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The PDZ-interacting Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is Required for Functional Expression in the Apical Plasma Membrane*

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Polarization of cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel to the apical plasma membrane in epithelial cells is critical for vectorial chloride transport. Previously, we reported that the C terminus of CFTR constitutes a PDZ-interacting domain that is required for CFTR polarization to the apical plasma membrane and interaction with the PDZ domain-containing protein EBP50 (NHERF). PDZ-interacting domains are typically composed of the C-terminal three to five amino acids, which in CFTR are QDTRL. Our goal was to identify the key amino acid(s) in the PDZ-interacting domain of CFTR with regard to its apical polarization, interaction with EBP50, and ability to mediate transepithelial chloride secretion. Point substitution of the C-terminal leucine (Leu at position 0) with alanine abrogated apical polarization of CFTR, interaction between CFTR and EBP50, efficient expression of CFTR in the apical membrane, and chloride secretion. Point substitution of the threonine (Thr at position −2) with alanine or valine had no effect on the apical polarization of CFTR, but reduced interaction between CFTR and EBP50, efficient expression of CFTR in the apical membrane as well as chloride secretion. By contrast, individual point substitution of the other C-terminal amino acids (Gln at position −4, Asp at position −3 and Arg at position −1) with alanine had no effect on measured parameters. We conclude that the PDZ-interacting domain, in particular the leucine (position 0) and threonine (position −2) residues, are required for the efficient, polarized expression of CFTR in the apical plasma membrane, interaction of CFTR with EBP50, and for the ability of CFTR to mediate chloride secretion. Mutations that delete the C terminus of CFTR may cause cystic fibrosis because CFTR is not polarized, complexed with EBP50, or efficiently expressed in the apical membrane of epithelial cells.

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] is a cAMP-activated chloride channel that is polarized to the apical plasma membrane in epithelial cells (1–3). Mutations in the CFTR gene lead to the genetic disease cystic fibrosis (CF), a lethal autosomal recessive disorder (4, 5). Nearly 70% of individuals with CF are homozygous for the ΔF508 mutation, which accounts for approximately 90% of all mutant CFTR alleles (4). Anterograde trafficking of CFTR-ΔF508 from the endoplasmic reticulum to the plasma membrane is inefficient; thus, little CFTR-ΔF508 reaches the apical plasma membrane (6, 7). Because CFTR-ΔF508 retains function as a cAMP-activated chlorine channel (8), identification of strategies for increasing delivery of CFTR-ΔF508 to the apical plasma membrane would have important implications for the treatment of CF. With the long term goal of identifying compounds that increase the expression of CFTR-ΔF508 in the apical plasma membrane, we have initiated a series of experiments to identify the sorting determinants and elucidate the mechanisms that localize wild-type (wt) CFTR to the apical plasma membrane in polarized epithelial cells. Once the signals, proteins, and mechanisms that localize wt-CFTR to the apical membrane are understood, rationale methods can be developed for increasing the apical membrane expression of CFTR-ΔF508.

Recent studies (9–11) have demonstrated that PDZ domains, which are named for three proteins in which this domain was first described (PSD-95, Dlg and ZO-1), play an essential role in determining cell polarity. 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 (10, 12–14). PDZ domain-PDZ-interacting domain interaction localizes some proteins to the basolateral plasma membrane (15–20). A role for PDZ-interacting domains in the localization of proteins to the apical membrane is also emerging (21, 22). Previously, we and others (23–27) reported that the C terminus of CFTR comprises a PDZ-interacting domain, which is required for apical polarization (23) and interaction with the apical membrane PDZ domain-containing protein ezrin-radixin-moesin phosphoprotein 50 (EBP50). Deletion of the three C-terminal amino acids of CFTR (CFTR-ΔTRL) abrogated polarization of CFTR to the apical membrane and interaction with EBP50 (NHERF) (23). Thus, interaction between CFTR

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[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; GFP, green fluorescent protein; EBP50, ezrin-radixin-moesin phosphoprotein 50; wt, wild type; MDCK, Madin-Darby canine kidney; HA, hemagglutinin.

and EBP50 may be required for the apical polarization of CFTR.

The objectives of the present study were to determine which residues in the PDZ interacting domain (QDTRL) of CFTR are most crucial for the apical polarization of CFTR and to begin to explore the mechanism(s), whereby the C terminus localizes CFTR to the apical membrane in polarized epithelial cells. To these ends we made chimeric constructs in which the green fluorescent protein (GFP) was linked to either wt-CFTR or CFTR with point substitutions in the C terminus and expressed these proteins in polarized Madin-Darby canine kidney (MDCK) epithelial cells, which express low levels of endogenous CFTR (28), and COS-7 cells. We examined the polarized expression of CFTR in MDCK cells by confocal fluorescence microscopy and cell surface biotinylation, CFTR interaction with EBP50 by co-immunoprecipitation in COS-7 cells, and CFTR-mediated transepithelial chloride secretion across MDCK cells by short circuit current analysis. We report that the PDZ-interacting domain, in particular the leucine (position 0) and threonine (position −2) residues, are required for the efficient, polarized expression of CFTR in the apical plasma membrane, interaction of CFTR with EBP50, and for the ability of CFTR to mediate transepithelial chloride secretion.

