basolateral surface before surface biotinylation. After the biotinylated proteins were released from the basolateral surface for the indicated time period, the transcytosed and the basolaterally retained m135dC mutants were recovered by surface immunoprecipitation from the apical and basolateral surfaces, respectively, in 50 µg/ml anti-Myc antibody on ice for 2 h. The endocytosed m135dC was recovered by immunoprecipitation of the cell extracts after applying trypsin (0.00625%) to the monolayer and incubated at 4°C for 1 h to remove the surface-associated proteins. In the meantime, the degradation of basolateral m135dC was estimated by calculating the recovery of total immunoprecipitated proteins at each time point. It is worth noting that our assay included immunoprecipitation of the medium from either the apical or basolateral compartment to examine the possibility that the m135dC protein is cleaved at its extracellular domain and shed into the culture medium. However, we never detected any m135dC protein in the medium (unpublished data), and therefore we have omitted these data in the final presentation.

Lipid Rafts Floatation. Confluent MDCK cells grown on 100-mm dishes were lysed for 20 min on ice in 2 ml of TNE buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% TX-100 and passed five times through a 23-gauge needle. The 2 ml lysate was mixed with 2 ml 80% sucrose in TNE and placed at the bottom of a centrifuge tube. A discontinuous sucrose gradient (5–30% in TNE) was layered on top of the lysates, and the samples were centrifuged at 39,000 rpm for 18 h in an ultracentrifuge (SW41; Beckman Coulter, Fullerton, CA). One-milliliter fractions were harvested from the gradient and TCA precipitated. The precipitated proteins were processed for Western blotting analysis to reveal the distribution profiles of each Gp135 mutant proteins. To reveal the distribution of ganglioside GM1 in the gradient, 30 µl of each fraction was spotted on nitrocellulose membrane and detected with HRP-conjugated cholera toxin B subunit (Sigma-Aldrich). For pulse-chase experiments, confluent MDCK cells on 100-mm dishes were starved of Met/Cys for 16 h, pulse-labeled with 200 µCi ³⁵S-Met/Cys (NEN) for 10 min, and incubated with chase medium for the indicated time. Fractions of 1.5 ml were harvested from the top of the gradient and immunoprecipitated with anti-Gp135 cytoplasmic domain pAb (wild-type MDCK cells) or anti-Myc (mutant Gp135-expressing cells) mAb for detecting the fully mature and underglycosylated Gp135. The 3F2/D8 mAb was not used in this experiment because it could only detect the fully glycosylated protein (Cheng *et al.*, 2005).

Cross-Linking. BS3 (1 mM; Pierce) in Dulbecco's PBS buffer was added to confluent MDCK cells expressing Myc-tagged Gp135 grown on Transwells for 2 h on ice and quenched in 20 mM Tris, pH 7.5, for 20 min. The domain-specific surface proteins were further labeled with biotin as described in steady state surface biotinylation. The target proteins were immunoprecipitated with Myc antibody, separated on SDS-PAGE, and revealed by HRP-conjugated streptavidin.

m135.7) or anti-CD7 (CD7 and CD7.135) mAb. The percentage of each newly synthesized chimera targeted to the apical domain was shown as a bar histogram (light blue, 15-min chasing; deep blue, 30-min chasing). The values are shown as the means and the SEs of three samples. A representative fluorogram was shown beside the histograms. 15(A/B)/30(A/B), 15 min/30-min chasing before apical or basolateral biotinylation.

RESULTS

Both Extracellular and Intracellular Domains of Gp135 Contain Apical Targeting Signals

To identify the apical sorting determinants of Gp135, we started with constructing various domain swapping mutants of Gp135 and CD7. Because CD7 was previously reported to display a nonpolarized membranous expression pattern in MDCK cells (Haller and Alper, 1993), we reasoned that the steady state distribution of these chimeras in polarized MDCK cells would reveal the apical sorting signals of Gp135. Indeed, steady state biotinylation and confocal section imaging confirmed that CD7 was evenly distributed at the apical and the basolateral domains (Figure 1A). Further analysis revealed substitution of the extracellular or the intracellular domain of Gp135 with those domains of CD7 resulted in chimeras that still predominantly reside at the apical domain (m135.7 or CD7.135 in Figure 1A). To determine the influence of the extracellular and intracellular domains on the apical sorting of newly synthesized Gp135, we monitored surface delivery of these chimeric proteins using a biotin targeting assay (Le Bivic *et al.*, 1989). In contrast to the steady state nonpolarized distribution, the metabolically labeled CD7 proteins were preferentially targeted to basolateral domain (Figure 1B). A previous report showed a similar finding (Chuang and Sung, 1998). Despite the basolateral targeting tendency of CD7, exchange of either the extracellular or intracellular domains of CD7 and Gp135 resulted in increased apical surface delivery of the newly synthesized chimeric proteins (~60% and ~50% of the metabolically labeled m135.7 and CD7.135 chimeric proteins arrived at the apical surface respectively; Figure 1B). These findings indicate that both extracellular and intracellular domains of Gp135 harbor an apical sorting signal.

Figure 1. Both extracellular and intracellular domains of Gp135 confer nonpolarized CD7 with an apical targeting signal. (A) Domain specific steady state distribution of Gp135/CD7 chimeric proteins. Left, the diagram shows schematically the structural features of the domain swapping constructs. m, Myc epitope tag; Ex, extracellular domain; TMC, transmembrane and cytosolic domains. ■, Myc tag; □, Gp135; empty ellipse, CD7. Middle, steady state surface biotinylation, immunoprecipitation, SDS-PAGE separation, nitrocellulose filter transferring, and HRP-avidin hybridization were performed as described in *Materials and Methods*. A representative fluorography was shown. AP, apical; BL, basolateral. The fluorographic signals were quantified by ImageGauge software program. The percentage of each chimera distributed at the apical domain was analyzed by quantifying the fluorographic signals and is shown as the mean of a triplicate experiment. Right, the representative confocal X-Z section images of the Myc-tagged Gp135/CD7 chimeric proteins. Green, anti-Myc (m135 and m135.7) or anti-CD7 (CD7 and CD7.135) staining; red, staining for tight junction marker ZO-1, and propidium iodide, staining for nuclei. Bar, 10 μ m (B) The plasma membrane delivery of newly synthesized Gp135/CD7 chimeric proteins was examined as described in *Materials and Methods*. The immunoprecipitation was performed using anti-Myc (m135 and

The O-Glycan-rich Region of the Extracellular Domain Contains an Apical Sorting Signal for Gp135

