PDZ-Domain Interaction Controls the Endocytic Recycling of the Cystic Fibrosis
Transmembrane Conductance Regulator

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Running title: The PDZ Interacting Domain in CFTR.

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SUMMARY

The C-terminus of CFTR contains a PDZ interacting domain that is required for the polarized expression of CFTR in the apical plasma membrane of polarized epithelial cells. To elucidate the mechanism whereby the PDZ interacting domain mediates the polarized expression of CFTR, MDCK cells were stably transfected with wild-type (wt-CFTR) or C-terminally truncated human CFTR (CFTR-ΔTRL). We tested the hypothesis that the PDZ interacting domain regulates sorting of CFTR from the Golgi to the apical plasma membrane. Pulse-chase studies in combination with domain-selective cell surface biotinylation revealed that newly synthesized wt-CFTR and CFTR-ΔTRL were targeted equally to the apical and basolateral membranes in a nonpolarized fashion. Thus, the PDZ interacting domain is not an apical sorting motif. Deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane from ~24 hrs to ~13 hrs, but had no effect on the half-life of CFTR in the basolateral membrane. Thus, the PDZ interacting domain is an apical membrane retention motif. Next, we examined the hypothesis that the PDZ interacting domain affects the apical membrane half-life of CFTR by altering its endocytosis and/or endocytic recycling. Endocytosis of wt-CFTR and CFTR-ΔTRL did not differ. However, endocytic recycling of CFTR-ΔTRL was decreased when compared with wt-CFTR. Thus, deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane by decreasing CFTR endocytic recycling. Our results identify a new role for PDZ proteins in regulating the endocytic recycling of CFTR in polarized epithelial cells.
INTRODUCTION

The selective expression of transport proteins in either the apical or basolateral membrane is essential for polarized epithelial cells to carry out vectorial transport of ions and water (1-4). For example, polarization of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) to the apical plasma membrane is required for vectorial Cl⁻ secretion across a variety of epithelial cells including those in airway, kidney, intestine, and pancreas (4-7). In the genetic disease Cystic Fibrosis (CF) the most common mutation in the CFTR gene, ΔF508, causes CFTR to fold incorrectly and to be retained in the endoplasmic reticulum (6-9). Because ΔF508-CFTR does not reach the apical plasma membrane, epithelial cells in the airway, pancreas, and intestine do not secrete Cl⁻ (6,7,10).

Transport proteins contain amino acid motifs that direct and/or localize proteins to the appropriate membrane domains (1-4). Highly conserved motifs that direct the polarized expression of transport proteins to the basolateral membrane of epithelial cells include tyrosine and dileucine based motifs (1-4). PDZ domains, which are named for three proteins in which this domain was first described (PSD-95, Dlg and ZO-1), also determine the polarized expression of proteins in epithelial cells and neurons (11-22). PDZ domains are modular 70-90 amino acid domains that bind to short peptide sequences at the C-termini of other proteins, called PDZ interacting domains (15,21,23-25). Previously, we demonstrated that a C-terminal, PDZ interacting domain is required for the polarization of CFTR to the apical plasma membrane in airway and kidney epithelial cells (16,18,19). Deletion of the PDZ interacting domain (TRL, using the single letter amino acid code) abrogated the polarized expression of CFTR in the apical membrane and eliminated CFTR-mediated transepithelial Cl⁻ secretion. However, the mechanism whereby the PDZ interacting domain directs the polarized expression of CFTR to the apical plasma membrane is unknown.

The objectives of the present study were to test whether the PDZ interacting domain of CFTR is an apical membrane sorting motif that directs the trafficking of CFTR to the apical membrane or is a retention motif that results in the polarization of CFTR by selectively retaining CFTR in the apical membrane by interacting with an apical PDZ protein. Pulse-chase studies in combination with
domain-selective cell surface biotinylation revealed that newly synthesized wt-CFTR and CFTR-
ΔTRL were targeted to the apical and basolateral membrane domains in a nonpolarized fashion. Thus, the PDZ interacting domain is not an apical membrane sorting motif. To determine if the PDZ interacting domain is a membrane retention motif we measured the half-life of wt-CFTR and CFTR-ΔTRL in the apical and basolateral membranes. Deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane from ~24 hrs to ~13 hrs, but had no effect on the half-life of CFTR in the basolateral membrane. Thus, the PDZ interacting domain is an apical membrane retention motif. Deletion of the PDZ interacting domain did not affect apical membrane endocytosis of CFTR. By contrast, deleting the PDZ interacting domain decreased apical endocytic recycling of CFTR. Thus, deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane by decreasing CFTR endocytic recycling. Our results identify a new role for PDZ proteins in regulating the endocytic recycling of CFTR in polarized epithelial cells.
EXPERIMENTAL PROCEDURES

Cell culture and stable cell lines. MDCK cells stably expressing GFP-CFTR fusion proteins were established and maintained in culture at 37°C in MEM complete medium containing penicillin, streptomycin, L-glutamine, FBS (10%) and G418 (150 µg/ml) as described previously (16,26). Addition of GFP to the N-terminus of CFTR had no effect on CFTR localization, trafficking, function as a Cl⁻ channel, or on its degradation (26,27).

