

PDZ-binding motifs are unable to ensure correct polarized protein distribution in the absence of additional localization signals

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Abstract The C-terminal PDZ-binding motifs are required for polarized apical/basolateral localization of many membrane proteins. To determine the specificity of the PDZ-binding motifs in establishing cellular distribution, we utilized a 111-amino acid region from the C-terminus of cystic fibrosis transmembrane conductance regulator (CFTR) that is able to direct apical localization of fused reporter proteins. Substitution of the C-terminal PDZ-binding motif of CFTR with corresponding motifs necessary for basolateral localization of other membrane proteins did not lead to the redistribution of the fusion protein to the basolateral membrane. Instead, some fusion proteins remained localized to the apical membrane, whereas others showed no specific distribution. The specificity of the PDZ-based interactions was substantially increased when specific amino acids located upstream of the classical PDZ-binding motifs were included. However, even the presence of a longer C-terminal motif from a basolateral protein could not ensure basolateral distribution of the fusion protein. Our results indicate that the C-terminal PDZ-binding motifs are not the primary signals for polarized protein distribution, although they are required for targeting and/or stabilization of protein at the given location.
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1. Introduction

PDZ domains are protein–protein interaction modules that bind to specific C-terminal motifs of their binding partners [1]. Many PDZ-containing proteins contribute to the formation of multi-protein complexes that target proteins to specific submembrane sites and it has been established that the PDZ domain-based interactions are essential for polarized distribution of numerous membrane proteins in neurons and epithelial cells [2–4].

Variation in the last four amino acid residues of proteins seems to provide sufficient diversity to ensure correct polarized localization of proteins containing different PDZ-binding motifs. However, apparent sequence similarities between the PDZ-binding motifs of selected apical and basolateral membrane proteins suggest that either the process of recognizing the C-terminal “zip codes” is extremely specific or other pro-

tein sequences contribute to observed specificity by forming multi-component localization signals. For example, distinguishing between the “apical” E–T–R–L> motif of mouse cystic fibrosis transmembrane conductance regulator (CFTR) and the E–T–H–L> motif of the basolateral γ -aminobutyric acid transporter BGT1 would require a PDZ-based interaction that is characterized by extreme sensitivity in respect to the position –1 of the PDZ-binding motif. Though this particular position has been considered to be the least crucial in the PDZ-mediated ligand binding, it may play a significant role in establishing required specificity of these interactions [5,6]. On the other hand, the observation that other localization signals are sometimes required to ensure polarized protein distribution mediated by individual C-terminal PDZ-binding motifs suggests that additional sequences contribute to the specificity of PDZ-based interactions [7–9].

Integral membrane proteins include transmembrane domains that anchor the polypeptide in the lipid bilayer and secure the association of these proteins with the membrane, even in the absence of specific targeting signals. Thus, studying the function of specific localization signals in native membrane proteins presents some difficulty. For example, it is difficult to distinguish the non-specific apical/basolateral membrane localization from specific apical or basolateral distribution. To overcome this obstacle, we have developed a model system that employs a CFTR-derived peptide devoid of transmembrane domains but containing all signals required for apical protein distribution, including the C-terminal PDZ-binding motif [9]. This model was used to study the specificity of the PDZ domain-based interactions responsible for polarized protein distribution in epithelial cells. To determine the extent to which PDZ interactions mediate protein localization, we investigated the effect of replacing the PDZ-binding motif of CFTR with different C-terminal PDZ-binding motifs derived from apical or basolateral membrane proteins.

2. Materials and methods

2.1. Plasmid constructions

The construction of the GFP-tagged full-length CFTR protein and the shorter version of this chimera (GFP–CFTR 1370–1480) was described elsewhere [9,10]. Also, the introduction of the H1402A and R1403A mutations, reducing the aggregation rate of the CFTR C-terminus, was previously described [9]. Site-directed mutagenesis system “Transformer” (Becton Dickinson) was used to replace the C-terminal CFTR codons with sequences encoding different PDZ-binding motifs. The nucleotide sequences of selection primers and mutagenic primers used to introduce those changes are available upon request.