Given that both N- and O-glycans have been proposed as apical targeting signals (Scheiffele *et al.*, 1995; Yeaman *et al.*, 1997) and that Gp135 is heavily glycosylated with two thirds of its apparent molecular weight on SDS-PAGE coming from posttranslational modifications, we determined to find out the contribution of glycosylation on apical targeting of Gp135. Sequence analysis (Eukaryotic Linear Motif resource, <http://elm.eu.org/>) revealed four potential N-glycosylation sites (NXS/T as the predicted N-glycosylation motif) in the extracellular portion of Gp135. We used site-directed mutagenesis to convert the asparagine at each potential N-glycosylation site to a glutamine residue. Only mutation of amino acids 199, 373, and 383 resulted in a decrease of the apparent molecular weights for both the mature and underglycosylated products (Supplementary Figure S1). Therefore, we concluded that only asparagines at these three sites could be N-glycosylated *in vivo*. We studied the contribution of each N-glycosylation on the apical surface distribution of Gp135 and found none of these single N-glycosylation mutations compromised its apical distribution (unpublished data). To exclude the possibility that these N-glycosylated residues could work in a redundant way to specify apical delivery of Gp135, we converted all three asparagines at the N-glycosylation sites to the homologous glutamine residues. This triple N-glycosylation defective mutant (NQ mutant in Figure 2) was also exclusively expressed at the apical surface of polarized MDCK monolayer similar to the wild-type protein (m135 in Figure 1) implying that N-glycosylation does not play a significant role in the polarity of Gp135. Besides N-glycosylation, heavy O-glycosylation and a juxta-membranous globular domain are two prominent features in the extracellular domain of Gp135. We then created several deletion mutants spanning these domains and examined both their steady state surface distri-

Figure 2. Deletion of the O-glycosylated domain disrupts the apical sorting of Gp135. (A) Left, the schematic diagram of Myc epitope (m)-tagged wild-type Gp135, triple N-glycosylation mutant, and four extracellular domain deletion constructs. M, Myc tag; TM, transmembrane domain; trident sign, the N-glycosylated sites; pink color region, a predicted membrane-distal O-glycosylation region; red color region, a predicted membrane-proximal O-glycosylation rich region; G, membrane proximal globular domain; orange color region, the PDZ-domain-binding motif. Middle, steady state distribution of Gp135 extracellularly mutated proteins. MDCK clones expressing the various mutant constructs were stably selected. The experiments were carried out and the results were presented as described in Figure 1A. Only the dN122 mutant protein was significantly located at the basolateral membrane. Right, the representative confocal X-Z section images of the Myc-tagged mutant Gp135 proteins were shown. Green channel, anti-Myc staining; red, staining for tight junction marker ZO1, and propidium iodide, staining for nuclei. Bar, 10 μ m. (B) Plasma membrane delivery of newly synthesized extracellularly deleted Gp135 proteins. The experiments were performed, and the results were presented as described in Figure 1B.

bution and the targeting of the newly synthesized proteins. Deletion of the juxta-membranous globular domain did not affect either the delivery of newly synthesized protein or the steady state distribution of the protein at the apical surface (dN342 mutant in Figure 2). Similarly, deletion of the first 50 amino acids after signal recognition peptide, which contained a sialic acid modification and the antigenic epitope for the 3F2/D8 mAb used for the initial identification, characterization, and biochemical purification of Gp135 (Cheng *et al.*, 2005), did not affect the apical polarity of Gp135 (dN121 mutant in Figure 2). With the aid of bioinformatics analysis (<http://www.cbs.dtu.dk/services/NetOGlyc/>), numerous potential O-glycosylation sites (Supplementary Figure S2) were predicted in the extracellular domain of Gp135, which are mainly clustered in a region distal to the globular domain (red-color region in Figure 2). Deletion of a large portion of the heavily O-glycosylated region affected neither the steady state distribution nor the apical targeting of newly synthesized protein (dOL mutant in Figure 2, A and B). Besides the juxta-globular domain region, there is a membrane distal region which is also predicted to be O-glycosylated (pink-color region in Figure 2). When both O-glycosylation-rich regions were deleted, both the steady state and newly synthesized mutant proteins were found to be missorted (dN122 mutant in Figure 2). Interestingly, although the dN122 mutant showed defects in apical sorting, deleting only the membrane-distal O-glycosylation region did not display any mislocalization (dN121 mutant in Figure 2). The results from deletion mutants covering the O-glycosylation region imply the membrane-distal and proximal O-glycosylation regions seem to function in a compensatory way, and only deletion of both could affect the fidelity of Gp135 sorting. An attempt to make longer deletion of O-glycosylation region was hampered by the fact that the mutant protein accumulated in an undefined endosomal compartment (unpublished data). Nevertheless, these results demonstrate that the O-glycosylated extracellular do-

main, but not the N-glycosylated residues, contains apical signals for Gp135.

The PDZ-binding Motif in the Cytoplasmic Tail Contains an Apical Sorting Signal for Gp135

Although O-glycosylation is important for the apical delivery of gp135, it is clear from our data that it is not the only determinant for correct delivery. Therefore we have examined the role of cytoplasmic domain of Gp135 in apical sorting. The cytoplasmic domain of Gp135 is highly conserved among different species and contains a PDZ-domain-binding motif (DTHL) at its C-terminal end, which serves as the binding site for Ezrin-binding phosphoprotein 50 (EBP50, also known as NHERF1, sodium hydrogen exchanger regulatory factor 1; Orlando *et al.*, 2001). To analyze the role of EBP50 binding in apical sorting of Gp135, we generated a PDZ-domain-binding motif deleted mutant (m135dC mutant in Figure 3A). This mutant, however, was perfectly located at the apical domain (Figure 3A). In contrast, 20% of a mutant depleted of most of the cytoplasmic domain localized to the basolateral domain (m135sC mutant in Figure 3A). However, when we metabolically labeled MDCK stable clones expressing m135dC and m135sC and followed the arrival of newly synthesized proteins at the plasma membrane by domain specific biotinylation and immunoprecipitation, both mutants showed a similar degree of mistargeting to the basolateral domain (Figure 3B). This result shows that the PDZ-domain-binding motif in the cytoplasmic tail of Gp135 contains an apical sorting signal.