EXPERIMENTAL PROCEDURES

Expression Vectors—pGFP-CFTR, pGFP-CFTR-ΔTRL, and pGFP-CFTR-S1455X, encoding enhanced GFP fused to the N terminus of wt-CFTR, CFTR lacking the 3 C-terminal amino acids, and CFTR lacking the 26 C-terminal amino acids, respectively, were constructed as described previously (23, 29). Previously we demonstrated that addition of GFP to the N terminus of wt-CFTR had no effect on CFTR localization, trafficking, or function (29). Moreover, others (30) have shown that GFP has no effect on the degradation of wt-CFTR. Additional GFP-CFTR cDNAs encoding CFTR with point substitutions in the C terminus (substitutions are underlined: pGFP-CFTR-QDTRA, pGFP-CFTR-QDTAL, pGFP-CFTR-QDARL, pGFP-CFTR-QDVRL, pGFP-CFTR-QATRL, and pGFP-CFTR-ADTRL) were constructed using polymerase chain reaction-based mutagenesis as described in detail previously (23). For example, to construct pGFP-CFTR-QDTRA, oligonucleotide primers complementary to sequences prior to NBD2 (CFTR 4917 sense primer: 5′ TAA ACC TAC CAA GTC AAC CAA ACC ATA C 3′) and at the CFTR C terminus (CFTR 5803TRA antisense primer: 5′ CGA TAT **CTA** ***AGC*** CCT TGT ATC TTG CAT CTC TTC TTC TGT CTC CTC 3′) were used to amplify a 931-base pair fragment from pGFP-CFTR plasmid DNA. Bold sequences correspond to the natural CFTR stop codon. Bold and italicized sequences correspond to the CFTR codon mutated from Leu to Ala, and underlined sequences correspond to an artificial *Eco*RV site appended to facilitate cloning. Polymerase chain reaction products were subcloned into the pCR 2.1 vector using the TA Cloning Kit (Invitrogen, San Diego, CA), sequenced to verify the desired mutation, and inserted into the pGFP-CFTR expression vector as described previously (23). pHA-EBP50 encodes mouse EBP50 tagged with the hemagglutinin epitope at the N terminus and was constructed as described previously (24).

Cell Culture and Stable Cell Lines—MDCK type I cells (C7 clone) stably expressing GFP-CFTR fusion proteins were established and maintained in culture as described previously (23, 29). Three stable cell lines were studied for each C-terminal mutant to exclude the possibility that results were attributable to clonal variation. COS-7 cells were obtained from the American Type Tissue Collection (CRL-1651; Manassas, VA) and grown as described previously (23).

Immunocytochemistry and Semiquantitative Confocal Microscopy—Cells were fixed and stained for the tight junction protein ZO-1, as described previously (29). We quantitated the relative distribution of GFP-CFTR fluorescence in the apical and lateral membranes in randomly acquired confocal fluorescence xz vertical sections using NIH Image v1.62 software as described previously (23, 31).

Selective Cell Surface Biotinylation, Immunoprecipitation, SDS-Polyacrylamide Gel Electrophoresis, and Western Blotting—Selective apical and basolateral membrane biotinylations and immunoprecipitations were performed as described in detail previously (23, 29). All steps were performed at 4 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes as described previously(29). GFP-CFTR fusion proteins were detected on membranes blocked with 5% nonfat dry milk in Tris-buffered saline, 0.02% Tween 20 using a polyclonal GFP antibody (1:1000; CLONTECH, Palo Alto, CA) followed by anti-mouse horseradish peroxidase (1:5,000; Amersham Pharmacia Biotech). HA-EBP50 was detected on membranes blocked with Superblock (Pierce) supplemented with 0.02% Tween 20 using biotinylated anti-HA monoclonal antibody 12CA5 (1:200) followed by neutravidin-horseradish peroxidase (1:80,000; Pierce). Both HA antibody and neutravidin-horseradish peroxidase were prepared in Superblock containing 0.02% Tween 20.