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2.2. Cell culture and transfection

Madin–Darby canine kidney (MDCK) cells type II (gift from A. Hubbard) were grown in DMEM supplemented with 10% fetal bovine serum (Biofluids) and 0.37% sodium bicarbonate. For protein localization analysis, cells were grown on non-coated glass coverslips and transiently transfected using the Lipofectin reagent (Invitrogen). To increase the transfection rates, the DNA/lipofectin mixture was added to the trypsinized MDCK cells. The cells were then seeded by placing the whole mixture on the coverslips. Remaining transfection steps were carried out according to the manufacturer's instructions.

2.3. Antibodies and immunofluorescence staining

Monoclonal anti- Na^+/K^+ ATPase antibodies (Upstate Biotechnology) were used to stain the basolateral plasma membrane in MDCK cells. Prior to immunostaining, the cells were fixed with 4% PFA for 20 min, permeabilized with 0.5% Triton X-100 for 5 min, and washed with PBS. Non-specific binding sites were blocked with 2.5% goat serum (Sigma). Staining was performed by two sequential incubation steps. Cells were first incubated with the primary anti- Na^+/K^+ ATPase antibody for 1.5 h and then with the secondary anti-mouse antibody conjugated to Cy3 fluorescent dye (Sigma) for 30 min. After staining, the coverslips with cells were washed in PBS and mounted in SlowFade (Molecular Probes) containing 0.1 mg/ml of DAPI (Sigma).

2.4. Confocal laser microscopy

The fluorescence label was examined with laser scanning confocal imaging system (LSM Carl Zeiss). Only cells that expressed the fusion protein at relatively low level (at the minimal level that enabled the localization analysis) were tested for protein localization. Images were generated using 16-fold line averaging and the xz cross-sections were produced using a 0.2- μm motor step. At least 25 transfected cells were tested for each construct. The results were considered unequivocal, when at least 90% of cells showed the same distribution pattern (apical, basolateral or cytoplasmic). To examine the relative distribution of the GFP fusion protein, the semi-quantitative analysis was performed, as described elsewhere [11]. The apical to cytoplasmic ratio was calculated

as d_a/d_c , where d_a was a fluorescence signal density measured in the apical and subapical regions of the cell and d_c was a signal density in the remaining cell body (cytoplasm). Images were prepared for publication with LSM Carl Zeiss Software and Adobe Photoshop.

3. Results and discussion

3.1. Limited specificity of the four amino acid PDZ-binding motifs

To determine whether the PDZ-binding motifs are the key elements responsible for specificity of polarized localization, we tested whether replacing the PDZ-binding motif D–T–R–L> with corresponding motifs from apical and basolateral proteins alters the localization of the C-terminus of human CFTR. The GFP fusion protein containing the C-terminal amino acids 1370–1480 of human CFTR was used as a model for constructing a series of proteins differing only in their PDZ-binding motifs. Despite lacking the CFTR transmembrane domains, this fusion protein contained all signals required for apical localization (Fig. 1). Seven C-terminal motifs that were reported to be required for basolateral or apical localization of different membrane proteins and one that could be potentially involved in the polarized protein distribution (Table 1) were used to replace the original PDZ-binding motif in the fusion protein. Additionally, the original D–T–R–L> motif of human CFTR was replaced with the similar E–T–R–L> motif found in several other species [12]. All these motifs were known to bind PDZ domains of specific proteins (see Table 1) and/or matched the broad consensus for PDZ-binding sequences [13].