Pulse-chase and selective cell surface biotinylation studies. Pulse-chase and selective cell surface biotinylation studies were conducted essentially as described by Lissanti et al (28) to determine if the PDZ interacting domain of CFTR is a sorting and/or a membrane retention motif. The day before pulse-chase studies G418 was removed from the cell culture medium and sodium n-butyrate (5 mM) was added to stimulate CFTR expression as described previously (26). Confluent, polarized MDCK cells grown on Transwell permeable growth supports (24 mm diameter, 0.4 µm pore size; Corning Corporation, Corning, NY: #3412) were washed extensively in PBS and incubated in complete MEM without methionine (Met) and cysteine (Cys) for 60 min at 37°C. Subsequently, cells were metabolically labeled with Tran³⁵S-Label Reagent (250 µCi/ml: ICN Pharmaceuticals, Inc., Cosa Mesa, CA) in complete MEM without Met and Cys for 30 min at (37°C). The filters were washed at 4°C in complete MEM containing an excess (10 mM) of unlabeled Met and Cys and chased for variable periods of time in complete MEM containing an excess (10 mM) of unlabeled Met and Cys at 37°C.

To measure the half-life of wt-CFTR and CFTR-ΔTRL in cell lysates, cells were metabolically labeled with Tran³⁵S-Label Reagent as described above, cooled to 4°C and lysed in lysis buffer described previously (26). CFTR was immunoprecipitated by incubation with either a GFP polyclonal antibody (5 µg: Clontech, Palo Alto, CA: #8372-2) or a cocktail of monoclonal antibodies M3A7 and L12B4A (29) (2 µg each; Upstate Biotechnology, Inc., Waltham, MA) followed by a second incubation with protein A or Protein G (as appropriate) conjugated to Sepharose beads (Pierce Chemical Co., Rockford, IL). Immunoprecipitated CFTR was eluted from the protein A or G Sepharose beads by incubation at 95°C for 5 minutes in SDS-sample
buffer and centrifuged for 1 minute at 14,000g. Immunoprecipitated and \(^{35}\text{S}\)-labeled wt-CFTR and CFTR-\(\Delta\)TRL were separated by 7.5% SDS-PAGE.

To test the hypothesis that the PDZ interacting domain is a sorting motif, cells were pulse-labeled with Tran\(^{35}\text{S}\)-Label Reagent (Met/Cys: 250 $\mu$Ci/ml) for 30 minutes, chased for 0, 30, 60, 90 or 120 minutes and then cooled to 4°C. Proteins in the apical and basolateral membranes were selectively biotinylated (EZ-Link™ Biotin-LC-Hydrazide, Pierce) as described in detail previously (16,26). Subsequently, cells were lysed, biotinylated proteins were isolated by streptavidin beads and biotinylated CFTR was immunoprecipitated using a GFP monoclonal antibody (26) or a cocktail of M3A7/L12B4 monoclonal antibodies (29). Immunoprecipitated and biotinylated \(^{35}\text{S}\)-labeled wt-CFTR and CFTR-\(\Delta\)TRL were separated by 7.5% SDS-PAGE.

Detection of \(^{35}\text{S}\)-labeled wt-CFTR and CFTR-\(\Delta\)TRL was conducted by placing gels on a general purpose storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) and, 24 to 72 hours later, detection using the Storm 860 PhosphorImager system (Molecular Dynamics). Measurement of \(^{35}\text{S}\)-labeled wt-CFTR and CFTR-\(\Delta\)TRL was conducted using a Dell OptiPlex GX1 computer and ImageQuant 5.1 software (Molecular Dynamics).

**Transcytosis assay.** Studies were conducted to determine if apical polarization of CFTR was mediated in part by transcytosis of CFTR from the basolateral to the apical plasma membrane according to a method described in detail previously (30). In brief, basolateral membrane proteins were biotinylated at 4°C using a derivative of biotin (EZ-Link™ Sulfo-NHS-SS-Biotin; Pierce) that can be reduced by glutathione (GSH). Subsequently, cells were warmed to 37°C for 0, 60 or 120 minutes and the disulfide bonds on Sulfo-NHS-SS-biotinylated proteins in the apical or basolateral membranes were reduced by GSH added to the apical or basolateral solutions, respectively, for 30 minutes at 4°C. In preliminary studies we demonstrated that GSH only reduces the disulfide bonds of biotinylated proteins in plasma membranes and does not cross membranes or monolayers of polarized MDCK cells. Biotinylated CFTR was analyzed by Western blotting using a GFP monoclonal antibody (26) or a cocktail of M3A7/L12B4 monoclonal antibodies (29) and an anti-
mouse HRP antibody using the Western Lightning™ Chemiluminescence Reagent Plus detection system (ECL).