Short Circuit Current—Short circuit current (I_{sc}) was measured across MDCK monolayers stable expressing GFP-CFTR fusion proteins as described previously (29) with the exceptions that cells were treated with CPT-cAMP (100 μM) instead of a cAMP-stimulating mixture to stimulate chloride secretion and then treated with glybenclamide (100 μM) instead of DPC to inhibit chloride secretion. In all experiments, amiloride (10^{-5} M) was present in the apical bath solution to inhibit electrogenic sodium absorption. Under these conditions, cAMP-stimulated I_{sc} across monolayers of MDCK cells is referable to CFTR-mediated vectorial chloride secretion from the basolateral to the apical solution (29, 32).

RESULTS

The C-terminal Leucine Is the Most Critical Residue for the Polarization of CFTR to the Apical Membrane—In a previous study (23) we demonstrated that deletion of the last three amino acids of the PDZ-interacting domain of CFTR (CFTR-ΔTRL) abrogated its polarization to the apical plasma membrane in MDCK and human airway epithelial cells. PDZ-interacting domains are generally localized to the C termini of proteins and comprise as many as five amino acids (10, 12, 13). In CFTR the last five residues, QDTRL, are highly conserved across species and closely match the PDZ-interacting domain consensus sequence S/T-*X*-L/V/I (where *X* is any amino acid) found in proteins that bind to type I PDZ domains (12, 24, 33). In general, the C-terminal-most residue (position 0) and the second residue from the C-terminal position (position −2) are the most critical for interacting with PDZ domains in other proteins; however, residues in position −3 and −4 may also modify this interaction (33–36). By contrast, the amino acid at position −1 is not thought to play a major role in protein-protein interaction (33–36). However, the role of each amino acid within the CFTR PDZ-interacting domain in determining the apical polarization of CFTR has not been examined. Accordingly, we generated GFP-CFTR fusion proteins containing alanine point substitutions within the QDTRL motif and determined the polarized expression of these fusion proteins in MDCK cells by confocal fluorescence microscopy and selective cell surface biotinylation.

As shown previously, wt-CFTR was polarized to the apical membrane in a plane parallel to the tight junctions, which separate apical and basolateral membrane domains (Fig. 1*A*). By contrast, CFTR-ΔTRL, which lacks the three C-terminal amino acids, was nonpolarized and equally expressed in apical and lateral membrane domains (Fig. 1*B*) (23). Semiquantitative confocal fluorescence microscopy and cell surface biotinylation analyses revealed that wt-CFTR was polarized to the apical membrane (apical to basolateral ratio > 10), whereas CFTR-ΔTRL was not polarized (apical to basolateral ratio ≈ 1, Table I). Alanine substitution at position 0 (QDTRL to QDTRA, mutation underlined) disrupted apical polarization of CFTR and resulted in an apical to basolateral ratio close to 1, similar to CFTR-ΔTRL (Fig. 1*C* and Table I). As predicted, alanine substitution at position −1 (QDTRL to QDTAL) had no effect on the polarized distribution of CFTR (Fig. 1*D* and Table I). Alanine substitution for threonine at position −2 (QDTRL to QDARL) also had no effect on the polarization of CFTR (Fig. 1*E* and Table I). Similar results were obtained when valine (Val) was substituted for Thr at position −2 (QDTRL to QDVRL, Table I). Alanine point substitutions at the −3 position

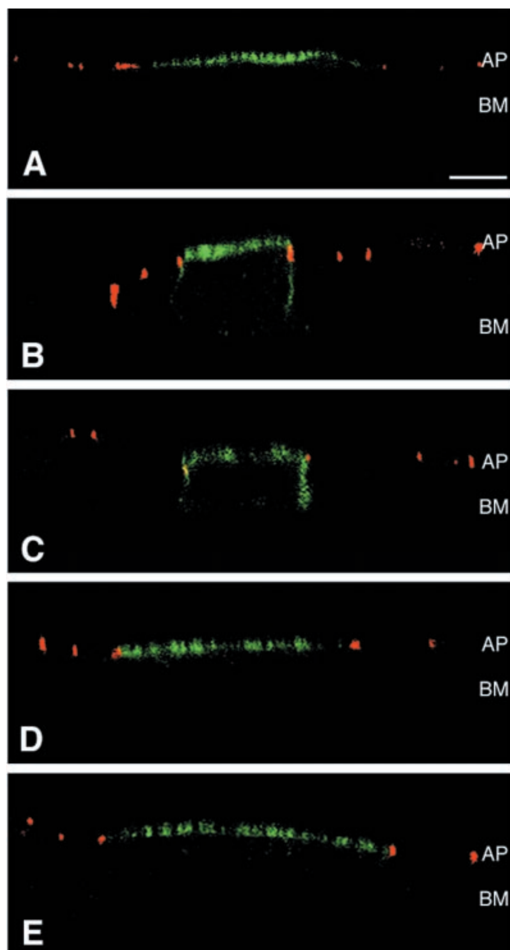

FIG. 1. **The C-terminal amino acid (leucine) is the most critical residue for polarization of CFTR to the apical membrane.** Confocal fluorescence micrographs (xz vertical sections) of MDCK cells expressing: wt-CFTR (apical membrane expression) (*A*), CFTR-ΔTRL (apical and lateral membrane expression) (*B*), CFTR-QDTRA (apical and lateral membrane expression) (*C*), CFTR-QDTAL (apical membrane expression) (*D*), and CFTR-QDARL (apical membrane expression) (*E*). Point mutations are *underlined*. GFP-CFTR fluorescence is *green* and ZO-1, a protein in tight junctions that separate apical and basolateral membrane domains, is *red*. *AP* = location of apical membrane; *BM* = location of basal membrane. *Scale bar* = 10 μm.

