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A PDZ-Binding Motif is Essential but Not Sufficient to Localize the C Terminus of CFTR to the Apical Membrane

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SUMMARY

Localization of ion channels and transporters to the correct membrane of polarized epithelia is important for vectorial ion movement. Prior studies have shown that the cytoplasmic carboxyl terminus of the cystic fibrosis transmembrane conductance regulator (CFTR) is involved in the apical localization of this protein. Here we show that the C-terminal tail alone, or when fused to the green fluorescent protein (GFP), can localize to the apical plasma membrane, despite the absence of transmembrane domains. Co-expression of the C terminus with full-length CFTR results in redistribution of CFTR from apical to basolateral membranes, indicating that both proteins interact with the same target at the apical membrane. Amino acid substitution and deletion analysis confirms the importance of a PDZ-binding motif D-T-R-L> for apical localization. However, two other C-terminal regions, encompassing amino acids 1370-1394 and 1404-1425 of human CFTR, are also required for localizing to the apical plasma membrane. Based on these results, we propose a model of polarized distribution of CFTR, which includes a mechanism of selective retention of this protein in the apical plasma membrane and stresses the requirement for other C-terminal sequences in addition to a PDZ-binding motif.

Key words: Apical polarization, CFTR, PDZ domain, Localization signal, Cystic fibrosis

INTRODUCTION

Achieving the correct cellular location of ion channels and transporters is essential for ion movement across epithelial cells. Elucidation of the mechanism of polarized protein distribution is difficult without identification of signals required for targeting and/or retention of these proteins in specific subdomains of the plasma membrane. Cystic fibrosis (CF), a common lethal genetic disorder characterized by abnormal transepithelial ion transport (Welsh et al., 1995; Cutting, 1997), is caused by mutations in an apical membrane protein called CFTR (cystic fibrosis transmembrane conductance regulator) (Kerem et al., 1989). The apical localization of CFTR is critical for its channel and regulatory function. However, the mechanism of creating and maintaining the polarized distribution of this protein remains unknown.

CFTR belongs to a superfamily of ABC (ATP-Binding Cassette) transporters that share a common structural organization consisting of two nucleotide-binding domains (NBD) and two membrane-spanning domains (MSD) (Hyde et al., 1990). The region of CFTR extending from the second nucleotide binding domain (NBD2) to the C terminus is unique among ABC transporters, and contains a region rich in positively charged amino acids followed by a short stretch of negatively charged residues (Riordan et al., 1989). The charged amino acids, as well as a very C-terminal motif T-R/K-L>, are highly conserved across species. Studies of the distribution of CFTR fused to GFP revealed that the very C-terminal sequence of CFTR is involved in its apical localization in polarized kidney epithelial cells (Moyer et al., 1999). In contrast to the apically located normal CFTR, the protein with a truncated C terminus showed both basolateral and apical localization in MDCK cells. The presence of a PDZ-binding motif in the very C terminus of CFTR suggests that confinement to the apical membrane is dependent on interaction with proteins containing PDZ domains (Wang et al., 1998; Short et al., 1998; Hall et al., 1998). However, whether this sequence constitutes the only signal required for preferential apical localization of CFTR remains unknown.

The accepted view is that epithelial cell polarity is a result of protein sorting in the trans-Golgi network and endosomes (Mostov and Cardone, 1995; Zegers and Hoekstra, 1998; Yeaman et al., 1999; Matter, 2000). However, there are a growing number of known integral membrane proteins that maintain their polarized distribution by selective retention in specific domains of plasma membrane rather than by sorting to specific transport vesicles. Examples of such mechanism include basolateral stabilization of β_1 integrin (Gut et al., 1998) and γ-aminobutyric acid transporter BGT1 (Perego et al., 1999), with the latter involving PDZ-based interactions. These two general strategies of polarized protein distribution are likely to utilize different localization signals. Thus, defining the minimal CFTR sequence required for polarized distribution and characterizing the sequences responsible for apical

localization should bring us closer to the understanding of how the polarized distribution of CFTR is established.

Many deletions and point mutations in the CFTR gene lead to the synthesis of a misfolded protein that is unable to reach the cell surface (Cheng et al., 1990; Rich et al., 1993; Gentzsch and Riordan, 2000). This makes the identification of sequences required for apical localization difficult. Therefore, we took a different approach that focused on one particular portion of CFTR suspected by us of containing signals required for localization to the apical membrane. Indeed, we were able to demonstrate that the CFTR C-terminal region alone was capable of acquiring apical localization in polarized MDCK cells. This discovery enabled the characterization of sequences within the C terminus that are required for polarized apical distribution of CFTR.

MATERIALS AND METHODS

Plasmid constructions

The GFP-CFTR 1370-1480 chimera was created by PCR amplification using the 'band-aid' method (Rubin et al., 1992). The CFTR-containing vector pBQ4.7 (gift from J. Rommens and L.-C. Tsui) and the pTR-UF5 plasmid containing 'humanized' GFP cDNA (gift from N. Muzyczka) were used as templates for PCR reactions. TA cloning was used to clone the PCR product into pCR2.1 vector (Invitrogen). The insert was sequenced and subcloned into the mammalian expression vector pAvS6 containing a Rous sarcoma virus (RSV) promoter (Mittereder et al., 1994). A similar procedure was used to create control plasmid containing the GFP cDNA. The correct size of GFP fusion proteins encoded by these DNA constructs was examined by western blotting analysis. To obtain better transfection rates and higher expression level in mammalian cells, the inserts were also subcloned into a cytomegalovirus (CMV) promoter-driven vector pRK5-SK. These constructs in CMV promoter-driven vector were used to obtain the localization data presented here, although very similar results were observed when constructs in RSV promoter-driven vectors were used. The construction of mammalian expression plasmid containing full-length CFTR fused to GFP was described elsewhere (Moyer et al., 1998).

Site-directed mutagenesis

The mutant versions of the GFP-CFTR C-ter were created using the site-directed mutagenesis system 'Transformer' (Clontech). 'Transformer' mutagenesis system was also used to create the CFTR C-terminal constructs tagged with influenza hemagglutinin (HA) epitope. The sequences of selection primers and mutagenic primers used to create point mutations, deletions and insertions in the GFP-CFTR C-ter construct and in its derivatives are available upon request.