**Endocytic assay.** Studies were conducted to determine whether the reduced half-life of CFTR-ΔTRL compared to wt-CFTR in the apical membrane was due to a difference in endocytosis according to a method described in detail previously (26,31). In brief, apical membrane proteins were biotinylated at 4°C using EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce). Subsequently, cells were warmed to 37°C for 1, 3, 5, or 10 minutes and the disulfide bonds on Sulfo-NHS-SS-biotinylated proteins remaining in the apical membrane were reduced by GSH added to the apical solution for a total of 90 minutes at 4°C. At this point in the protocol, biotinylated proteins reside within the endosomal compartment. Subsequently, cells were lysed, biotinylated proteins were isolated by streptavidin-agarose beads, eluted into SDS-sample buffer, and separated by 7.5% SDS-PAGE. Biotinylated CFTR was analyzed by Western blot analysis as described above.

**Endocytic recycling assay.** Studies were conducted to determine whether the reduced half-life of CFTR-ΔTRL compared to wt-CFTR in the apical membrane was due to a difference in endocytic recycling according to a method described in detail previously (32,33). Briefly, apical membranes were biotinylated at 4°C, and then warmed to 37°C for 3 minutes to load endocytic vesicles with biotinylated proteins, including CFTR. Subsequently, cells were cooled to 4°C and the disulfide bonds on Sulfo-NHS-SS-biotinylated proteins in the apical membranes were reduced by GSH, as described for the endocytic assay. Subsequently, cells were either lysed or warmed again to 37°C for 3 or 5 minutes (to allow internalized, biotinylated CFTR to recycle to the apical membrane). Cells were then cooled again to 4°C, and the disulfide bonds on Sulfo-NHS-SS-biotinylated proteins in the apical membranes were reduced with GSH and cells were lysed. Biotinylated proteins were isolated by streptavidin-agarose beads, eluted into SDS-sample buffer, and separated by 7.5% SDS-PAGE. Biotinylated CFTR was analyzed by Western blot analysis as described above. Endocytic recycling of CFTR was expressed as the difference between the amount of biotinylated CFTR after the first and second warming to 37°C. Biotinylated CFTR was analyzed as described above.
**Data analysis and statistics.** Each experiment was repeated a minimum of three to six times. In each experiment 3-6 filters were studied at each time point. Calculation of the half-life of CFTR was performed using GraphPad Prism version 3.0a for Macintosh, GraphPad Software, San Diego California USA. Statistical analysis of the data was performed using GraphPad Instat version 3.0a for Macintosh, GraphPad Software, San Diego California USA. Means were compared by the unpaired t-test. A P value <0.05 was considered significant. Data are expressed as mean ± SEM.
**RESULTS**

The PDZ interacting domain of CFTR is not an apical membrane sorting motif. To test the hypothesis that the PDZ interacting domain (TRL) is a sorting motif, studies were conducted in MDCK cells using a pulse-chase and domain-specific cell surface biotinylation protocol. MDCK cells stably expressing GFP-wt-CFTR or GFP-CFTR-ΔTRL were washed in a methionine (Met) and cysteine (Cys)-free MEM solution for 30 minutes, and then pulsed with Tran$^{35}$S-Labeled Met/Cys for 30 minutes to label newly synthesized proteins, and chased for 0, 30, 60, 90 and 120 minutes in MEM containing an excess of unlabeled Cys/Met. Subsequently, apical and basolateral membrane proteins were selectively biotinylated at 4°C, cells were lysed and the biotinylated proteins were isolated by streptavidin beads. Biotinylated CFTR was immunoprecipitated using either a GFP monoclonal antibody or a cocktail of M3A7/L12B4 monoclonal antibodies. Similar results were obtained using either the GFP or the M3A7/L12B4 antibodies. $^{35}$S-labeled and biotinylated CFTR was separated by SDS-PAGE and detected by phosphorimage analysis (see Experimental Procedures for details). If the PDZ interacting domain is an apical membrane-sorting motif, newly synthesized wt-CFTR should be sorted directly from the trans Golgi network (TGN) to the apical membrane with little or no wt-CFTR appearing in the basolateral membrane. By contrast, CFTR-ΔTRL should be delivered to the apical and basolateral membranes in equal amounts at each time point.

Newly synthesized wt-CFTR appeared in the apical and basolateral membranes at the same rate at 0, 30, 60 and 90 minutes into the chase period (Figure 1A). The ratio of wt-CFTR in the apical/basolateral membrane was ~1 at all time points between 0 and 90 minutes into the chase period (Figure 2). Thereafter (120 minutes) wt-CFTR accumulated preferentially in the apical plasma membrane. At 120 minutes into the chase period the ratio of wt-CFTR in the apical to basolateral membrane was ~2 (Figures 1A and 2). Similar results were obtained in pulse-chase studies on wt-CFTR in Calu-3 cells, a human airway epithelial cell line expressing native wt-CFTR (data will be presented in a separate study). These data suggest that the polarized expression of wt-
CFTR to the apical membrane in the steady-state does not result from the selective sorting of wt-CFTR from the TGN directly to the apical plasma membrane.