(QDTRL to QATRL) and at the −4 position (QDTRL to ADTRL) had no significant effect on the polarization of CFTR to the apical plasma membrane (Table I). Taken together, our point substitution studies suggest that the C-terminal leucine is the most critical residue in the PDZ-interacting domain for the apical polarization of CFTR.

Interaction between CFTR and EBP50, a PDZ Domain-containing Protein, Is Required for CFTR Apical Polarization—Recently, we and others (23–26) reported that the PDZ domain-containing protein EBP50 (*i.e.* NHERF) interacts and co-localizes with wt-CFTR at the apical membrane of MDCK and human airway epithelial cells. Deletion of the C-terminal three amino acids of CFTR eliminated co-localization and co-immunoprecipitation of CFTR and EBP50 (23). These studies suggest that EBP50, or some other PDZ domain-containing protein, may be responsible for the apical polarization of CFTR in MDCK cells. To further test this hypothesis, we performed co-immunoprecipitation studies to determine which C-terminal amino acid(s) in the PDZ-interacting domain of CFTR is most critical for interaction with EBP50 *in vivo*. COS-7 cells were transiently co-transfected with EBP50 and either wt-CFTR or CFTR containing C-terminal point substitutions (Fig. 2 and

TABLE I

Summary of effects of C-terminal mutations on the polarized expression of CFTR, CFTR-EBP50 interaction, and CFTR-mediated chloride secretion

AP/BL ratio is the ratio of CFTR expressed in the apical *versus* the basolateral plasma membrane as determined by domain selective cell-surface biotinylation and/or semi-quantitative confocal microscopy as described under "Experimental Procedures" ($n \geq 6$ monolayers/wt or mutant CFTR). Data on ΔTRL and 1455X, published previously, are shown for comparison (23). Results were similar using both techniques as described previously (23). Co-IP EBP50 is expressed as a percent of EBP50 that co-immunoprecipitated with wt-CFTR ($n = 4$ experiments/wt or mutant CFTR: see Fig. 2*B* for S.E.). I_{sc} is the CPT-cAMP (100 μM)-stimulated transepithelial chloride secretory current (see Fig. 3). Point substitutions in CFTR are underlined.

CFTR	AP/BL ratio	Co-IP EBP50	I_{sc}
		%	$\mu A/cm^2$
wt-CFTR	10.4 ± 2.0	100	8.3 ± 0.8
ΔTRL	0.6 ± 0.3[a]	0[a]	2.6 ± 0.4[a]
QDTRA	0.7 ± 0.2[a]	0[a]	2.0 ± 0.3[a]
QDTAL	10.8 ± 1.8	40[a]	—
QDARL	10.9 ± 2.0	10[a]	3.6 ± 0.6[a]
QDVRL	11.2 ± 1.9	16[a]	4.8 ± 0.5[a]
QATRL	15.2 ± 2.0	74	8.6 ± 0.9
ADTRL	11.4 ± 2.0	78	7.6 ± 1.5
S1455X	0.2 ± 0.1[a]	0[a]	1.7 ± 0.2[a]

[a] Indicates significantly different from wt-CFTR ($p < 0.001$).

Table I). EBP50 was co-immunoprecipitated with wt-CFTR (QDTRL), CFTR-QDTAL, CFTR-QDARL, CFTR-QDVRL, CFTR-QATRL, and CFTR-ADTRL, all of which were polarized to the apical membrane. However, it should be noted that the ability of CFTR-QDARL and CFTR-QDVRL to be co-immunoprecipitated with EBP50 was reduced by 80–90% compared with wt-CFTR. By contrast, EBP50 could not be co-immunoprecipitated with CFTR-QDTRA or CFTR-ΔTRL, which are nonpolarized, and, as shown previously, CFTR-S1455X (23) (Fig. 2 and Table I). CFTR-S1455X, a naturally occurring mutation that lacks the 26 C-terminal amino acids, is polarized to the lateral membrane (23). Thus, C-terminal mutants of CFTR that did not polarize to the apical membrane did not co-immunoprecipitate with EBP50. Taken together, these findings suggest that efficient CFTR-EBP50 interaction requires the C-terminal leucine (position 0) and threonine (position −2) residues of CFTR.