Cell cultures and transfection

All cell lines were grown in tissue culture treated polystyrene flasks in 5% CO_2-balanced air at 37°C. Madin-Darby canine kidney (MDCK) cells type II were a generous gift of A. Hubbard. The cells were grown in DMEM containing 10% FBS (Biofluids) and 0.37% sodium bicarbonate. Human embyonic kidney cells HEK293, epithelioid cervix carcinoma cells HeLa, and MDCK cells type I, all obtained from American Type Tissue Collection, were grown in EMEM supplemented with 10% FBS. Porcine kidney epithelial cells LLC-PK_1 cells (gift from M. Li) were grown in Medium 199 (Life Technologies) containing 10% FBS. IB3-1 bronchial epithelial cells, derived from a CF patient, were cultured as described (Zeitlin et al., 1991).

For protein localization analysis, cells were grown on non-coated (MDCK, HeLa and LLC-PK1 cells) or collagen-coated (IB3-1, HEK293 cells) glass coverslips, and transiently transfected using the Lipofectin reagent (Life Technologies). To increase the transfection rates for polarized epithelial cells (MDCK, LLC-PK_1), the DNA/lipofectin mixture was added immediately after the cells were seeded on the coverslips. Remaining transfection steps were carried out according to manufacturer's instructions. In the co-transfection experiments, a 5:1 ratio was used for plasmids encoding the HA-tagged C-terminal constructs and GFP-tagged full-length CFTR, respectively. 48-72 hours after transfection, the cells were fixed with 4% PFA for 20 minutes and, if not immunostained, mounted in SlowFade (Molecular Probes) containing 0.1 mg/ml of DAPI (Sigma).

Antibodies and immunofluorescent staining

Antibodies for ZO-1 (Zymed) and Na^+/K^+ ATPase (Upstate Biotechnology) were used to stain the tight junctions and basolateral plasma membrane, respectively. HA-tagged proteins were detected by immunostaining with an anti-HA 12CA5 antibody (Boehringer Mannheim). Prior to immunostaining, the cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 for 5 minutes, and washed with PBS. Non-specific binding sites were blocked with 2.5% goat serum in PBS. Staining was performed by two sequential incubation steps. Cells were first incubated with the primary antibody (anti-ZO-1, anti-Na^+/K^+ ATPase or anti-HA) for 1.5 hours, and then with appropriate secondary antibody conjugated to Cy3 fluorescent dye (Sigma) for 30 minutes. After staining, the coverslips with cells were washed in PBS and mounted in SlowFade with DAPI.

Western blotting analysis

The MDCK II cells transiently transfected with DNA constructs encoding GFP fusion proteins of different length were lysed overnight in 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, 20 mM Hepes, pH 7.0. The lysates were spun at 20,000 ***g*** for 3 minutes to pellet the insoluble material, and the proteins from supernatant were separated by the SDS-PAGE (12%) and electrophoretically transferred to PVDF membranes (Amersham Pharmacia Biotech). The probing with monoclonal anti-GFP antibody (Clontech) and subsequent detection with ECL+plus system (Amersham Pharmacia Biotech) was performed according to the manufacturers' instructions.

Confocal laser microscopy

The fluorescence label was examined with laser scanning confocal imaging system (LSM Carl Zeiss). Images were generated using 16-fold line averaging. Contrast and brightness settings were chosen to ensure that all pixels were within the linear range. The xz cross-sections were produced using a 0.2 μm motor step. For protein localization analysis, at least 20 transfected cells were tested for each GFP or HA-tagged construct. The results were considered conclusive only if 90% or more cells showed specific (apical or cytoplasmic) pattern of distribution. For co-expression analysis, the semi-quantitative confocal microscopy was used to examine the relative distribution of GFP-CFTR in the apical and basolateral membranes, as described elsewhere (Moyer et al., 1999). Images were prepared for publication with LSM Carl Zeiss Software and Adobe Photoshop.

Electron microscopy

For transmission electron microscopy, transfected cells were grown on etched grid glass coverslips (Bellco). The grid coordinates were used to determine the precise position of selected cells showing intracellular accumulations of GFP fusion protein, as seen in standard epifluorescent microscopy. The cells were fixed at room temperature for 30 minutes in 2% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.4). They were then rinsed in 0.1 M sodium cacodylate buffer, postfixed in reduced osmium (1% OsO_4, 2% KFeCN) in the same buffer for 1 hour, incubated in 2% aqueous uranyl acetate for 30 minutes, rinsed in distilled water, dehydrated in a graded series of ethanol, and embedded in Eponate 12 (Ted Pella). Polymerization was carried out overnight at 55°C. The coverslips were separated from

embedded cells by immersing in liquid nitrogen. The grid traces, imprinted on the surface of the Eponate block, were used to locate the previously selected cells. Ultra-thin sections were cut, mounted on nickel grids, and examined at 60 kV in a Zeiss TEM 10A electron microscope.

RESULTS

Intracellular aggregation of the C terminus of CFTR

To identify specific localization signals in the C terminus of CFTR, the carboxyl terminus of GFP was fused in-frame to the last 111 amino acids (a.a.) of human CFTR, starting from the last amino acid of the conserved Walker B motif in the second nucleotide binding domain (NBD2) at position 1370. The subcellular distribution of this chimeric protein was examined in transiently transfected IB3-1 cells. In contrast to the diffuse cytoplasmic and nuclear localization of GFP protein, the GFP fused to a.a. 1370-1480 of CFTR accumulated in discrete intracellular aggregates, usually in a perinuclear distribution (Fig. 1a). Transmission electron microscopy of selected cells expressing the fusion protein revealed that the accumulations were large electron-dense bodies (Fig. 1b) that were not observed in non-transfected cells (data not shown). The ultrastructure of the accumulations resembled aggresomes (Johnston et al., 1998), indicating that they were aggregates of misfolded fusion protein.

Since aggregation of the fusion protein may have interfered with its targeting to specific subcellular locations, we sought to identify the CFTR region that facilitated intracellular accumulation. A series of C-terminal truncations and deletions were created to determine whether a specific protein sequence was responsible for aggregation of fusion protein. The truncated fusion protein containing a.a 1370-1425 of CFTR accumulated in aggregates, whereas no intracellular accumulation was observed for a construct with a.a. 1370-1385 (Fig. 1c, the upper panel). This suggested that the 40 amino acids region between residues 1386 and 1425 contained sequence responsible for aggregation. A series of nested deletions were made to further define the region causing protein aggregation (Fig. 1c, the lower panel). Deletion of a.a. 1395-1403 (Δag) eliminated intracellular accumulation, indicating that this nine amino acid region facilitated protein aggregation.