Similar pulse-chase and selective cell surface biotinylation studies were conducted in MDCK cells stably expressing CFTR-ΔTRL, which does not polarize in the steady state to the apical or basolateral membranes (16,18). Newly synthesized CFTR-ΔTRL appeared in the apical and basolateral membranes at the same rate at 0, 30, 60, 90 and 120 minutes into the chase period (Figure 1B). The ratio of CFTR-ΔTRL in the apical/basolateral membrane was ~1 at all time points between 0 and 120 minutes into the chase period (Figure 2). Taken together, our studies with wt-CFTR and CFTR-ΔTRL indicate that the PDZ interacting domain is not an apical membrane sorting motif. Thus, the polarized expression of wt-CFTR in the apical membrane of MDCK cells in the steady state does not result from the selective sorting of wt-CFTR from the TGN directly to the apical plasma membrane.

The PDZ interacting domain of CFTR is an apical membrane retention motif. Additional studies were conducted to test the hypothesis that the PDZ interacting domain is an apical membrane retention motif and that selective retention in the apical membrane of wt-CFTR leads to its apical polarization in the steady-state. According to this hypothesis, the half-life of wt-CFTR in the apical membrane should be greater than its half-life in the basolateral plasma membrane. Moreover, deletion of the PDZ interacting domain should reduce the half-life of CFTR in the apical but not in the basolateral membrane. To test this hypothesis, pulse-chase and domain-specific cell surface biotinylation studies were conducted as described above except that the chase periods were 2, 6, 18, and 24 hours.

As illustrated in Figures 3 and 4, the half-life of wt-CFTR in the apical membrane (24.1 hrs.) was significantly longer than the half-life of wt-CFTR in the basolateral membrane (12.9 hrs.). Deletion of the PDZ interacting domain significantly reduced the half-life of CFTR in the apical membrane from 24.1 to 12.6 hrs. By contrast, deletion of the PDZ interacting domain had no effect on the half-life of CFTR in the basolateral membrane (12.9 hrs. for wt-CFTR and 11.2 hrs. for CFTR-ΔTRL). Taken together, these data are consistent with the view that the PDZ
interacting domain in CFTR is an apical membrane retention motif and that wt-CFTR is selectively retained in the apical membrane via interaction with a PDZ protein(s).

To examine the role of the PDZ interacting domain in the degradation and stability of CFTR in the intracellular compartment(s), pulse chase studies were conducted, essentially as described above, except that CFTR was immunoprecipitated from cell lysates after biotinylated proteins had been removed by streptavidin isolation. Thus, non-biotinylated CFTR was immunoprecipitated and separated by SDS-PAGE. The half-life of maturely glycosylated (C band) wt-CFTR was 12.5 hrs. and the half-life of maturely glycosylated CFTR-ΔTRL was 11.9 hrs. (Figure 5). These results confirmed previous studies demonstrating that short truncations of the C-terminus of CFTR (<26 amino acids) have no effect on the degradation of the maturely glycosylated (C band) CFTR (34,35).

Apical membrane polarization of wt-CFTR is not mediated by transcytosis from the basolateral to the apical plasma membrane. The data presented above do not rule out the possibility that apical membrane polarization of wt-CFTR results in part from the transcytosis of wt-CFTR from the basolateral to the apical membrane. Transcytosis of wt-CFTR may be a mechanism that, in addition to apical retention, leads to the apical polarization of wt-CFTR. To test the hypothesis that CFTR is transcytosed from the basolateral to the apical plasma membrane we conducted a transcytosis assay as described previously (30). Plasma membrane proteins were biotinylated at 4°C with EZ-Link™ Sulfo-NHS-SS-Biotin. After reduction by GSH the biotin is no longer attached to membrane proteins. After biotinylation at 4°C, cells were warmed to 37°C for 0, 60 or 120 minutes, a time at which newly synthesized wt-CFTR begins to polarize to the apical membrane (see Figures 1 and 2). Subsequently, cells were cooled again to 4°C and then GSH (or vehicle) was added to the solutions bathing either the apical or basolateral side of the monolayers to reduce the disulfide bond of NHS-SS-Biotin attached to proteins in the plasma membrane on the cis but not the trans side of monolayers. Thus, in experiments in which basolateral membrane proteins were biotinylated, GSH added to the basolateral solution will reduce only the disulfide bond of NHS-SS-Biotin attached to proteins in the basolateral membrane but will
not reduce the disulfide bond in NHS-SS-Biotin attached to proteins that were endocytosed to an intracellular compartment or to proteins that were biotinylated in the basolateral membrane and transcytosed to the apical membrane. By contrast, addition of GSH to the apical solution will only reduce the disulfide bond of NHS-SS-Biotin attached to proteins that were biotinylated in the basolateral membrane and transcytosed to the apical membrane and not reduce the disulfide bond in NHS-SS-Biotin attached to proteins that were retained in the basolateral membrane or endocytosed. Subsequent to GSH (30 minutes) or vehicle treatment at 4°C, cells were lysed, biotinylated proteins were isolated with streptavidin beads and biotinylated proteins were separated by SDS-PAGE, proteins transferred to a PVDF membrane and biotinylated CFTR was detected using a GFP monoclonal antibody.