The importance of PDZ-domain-binding motif in guiding the newly synthesized Gp135 to the apical domain is especially evident for the underglycosylated m135dC protein. Although we could not detect any immature m135 proteins by surface biotinylation and immunoprecipitating metabolically labeled sample (left panel in Figure 3C), fast migrating signals were noticeably found in a similarly processed m135dC sample (right panel in Figure 3C). These proteins

exhibited the characteristic triplet pattern and comparable molecular weight of N-glycosylated Gp135 on SDS-polyacrylamide gels (Supplementary Figure S1). In contrast to the fully glycosylated m135dC proteins, 80% of which was apically sorted, the underglycosylated m135dC proteins were equally targeted to apical and basolateral domains underlying the importance of the PDZ-binding motif when the O-glycan dependent apical sorting signal was absent. This result suggests that O-glycosylated, but not the N-glycosylated, signals in the luminal domain of Gp135 regulate efficient apical sorting of Gp135. Having identified the O-glycosylated region and the PDZ-binding motif as the apical sorting signals contained in the extracellular and intracellular domains of Gp135 respectively, we set up to create double mutants in order to determine the interaction of these two signals in directing the apical targeting of Gp135. A MDCK cell line stably expressing a Myc-tagged mutant protein containing both deletions of the O-glycosylation-rich region and the C-terminal PDZ-binding motif was found to be 80% confined to the apical domain at steady state (dN122dC in Figure 3A). Another mutant possessing deletions at the O-glycosylation-rich region and most of the cytoplasmic domain was evenly distributed to the apical and basolateral domains (dN122sC in Figure 3A). The nonpolarized expression pattern of this latter mutant implies the effects of the extracellular and intracellular deletions on the steady distribution of Gp135 are additive because only 20% of the steady state the dN122 and m135sC mutant proteins are missorted to basolateral domains respectively (Figures 2 and 3). In agreement with the independent effect of both

Figure 3. Deletion of PDZ-binding motif disrupts an apical sorting signal of Gp135, which operates independently with the extracellular sorting signal. (A) Left, the schematic diagram shows Myc epitope (m)-tagged cytoplasmic domain deleted constructs; m135dC, the C-terminal PDZ domain binding motif was deleted; m135sC, all cytoplasmic domain but the five juxta-membranous amino acids (*) was deleted; dN122dC, double mutant composed of dN122- and m135dC-type deletion; dN122sC, double mutant composed of dN122- and m135sC-type deletion. Middle, steady state surface biotinylation was performed, and the results were presented as described in Figure 1A. Right, the confocal X-Z section images of the cytoplasmic domain mutant expressing stable MDCK clones are shown. Green channel, anti-Myc staining; red, staining for tight junction marker ZO1; and propidium iodide, staining for nuclei. Bar, 10 μ m. (B) Plasma membrane delivery of newly synthesized Gp135 cytoplasmic domain-deleted mutant proteins. The experiments were performed, and the results were presented as described in Figure 1B. (C) Apical targeting assays were performed as described in Figure 1B for m135 and m135dC, except full-size fluorography was shown to reveal the targeting behaviors of fully glycosylated and underglycosylated m135dC proteins. The arrowheads and arrows denote the positions of the fully glycosylated and underglycosylated proteins, respectively. Molecular weight standards are indicated in kilodaltons.

deletions on the steady state proteins distribution, targeting of metabolically labeled dN122dC and dN122sC was affected also by the accumulative effects from both the extracellular and intracellular mutations as these proteins were delivered nearly equally to both cellular domains (Figure 3B), whereas individual mutation could only account for ~20% missorting of the newly synthesized proteins (Figures 2 and 3).

Because ezrin was recently found to bind directly to the proximal region of the cytoplasmic domain of Gp135/PC (Schmieder *et al.*, 2004), we next examined whether the m135dC mutant harbors a cryptic binding site for EBP50. This is however not the case. Both in vitro pulldown (Supplementary Figure S3A) and in vivo coimmunoprecipitation assay (Supplementary Figure S3B) exclude a secondary binding site in dC mutant for EBP50. Therefore, the distinct steady state expression patterns between m135dC and m135sC must be due to an EBP50-independent sequence information residing in the remaining part of the cytoplasmic domain.

The Basolaterally Mistargeted m135dC Mutant Was Rapidly Endocytosed in a PKC-dependent Manner

To address the discrepancy in the steady state distribution of the m135dC and m135sC, we then examined the stability of S35-labeled membranous protein by using the membrane-impermeable and reducible biotinylating agent, sulfo-NHS-S-S-biotin. The half-life of the apical Gp135 was estimated to be ~2 h by this assay, and the apically located m135dC, m135sC, and dN122 mutants all had similar turnover rates

Figure 4. Basolaterally missorted mutants of Gp135 are rapidly endocytosed in a PKC-dependent manner. (A) To examine the membranous retention kinetics for the basolaterally mistargeted Gp135 mutant proteins, MDCK cells stably expressing the mutant proteins were processed as described in Figure 1B except the polarized cells were labeled with ^{35}S -Met/Cys for 2 h and then chased for 0, 1, or 2 h before domain-specific surface biotinylation to allow the fate of the saturationally labeled membranous proteins to be tracked. The percentage of each metabolically labeled mutant protein remained at the apical (AP) or basolateral (BL) domain was shown. The values shown are the means and the SEs of three samples. (B) The basolaterally mistargeted m135dC mutant protein is endocytosed in a PKC-dependent way. MDCK cells stably expressing m135dC mutant protein were cultured on Transwells and allowed for polarization. The polarized cells were pretreated for 2 h at 37°C with DMSO or PKC inhibitor Gö6976 and then incubated with rabbit anti-MYC antibody from the basolateral compartment for 2 h at 4°C with DMSO or PKC inhibitor Gö6976. After the unbound antibodies were washed off, the cells were warmed up to 37°C in the absence of PKC inhibitor for the indicated time period to resume endocytic activities. The cells were then processed for immunofluorescence and confocal microscopic examination. The early endosomal marker EEA1 was also stained (red), and the rabbit anti-MYC antibody was labeled with FITC-conjugated secondary antibody (green). The focal section was selected through the middle of the nucleus. Bar, 5 μm . (C) MDCK cells stably expressing m135dC mutant proteins were processed as described in *Postsecretory Fate Study of the Basolaterally Missorted m135dC Mutant*. After extensive quenching, the biotinylated proteins were released from the basolateral surface for the indicated time periods, the apically transcytosed (AP) and the basolaterally retained (BL) m135dC mutants were recovered by surface immunoprecipitation from the apical and basolateral surfaces, respectively. The endocytosed (Endo) m135dC was recovered by immunoprecipitation of the cell extracts after applying diluted trypsin to monolayer at 4°C for 1 h to strip the surface-associated proteins. In the meantime, the degradation of basolateral m135dC was estimated by calculating the recovery of m135dC proteins from total lysates (Total) at each time point. This experiment was repeated five times, and a representative result is shown. The fluorographic intensity of each signal was scanned and plotted as the percentage of the total recovered protein at 0 min. The values were the means and SEs of three samples.