The C Terminus Is Required for CFTR to Mediate Transepithelial Chloride Secretion in Polarized MDCK Cells—A search of the CF mutation data base reveals that several naturally occurring mutations truncate the cytoplasmic, C terminus of CFTR. However, these and other C-terminal truncations do not affect the ability of CFTR to mediate chloride efflux in nonpolarized cells (37–40). Thus, we tested the hypothesis that C-terminal truncations and deletions that disrupt CFTR apical polarization may cause CF, in part, due to an inability of CFTR to mediate transepithelial chloride transport across polarized epithelial cells. To this end, we measured cAMP-stimulated chloride secretion across polarized MDCK cells stably expressing wt or mutant CFTR by short circuit current analysis. In parental, untransfected cells, CPT-cAMP had a small stimulatory effect on chloride secretion, most likely due to stimulation of endogenous CFTR chloride channels (28, 29, 32) (Fig. 3). In cells in which CFTR was stably expressed and polarized to the apical plasma membrane, including cell lines expressing wt-CFTR, CFTR-QATRL, and CFTR-ADTRL, CPT-cAMP significantly increased chloride secretion compared with parental, nontransfected cells (Fig. 3). In addition chloride secretion in cells expressing wt-CFTR, CFTR-QATRL, and CFTR-ADTRL was similar. By contrast, in cells stably expressing mutant CFTRs that were not polarized to the apical plasma membrane, including CFTR-ΔTRL, CFTR-S1455X and CFTR-QDTRA,

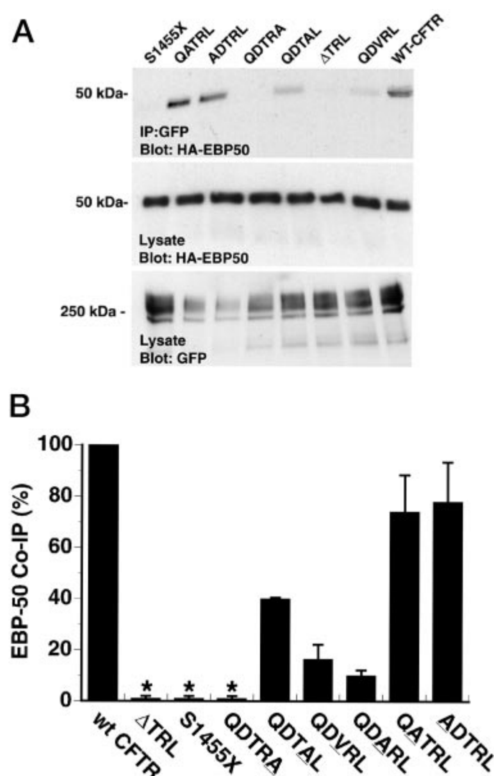

FIG. 2. **Co-immunoprecipitation of EBP50 with wt and mutant CFTRs in transiently transfected COS-7 cells.** *A*, representative Western blots. *Top blot*, cell lysates were immunoprecipitated with a polyclonal GFP antibody (to immunoprecipitate CFTR), and blots were probed with a monoclonal EBP50 antibody (to detect EBP50 associated with CFTR). *Middle blot*, cell lysates blotted with an anti-HA antibody to detect EBP50. In four experiments EBP50 expression was similar in all transfectants. *Bottom blot*, cell lysates blotted with an anti-GFP antibody to detect GFP-CFTR. In four experiments CFTR expression was similar in all transfectants. *B*, summary of co-immunoprecipitation experiments ($n = 4$ co-immunoprecipitations/mutation). EBP50 co-immunoprecipitated with QDTRL (*i.e.* wt-CFTR), ADTRL, QATRL, QDARL, QDVRL (not shown in *A*), and QDTAL. As demonstrated in Fig. 1, wt-CFTR and these mutant CFTR proteins were polarized to the apical plasma membrane. By contrast, CFTR proteins that did not associate with EBP50, including QDTRA (* indicates protein could not be detected) and, as shown previously, S1455X and ΔTRL (23), were not polarized to the apical membrane (Fig. 1). Point mutations are *underlined*. CFTR immunoprecipitation efficiencies, which corresponded to recoveries of over 60% of total cell lysate CFTR, were similar in all transfectants. The amount of EBP50 that co-immunoprecipitated with QDTRL (wt) was equal to ~5% of total cell lysate EBP50. This amount was arbitrarily assigned a value of 100%.

cAMP-stimulated chloride secretion was not significantly different from parental, untransfected cells (Fig. 3). Interestingly in cells stably expressing CFTR-QDVRL and CFTR-QDARL cAMP-stimulated chloride secretion was increased *versus* untransfected, parental cells but was significantly less than in cells expressing wt-CFTR (Fig. 3). Although CFTR-QDVRL and CFTR-QDARL were polarized to the apical plasma membrane, these mutants did not efficiently associate with EBP50 (Table I). Taken together these data suggest that the leucine (position 0) and threonine (position −2) in the PDZ-interacting domain of CFTR are required for CFTR-mediated transepithelial chloride secretion across polarized MDCK epithelial cells. Thus, loss or reduction of transepithelial chloride secretion is correlated with loss or reduction of interaction with EBP50.