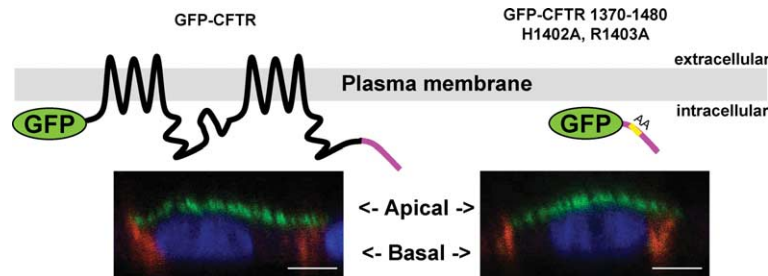


Fig. 1. Apical localization of the GFP-tagged C-terminal fragment of CFTR in MDCK cells. A diagram illustrating the structure and subcellular localization of the GFP fusion proteins containing either the full-length CFTR protein or its C-terminal fragment (a.a. 1370–1480) with two alanine substitutions (H1402A and R1403A), eliminating the aggregation of the CFTR C-terminus. The green signal corresponds to the GFP fusion proteins, whereas the localization of the basolateral marker (Na^+/K^+ ATPase) is represented by the red fluorescence. Nuclei were stained blue with DAPI. Bars – 10 μm .

Table 1
PDZ-binding motifs involved in polarized protein distribution in epithelial cells

Protein	C-terminal a.a.	Interacting PDZ proteins	Localization	References
SYN2	E–F–Y–A>	LIN2; syntenin; synectin	Basolateral	[22,23]
GLUT1 ^a	D–S–Q–V>	GLUTICBP	Basolateral ^b	[24]
GLR1	N–T–A–V>	LIN10	Basolateral	[25]
MCP	F–T–S–L>	? ^c	Basolateral ^b	[8,26,27]
LET23	E–T–C–L>	LIN7	Basolateral ^b	[17,28,29]
BGT1	E–T–H–L>	LIN7	Basolateral ^b	[18,30]
GAT3	E–T–H–F>	? ^c	Apical ^b	[7]
CFTR	D–T–R–L>	NHERF; E3KARP; CAP70; CAL	Apical ^b	[9,11,14,31–36]

^aThe PDZ-binding motif of GLUT1 has not been demonstrated to be required for polarized distribution.

^bThe basolateral/apical localization of the full-length protein has been confirmed in MDCK cells.

^cNo interacting PDZ protein has been reported, although the C-terminal sequence matches the PDZ-binding consensus.

If the PDZ-binding motif was the signal determining the apical distribution of CFTR, replacing it with a corresponding signal from basolateral protein should alter the apical distribution of the GFP fusion protein. Indeed, three “basolateral” motifs E-F-Y-A> (SYN2), N-T-A-V> (GLR1) and D-S-Q-V> (GLUT1) excluded the fusion protein from the apical membrane (Fig. 2). However, they were unable to mediate the basolateral membrane localization, leading instead to diffuse cytoplasmic distribution of the GFP fusion protein lacking the transmembrane domains. Most importantly, the fusion protein containing the “basolateral” C-terminal motif E-T-H-L (BGT1) was still localized to the apical membrane, as were the proteins with the “apical” motifs E-T-H-F> (GAT3) and E-T-R-L> (mouse, bovine and dogfish CFTR). Also, a significant proportion of cells expressing the fusion protein containing the “basolateral” F-T-S-L> motif from MCP (68%) or another “basolateral” motif E-T-C-L> from LET23 (72.5%) showed predominant apical distribution of green fluorescence signal. These five motifs, associated with