(Figure 4A). The mistargeted m135sC and dN122 also displayed a comparable stability at the basolateral domain. Intriguingly, the metabolically labeled m135dC disappeared rapidly from the basolateral surface ($T_{1/2} \sim 30$ min, Figure 4A). Confocal microscopic analysis of m135dC-expressing cells showed the presence of punctate staining in the cytosol, reminiscent of endosome-like structures (Supplementary Figure S4). These small cytosolic punctate structures seen in m135dC were reminiscent of the endocytosed basolateral domain marker, E-cadherin, which has been demonstrated to be endocytosed in a PKC-dependent pathway (Le *et al.*, 2002). We then set up to examine whether similar mechanism also operates on the basolaterally missorted m135dC. Although antibody surface labeling at 4°C could not detect any mistargeted m135dC at the basolateral surface in DMSO-pretreated cells, there were pronounced signals after anti-Myc antibody labeling in the presence of Gö6976, an efficient pharmacological conventional PKC inhibitor (0' in Figure 4B). The effect of Gö6976 was rather transient as the basolaterally retained antibody rapidly disappeared and the cytosolic puncta, which showed significant colocalization with the early endosomal marker EEA1, accumulated quickly after the cells were warmed to 37°C for only 15 min in the absence of Gö6976 to resume endocytosis (15 min in Figure 4B). After 30 min at 37°C, most of the punctate signals disappeared in the m135dC mutant, pre-

sumably due to degradation in the lysosomal pathway (30 min in Figure 4B). These results indicate the basolaterally mistargeted m135dC mutant proteins are endocytosed in a PKC-dependent process and that surface antibody labeling is an effective way to trace the fate of the endocytosed proteins. To quantify the fraction of the basolateral m135dC mutant getting transcytosed versus degraded, we followed the fate of the endocytosed mutant by a biochemical assay. Basolaterally missorted m135dC mutants were accumulated first with Gö6976 and biotinylated with the nonreducible sulfo-NHS-biotin as described in *Materials and Methods*. The results showed that the basolaterally missorted m135dC proteins disappeared from the basolateral surface rapidly (BL in Figure 4C), similar to the result as disclosed by using the reducible biotinylating agent, sulfo-NHS-S-S-biotin (Figure 4A). Although there was a trace amount of endocytosed proteins being detected, we could not detect any apical transcytotic event. Taken together, we conclude that the missorted m135dC was rapidly removed from the basolateral surface by a PKC-dependent endocytosis signal in the cytosolic tail of m135dC, which is missing in m135sC, and that these endocytosed proteins were efficiently degraded before they reached the apical domain. The nature of this sequence is now under investigation in our laboratory.

loading control; triplicated experiments). The experiments presented in B and C were performed, and the results are presented as described in Figure 1B.

Fusion of EBP50 to the m135dC Mutant Restored the Apical Sorting Signal

We demonstrated that m135dC mutant does not bind EBP50 (Supplementary Figure S3) and as a consequence cannot be sorted correctly to the apical membrane (Figure 3). To confirm the role of EBP50 in the apical sorting of Gp135, we generated a fusion protein composed of the m135dC and canine EBP50 (m135dCE50 in Figure 5). Both the steady state and newly synthesized m135dCE50 were targeted to the apical domain normally as the endogenous Gp135/PC protein (Figure 5, A and B). These results confirm that the interaction through EBP50 is the missing information in m135dC for accurate apical sorting. Interestingly, the fusion of EBP50 could only partially rescue the missorting of dN122dC (dN122dCE50 in Figure 5), indicating again that the extracellular and the intracellular sorting signals of Gp135 apparently operate in an independent manner. EBP50 has two tandem PDZ domains followed by an Ezrin-binding domain in its C-terminal end that mediates actin cytoskeleton connection (Bretscher *et al.*, 1997; Reczek *et al.*, 1997). To examine whether the Ezrin and actin cytoskeleton have any effect on the apical sorting of Gp135, we truncated the Ezrin-binding domain in m135dCE50 (m135dCdEB in Figure 5) and followed both the steady state distribution (Figure 5A) and the apical sorting of newly synthesized protein (Figure 5B). The targeting assay shows that the newly synthesized m135dCdEB is predominantly apically sorted. To test the role of the PDZ domains of EBP50 in apical sorting of Gp135, we deleted the two PDZ domains and fused the remaining Ezrin binding domain to the m135dC mutant (m135dCEB in Figure 5). This chimeric protein was missorted to the basolateral surface, indicating the importance of EBP50 PDZ

Figure 5. The missorted m135dC protein is completely rescued by fusion with the PDZ domains of EBP50. (A) Left, the diagram shows m135dC and dN122dC fused with the PDZ-domain-containing protein EBP50 (blue box). EBP50dEB, the Ezrin/Radixin/Moesin-binding domain (EB) was deleted from EBP50. m135dCPDZ1, m135dCPDZ2, and m135dCEB are chimeric proteins with the m135dC mutant fused with the first PDZ domain, the second PDZ domain, and the Ezrin binding domain of EBP50, respectively. Middle, the percentage of each chimera distributed at the apical domain was shown, which was the mean of a triplicate experiment. A representative fluorography was shown. Right, the confocal X-Z section images of the Myc-tagged EBP50 chimeras are shown. Green channel, anti-Myc staining; red, staining for tight junction marker ZO-1; and propidium iodide, staining for nuclei. Bar, 10 μ m. (B) Plasma membrane delivery of newly synthesized m135dC/EBP50 chimeric proteins. Please note that fusion with EBP50 cannot completely rescue the missorting of dN122dCE50, and fusion with the Ezrin-binding domain alone cannot correct the missorting of m135dC. (C) The sorting of newly synthesized m135 prototypic protein (bottom panel) was also analyzed in the condition when Ezrin was knocked down by siRNA approach (top panel, siEzrin). This approach resulted in significant down-regulation of ezrin ($10.0 \pm 4.6\%$ of the Ezrin level in mock-transfected cells after normalization by the actin

domains in the apical sorting of Gp135 (Figure 5B). To further characterize the involvement of Ezrin in the apical delivery of Gp135, we analyzed the apical sorting of the full-length m135 protein under the condition when endogenous Ezrin was knock-downed to $\sim 10\%$ of the control situation (siEzrin, Figure 5C). The result showed the m135 protein was still apically delivered. Taken together, these data suggest that Ezrin and the actin cytoskeleton may not play a significant role in Gp135 apical sorting. To test the role of the individual PDZ domain of EBP50 in regulating the apical sorting of Gp135, we generated additional stable clones expressing m135dC fused to either the first or second PDZ domain of EBP50 (m135dCPDZ1 and m135dCPDZ2 in Figure 5A). Studies of the delivery of the newly synthesized protein to the surface showed that these two mutants were perfectly sorted to the apical domain (Figure 5B). This result indicates that the two PDZ domains in EBP50 perform a redundant role in regulating the apical sorting of Gp135.

