The emerging role of PDZ adapter proteins for regulation of intestinal ion transport

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Lamprecht, G., and U. Seidler. The emerging role of PDZ adapter proteins for regulation of intestinal ion transport. Am J Physiol Gastrointest Liver Physiol 291: G766–G777, 2006. First published June 22, 2006; doi:10.1152/ajpgi.00135.2006.—In the gastrointestinal tract, CFTR, in conjunction with one or several members of the SLC26 anion exchanger family, mediates electrogenic Cl⁻ and HCO₃⁻ secretion. Na⁺/H⁺ exchanger isoform NHE3, on the other hand, coupled to one or several of the SLC26 isoforms, mediates electroneutral NaCl absorption. The agonist-induced activation of anion secretion and inhibition of salt absorption causes secretory diarrhea. Current dogma sees the formation of a multiprotein complex of transport proteins, postsynaptic density-95/discs large/zonula occludens-1 (PDZ) adapter proteins, anchoring proteins, the cytoskeleton, and the involved protein kinases as one crucial step in the regulation of these transport processes. Data obtained in heterologous expression studies suggest an important role of these PDZ adapter proteins in trafficking, endocytic recycling, and membrane retention of the respective transmembrane proteins. This article reviews recent advances in our understanding of the role of the PDZ adapter proteins NHERF, E3KARP, PDZK1, IKEPP (NHERF-1 to NHERF-4), CAL, and Shank-2 that bind to CFTR, NHE3, and the intestinal SLC26 members in the regulation of intestinal fluid transport. Current concepts are mostly derived from heterologous expression studies and studies on their role in organ physiology are still in infancy. Recently, however, PDZ adapter protein-deficient mice and organ-specific cell lines have become available, and the first results suggest a more cell-type and possibly signal-specific role of these adapter proteins. This opens the potential for drug development targeted to PDZ domain interactions, which is, in theory, one of the most efficient antidiarreal strategies.

gastrointestinal ion transport; chloride secretion; bicarbonate secretion; sodium chloride absorption

DURING THE NORMAL DIGESTIVE PROCESS, the gastrointestinal tract secretes and absorbs large quantities of electrolytes and water. Both processes involve the concerted action of a number of ion transport proteins, and derangement of either function causes severe disease.

It is currently dogma that fluid secretion in the intestine is secondary to anion secretion and that fluid absorption is secondary to sodium absorption (100). The best known and most studied of the ion transport proteins involved in anion secretion is the CFTR Cl⁻ channel. This channel mediates electrogenic Cl⁻ as well as HCO₃⁻ secretion throughout the gut (2, 37) and, although able to mediate the secretion of both ions by itself (87, 91), appears to work optimally in conjunction with apical Cl⁻/HCO₃⁻ exchangers (58, 59, 71).

Sodium absorption is mediated by several brush-border ion transport proteins, which are operative in different segments and during different phases of the digestive process. Postprandially, sugar- and amino acid-coupled sodium absorption prevails in the upper small intestine (100). Electroneutral sodium absorption is mediated predominantly by the Na⁺/H⁺ exchanger isoform NHE3, with some contribution of other ill-defined sodium uptake mechanisms. It has the highest net sodium absorptive capacity and is present along the total length of the gut, with predominance in the ileum and proximal colon (100). Electrogenic sodium absorption is present in the distal colon and can reduce the sodium concentration in the stool to a few millimolar (64). Electroneutral Cl⁻ uptake parallels Na⁺ uptake in a one-to-one ratio and is believed to be mediated to a large part by the operation of apical anion exchangers of the SLC26 gene family (79). However, the contribution of trans- vs. paracellular Cl⁻ uptake is ill-defined at the present moment.

To provide driving force, ensure electroneutrality, preserve cellular ion homeostasis, and maintain cellular volume, each transport process involves a coordinated action of a number of ion transporters in the apical as well as basolateral membrane. Originally thought to be regulated by changes in membrane

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potential and transepithelial ion concentration gradients, it has become clear that changes in ion flux involve extremely complicated signal-transduction networks regulating the trafficking and interaction of multiple proteins involved in a regulatory process with more diversity than we ever thought of. In this review, we will focus on the recent progress that has been made in the understanding of the role of PDZ domain proteins in regulating the position, the interaction with other proteins, and the activity of the major players in intestinal anion secretion and salt absorption. After briefly reviewing the basic properties of this subset of PDZ adapter proteins, we discuss the role of PDZ adapter proteins in regulating trafficking, control of transport activity, and interaction of different transporters. Finally, we summarize recent findings on anion secretory and salt absorptive regulatory dysfunctions seen in knockout mice for several of these PDZ adapter proteins.

**PDZ Domain Proteins That Bind to CFTR, NHE3, SLC26A3, and SLC26A6**

PDZ domains are the most abundant protein-protein interaction modules in the human genome. Their name is derived from the first three proteins in which such domains were detected, the postsynaptic density protein PSD95, the *Drosophila* homolog Disc-large, and the tight junction protein ZO-1. PDZ domains are 90- to 100-amino acid-long globular structures that adopt a /H9252-/H9251-/H9251-/H9252-/H9252/-sheet and two /H9252-/H9251-/H9251-/H9252/-helices (41). They bind the PDZ motif of their ligands in a hydrophobic binding pocket between the second /H9252/-chain and the second /H9251/-helix. The affinity of the ligand-PDZ domain binding can be regulated by phosphorylation and other conformational changes both in the PDZ binding motif of the ligand (7, 15, 17, 72) as well as the PDZ domain protein (74, 89, 90).