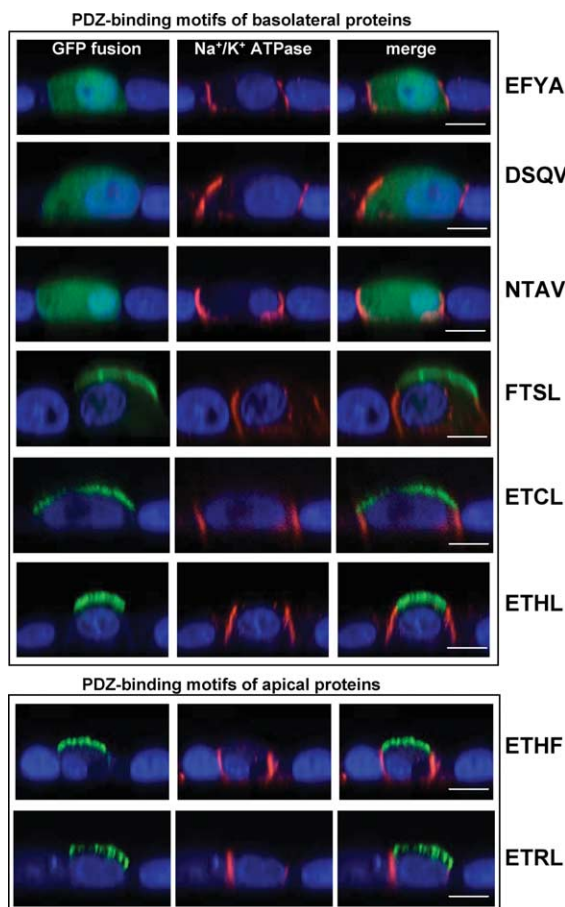


Fig. 2. Subcellular localization of the GFP-CFTR C-terminal constructs containing different PDZ-binding motifs. The green signal corresponds to the GFP fusion protein and the basolateral marker (Na^+/K^+ ATPase) is seen in red. Cytoplasmic distribution (i.e., no distinctive plasma membrane association) of the GFP fusion protein was seen in all cells expressing the EFYA (28 cells tested), DSQV (25), or NTAV (25) constructs. Predominant apical localization of the GFP signal was observed in the majority of cells expressing the FTSL (34/50, 68%), ETCL (29/40, 72.5%), ETHL (24/25, 96%), ETHF (25/25, 100%) or ETRL (23/25, 92%) fusion proteins. Nuclei were stained blue with DAPI. Bars – 10 μm .

partial (F-T-S-L>; E-T-C-L>) or unequivocal (E-T-H-L>; E-T-H-F>; E-T-R-L>) apical localization of the fusion protein, showed substantial sequence similarity to the D-T-R-L> motif, as all of them had a bulky hydrophobic residue (leucine or phenylalanine) at position 0 and threonine at position -2. Also, most of those motifs, with one exception of F-T-S-L>, had an acidic amino acid (aspartate or glutamate) at position -3. These results were consistent with our previous reports on the specificity of PDZ interactions involved in polarization of full-length CFTR, where we demonstrated that the residue at position 0 was the most critical for apical localization of CFTR, with position -3 also playing an important role [14]. Together, these results indicated that the specificity of PDZ interactions was insufficient to determine the correct localization of apical or basolateral proteins.

3.2. Contribution of amino acids upstream of the “classical” PDZ-binding motif

We have previously shown that the amino acids immediately upstream of the PDZ-binding motif of CFTR were not required for the apical membrane localization [9]. However, it has been suggested that some PDZ domains recognize more than four C-terminal residues, with the amino acids further upstream contributing to the affinity of PDZ interactions [6,15,16]. To test whether polarized distribution of certain basolateral proteins requires the presence of longer (complete) PDZ-binding motifs, we investigated whether specific amino acids upstream of the last four residues of the basolateral proteins BGT1, LET23 and MCP could affect the localization of the GFP fusion protein.

The only amino acid residue that was shared by the C-termini of these three basolateral proteins was a lysine at position -4, which was missing in CFTR (Fig. 3A). The basolateral localization of two of these proteins (BGT1 and LET23) has been