EBP50 Binds to Newly Synthesized Gp135

EBP50 has been postulated as a linker protein providing cell surface podocalyxin molecules a connection to the subcortical cytoskeleton through Ezrin (Li *et al.*, 2002). This scenario does not implicate a functional role of EBP50 in the sorting of newly synthesized podocalyxin at the TGN level. On the basis of the evidence that EBP50 regulates the apical sorting of newly synthesized Gp135/PC (Figure 5), we hypothesized that EBP50 interacts with newly synthesized Gp135 at the Golgi-TGN level. To address this possibility, we performed both biochemical and imaging analysis at 19.5°C , which had been demonstrated to halt the transport of the newly synthesized proteins at TGN (Matlin and Simons,

Figure 6. EBP50 binds to Gp135 during its early biosynthetic stage in MDCK cells. (A) MDCK cell line stably expressing siRNA targeted for Gp135 (Si) and its daughter clone stably expressing the siRNA-resistant Myc-tagged full-length Gp135 construct (FL) were processed for metabolically labeling experiment. Both cell clones were either incubated in Met⁻/Cys⁻ medium for 3 h and labeled with ³⁵S-Met/Cys at 19.5°C for 3 h before harvest (19.5°C), or they were first pulse-labeled at 19.5°C for 3 h and then chased at 37°C for another hour before harvest (37°C). The extracts were evenly divided for immunoprecipitation of the Myc-tagged Gp135 (Myc) or endogenous EBP50 (EBP50). The immunoprecipitates were then separated by a 5–15% linear gradient SDS-PAGE, blotted onto nitrocellulose membrane, and processed for immunoblotting (IB) using anti-Myc and anti-EBP50 antibodies. The arrowhead denotes the position of the underglycosylated Gp135, which has the estimated molecular weight at about 90 kDa. The double arrowheads denote the mature fully glycosylated Gp135 at 170-kDa region. Molecular weight standards are indicated in kilodaltons. (B) After the immunoblotting was performed, the same blot as shown in A was air-dried and processed for fluorography to show the metabolically labeled Gp135. (C–J) The Gp135 siRNA expressing clone was cotransfected with a construct expressing Gp135 with an amino-terminally tagged EGFP (EGFP-Gp135 in C–E and G–J) or a similar construct deleted of the C-terminal PDZ-binding motif (EGFP-Gp135dC in F) and a plasmid construct expressing the Flag-tagged EBP50 (F-E50 in C–J). Three hours after incubation at 37°C, the cells were further incubated at 19.5°C for 2 h to trap the newly synthesized Gp135 at the TGN. The cells were then processed for immunofluorescence double (C–F) or triple labeling (G–J) and confocal section imaging study using rabbit anti-Flag antibody and the mouse anti-TGN marker, p230. Note that deletion of the PDZ-binding motif in Gp135 prevents colocalization with EBP50 at the TGN. Bar, 5 μm.

1983). We reasoned that this manipulation could enhance the possibility of detecting the interaction between EBP50 and newly synthesized Gp135.

We used a pair of previously characterized MDCK stable clones: one was a Gp135 knockdown expressing <10% Gp135 protein of the wild-type control (Si in Figure 6A) and the other was a derivative from this knockdown mutant in which a full-length, but small interfering RNA (siRNA)-resistant Myc-tagged Gp135 construct was stably transfected to restore the expression of wild-type protein (FL in Figure 6A; Cheng *et al.*, 2005). To demonstrate Gp135 interaction

with EBP50 during early biosynthetic transport, we performed coimmunoprecipitation experiments using metabolically labeled cells incubated at either 19.5 or 37°C. By Western blotting analysis, we demonstrated that Gp135 and EBP50 associated with each other in the reciprocal immunoprecipitations. Significantly, both the fully and underglycosylated Gp135 (denoted as double arrowheads and single arrowhead, respectively, in Figure 6A) existed in the EBP50 immunoprecipitates, implying Gp135 interacts with EBP50 early in the secretory pathway. The underglycosylated Gp135 migrated around the 90-kDa region, corresponding to signals representing the nascent protein core and N-glycosylated forms of Gp135 (Supplementary Figure S1). After Western blotting analysis, the same nitrocellulose membrane containing the metabolically labeled material was air-dried and exposed to x-ray film for fluorography (Figure 6B). By comparing the fluorographic patterns of Myc-immunoprecipitates from the cells incubated at 19.5 and 37°C, little signal was found at the 170-kDa range in the sample incubated at 19.5°C, indicating that transport of Gp135 from TGN was efficiently blocked at this temperature. Furthermore, when EBP50 was immunoprecipitated, a 90-kDa signal specifically was found in the lysate of FL cells incubated at 19.5°C. This signal was missing from both the Myc and EBP50 immunoprecipitates of the Gp135 knockdown lysate. It is likely that this signal came from newly synthesized Gp135 because it migrated to the same position of the underglycosylated Gp135, as revealed by the Myc-specific Western blotting analysis. The specific interaction of EBP50 and the newly synthesized Gp135 was further confirmed by the observation that the 90-kDa Myc-specific signal diminished greatly by shifting the incubating temperature to 37°C (Figure 6B). All together, these data support the interaction of Gp135 and EBP50 at the early biosynthetic pathway.

To visualize the association between Gp135 and EBP50, we cotransfected MDCK cells with plasmids expressing an amino-terminal tagged EGFP-Gp135 fusion protein and the Flag-tagged EBP50. After incubation of the transfected cells at 37°C for 3 h to facilitate the endocytosis of the DNA-liposomal complex and expression of the constructs, we shifted the incubation temperature to 19.5°C for another 3 h to accumulate the newly synthesized proteins at TGN (Matlin and Simons, 1983). In the end, the green fluorescence signals were mostly found at a perinuclear compartment (Figure 6, C, E, G, and J), which colocalized with TGN marker p230 (Figure 6I), and overlapped extensively with the fluorescence of the Flag-tagged EBP50 protein (Figure 6, D, F, H, and J). This finding demonstrated the newly synthesized Gp135 could be trapped at Golgi apparatus by the experimental protocol, and there was a significant amount of EBP50 proteins colocalized with the newly expressed Gp135. We noticed the colocalization between Gp135 and EBP50 no longer existed, when the EGFP moiety was homologously fused to the PDZ-binding motif deletion mutant m135dC (Figure 6F). Although the green fluorescent signal was still retained in a tubulovesicular structure, EBP50 was found dispersed throughout the cytosol. Note that this experiment was performed in a previously characterized MDCK cell line expressing siRNA, which diminished the expression of endogenous Gp135 down to 10% of the wild-type cells (Cheng *et al.*, 2005) to minimize the interaction of endogenous Gp135 with the transfected proteins. We noticed that the transiently expressed EGFP-tagged Gp135 proteins in wild-type MDCK cells were fast sent to the plasma membrane, which made impossible for us to see any retention signal of EGFP-tagged Gp135 at TGN compartment (unpublished data). Taken together, these results further support our conclusion that the

Figure 7. Mutants of Gp135 deficient in EBP50 binding are found in detergent-resistant microdomains (DRM). (A) MDCK cells stably expressing the indicated proteins were lysed in TNE/TX-100 buffer at 4°C and separated through 5–40% sucrose gradients. Fractions of 1 ml were collected from top to bottom of the centrifuge tube after centrifugation to equilibrium. After TCA precipitation samples were run on SDS-PAGE and detected by mouse anti-Gp135 antibody 3F2/D8 (wild-type MDCK cells) or anti-Myc antibody 9E10 (the other mutants). An aliquot of each fraction was spotted on the nitrocellulose membrane, and GM1 was detected by HRP-conjugated cholera toxin B (CTxB) to indicate the fractions of DRM. (B) MDCK cells stably expressing Myc-tagged dN122dC proteins were grown in Transwell for polarization. Myc Ab (9E10), 10 µg/ml, was added either to the apical (AP + Ab) or basolateral (BL + Ab) compartment at 4°C for 2 h before lipid raft floatation assay was conducted as described in A.

newly synthesized Gp135 is associated with EBP50 and this interaction depends on the PDZ-binding motif in Gp135.