The representative experiment illustrated in Figure 6 demonstrates that transcytosis of wt-CFTR could not be detected after 60 or 120 minutes. In monolayers in which basolateral membranes were biotinylated, addition of GSH to the solution bathing the basolateral side of the monolayers reduced the amount of wt-CFTR pulled down by streptavidin by more than 95% (compare lanes a-c with lanes d-f in Figure 6A). Moreover, addition of GSH to the solution bathing the apical side of the monolayers had no effect on the amount of wt-CFTR pulled down by streptavidin (compare lanes a-c with lanes g-i in Figure 6A). Figure 6B demonstrates that the total wt-CFTR expression was similar in each set of monolayers. These data suggest that wt-CFTR was not transcytosed from the basolateral to the apical membrane. Thus, apical polarization of wt-CFTR does not involve transcytosis of wt-CFTR from the basolateral to the apical membrane.

**The PDZ interacting domain does not regulate apical membrane endocytosis of CFTR.** To determine the mechanism whereby deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane, we monitored the endocytosis of wt-CFTR and CFTR-ΔTRL as described in Experimental Procedures. As illustrated in Figure 7A, we observed a linear increase in the endocytic uptake of wt-CFTR between 0 and 3 minutes. Thereafter, the amount of CFTR in endocytic vesicles failed to increase because, as shown below, CFTR is rapidly recycled back to the plasma membrane. The amount of CFTR-ΔTRL endocytosed
between 0 and 3 minutes was similar to wt-CFTR. Thus, deletion of the PDZ interacting domain had no effect on apical membrane endocytosis of CFTR.

The PDZ interacting domain of CFTR is an apical membrane endocytic recycling motif. Additional studies were conducted to determine whether deletion of the PDZ interacting domain reduced the half-life of CFTR in the apical membrane by decreasing its endocytic recycling. In preliminary studies we observed that endocytic recycling of CFTR was linear between 0 and 3 minutes. Thereafter, the amount of CFTR that recycled back to the apical plasma membrane failed to increase because, as shown above, CFTR is rapidly endocytosed. The endocytic recycling of CFTR was dramatically reduced by deletion of the PDZ interacting domain (Figure 7B). Thus, the PDZ interacting domain of CFTR is an endocytic recycling motif. Taken together, these data reveal that the PDZ interacting domain selectively retains CFTR in the apical membrane by facilitating the endocytic recycling of CFTR.
DISCUSSION

Our data demonstrate that the PDZ interacting domain of CFTR is an apical membrane, endocytic recycling motif. CFTR polarizes to the apical membrane in MDCK cells because a CFTR–PDZ protein interaction facilitates the endocytic recycling of CFTR to the apical membrane, and thereby, dramatically and selectively increases the half-life of CFTR in the apical plasma membrane. When the CFTR–PDZ protein interaction is eliminated, by deleting the PDZ interacting domain of CFTR, endocytic recycling is less efficient, the half-life of CFTR in the apical membrane is reduced and CFTR no longer polarizes to the apical plasma membrane. Because deletion of the PDZ interacting domain did not affect the delivery of CFTR from the TGN to the apical or basolateral membranes, our data also demonstrate that the PDZ interacting domain of CFTR is not an apical targeting or sorting signal. Moreover, the PDZ interacting domain is not involved in transcytosis of CFTR from the basolateral to the apical plasma membrane.

Three basic mechanisms are utilized to direct the polarized expression of proteins in the apical membrane of epithelial cells: (1) Sorting from the TGN directly to the apical membrane; (2) Sorting from the TGN directly to the basolateral membrane and then transcytosis to the apical membrane; and (3) Random sorting to the apical and basolateral membranes with selective retention in the apical membrane (2). Most apical membrane proteins expressed in MDCK cells are sorted directly to the apical membrane (2, 36, 37). Our data indicate that CFTR is randomly sorted to the apical and basolateral membranes and retained in the apical membrane via interaction with a PDZ protein. Like CFTR, the Na\(^+\)-K\(^+\)-ATPase is randomly sorted from the TGN to the apical and basolateral membranes in MDCK cells. The Na\(^+\)-K\(^+\)-ATPase is selectively retained in the basolateral membrane via interaction with the actin-based cytoskeleton, which retains the Na\(^+\)-K\(^+\)-ATPase in a polarized state (38). Apical Na\(^+\)-K\(^+\)-ATPase does not interact with actin and is removed from the apical membrane and degraded. Transcytosis of wt-CFTR from the basolateral to the apical membrane does not appear to be a major mechanism whereby wt-CFTR is polarized to the apical plasma membrane in MDCK cells (Figure 6). Because MDCK cells are a well described
model to study transcytosis, it is reasonable to conclude that our inability to detect significant transcytosis of wt-CFTR from the basolateral to the apical membrane is not due to the inability of MDCK cells to transcytose proteins. However, we cannot completely exclude the possibility that a small amount of wt-CFTR, below the detection limit of our assay, may undergo transcytosis.