The PDZ-interacting Domain Is Required for Efficient Expression of CFTR in the Plasma Membrane—Previous studies have shown that C-terminal amino acids play a critical role in the efficient export from the endoplasmic reticulum and expression in the plasma membrane (41–44). Accordingly, we

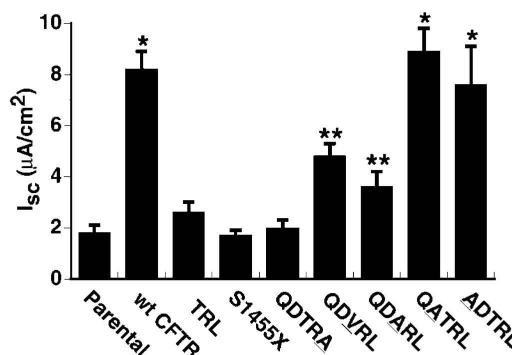

FIG. 3. **Summary of cAMP-stimulated short circuit current (I_{sc}) across parental, nontransfected MDCK cells or cells stably expressing wt or mutant CFTRs.** Expression of CFTR in all clones was confirmed by Western blot analysis. CFTR protein expression was similar in all clones. Data are expressed as the CPT-cAMP-stimulated (100 μM) I_{sc} inhibited by the CFTR chloride channel blocker glybenclamide (100 μM) added to the apical bath solution. In previous studies we showed that cAMP-stimulated I_{sc} in MDCK stably expressing GFP-CFTR is referable to chloride secretion (29, 32). wt and CFTR mutants, including QATRL, and ADTRL, polarized to the apical membrane and produced cAMP-stimulated chloride currents significantly greater than cAMP-stimulated currents in untransfected parental cells. By contrast, CFTR mutants that were not polarized to the apical membrane, including ΔTRL, S1455X, and QDTRA, did not produce cAMP-stimulated chloride currents significantly different from cAMP-stimulated currents in untransfected, parental cells. * indicates significantly different from parental cells ($p < 0.001$ by two-tailed, unpaired t test). ** indicates significantly different from cells expressing wt-CFTR and parental cells ($p < 0.001$). Number of monolayers studied was: 14 for parental cells, 22 for wt-CFTR, 14 for ΔTRL, 8 for S1455X, 21 for QDTRA, 14 for QDVRL, 17 for QDARL, 5 for QATRL, and 4 for ADTRL.

conducted cell surface biotinylation experiments to determine whether the PDZ-interacting domain of CFTR regulates the expression of CFTR in apical and basolateral membranes. As shown in Fig. 4, 30% of total cellular wt-CFTR, which was polarized to the apical membrane, was expressed in the apical and basolateral plasma membranes of MDCK cells. Similar surface expression levels for CFTR-QATRL (42%) and CFTR-ADTRL (28%), which were also polarized to the apical membrane, were observed. By contrast, only 5% of total cellular CFTR-ΔTRL, which was not polarized, was expressed in the apical and basolateral membranes. Similar inefficient expression was observed for CFTR-S1455X, CFTR-QDTRA, CFTR-QDVRL and CFTR-QDARL (2–5% of total CFTR was expressed in the plasma membranes). Thus, in addition to playing a key role in apical polarization, interaction with EBP50, and transepithelial chloride secretion, the C-terminal PDZ-interacting domain, in particular the C-terminal leucine (position 0) and threonine (position −2) residues, is required for the efficient expression of CFTR in the apical plasma membrane.

DISCUSSION

Our results demonstrate that the C-terminal PDZ-interacting domain in CFTR plays a key role in (*a*) the polarized expression of CFTR in the apical plasma membrane, (*b*) the interaction of CFTR with EBP50, and (*c*) the ability of CFTR to mediate transepithelial chloride secretion across polarized MDCK epithelial cells. Because deletions of the C terminus of CFTR (61 amino acids) do not affect the ability of CFTR to conduct chloride in nonpolarized cells (37–40), our data suggest that mutations that delete the C terminus may cause CF in part because CFTR is not polarized, complexed with EBP50, or efficiently expressed in the apical membrane of epithelial cells.