Lipid Raft Association Is Required, but Not Sufficient, for Apical Sorting of Gp135

Lipid raft association is thought to be a critical characteristic shared by many apically targeted membrane proteins (Lisanti *et al.*, 1989; Brown and Rose, 1992; Carrasco *et al.*, 2004), but whether Gp135 is sent to apical domain by a lipid raft-dependent mechanism has not been previously addressed. To clarify this issue, we performed the floatation assay (Brown and Rose, 1992) in wild-type MDCK cells. It appeared that the endogenous Gp135 was not associated with lipid rafts because the distribution of the endogenous Gp135 was distinct from the cholera toxin B-labeled detergent-resistant microdomain (DRM) in the sucrose gradient fractions (MDCK in Figure 7A). This result was also reproduced in a stable cell line expressing Myc-tagged Gp135 (m135 in Figure 7A). Surprisingly, the DRM association became more evident when the floatation assay was carried out in MDCK cell lines stably expressing various deletion mutant constructs. The mutants that could not bind directly to EBP50 (m135dC, m135sC, and dN122dC in Figure 7A), were found partially distributed in DRM. However, when our analysis was extended to those mutants with EBP50

fused to the C-terminally deleted PDZ-binding motif defective clones, the DRM association patterns reverted to the ones seen in wild-type Gp135 or the Myc-tagged Gp135 construct (m135dCE50 and dN122dCE50 in Figure 7A). This implies the DRM participation of Gp135 is regulated by EBP50 binding to the C-terminal of Gp135. The contrasting behaviors between wild-type and PDZ-binding motif deletion mutant proteins could be reconciled by the following hypothesis. The newly synthesized Gp135 is transiently associated with DRM but later is segregated from the traditionally defined lipid raft to form a distinct population as the protein becomes fully mature by advanced glycosylation and associates with its cytoplasmic adaptor protein EBP50. To test this possibility, we performed the lipid raft association assay with metabolically labeled cells. When the 10-min-pulsed wild-type MDCK was chased and analyzed in the sucrose gradient after 1% Triton X-100 treatment at 4°C, the underglycosylated and Endoglycosidase-H (Endo-H)-sensitive immature proteins first appeared in the 40% sucrose fractions (Gp135 in Figure 8). As the chase continued, the proteins became fully glycosylated and resistant to Endo-H as expected. By contrast, the nascent m135dC first appeared in the DRM and later shifted to 40% sucrose fractions only when the proteins became fully glycosylated (m135dC in Figure 8). Note that the time kinetics of the maturation and glycosylation between the wild-type endogenous Gp135 and the m135dC mutant were not much different from each other, implying that the glycosylation and protein folding machineries work equally efficiently in the luminal domain for these two proteins. We hypothesized the lack of association to DRM for the endogenous Gp135 is because its participation in the lipid raft is very transient so that even the short pulse-chase time interval could not disclose it. To test this hypothesis, we treated wild-type cells with CHX (Figure 8, CHX-1 and CHX-2), which blocks the biosynthesis of membrane proteins at the cotranslational transport stage. Indeed, a transient association of Gp135 with DRM could be demonstrated in the CHX-treated cells. When the CHX was added during the chase period, immature and underglycosylated Gp135 proteins were found in the DRM fraction, presumably due to delayed exit of the nascent polypeptides off the ribosomal structure, which blocks the binding of EBP50 (Figure 8, 0 and 10 min in the CHX-1 panels). As the chase continued to later time points, the effect of CHX became even more evident (the proportion of mature to underglycosylated Gp135 is smaller at 20 min in CHX-1 than that of 20-min chase sample in untreated Gp135) that even the fully glycosylated proteins were still in the DRM fractions, similar to the situation found in m135dC mutant (Figure 8, 20, and 40 min in the CHX-1 panels). When the CHX was added before the pulse period but washed out during the pulse and chase periods, both immature and mature Gp135 proteins were still associated with DRM (Figure 8, CHX-2 panels). On the basis of these findings, we propose that the nascent Gp135 associated with DRM is initially monomeric and is later oligomerized as it is integrated into an apical sorting platform. Although detergent extraction and sucrose density analysis remains a classical method to determine lipid rafts association, it does not measure all cases of association with lipid rafts (Shvartsman *et al.*, 2003). Thus, lack of DRMs participation does not preclude the possibility of lipid rafts association for Gp135 because the association of gp135 with rafts may be changed in its biophysical properties upon clustering by EBP50.

Figure 8. Gp135 is transiently associated with the detergent-resistant microdomains during its biosynthesis. Wild-type MDCK (Gp135), m135dC expressing, cycloheximide (CHX)-treated cells, and cells treated with small interfering RNA targeted at EBP50 (siE50) were pulsed-labeled for 10 min with ^{35}S -Met/Cys and chased for the indicated times. At the end of each chase, the cells were lysed in TNE/TX-100 buffer at 4°C and separated on sucrose density gradients. For each chase time an aliquot of lysate was immunoprecipitated and treated with Endoglycosidase H (Endo-H). Cycloheximide ($25\text{ }\mu\text{g/ml}$) was either added in the medium during the metabolic labeling period (CHX-1) or added in the medium for 1 h before the metabolic labeling (CHX-2).

Basolaterally Missorted dN122dC Mutant Proteins Are Monomeric and Are Associated with the DRM

If our hypothesis is correct, it is likely the basolaterally missorted Gp135 mutant would exist in a monomeric form and continue to retain in the DRM at steady state. To test this, we plated the MDCK cells stably expressing Myc-tagged dN122dC mutant, which was distinctly distributed over basolateral domain at steady state (Figure 3A), in Transwells and let them become confluent and polarized. We then added Myc antibody to either the apical or basolateral surface before harvesting the cells for the lipid raft floatation assay. We took advantage of the fact that the bivalent Myc antibody could cluster surface proteins and drive the distribution of the monomeric dN122dC proteins to a still-uncharacterized fraction where oligomerized Gp135 proteins reside. When the clustering antibody was applied from the apical pole, the protein distribution pattern through the sucrose gradients was no difference from the control experiment without antibody clustering (Figure 7B, left). This result was similar to that from an experiment when antibody was applied to a monolayer grown on plastic plate (unpublished data). By contrast, when the clustering antibody was applied from the basolateral pole of the cells, the proteins relocated to the 40% sucrose fractions as the wild-type Gp135 proteins (Figure 7B, right). These results confirmed our hypothesis that the basolaterally missorted mutant proteins are monomeric and retain in an operationally defined lipid raft-associated fraction.