CFTR C terminus can target GFP to the apical membrane

To test whether the CFTR C terminus, devoid of sequences responsible for protein aggregation, contains specific localization signals, we examined the subcellular distribution of the GFP-CFTR 1370-1480 Δag fusion protein in two different cell lines. The IB3-1 cells were used as an example of non-polarized cells of epithelial origin, and the MDCK cell line, a commonly used model of polarized epithelia, was chosen to study the subcellular localization of the fusion protein in polarized cells. The polarization status of MDCK cells growing on glass coverslips was assessed by immunolocalization of endogenous ZO-1 and Na^+/K^+ ATPase, markers for tight junctions and basolateral plasma membrane, respectively (Fig. 2a,b). The apical membrane localization of full-length CFTR fused to GFP was another indicator of cell

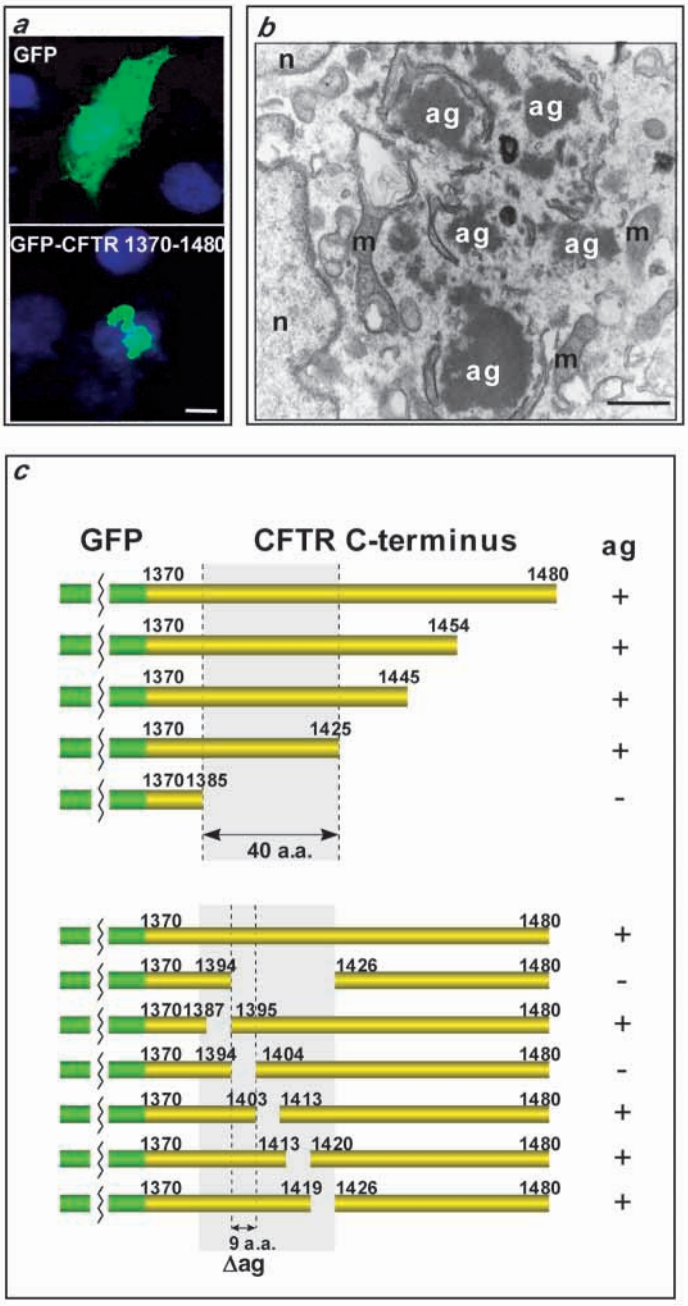

Fig. 1. Intracellular aggregation of the C terminus of CFTR fused to GFP. (a) Confocal microscope analysis of IB3-1 cells transfected with GFP (green) or GFP-CFTR 1370-1480 (green). Cell nuclei were stained blue with DAPI. Bar, 10 μm. (b) Ultrastructure of intracellular aggregates formed by GFP-CFTR 1370-1480 in an IB3-1 cell, as seen by transmission electron microscopy. Selection of cells showing fluorescent intracellular accumulations was performed as described in Materials and Methods. ag – aggregates, n – nucleus, m – mitochondria. Bar, 0.2 μm. (c) CFTR C-terminal constructs created to identify the sequence responsible for protein aggregation (ag). The GFP portion of fusion protein is shown in green and the CFTR C-terminal portion is shown in yellow. Numbering of CFTR amino acids was used according to published sequence (Riordan et al., 1989). The construct with deletion of a.a. 1395-1403 (Δag) that eliminated intracellular aggregation was utilised in subsequent experiments.

membrane polarity (Moyer et al., 1998) (Fig. 2c). The subcellular localization of GFP-CFTR did not differ between polarized MDCK type I and polarized MDCK type II cells (data not shown). Since transfection rates were significantly higher for the MDCK II cells, this cell line was used for most of our experiments on polarized protein distribution.

Confocal microscope analysis of non-polarized IB3-1 cells transiently transfected with the GFP fusion protein containing the Δag deletion showed diffuse cytoplasmic distribution of fluorescence signal (Fig. 2d). In contrast to full-length CFTR, the C-terminal construct lacks transmembrane domains, so the protein should not associate with the plasma membrane unless other signals for membrane localization are present. Thus, the diffuse cytoplasmic distribution of GFP fusion protein in IB3-1 cells suggested that the C-terminal portion of CFTR did not contain an active membrane localization signal. However, in polarized MDCK cells, this fusion protein localized to the apical plasma membrane (Fig. 2e). Since the GFP alone showed cytoplasmic and nuclear distribution in MDCK cells, this result suggested that the C terminus of CFTR contains a signal that can alter the subcellular distribution of attached protein in polarized cells. The polarization-specific nature of this localization signal was further confirmed by apical localization of the fusion protein in another polarized epithelial cell line LLC-PK_1 and by diffuse cytoplasmic distribution of the same protein in non-polarized HEK293 and HeLa cells (data not shown).

The horizontal sections through the apical membrane of MDCK cells showed that protein was not evenly distributed on the cell surface but it was rather concentrated in discrete regions (Fig. 2e). This pattern resembled the distribution of full-length CFTR in this cell line and might correspond to clustering, perhaps within microvilli.