Using two independent techniques, domain-selective cell surface biotinylation and quantitative confocal fluorescence microscopy, we demonstrate that the PDZ-interacting domain,

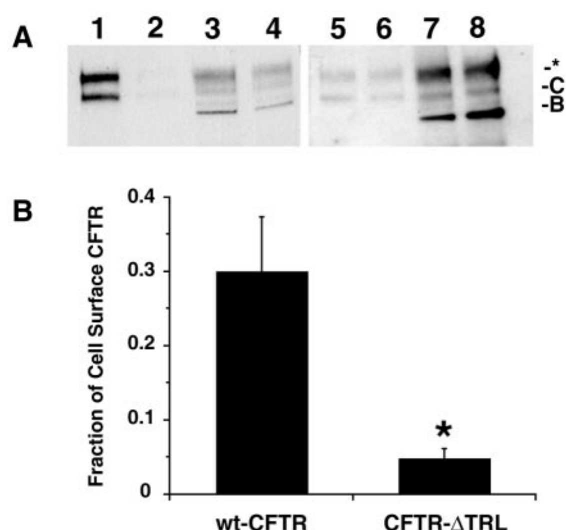

FIG. 4. **Deletion of the C-terminal three amino acids decreases the fraction of total cell CFTR expressed in the plasma membrane of MDCK cells.** *A*, representative Western blots. *Lanes 1–4*, wt-CFTR. *Lane 1* is wt-CFTR biotinylated in the apical membrane, and *lane 2* is wt-CFTR biotinylated in the basolateral membrane. Results demonstrate that wt-CFTR is polarized to the apical membrane. *Lanes 3* and *4* were loaded with one-fifth of total cell lysate isolated from cells expressing wt-CFTR whose apical (*lane 3*) or basolateral (*lane 4*) membranes were biotinylated. Results indicate that both groups of cells expressed equal amounts of wt-CFTR. *Lanes 5–8*, CFTR-ΔTRL. *Lane 5* is CFTR-ΔTRL biotinylated in the apical membrane, and *lane 6* is CFTR-ΔTRL biotinylated in the basolateral membrane. Results demonstrate that CFTR-ΔTRL is not polarized. *Lanes 7* and *8* were loaded with one-fifth of the total cell lysate isolated from cells expressing CFTR-ΔTRL whose apical (*lane 7*) or basolateral (*lane 8*) membranes were biotinylated. Results indicate that both groups of cells expressed equal amounts of CFTR-ΔTRL. * = high molecular weight CFTR as described previously (23, 29). *C* = mature glycosylated band C form of CFTR. *B* = core glycosylated band B form of CFTR. The band that runs below the B band is a nonspecific protein that cross-reacts with the GFP antibody. *B*, fraction of wt-CFTR and CFTR-ΔTRL expressed in the plasma membrane (apical and basolateral) referenced to total cellular wt-CFTR and CFTR-ΔTRL, respectively. Whereas 30% of total cellular wt-CFTR was expressed in the plasma membrane, only 5% of total cellular CFTR-ΔTRL was expressed in the plasma membrane. $n = 5$ for wt-CFTR and 9 for CFTR-ΔTRL. * indicates significantly different from wt-CFTR ($p < 0.001$).

and in particular the C-terminal leucine residue, is required for apical polarization of CFTR. Deletion of the three C-terminal amino acids (ΔTRL) or alanine point substitution of the C-terminal leucine (position 0) resulted in nonpolarized expression of CFTR at apical and basolateral membrane domains. In addition, these mutations eliminated interaction between CFTR and EBP50 and reduced the efficient expression of CFTR in the apical plasma membrane as well as transepithelial chloride secretion. In light of the importance of residue −2 in PDZ-interacting domains (33–36, 45), it was initially surprising that the alanine or valine substitution at position −2 had no effect on the polarized distribution of CFTR between the apical and basolateral membranes. However, further experimentation revealed that these mutations significantly reduced the interaction between CFTR and EBP50, the efficiency of CFTR expression in the apical membrane, and the ability of CFTR to mediate transepithelial chloride secretion. Thus, we conclude that the threonine residue at the position −2 is also required for efficient interaction of CFTR with EBP50, as well as for the efficient functional expression of CFTR in the apical membrane. By contrast, alanine substitution at positions −1, −3, and −4 did not affect the apical polarization of CFTR, interaction between CFTR and EBP50, or chloride secretion. Positions −1, −3, and −4 in PDZ-interacting domains are generally not as important for interaction with PDZ domains as positions 0 and −2 (33–36). Taken together, our data are consistent with the literature that positions 0 and −2 are the key amino acids that determine PDZ-interacting domain-PDZ domain interactions.