EBP50 Knockdown Results in a Failure of Oligomerization, Retention in the Lipid Raft, and Basolaterally Missorting of the Newly Synthesized Gp135/PC

Given the correlation between Gp135 oligomerization after lipid raft association, and its correct apical sorting, we reasoned that lipid raft participation is a prerequisite for apical targeting of newly synthesized Gp135 molecules, which

need to be converted into oligomers before the proteins are integrated into a functional apical sorting platform. This scenario has been demonstrated for GPI-anchored proteins (Paladino *et al.*, 2004), but never for transmembrane proteins. EBP50 molecules could oligomerize by themselves through their two N-terminal PDZ domains (Lau and Hall, 2001). We therefore postulated that EBP50 facilitates Gp135 oligomerization and that this oligomerizing ability could counteract the ensuing repulsive force generated when the highly negative-charged Gp135/PC is clustering with each other in a close proximity. To test this hypothesis, we took an siRNA approach to knock down the endogenous EBP50 in MDCK cells. Using siRNA designed for the canine EBP50 gene, we could knock down the EBP expression level to $<10\%$ of the endogenous level found in the mock-treated MDCK cells (Figure 9A). When the EBP50 knockdown cells were grown on Transwell filters as a monolayer, the staining intensity of EBP50 diminished significantly compared with that in the mock-treated control, demonstrating again the efficacy of the siRNA knock down (Figure 9B). The mock-treated cells had an apical EBP50 staining pattern similar to that of Gp135, indicating they were associated at the apical domain of confluent MDCK cells (Figure 9B). As shown in Figure 4B, some basolaterally missorted Gp135 mutants can be rapidly endocytosed in a PKC-dependent manner. Indeed, when the EBP50 knockdown cells were treated with the PKC inhibitor Gö6976, intense basolateral Gp135 staining was observed (Figure 9B). This indicates that when endogenous EBP50 is down-regulated, the full-length Gp135 protein would be mistargeted. Furthermore, when the EBP50 knockdown cells were metabolically labeled and analyzed for their lipid raft association, a significant retention of the newly synthesized Gp135 protein in the DRM fractions was noted (siE50 in Figure 8). To provide further evidence of the effect of EBP50 knockdown on the oligomerization of Gp135, the PKC inhibitor-treated EBP50 knockdown cells were incubated with BS3, a membrane-impermeable and nonreducible cross

processed for domain-specific biotinylation as described in *Materials and Methods*. AP or BL, apical or basolateral biotinylation, respectively. Arrowhead, Gp135 monomer; double arrowheads, Gp135 oligomer. An autoradiography of the siE50-treated sample with longer exposure (siE50*) was also shown to facilitate comparison between the apical signal in the mock-treated cells and the basolateral signal in the siE50-transfected cells. (D) Plasma membrane delivery of the newly synthesized Gp135 proteins in EBP50 knockdown (siE50) and mock-treated cells. The experiments were performed, and the results are presented as described in Figure 1B.

linker. The cells were lysed after cross-linking and domain-selective biotinylation. The ratio of Gp135 oligomer (Figure 9C, double arrowheads) to monomer (Figure 9C, single arrowhead) arriving at the apical surface was about the same for the mock and the EBP50 knockdown cells (Figure 9C). However, the basolaterally missorted Gp135 under EBP50 knockdown condition was predominantly monomeric (Figure 9C). Even after longer exposure (Figure 9C, siE50*), when the signal of basolateral gp135 in the siE50 cells was similar to the monomeric gp135 in apical mock-treated cells on the fluorography, we did not observe any gp135 oligomers. We finally examined the effect of EBP50 knockdown on the delivery of newly synthesized gp135. Therefore, we metabolically labeled and processed the EBP50 knockdown cells for targeting assay by domain-specific biotinylation and immunoprecipitation. The result show that EBP50 knockdown leads to ~20% missorting (Figure 9D), similar to the extent found in the mutant Gp135 proteins, which lacked the EBP50 binding motif (Figure 3B, m135dC and m135sC).

DISCUSSION

In this study we carefully characterized the apical targeting of Gp135. We show that besides O-glycosylation, the binding of EBP50 plays an important role in the correct localization of Gp135. However, to our surprise, EBP50 binds Gp135 at the TGN, and the function of EBP50 in apical delivery of Gp135 is not related to binding to ERM proteins and the actin cytoskeleton, but depends on its ability to cluster Gp135 into oligomers during the sorting process. This unexpected role of EBP50 is in stark contrast to the previous finding that PDZ domain proteins serve as an apical retention regulator (Harris and Lim, 2001; Brone and Eggermont, 2005). This negligence of identifying EBP50 as an apical sorting modulator was possibly because the intracellular trafficking of the newly synthesized Gp135 had not been examined utilizing metabolically labeled cell materials (Takeda *et al.*, 2001; Li *et al.*, 2002; Meder *et al.*, 2005). Although the EBP50 homologous protein, NHERF2, has been

Figure 9. EBP50 knockdown causes missorting of Gp135. (A) MDCK cells were transfected with siRNAs targeted at canine EBP50 gene (siE50) or mock-treated. Western blotting analysis using rabbit anti-EBP50 antibody showed efficient knockdown of the EBP50 expression ($5.8 \pm 0.7\%$ of the EBP level in mock-treated cells after normalization by the actin-loading control signal; triplicated experiments). (B) MDCK cells expressing Myc-tagged Gp135 were transfected with siRNA targeted against canine EBP50 (siE50) or mock-treated and were plated on Transwells filters. The polarized monolayers were then treated with DMSO (–) or $2 \mu\text{M}$ G66976 (+) for 2 h before double immunofluorescence using mouse anti-Myc (green channel) and rabbit anti-EBP50 (red channel), and confocal section imaging analysis was performed. Bar, $10 \mu\text{m}$. (C) MDCK cells transfected with siRNAs targeted at canine EBP50 gene (siE50) or mock-treated (mock) were plated on Transwell filters. The polarized monolayers were then treated with $2 \mu\text{M}$ G66976 for 2 h or BS3 for 2 h on ice and

shown to participate with Gp135 in the formation of a preapical complex (Meder *et al.*, 2005), we cannot examine whether endogenous NHERF2 performs an apical sorting function for Gp135 because we (unpublished data) and others (Schmieder *et al.*, 2004) cannot detect the expression of NHERF2 in MDCK cells.