A PDZ-binding motif is required for apical targeting

To determine the role of the C-terminal PDZ-binding motif in apical localization of the CFTR C terminus, we examined the subcellular distribution of GFP fusion proteins lacking the very C-terminal amino acids. Truncation of the GFP fusion protein by 26 amino acids abolished its apical localization (Fig. 3a). This truncation corresponds to the naturally occurring CFTR mutation S1455X that was associated with sweat gland dysfunction (Mickle et al., 1998). Also, deletion of the C-terminal three amino acids (ΔTRL) or their substitution with alanines (TRL→AAA) resulted in diffuse cytoplasmic distribution of the fusion protein. This suggested that the very C-terminal amino acids forming a PDZ-binding domain (Wang et al., 1998; Short et al., 1998; Hall et al., 1998) are required for apical localization of the fusion protein.

To identify the amino acids within the PDZ-binding

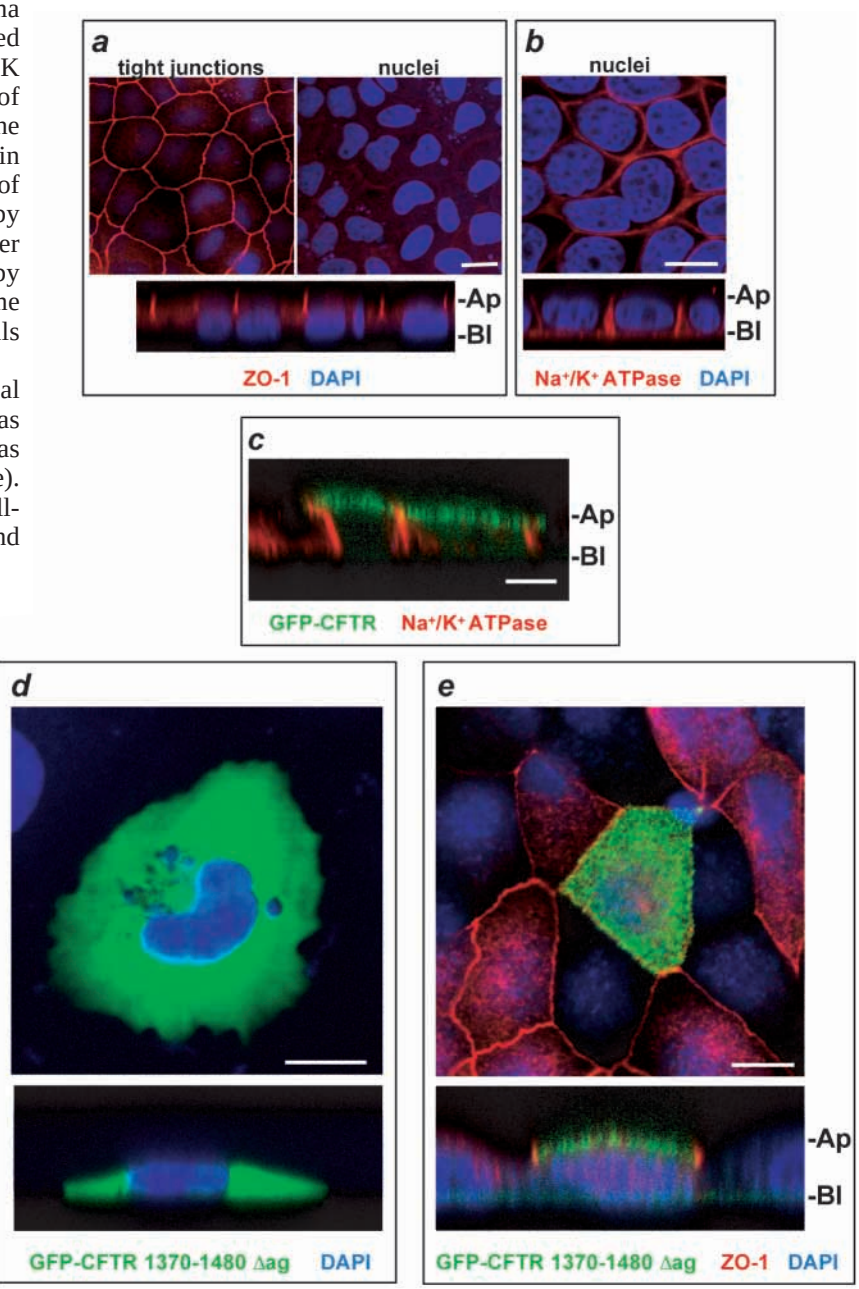

Fig. 2. Subcellular distribution of the GFP-tagged CFTR C terminus, devoid of the sequence responsible for aggregation, in non-polarized and polarized epithelial cells. (a-b) Analysis of polarization status of MDCK type II cells. Horizontal sections through tight junctions or through nuclei, and vertical reconstructions (below) are shown. Nuclei were stained blue with DAPI. Ap – apical surface, Bl – basal surface. (a) Tight junctions stained red with ZO-1 antibody. (b) Basolateral membranes stained red with Na^+/K^+ ATPase antibody. (c) Vertical reconstruction of MDCK II cells transiently expressing full-length CFTR fused to GFP (green). GFP-CFTR demonstrated apical plasma membrane localization that did not overlap with the basolateral localization of Na^+/K^+ ATPase. (d) Cytoplasmic distribution of GFP-CFTR 1370-1480 Δag (green) in non-polarized IB3-1 cells. e, Apical membrane localization of GFP-CFTR 1370-1480 Δag (green) in polarized MDCK II cells. Tight junctions were stained with ZO-1 antibody (red). Bars, 10 μm. Cell nuclei were stained blue with DAPI.