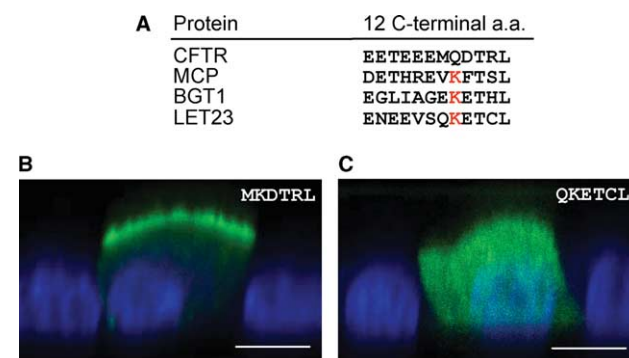


Fig. 3. Contribution of the -4 lysine to the specificity of the PDZ-dependent localization signals. (A) The C-terminal sequences of CFTR and three basolateral proteins (MCP, BGT1 and LET23) are compared. The CFTR sequence, originating from the cDNA-containing pBQ4.7 vector (gift from J. Rommens and L.-C. Tsui), includes the variant methionine at position -5, instead of the most frequent valine residue. Both residues at this position have been found to be non-essential for apical localization in MDCK cells ([9] and data not shown). Lysines at position -4 are marked in red. (B) Apical localization of the GFP-CFTR 1370–1480 H1402A, R1403A fusion protein containing the C-terminal M-K-D-T-R-L> motif. (C) Predominant cytoplasmic distribution of the GFP-CFTR 1370–1480 H1402A, R1403A fusion protein containing the extended Q-K-E-T-C-L> PDZ-binding motif of LET23. Green signal corresponds to the GFP fusion protein. Nuclei were stained blue with DAPI. Bars – 10 μm .

previously demonstrated to depend on their interaction with the PDZ protein LIN7 (or its mammalian counterpart VELI) [17,18]. Therefore, it seemed possible that the lysine residue at position –4 was critical for the specificity of this particular interaction. However, replacing the corresponding amino acid of CFTR (glutamine at position –4) with lysine did not abolish the apical localization of the GFP fusion protein (Fig. 3B). Also, the original C-terminal sequence of the full-length GAT3 protein already included the lysine residue at this particular position, which together indicated that this particular amino acid residue did not interfere with the polarized distribution of apical proteins. On the other hand, introduction of the last six amino acids of LET23 (Q–K–E–T–C–L>), which included the lysine residue at position –4, into the GFP fusion protein led to its partial exclusion from the apical membrane (Fig. 3C). Only 16% of the transfected cells showed predominant apical localization of this fusion protein. Also, the average apical to cytoplasmic signal ratio was significantly lower (1.12 ± 0.24) than in the case of the GFP fusions containing the D–T–R–L> (2.48 ± 0.71 , $P < 0.001$), K–D–T–R–L> (2.31 ± 0.70 , $P < 0.001$) or E–T–C–L> (1.50 ± 0.36 , $P < 0.025$) motifs, respectively. This suggested that the amino acids located upstream of the “classical” (four residue) motif contributed to the specificity of the PDZ-based interactions and that the lysine at position –4, as well as the cysteine at position –1, could constitute important components of the “basolateral” motif of LET23. Also, these two residues were likely to act together to ensure required specificity. Otherwise, the motif lacking just one of them could be easily mistaken for the apical localization motif, as can be judged from the apical localization of the fusion protein containing the short E–T–C–L> motif.

Importantly, even the presence of a longer PDZ-binding motif could not guarantee the specific (in this case basolateral) polarized protein distribution. Thus, the use of an extended PDZ-binding motif of LET23 seemed to decrease interaction with the PDZ protein responsible for the apical localization of CFTR, but was unable to facilitate the basolateral distribution of the fusion protein. This suggested that the LIN7/VELI protein, a component of the epithelial multi-protein complex involved in basolateral localization of LET23, was unable to direct the fusion protein to the basolateral membrane in the absence of additional localization signals accompanying the “basolateral” PDZ-binding motif.

4. Concluding remarks

The above results confirm and extend our contention that PDZ-based interactions alone are not sufficient to ensure correct polarized localization of membrane proteins. We conclude that the specific apical/basolateral protein distribution must be determined by the presence of additional localization signal(s) that may cooperate with the PDZ-binding motif during the process of targeting and/or stabilization at given location. As these additional elements remain poorly characterized, future efforts should be focused on their identification and elucidation of their relationship with the PDZ-based interactions.

As our results support the presence of multiple localization signals, it follows that the absence of a single signal may not necessarily lead to the protein mislocalization. The presence of the remaining signals may be sufficient to ensure the correct

protein localization in certain cell types or under certain conditions. This could at least partially explain why the deletion of the C-terminal PDZ-binding localization signal does not always alter the function and localization of the polarized membrane protein, as is the case with CFTR [19–21].

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