Our data are consistent with a growing body of evidence to support the view that PDZ domains direct the polarized expression of transport proteins in epithelial cells and neurons (11-20,22,39). Previously, we demonstrated that deletion of the PDZ interacting domain of CFTR abrogates binding to EBP50 and CAP70, eliminates the polarized expression of CFTR to the apical membrane of kidney (MDCK) and human airway epithelial cells (16HBE14o-), and eliminates CFTR-mediated transepithelial Cl secretion in MDCK cells (16,18,19). The polarized expression of another apical membrane protein, podocalyxin, also requires an interaction between its C-terminal PDZ interacting domain and an apical PDZ protein in MDCK cells (39). Moreover, deletion of the PDZ interacting domain of podocalyxin decreased its stability in the apical membrane. In addition, the polarized expression of the γ-aminobutyric acid transporter (BGT-1) to the basolateral membrane in MDCK cells requires an interaction between its C-terminal PDZ interacting domain and the basolateral PDZ protein Lin-7 (12). Deletion of the PDZ interacting domain does not effect BGT-1 targeting from the TGN to the basolateral membrane but decreases BGT-1 retention in the basolateral membrane. Similarly, the expression of the inwardly rectifying potassium channel, Kir 2.3, in the basolateral membrane of MDCK cells also involves stabilization in the membrane via interaction with the PDZ protein hLin-7b (40). Thus, PDZ proteins determine the polarized expression of polytopic membrane proteins in MDCK epithelial cells.

It is becoming increasing clear that PDZ proteins regulate endocytosis and endocytic recycling of membrane proteins in epithelial cells (41-43). The PDZ interacting domains in both the β2-adrenergic and the κ-opioid receptors interact with PDZ domains in EBP50 and regulate endocytic recycling (41,42). Deletion of the PDZ interacting domain eliminates the endocytic recycling of the β2-adrenergic receptor without altering the recycling of the transferrin receptor (41). In addition, fragmentation of actin or expression of a dominant negative EBP50 that lacks the
ezrin binding domain also reduces endocytic recycling of the β2-adrenergic receptor. Moreover, addition of a PDZ interacting domain to the C-terminus of the δ-opioid receptor, which is normally endocytosed and degraded, causes the receptor to enter the endocytic recycling pathway (43). Another PDZ protein, PSD95, inhibits the endocytosis of NMDA receptors by tethering the receptors to the cytoskeleton (44). Taken together, these observations demonstrate that PDZ proteins regulate both endocytosis and the endocytic recycling of a variety of membrane proteins including CFTR. The mechanism whereby PDZ proteins regulate endocytic trafficking is not clear. However, recent studies suggest that PDZ proteins may provide the molecular link between receptors (i.e., cargo, including CFTR) in endocytic vesicles and myosin VI molecular motors, which bind directly to F-actin and produce unidirectional movement along actin filaments in an ATP dependent manner. Myosin VI and the PDZ protein SAP97 form a complex with the AMPA receptor subunit, GluR1 (45). The myosin VI-SAP97 complex has been implicated in the endocytic trafficking of GluR1 (45). In addition, the PDZ protein GIPC binds to both the C-terminus of myosin VI and to the glucose transporter Glut-1 (46). By linking Glut-1 containing vesicles to myosin VI and actin filaments, GIPC may be the molecular link that determines cargo selection for Glut-1 vesicles to enter the endocytic trafficking pathway. Thus, PDZ proteins may be a molecular link between cargo in vesicles and myosin motors, which bind to F-actin and produce unidirectional vesicular movement along actin filaments.

Because there are PDZ proteins in the apical and basolateral membrane of MDCK cells, it is reasonable to ask why is CFTR selectively retained in the apical membrane? There are at least four explanations that may not be mutually exclusive. First, EBP50, E3KARP and CAP70 - PDZ proteins that interact with CFTR - are abundantly expressed in the apical, but not in the basolateral membrane of Calu-3, T84 (47-49) and MDCK cells (Swiatecka-Urban et al., unpublished observations). Second, CFTR may bind to apical PDZ proteins with a higher affinity than to basolateral PDZ proteins. Third, although the PDZ interacting domains of CFTR (DTRL), an apical protein, and BGT-1 (ETHL), a basolateral protein, are similar, it is important to note that the C-terminal eight amino acids, which differ in CFTR and BGT-1, can determine the specificity of
PDZ protein interactions (21). Finally, our data indicate that PDZ proteins in the apical membrane that interact with CFTR regulate endocytic recycling, whereas PDZ proteins that may interact with CFTR in the basolateral membrane may not be involved in endocytic trafficking. Thus, selective retention of CFTR in the apical membrane of MDCK cells may result from a number of factors including the polarization of PDZ proteins, the binding affinity of PDZ proteins to CFTR, the amino acids upstream of the PDZ interacting domain and the function of the PDZ proteins that interact with CFTR. It will be interesting to identify which of the aforementioned possibilities are responsible for the polarized expression of CFTR in the apical membrane.