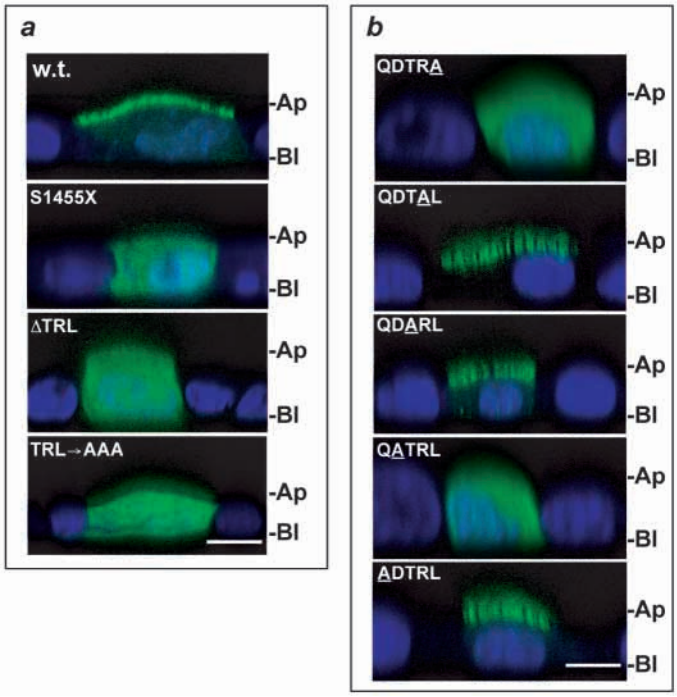

Fig. 3. Requirement for PDZ-binding motif in apical localization of the CFTR C terminus in polarized MDCK II cells. (a) Loss of apical localization of GFP-CFTR 1370-1480 Δag construct (green) after truncation of last 26 amino acids (S1455X), truncation of last three amino acids (ΔTRL), or substitution of last three amino acids with alanines (TRL→AAA). (b) Effect of alanine substitutions of single amino acids in the very C-terminal region on apical localization of GFP-CFTR 1370-1480 Δag (green). Substitution of amino acids at position 0 and –3, but not at positions –1, –2 and –4, resulted in loss of apical localization. Bars, 10 μm. Cell nuclei were stained blue with DAPI.

domain that are essential for apical localization, we examined the impact of single amino acid substitutions in this region (Fig. 3b). Substitution of the highly conserved leucine, located at position 0 (amino acid 1480), with alanine resulted in complete loss of apical localization. Also, the substitution of aspartic acid at position –3 with alanine led to substantial redistribution of the fusion protein from the apical membrane to the cytoplasm, although in this case the loss of polarized distribution was not complete with some cells (4/25) showing a moderate fluorescence signal at the apical membrane (data not shown). Similar substitutions of glutamate at position –4, threonine at position –2, or arginine at position –1 with alanine had no significant effect on apical localization of the GFP fusion protein. These results demonstrated that sequence specificity of the putative PDZ protein responsible for apical localization of the CFTR C-terminal fusion protein was similar to that shown previously for the full-length protein (Moyer et al., 2000). The only difference was observed at position –3, that was not critical for apical localization of full-length CFTR but seemed important for distribution of the CFTR C terminus

C terminus and full-length protein compete for the same target

Similar subcellular localization of the C terminus of CFTR and the full-length protein in polarized MDCK cells suggested that both proteins recognize the same target at the apical plasma membrane. However, their slightly different preferences toward the most efficient amino acid composition of a PDZ-binding motif raised the question whether apical localization of these two proteins is really a result of interaction with the same putative PDZ protein. To test whether the full-length CFTR and the C terminus of this protein can compete for the same target at the apical membrane, we co-expressed the two proteins in MDCK cells. To distinguish between the two constructs, the GFP tag in the C-terminal construct was replaced with an HA (hemagglutinin) epitope. When expressed alone in MDCK cells, the HA-tagged C terminus of CFTR (HA-CFTR 1370-1480 Δag) showed apical membrane localization similar to that observed for GFP-tagged full-length CFTR. Deletion of the last three amino acids from this HA-tagged protein resulted in loss of polarized distribution, indicating that HA-CFTR 1370-1480 Δag, like the corresponding GFP-tagged construct, required the PDZ-binding motif for apical localization (Fig. 4a).

If the C terminus of CFTR competed with the full-length protein for an apically located target, over-expression of the C-terminal construct should result in subcellular redistribution of the full-length CFTR. Indeed, when co-expressed with HA-

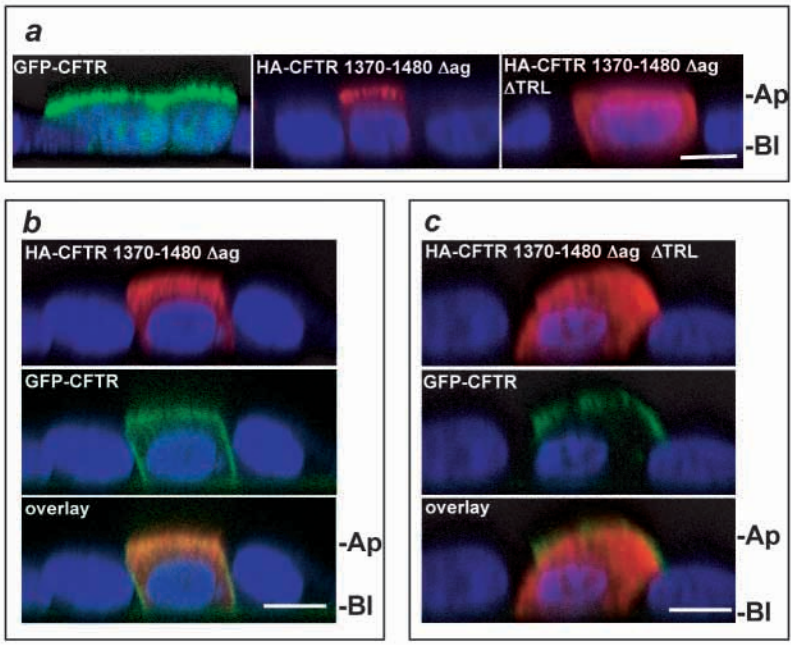

Fig. 4. Partial redistribution of full-length CFTR caused by co-expression with HA-tagged C terminus of CFTR. (a) Apical localization of full-length CFTR fused to GFP (green) and HA-CFTR 1370-1480 Δag (red), and cytoplasmic distribution of HA-CFTR 1370-1480 Δag ΔTRL (red), expressed separately in polarized MDCK II cells. (b) Apical and basolateral distribution of GFP-CFTR (green), when co-expressed with HA-CFTR 1370-1480 Δag (red). (c) Apical localization of GFP-CFTR (green), when co-expressed with HA-CFTR 1370-1480 Δag ΔTRL (red). Bars, 10 μm. Cell nuclei were stained blue with DAPI.

CFTR 1370-1480 Δag, the full-length CFTR was redistributed from the apical to the lateral domain of plasma membrane (Fig. 4b). However, some full-length protein remained in the apical membrane, which resembled the situation observed when the PDZ-binding motif was removed from GFP-tagged full-length CFTR (Moyer et al., 1999). The semi-quantitative fluorescence analysis showed that the apical/basolateral ratio in this case was significantly lower (0.92±0.52) than for GFP-CFTR expressed alone (7.21±6.12, $P<0.005$). Importantly, no protein redistribution was observed when full-length CFTR was co-expressed with the truncated HA-tagged C terminus that was unable to localize to the apical membrane (Fig. 4c). In this case, the apical/basolateral ratio for GFP-CFTR (6.03±4.89) was not significantly different from that in control experiment ($P>0.5$).