PC/Gp135 is thought to provide the glomerular podocytes an antiadhesive characteristic that is essential for the charge barrier function of the renal filtration apparatus (Kerjaschki *et al.*, 1984, 1985). The electric repulsive nature of PC is also postulated to be the biochemical basis for creating the slit space between the foot processes of glomerular podocytes, as PC knock-out mice died of anuria in early infancy, and the slit space was found obliterated in these mutant mice (Doyonnas *et al.*, 2001). More than two thirds of the apparent molecular weight of PC results from posttranslational modification, mainly by N- and O-linked glycosylation and the subsequent sialic acid addition, which is thought to confer PC the antiadhesive propensity. Once arriving on the apical surface, PC is thought to be linked to and stabilized by membrane cytoskeleton via EBP50 and Ezrin (Orlando *et al.*, 2001; Li *et al.*, 2002). Our findings that EBP50 binds to PC/Gp135 during the early phase of its biosynthetic pathway and facilitate its apical sorting shed new light on the mechanism by which a heavily negative charged sialomucin protein could be successfully packed in a sorting vesicle and sent to the correct destination (Figure 10).

Oligomerization has been proposed to be a stabilizing factor and a secondary requirement beside lipid raft partitioning for apical sorting of exogenously expressed GPI-APs in MDCK cells (Hannan *et al.*, 1993; Paladino *et al.*, 2004). However, a similar requirement has not been demonstrated for endogenous proteins in polarized epithelial cells. Furthermore, the interacting protein providing such a clustering effect has been proposed to be a raft-associated protein or a yet unidentified luminal lectin. Our result demonstrates, at least for the case of Gp135/PC, that this partner could be a cytosolic protein possessing a strong association with the cargo and an inclination to oligomerize by itself such as

Figure 10. Multistep model for the apical sorting of podocalyxin/Gp135 in polarized epithelial cells. (A) As the nascent polypeptide of Gp135 is being released from the membrane-associated ribosome, the signal peptide of Gp135 has directed its entrance into the ER. (B) The polypeptide is undergoing N-linked glycosylation (not shown in the diagram) and O-linked glycosylation along its biosynthetic route through the ER/Golgi apparatus. Although the putative luminal lectin receptor can recognize the O-glycan as an apical sorting signal and cluster the nearby Gp135, the highly negative charged nature of the superimposed sialic acid modifications would impede the coalescence of the single lipid rafts (shown in red) into a functional apical-sorting platform. (C) Binding of EBP50 to the cytoplasmic tail of Gp135 and the oligomerization of EBP50 would counteract the electric repulsive force by the sialic acid modifications incurred on the closely packed Gp135 and help clustering of multiple lipid rafts into a competent functional unit. Please note that the oligomerization of EBP50 could be through either homodimerization or heterodimerization between the first and second PDZ domains (Lau and Hall, 2001). For simplicity, only homodimerization between the first PDZ domains of adjacent EBP50 molecules is shown in this model.

EBP50. The confinement of PC/Gp135 into lipid raft seems to be a very transient phenomenon because the wild-type proteins appear in a fraction away from the conventional lipid microdomain at steady state and participation into the lipid raft could only be demonstrated when CHX was used to delay the transport of the newly synthesized Gp135 (Figure 8). Although short residency time in rafts has been similarly reported for GPI-APs (Sheets *et al.*, 1997; Zurzolo *et al.*, 2003; Simons and Vaz, 2004), the status after clustering of the apically sorted candidates seems to differ between Gp135 and GPI-APs. By floatation gradient separation, the apically sorted GPI-APs were noted to float to lower isopycnic density on sucrose gradients (Paladino *et al.*, 2004), whereas the apically sorted Gp135 as well as the antibody-clustered basolaterally missorted Gp135 mutant sedimented to a higher isopycnic fraction (Figure 7). We had found Gp135 and EBP50 comigrated in a supramolecular complex of molecular weight larger than 1000 kDa by FPLC analysis (unpublished data). This supramolecular complex could be the oligomerized Gp135/EBP50 and other cytoplasmic proteins. The capability of transmembrane proteins to incorporate PDZ domain proteins may explain the different behaviors of oligomerized Gp135 and GPI-APs by conventional raft floatation assay.

Many of the membrane proteins that bind EBP50 can be internalized and recycled from endosomes back to the plasma membrane. Strong association between these transmembrane proteins and EBP50 at the plasma membrane led to the suggestion that a potential role of EBP50 may tether specific membrane proteins in the plasma membrane and restrain them from entering the endocytic cycle (Bretscher, 1999). Consistent with this hypothesis was the finding that deletion of the EBP50-binding motif in the tail of the CFTR resulted in the receptor's relocation from the apical to basolateral membrane in airway epithelial cells with no effect on the apical localization of EBP50 (Moyer *et al.*, 1999). However, this result could be alternatively interpreted with missorting of CFTR during secretion or after endocytosis. Our data clearly show such a possibility by demonstrating the association of newly synthesized Gp135/PC with EBP50 along the biosynthetic pathway (Figure

6), and fusion with EBP50 corrects missorting of the tail-minus Gp135/PC (Figure 5).

It has been demonstrated that EBP50 is involved in the recycling of the internalized β 2-adrenergic receptor from an early endosome to the plasma membrane (Cao *et al.*, 1999). The β 2-adrenergic receptor which harbors cytoplasmic tail mutation that abolishes EBP50 binding is delivered to the lysosome for degradation instead of being recycled back to the plasma membrane in agonist-stimulated cells. Although these data are supportive of a role of EBP50 oligomerization in protein trafficking, there is also a clear discrepancy that their result suggests interaction of EBP50 and ERM proteins is necessary for receptor recycling, whereas ours excludes a similar requirement for ezrin to direct Gp135/PC trafficking (Figure 5). This discrepancy could be reconciled by the fact that an EBP50/ERM/F-actin linkage in the biosynthetic pathway remains to be elucidated although their association has been extensively characterized at the plasma membrane level.

The scenario for adaptor protein to regulate sorting of membrane protein is not without precedent. Basolateral delivery of E-cadherin has been demonstrated to depend partially on β -catenin (Chen *et al.*, 1999), and these two protein partners already associate at the endoplasmic reticulum (ER; Hinck *et al.*, 1994). However, there are several noticeable differences in these two examples: 1) β -catenin binding acts not only for a basolateral targeting signal but also for ER exiting signals for E-cadherin (Chen *et al.*, 1999), 2) E-cadherin is not known to be associated with lipid rafts, and 3) β -catenin is not known to form oligomers for E-cadherin transport. Whether any of these differences serves as the determinant for basolateral vs. apical targeting awaits further studies. Nevertheless, the novel role of EBP50 oligomerization in sorting apical polarity proteins should introduce a new perspective of the mechanism by which polarity is regulated by peripheral membrane proteins.

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