Protein targeting and sorting motifs are differentially interpreted by various cell types (50). For example, the β subunit of the H-K-ATPase is expressed in the apical membrane of LLC-PK1 cells and in the basolateral membrane of MDCK cells (51). Aquaporin 2 is expressed in the apical and basolateral membrane of MDCK cells but only in the apical membrane of LLC-PK1 cells (52). These observations demonstrate that it is important to study sorting and retention motifs within the context of the cellular environment in which the protein is normally expressed. Whereas we observed that CFTR is randomly sorted from the TGN to the apical and basolateral membranes in MDCK cells, we found that CFTR is sorted directly from the TGN to the apical membrane in LLC-PK1 cells, a cell line derived from the renal proximal tubule (Swiatecka-Urban et al., unpublished data). These data are consistent with the recent observation that LLC-PK1 cells lack the µ1B subunit of the AP1 complex, a protein that is required for proteins to traffic from the TGN to the basolateral membrane (53). Thus, it is not surprising that addition of an epitope tag to the C-terminus of CFTR (which should block interaction of CFTR with PDZ proteins) did not affect the apical polarization of CFTR in LLC-PK1 cells (54). Because CFTR is also expressed in airway epithelial cells we conducted pulse chase studies in Calu-3 cells, a well characterized human airway epithelial cell line that express CFTR in the apical plasma membrane (47,55,56). Similar to our data in MDCK cells, wt-CFTR was sorted in a non-polarized manner from the TGN to the apical and basolateral membrane (ratio apical/basolateral =1.0 from 0 to 90 minutes after the pulse: data to be presented in a separate manuscript). Our results in MDCK and Calu-3 cells suggest a common
mechanism for CFTR trafficking in polarized kidney (MDCK) and airway epithelial cells (Calu-3), but not in LLC-PK1 cells.

In conclusion, the data in this manuscript demonstrate that the PDZ interacting domain of CFTR is an apical membrane, endocytic recycling motif and that polarization of CFTR to the apical membrane in kidney (MDCK) epithelial cells is mediated by interaction with PDZ protein(s) that facilitate CFTR endocytic recycling.
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FOOTNOTES

1 Abbreviation used in the text are: CFTR - cystic fibrosis transmembrane conductance regulator; CF - cystic fibrosis; GFP - green fluorescent protein, GSH – glutathione; TGN – trans Golgi network; PDZ - (PSD-95, Dlg and ZO-1).
FIGURE LEGENDS

FIGURE 1. Representative pulse-chase experiments illustrating the amount of newly synthesized wt-CFTR and CFTR-ΔTRL in the apical and basolateral membranes during the chase period. The time (in minutes) after the end of the 30 minute pulse with Tran^{35}S-Labeled Met/Cys is indicated above each lane. Tran^{35}S-labeled CFTR isolated from apical or basolateral membranes was separated by SDS-PAGE and detected by Phosphorimage analysis. A. wt-CFTR. B. CFTR-ΔTRL. As described previously, the molecular mass of GFP (27 plus a 3 kDa flexible linker sequence) fused to maturely glycosylated CFTR (180 kDa) is predicted to run at ~210 kDa (26). The molecular mass of the maturely glycosylated “C-Band” of CFTR was ~210 kDa (26). As described previously, the maturely glycosylated “C” band of GFP-tagged CFTR (evident in this as well as subsequent figures) runs as a doublet on Western blots, possibly due to aggregation of CFTR (26). Neither band is sensitive to Endo H digestion but is digested to the unglycosylated “A” band by PNGase F (26).

FIGURE 2. Summary of the ratio of wt-CFTR and CFTR-ΔTRL in the apical/basolateral membranes as a function of time after the end of the 30 minute pulse with Tran^{35}S-Labeled Met/Cys. Data are expressed as the mean ± SEM where the number of experiments was 4 for wt-CFTR and 5 for CFTR-ΔTRL. The ratio of wt-CFTR in the apical/basolateral membrane was significantly different from unity at the 120 minute time point, as indicated by the asterisk (P<0.01). By contrast, the ratio of CFTR-ΔTRL in the apical/basolateral membrane was not different from unity at any time point.

FIGURE 3. Representative images from pulse-chase and selective plasma membrane biotinylation studies conducted to measure the half-life of wt-CFTR and CFTR-ΔTRL in the apical and basolateral membranes as described in Results. Cells were metabolically labeled with Tran^{35}S-Labeled Met/Cys for 30 minutes at 37°C and chased in an excess of unlabeled Met/Cys at 37°C for 2, 6, 18 and 24 hrs. Subsequently, monolayers were cooled to 4°C and the apical or basolateral
membranes were biotinylated at the times indicated. Biotinylated proteins were pulled down with streptavidin beads and biotinylated CFTR was immunoprecipitated with a GFP monoclonal antibody or a cocktail of monoclonal antibodies M3A7/L12B4 (29). Immunoprecipitated CFTR was separated by SDS-PAGE and detected by Phosphorimage analysis. In this experiment it is evident that the half-life of wt-CFTR in the apical membrane was significantly longer than the half-life of wt-CFTR in the basolateral membrane and than the half-life of CFTR-ΔTRL in the apical and basolateral membranes.