More than one sequence is required for apical localization

To determine whether the C-terminal PDZ-binding motif was the only signal required for targeting proteins to the apical membrane, we examined the subcellular distribution of the fusion proteins containing smaller portions of the CFTR C terminus fused to GFP. The western blotting analysis showed that these shorter constructs encode GFP fusion proteins of expected size (data not shown). Surprisingly, GFP fused to the last 26 or 55 amino acids of CFTR did not localize to the apical plasma membrane (Fig. 5a), suggesting that the PDZ-binding motif alone is not able to target GFP to the apical membrane. To exclude the possibility that the shorter C terminus of CFTR was not properly exposed in the GFP fusion protein and therefore it could not interact with the apically located target, we examined the distribution of HA-tagged C terminus of CFTR containing the last 55 amino acids of CFTR. This peptide showed only cytoplasmic distribution (Fig. 5b) and was not able to compete with the full-length CFTR for apical localization (data not shown). These results supported our hypothesis that the PDZ-binding motif is not the only C-terminal signal involved in localization to the apical membrane.

A series of deletions in the GFP fusion protein was created to identify the regions of the C terminus that contribute to apical membrane localization (Fig. 5c). The minimal construct that localized to the apical membrane of MDCK cells contained 52 amino acids of CFTR located in three separate regions of the C terminus, including amino acids 1370-1394, 1404-1425 and 1476-1480 of full-length CFTR. The amino acids 1370-1394 and 1404-1425 showed some similarity to the corresponding sequences of other ABC transporters (Riordan et al., 1989), although no obvious conserved motifs were present in these regions. Taken together, our results indicated that, in addition to the PDZ-binding motif, at least two other CFTR sequences are required for localizing to the apical plasma membrane. In context of the full-length protein, it is worth noting that large deletions in this particular region usually result in misfolding of CFTR (Rich et al., 1993; Haardt et al., 1999). However, results of the competition experiment, shown above, suggested that the sequences in question were required for preferential apical localization of the full-length protein.

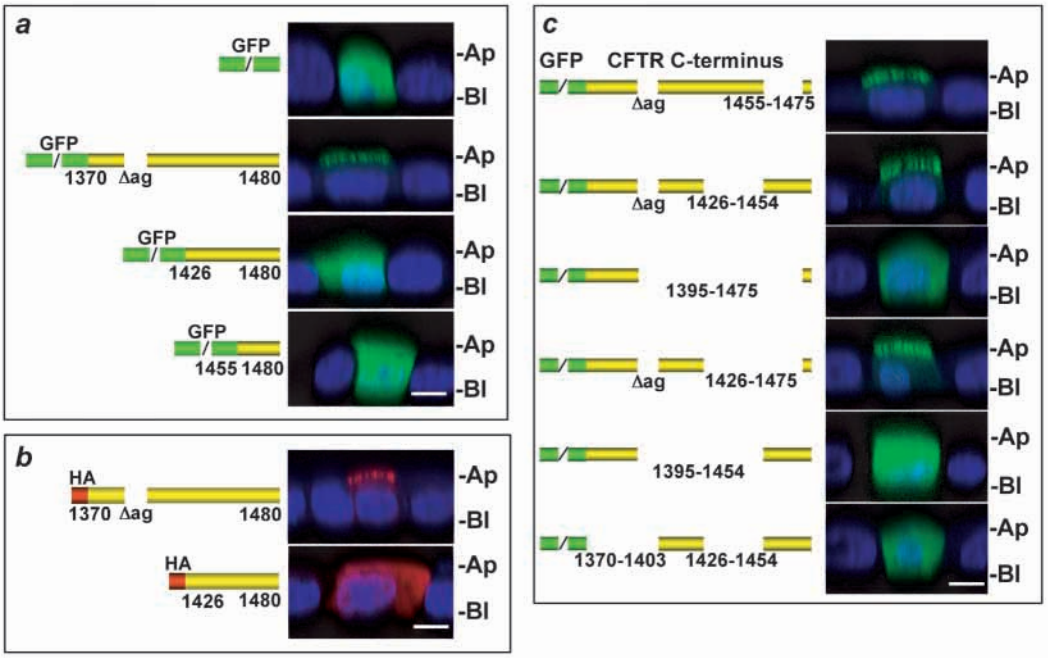

Fig. 5. Subcellular distribution of modified CFTR C-terminal constructs in polarized MDCK II cells. (a) Effect of different lengths of the CFTR C terminus on the localization of GFP-CFTR C-ter fusion protein (green). In contrast to apically located GFP-CFTR 1370-1480 Δag, the fusion proteins containing only amino acids 1426-1480 or 1455-1480 of CFTR showed cytoplasmic distribution, similar to that of GFP alone. (b) Subcellular localization of HA-tagged CFTR C-termini of different length (red). HA-CFTR 1370-1480 Δag localized to the apical membrane, whereas the HA-tagged CFTR C terminus containing amino acids 1426-1480 showed diffuse cytoplasmic distribution. (c) Effect of internal deletions within the C terminus of CFTR on subcellular localization of GFP-CFTR C-ter fusion protein (green). Removal of amino acids 1395-1403 (Δag) and 1425-1475 did not affect apical localization, whereas further deletions led to loss of polarized distribution. Bars: 10 μm. Cell nuclei were stained blue with DAPI.

DISCUSSION

In this study, we demonstrate that the cytoplasmic C-terminal tail of CFTR contains signals sufficient for its localization to the apical plasma membrane in polarized epithelial cells. Though our results confirm the involvement of the PDZ-interacting sequence in polarized distribution of CFTR, they also show that this C-terminal motif is not the only signal required for protein localization to the apical membrane. The carboxyl-terminal amino acids of several other integral membrane proteins are suspected of being an essential part of a multi-component signal that mediates apical or basolateral localization in epithelial cells. For example, the information contributed by the C-terminal PDZ-interacting domain-like sequence of apical γ-aminobutyric acid transporter GAT3 or by the carboxyl-terminal motif F-T-S-L> of basolateral membrane cofactor protein MCP is critical but not sufficient to mediate polarized distribution of these proteins (Muth et al., 1998; Teuchert et al., 1999).