The observation that a protein-based, cytoplasmic motif mediates the efficient polarization of CFTR to the apical membrane supports an emerging role for cytoplasmic-based determinants, in particular PDZ-interacting domains, in localizing proteins to the apical membrane in epithelial cells (21, 22, 46–48). For example, a splice variant of the voltage-gated potassium channel $K_{v3.2a}$, containing a C-terminal exon encoding a sequence resembling a PDZ-interacting domain, is polarized to the apical membrane (22). By contrast, the splice variant of $K_{v3.2a}$ containing a C-terminal exon lacking a recognizable PDZ-interacting domain is polarized to the basolateral membrane (22). In addition, the neuronal GABA-3 transporter (GAT-3) contains a C-terminal PDZ-interacting domain-like sequence and is polarized to the apical membrane in epithelial cells; deletion of the three C-terminal amino acids disrupts GAT-3 apical polarization and results in nonpolarized expression (21). Previously identified apical targeting motifs, including glycosylphosphatidylinositol membrane anchors, *N*- and *O*-linked oligosaccharides, and transmembrane domains, as well as partitioning into glycosphingolipid rafts, do not seem to be required for apical polarization of CFTR. CFTR is not a glycosylphosphatidylinositol-linked protein (5), and inhibition of CFTR *N*-linked glycosylation does not inhibit the polarization of CFTR to the apical membrane (49). Moreover, CFTR is not *O*-glycosylated (5, 7). Finally, CFTR and CFTR-ΔTRL were not detected in detergent-insoluble glycosphingolipid rafts, which frequently mediate trafficking of transmembrane proteins to the apical surface.[2]

In addition to directing the apical polarization of CFTR, we report that the C-terminal PDZ-interacting domain is required for the efficient expression of CFTR in the plasma membrane. Deletion of the PDZ-interacting domain (ΔTRL), or point substitution of alanine for leucine (position 0) or alanine or valine for threonine (position −2), decreased the expression of CFTR in the apical plasma membrane by at least 6-fold. Decreased expression of CFTR in the apical plasma membrane resulted in an inability of cAMP to stimulate chloride secretion across MDCK cells. The correlation between CFTR-EBP50 interaction and transepithelial chloride secretion suggests that EBP50 is involved in the efficient, polarized expression of CFTR at the apical membrane. However, we emphasize that the mechanism(s) by which the C-terminal PDZ-interacting domain of CFTR mediates apical polarization and plasma membrane expression are unknown. We are currently testing three hypotheses, which are not mutually exclusive. First, interaction of the C terminus of CFTR with a PDZ domain-containing protein, such as EBP50, CAL, or CAP70 (24, 50, 51), may regulate export of CFTR from the endoplasmic reticulum, as recently demonstrated for proTGF-α (42). Second, interaction of the C terminus of CFTR with a PDZ domain-containing protein may regulate the sorting of CFTR into vesicles destined for the apical membrane at the level of the trans-Golgi network. In support of this hypothesis, the PDZ-domain containing protein CAL binds to the C terminus of CFTR and is localized to the trans-Golgi network in nonpolarized cells (51). Finally, interaction of the C terminus of CFTR with a PDZ domain-containing protein, such as EBP50, may anchor CFTR in the apical membrane or regulate recycling of internalized CFTR to the

[2] B. D. Moyer, M. Duhaime, C. Shaw, J. Denton, D. Reynolds, K. H. Karlson, J. Pfeiffer, S. Wang, J. E. Mickle, M. Milewski, G. R. Cutting, W. B. Guggino, M. Li, and B. A. Stanton, unpublished observations.

apical membrane. In support of this hypothesis, CFTR proteins containing extensive C-terminal deletions encompassing the PDZ-interacting domain have been shown to be unstable and exhibit decreased residence times in the plasma membrane of nonpolarized cells (37). Moreover, EBP50 has been shown to regulate protein recycling to the cell surface (52). A similar mechanism involving selective retention and stabilization is utilized to anchor a GABA transporter to the basolateral membrane through its C-terminal PDZ-interacting domain (15).

In conclusion, we speculate that multiple PDZ domain-containing proteins (such as EBP50, CAL, CAP70, and others) may stabilize and retain CFTR in the apical plasma membrane as well as regulate the exocytic and endocytic trafficking of CFTR in polarized cells by interacting with the C-terminal PDZ-interacting domain of CFTR. Consistent with our proposal, point mutation of the threonine residue at position −2 did not affect CFTR polarization along the apical to basal axis but decreased the expression of CFTR in the apical membrane. Therefore, different PDZ domain-containing proteins are likely to modulate trafficking of CFTR through the biosynthetic pathway to the apical membrane region and the retention and recycling of CFTR in the apical membrane. The challenge that lies ahead is to identify all of these CFTR-binding proteins and elucidate the mechanisms whereby they regulate the trafficking and polarized expression of CFTR in epithelial cells as well the ability of CFTR to mediate chloride secretion.

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