**Figure 4.** Summary of studies conducted to determine the half-life of wt-CFTR and CFTR-ΔTRL in the apical and basolateral membranes. Data are reported as the percent of wt-CFTR and CFTR-ΔTRL remaining in the apical and basolateral membrane as a function time after the end of the 30 minute pulse with Tran35S-Labeled Met/Cys. Based on half-life of CFTR in the apical (24.1 hrs.) and basolateral membrane (12.9 hrs.) we estimate that, at steady-state, the ratio of CFTR in the apical/basolateral membrane should be ~2:1. This value is similar to the range of values measured for wt-CFTR previously in MDCK and human airway epithelial cells (i.e., 3:1 to 8:1)(16)). Moreover, the value of 2:1 is similar to the apical/basolateral ratio of a GFP tagged fusion protein (3:1) recently reported in MDCK cells by Simons and colleagues (37). Data are expressed as the mean ± SEM where the number of experiments was 6 for wt-CFTR and CFTR-ΔTRL.

**Figure 5.** Summary of studies conducted to determine the half-life of the maturely glycosylated (C band) of wt-CFTR and CFTR-ΔTRL in cell lysates (minus the plasma membrane biotinylated CFTR). In panel A data are reported as the percent of wt-CFTR and CFTR-ΔTRL remaining as a function time after the end of the 30 minute pulse with Tran35S-Labeled Met/Cys (100% value is the amount of CFTR at time 0, which is at the beginning of the chase period). Data are expressed as the mean ± SEM where the number of experiments was 6 for wt-CFTR and CFTR-ΔTRL. Panel B illustrates a representative experiment examining the amount of wt-CFTR or CFTR-ΔTRL is cell lysates as a function of time (in hours) after a 30 minute pulse with Tran35S-Labeled
Met/Cys. As determined by pulse-chase analysis, there appears to be two pools of CFTR: one in the apical plasma membrane (~20% of CFTR in the cell) with a half life of ~24 hrs (Figure 4) and one in an intracellular compartment(s) (80% of CFTR in the cell) with a half life of ~12.5 hrs (Figure 5). The difference in half-life suggests that CFTR in the apical membrane, endocytic trafficking pathway does not enter the degradative pathway as readily as does CFTR in the intracellular compartment(s). Thus, in can be predicted that deletion of the PDZ interacting domain should increase the amount of CFTR in the intracellular compartment(s) and, thus, the half life of CFTR-ΔTRL (11.2 hrs) should be less than half life of wt-CFTR (12.9 hrs.) in the intracellular compartment(s). The difference in half life did not achieve statistical significance, most likely because the endocytic recycling pool of CFTR is relatively small (~20%) and because the decrease in endocytic recycling with deletion of the PDZ interacting domain is also relatively small.

**Figure 6.** Transcytosis assay to determine if wt-CFTR is transcytosed from the basolateral to the apical plasma membrane.  **A.** Basolateral membranes were biotinylated (EZ-Link™ Sulfo-NHS-SS-Biotin) at time 0 at 4°C and then warmed to 37°C for 0, 60 or 120 minutes as indicated. Subsequently, monolayers were cooled to 4°C and GSH was added to the apical or basolateral bathing solutions as indicated by a + sign above the blot, or to neither side as indicated by the – sign above the blot. Subsequently, cells were lysed and biotinylated proteins were pulled down with streptavidin and separated by SDS-PAGE. Biotinylated proteins were transferred to PDVF membranes and CFTR detected by a GFP monoclonal antibody and ECL. The letters below each lane are to enhance the description in Results. **B.** CFTR in cell lysates demonstrating that monolayers in each set of experiments expressed similar amounts of CFTR.

**Figure 7.** Summary of studies conducted to determine if the reduced half-life of CFTR-ΔTRL compared to wt-CFTR in the apical membrane is due to a difference in endocytosis (A) and/or endocytic recycling (B). Data are expressed as the mean ± SEM of 3 experiments for both wt-CFTR and CFTR-ΔTRL.  **A**  Endocytic assay.  **B**  Recycling assay. At three minutes the percent
of endocytosed CFTR-ΔTRL recycled was significantly less than the percent of endocytosed wt-CFTR recycled (P<0.01).
Figure 1

A. wt-CFTR

B. CFTR-TRL
Figure 2

Ratio apical/basolateral vs Time (Minutes)

- wt-CFTR
- CFTR-ΔTRL

* Significance indicated by asterisk.
Figure 3
Figure 4
Figure 5

A. Percent CFTR Remaining

Time (Hours)

B. wt-CFTR

CFTR-ΔTRL

- 200 kDa

- 200 kDa
A. Basolateral membrane biotinylation

- Apical GSH: - - - - - + + +
- Basolateral GSH: - - - + + + - - -

200 kDa -

0 60 120 0 60 120 0 60 120
a b c d e f g h i

B. CFTR in lysates

200 kDa -

Figure 6
Figure 7
PDZ-domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator
Agnieszka Swiatecka-Urban, Marc Duhaime, Bonita Coutermash, Katherine H. Karlson, James Collawn, Michal Milewski, Garry R. Cutting, William B. Guggino, George Langford and Bruce A. Stanton

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