Some polarization signals are functional only when present on membrane-associated proteins. For example, the C-terminal motif Q-V-S/A-P-A>, that regulates sorting and post-Golgi trafficking of rhodopsin by interaction with the light chain of cytoplasmic dynein (Tai et al., 1999), is able to redirect basolateral membrane protein CD7 to the apical membrane, but cannot target the cytosolic protein, glutathione S-transferase (GST), to the apical membrane, unless the palmitoylation membrane anchor signal is provided by the rhodopsin sequence (Chuang and Sung, 1998). The C-terminal region of CFTR does not contain any predicted hydrophobic α-helical structure that could serve as a transmembrane domain. Furthermore, the diffuse cytoplasmic distribution of the CFTR C-terminal constructs lacking the PDZ-binding motif suggests that the remaining part of the C-terminal sequence does not associate with the membrane.

CFTR lacking the PDZ-binding motif localizes to both basolateral and apical plasma membranes. Co-expression of CFTR with competing C terminus produces the same result. This indicates that inactivation of apical localization signal does not prevent CFTR from leaving the Golgi compartment but instead leads to random distribution of CFTR in different domains of the plasma membrane. Since two different mechanisms are generally considered to contribute to polarized expression of plasma membrane proteins in epithelial cells (Matter, 2000), two hypothetical models can be proposed to explain selective apical localization of CFTR. The first model assumes that apical steady state localization of CFTR is a result of direct apical targeting that involves efficient protein sorting in the trans-Golgi network and may also include a mechanism that controls vesicle docking and fusion at the apical plasma membrane. A characteristic feature of this model is its association with vesicle transport, which implies that the efficiency of a putative apical localization signal may depend on the association of the transported protein with the membrane. To explain the basolateral and apical localization of CFTR after disruption of apical localization signal, this model must also include a possibility of non-specific CFTR transport to the plasma membrane through default pathway. According to the second model of polarized distribution of CFTR, the protein is randomly transported to different domains of plasma membrane and then selectively stabilized in the apical membrane. This selective retention model may be based solely on the protein-protein interactions, including PDZ-mediated protein binding, and therefore should be favored in view of our results demonstrating that apical localization signal of CFTR may function in absence of transmembrane domains. A combination of both models is also possible, in which CFTR is selectively targeted to the apical surface, and its retention requires interaction with apically located PDZ protein.

Besides the role in apical localization, the C terminus of CFTR appears also to be involved in CFTR processing. Reported here is the identification of a sequence responsible for aggregation (ag) of the C-terminal constructs, which suggests that the C terminus of CFTR is a part of larger folding domain. The participation of the C-terminal sequences in protein processing has also been recently suggested by others (Haardt et al., 1999; Gentzsch and Riordan, 2000). The role of the ag region (a.a. 1395-1403) in folding of full-length CFTR and other related ABC transporters is a subject of ongoing studies in our labs. Another group of functional motifs present in the C terminus of CFTR includes endocytosis signals. The role of the tyrosine-based motif Y-D-S-I in the regulation of CFTR internalization has been recently demonstrated by two independent studies (Prince et al., 1999; Weixel and Bradbury, 2000). The di-leucine motif present in the C-terminal region of CFTR (amino acids 1430 and 1431 of human CFTR) may also play a role in protein endocytosis. Additionally, the C-terminal amino acids 1420-1457, a region that includes these two putative endocytosis signals, have also been found to mediate interaction with α1 subunit of AMPK (AMP-activated protein kinase) (Hallows et al., 2000). The proximity of internalization and apical localization signals in the C terminus of CFTR (Fig. 6) suggests a possible regulatory relationship between both processes.

In summary, a number of PDZ proteins have been recently shown to interact with the carboxyl-terminal amino acids of CFTR (Wang et al., 1998; Short et al., 1998; Hall et al., 1998; Moyer et al., 1999; Cheng et al., 1999; Wang et al., 2000). However, none of these proteins have been shown to be responsible for CFTR apical localization. Our study demonstrates that the PDZ-binding motif alone is not sufficient for apical localization and that additional sequences in the C

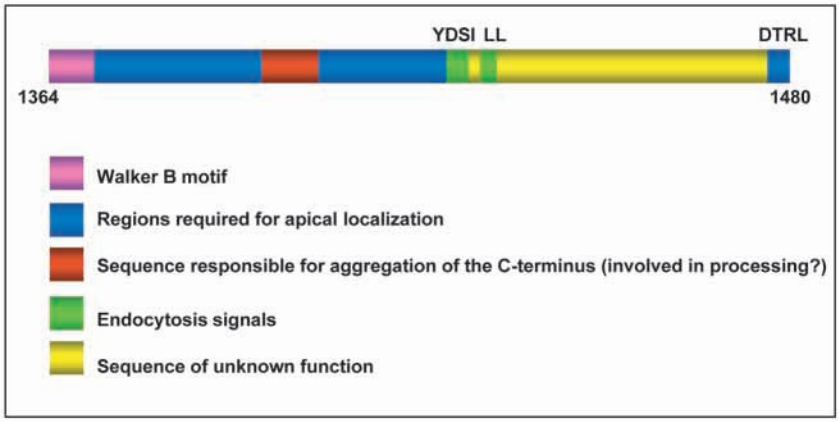

Fig. 6. Known motifs and functional regions in the C terminus of CFTR.

terminus mediate the process. This information will be critical in the identification of the binding partners of CFTR at the apical membrane.

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REFERENCES

Cheng, J., Moyer, B. D., Milewski, M., Hazama, A., Mickle, J. E., Cutting, G. R., Stanton, B. A. and Guggino, W. B. (1999). CFTR associates with the PDZ domain protein CAL at the trans-Golgi network. *Ped. Pulm. Supplement 19*, Abstract 15.

Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souzo, D. W., White, G. A., O'Riordan, C. R. and Smith, A. E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* **63**, 827-834.

Chuang, J. Z. and Sung, C. H. (1998). The cytoplasmic tail of rhodopsin acts as a novel apical sorting signal in polarized MDCK cells. *J. Cell Biol.* **142**, 1245-1256.

Cutting, G. R. (1997). Cystic Fibrosis. In *Emery and Rimoin's Principals and Practice of Medical Genetics*, (ed. D. L. Rimoin, J. M. Connor and R. D. Pyeritz), pp. 2685-2717. London: Churchill-Livingston.

Gentzsch, M. and Riordan, J. R. (2001). Localization of sequences within the C-terminal domain of cystic fibrosis transmembrane conductance regulator which impact maturation and stability. *J. Biol. Chem.* **276**, 1291-1298.

Gut, A., Balda, M. S. and Matter, K. (1998). The cytoplasmic domains of a beta1 integrin mediate polarization in Madin-Darby canine kidney cells by selective basolateral stabilization. *J. Biol. Chem.* **273**, 29381-29388.

Haardt, M., Benharouga, M., Lechardeur, D., Kartner, N. and Lukacs, G. L. (1999). C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis. A novel class of mutation. *J. Biol. Chem.* **274**, 21873-21877.

Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J. and Lefkowitz, R. J. (1998). A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. *Proc. Nat. Acad. Sci. USA* **95**, 8496-8501.

Hallows, K. R., Raghuram, V., Kemp, B. E., Witters, L. A. and Foskett, J. K. (2000). Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J. Clin. Invest.* **105**, 1711-1721.

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. L., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E. and Higgins, C. F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* **346**, 362-365.

Johnston, J. A., Ward, C. L. and Kopito, R. R. (1998). Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143**, 1883-1898.

Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M. and Tsui, L. C. (1989). Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**, 1073-1080.

Matter, K. (2000). Epithelial polarity: sorting out the sorters. *Curr. Biol.* **10**, R39-R42.

Mickle, J., Macek, M., Jr., Fulmer-Smentek, S. B., Egan, M., Schwiebert, E. M., Guggino, W., Moss, R. and Cutting, G. R. (1998). A mutation in the cystic fibrosis transmembrane conductance regulator gene associated with elevated sweat chloride concentrations in the absence of cystic fibrosis. *Hum. Mol. Genet.* **7**, 729-735.

Mittereder, N., Yei, S., Bachurski, C., Cuppoletti, J., Whitsett, J. A., Tolstoshev, P. and Trapnell, B. C. (1994). Evaluation of the efficacy and safety of *in vitro*, adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA. *Hum. Gene Therapy* **5**, 717-729.

Mostov, K. E. and Cardone, M. H. (1995). Regulation of protein traffic in polarized epithelial cells. *BioEssays* **17**, 129-138.

Moyer, B. D., Loffing, J., Schwiebert, E. M., Loffing-Cueni, D., Halpin, P. A., Karlson, K., Ismailov, I. I., Guggino, W. B., Langford, G. M. and Stanton, B. A. (1998). Membrane trafficking of the cystic fibrosis gene product, cystic fibrosis transmembrane conductance regulator, tagged with green fluorescent protein in Madin-Darby canine kidney cells. *J. Biol. Chem.* **273**, 21759-21768.

Moyer, B. D., Denton, J., Karlson, K. H., Reynolds, D., Wang, S., Mickle, J. E., Milewski, M., Cutting, G. R., Guggino, W. B., Li, M. and Stanton, B. A. (1999). A PDZ-interacting domain in CFTR is an apical membrane polarization signal. *J. Clin. Invest.* **104**, 1353-1361.

Moyer, B. D., Duhaime, M., Shaw, C., Denton, J., Reynolds, D., Karlson, K. H., Pfeiffer, J., Wang, S., Mickle, J. E., Milewski, M., Cutting, G. R., Guggino, W. B., Li, M. and Stanton, B. A. (2000). The PDZ interacting domain of CFTR is required for functional expression in the apical plasma membrane. *J. Biol. Chem. 275*, 27069-27074.

Muth, T. R., Ahn, J. and Caplan, M. J. (1998). Identification of sorting determinants in the C-terminal cytoplasmic tails of the gamma-aminobutyric acid transporters GAT-2 and GAT-3. *J. Biol. Chem.* **273**, 25616-25627.

Perego, C., Vanoni, C., Villa, A., Longhi, R., Kaech, S. M., Frohli, E., Hajnal, A., Kim, S. K. and Pietrini, G. (1999). PDZ-mediated interactions retain the epithelial GABA transporter on the basolateral surface of polarized epithelial cells. *EMBO J.* **18**, 2384-2393.

Prince, L. S., Peter, K., Hatton, S. R., Zaliauskiene, L., Cotlin, L. F., Clancy, J. P., Marchase, R. B. and Collawn, J. F. (1999). Efficient endocytosis of the cystic fibrosis transmembrane conductance regulator requires a tyrosine-based signal. *J. Biol. Chem.* **274**, 3602-3609.

Rich, D. P., Gregory, R. J., Cheng, S. H., Smith, A. E. and Welsh, M. J. (1993). Effect of deletion mutations on the function of CFTR chloride channels. *Receptors and Channels* **1**, 221-232.

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S. and Tsui, L. C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073.

Rubin, J. B., Verselis, V. K., Bennett, M. V. and Bargiello, T. A. (1992). A domain substitution procedure and its use to analyze voltage dependence of homotypic gap junctions formed by connexins 26 and 32. *Proc. Nat. Acad. Sci. USA* **89**, 3820-3824.

Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R., Stutts, M. J. and Milgram, S. (1998). An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J. Biol. Chem.* **273**, 19797-19801.

Tai, A. W., Chuang, J. Z., Bode, C., Wolfrum, U. and Sung, C. H. (1999). Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell* **97**, 877-887.

Teuchert, M., Maisner, A. and Herrler, G. (1999). Importance of the carboxyl-terminal FTSL motif of membrane cofactor protein for basolateral sorting and endocytosis. Positive and negative modulation by signals inside and outside the cytoplasmic tail. *J. Biol. Chem.* **274**, 19979-19984.

Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B. and Li, M. (1998). Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). *FEBS Lett.* **427**, 103-108.

Wang, S., Yue, H., Denn, R. B., Guggino, W. B. and Li, M. (2000). Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. *Cell* **103**, 169-179.

Weixel, K. M. and Bradbury, N. A. (2000). The carboxyl terminus of the cystic fibrosis transmembrane conductance regulator binds to AP-2 clathrin adaptors. *J. Biol. Chem.* **275**, 3655-3660.

Welsh, M. J., Tsui, L. C., Boat, T. F. and Beaudet, A. L. (1995). Cystic fibrosis. In *The Metabolic and Molecular Bases of Inherited Disease* (ed. C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle), pp. 3799-3876. New York: McGraw-Hill, Inc.

Yeaman, C., Grindstaff, K. K. and Nelson, W. J. (1999). New perspectives on mechanisms involved in generating epithelial cell polarity. *Physiol Rev.* **79**, 73-98.

Zegers, M. M. P. and Hoekstra, D. (1998). Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. *Biochem. J.* **336**, 257-269.

Zeitlin, P. L., Lu, L., Hwang, T. C., Rhim, J., Craig, R., Cutting, G. R., Stetton, G., Kieffer, K. A. and Guggino, W. B. (1991). A cystic fibrosis bronchial epithelial cell line: Immortalization by Adeno-12-SV40 infection. *Am. J. Respir. Cell Mol. Biol.* **4**, 313-319.