PDZ adapter proteins usually possess several PDZ domains and often contain other non-PDZ protein interaction domains. The multidomain structure of the PDZ adapter proteins allows them to interact simultaneously with multiple binding partners and theoretically to mediate the formation of large protein networks, although up to this point, such large networks have not been isolated or visualized directly.

As mentioned above, a large number of proteins containing PDZ domains are found in the human genome. Each of these is assumed to bind to a limited set of ligands. On the basis of our understanding of the requirements for PDZ binding motif-PDZ domain interaction, databases can be searched for potential PDZ binding motifs in the COOH terminus of a protein, corresponding to class 1–3 binding patterns (41). Concurrently, crystallographic studies have revealed a more complex nature of the PDZ binding motif-PDZ domain interaction, with more amino acids involved than just the last four (102). Furthermore, certain PDZ-domains can also interact with internal protein sequences that adopt a /H9252/-hairpin structure (43). This, for example, is the case for the NHERF-NHE3 interaction (123). Therefore, a new classification is being awaited in the near future.

In the following section, we briefly discuss the properties of PDZ proteins that are expressed in the intestinal tract and have been found to bind to the PDZ motif of CFTR (Table 1). Most of these have been found to also bind to NHE3 and to the anion exchangers SLC26A3 [downregulated in adenoma (DRA)] and SLC26A6 [putative anion transporter 1 (PAT1)], the likely interaction partners of CFTR in bicarbonate secretion and of NHE3 in salt absorption (1, 4, 33, 58, 59). From this binding pattern alone, it is tempting to speculate that this subset of PDZ proteins may play a dominant role in regulating intestinal anion secretion and salt absorption.

**NHERF, E3KARP, PDZK1, and IKEPP (NHERF-1 to NHERF-4)**. NHE3 regulatory factor (NHERF), also called NHERF-1 or EBP50; NHE3 kinase A regulatory protein (E3KARP), also called NHERF-2; as well as PDZ domain protein kidney 1 (PDZK1), also called CAP70, PDZ-dc1, or NHERF-3; and intestinal and kidney enriched PDZ protein (IKEPP), also called NHERF-4, are highly homologous (35, 123, 124). Both NHERF (NHERF-1) and E3KARP (NHERF-2) consist of two PDZ domains and a COOH terminal ezrin/radixin/moesin (ERM) binding domain, which links these proteins via ezrin to the cytoskeleton (110, 123). Both proteins bind to CFTR, NHE3, and SLC26A3 (DRA) in heterologous expression systems (66, 110, 123). NHERF is a phosphoprotein, whereas E3KARP is not (39, 67). Constitutive phosphorylation of NHERF at S289 by G protein-coupled receptor kinase 6A (GRK6A) enhances oligomerization of NHERF (68). Regulated phosphorylation of S289 in response to PKA or PKC has been demonstrated in kidney slices, resulting in decreased association of NHERF with NaPi-IIa (23). Furthermore, NHERF is phosphorylated by cyclin dependent kinase 2 (cdck2) and dephosphorylated by protein phosphatase 1 and/or 2A at serine 279 and serine 301 (42). Both NHERF and E3KARP have been shown to dimerize both homotypically and heterotypically (31, 68, 103). Dimerization or oligomerization may allow the formation of larger clusters of adapter proteins below the apical plasma membrane.

PDZK1 (NHERF-3) contains four PDZ domains, but no other protein interaction domains have been identified (60, 114). Phosphorylation of PDZK1 at serine 509 by PKA has recently been demonstrated (83). With regard to gastrointestinal ion transport, it is most relevant that PDZK1 binds CFTR (114), SLC26A3 (DRA) (97), and NHE3 (34, 125, 126). PDZK1 forms heterooligomers with NHERF in vitro (34), and as mentioned for NHERF and E3KARP, this may allow the formation of an entire network of PDZ adapter proteins underneath the plasma membrane.

IKEPP (NHERF-4) was identified as an interactor of guanylate cyclase C (GCC) (99). IKEPP contains four PDZ domains and is expressed in the kidney, in the small and in mouse large intestine, and in Caco-2 and in T84 cells (35, 99). In the intestine and in the proximal tubule, IKEPP is localized to subapical vesicular structures (28, 35), whereas PDZK1 resides in or immediately under the plasma membrane (28, 35). In COS7 cells, IKEPP inhibits the activity of GCC (99). Controversial results concerning the binding of IKEPP to NHE3 have

1 The PDZ adapter proteins dealt with in this report have been identified in different settings and different species and are thus known under various names. To minimize the number of acronyms, we use the most common name irrespective of the original reference. Synonyms and the names of orthologs from other species are summarized in Table 1. Recently, it has been suggested to rename these proteins as NHERF-1 to NHERF-4 (28a), and these new designations are given in brackets.

2 There is some indirect evidence though that SGK1 may phosphorylate E3KARP (16).
Overview of the PDZ adapter proteins

<table>
<thead>
<tr>
<th>PDZ Adapter Protein</th>
<th>Synonym/Ortholog</th>
<th>Multimerization</th>
<th>Phosphorylated</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHERF (117) (NHERF-1) Mouse, rat, and rabbit: NHERF (117) Human: EBP50 (95)</td>
<td>(31, 68, 103)</td>
<td>S289, S301, and S162</td>
<td>A: (104) B: (78)</td>
<td></td>
</tr>
<tr>
<td>E3KARP (124) (NHERF-2) NHERF-2</td>
<td>(68, 121)</td>
<td>Probably not but see Ref. 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDZK1 (61) (NHERF-3) Mouse: CAP70 (114) or NaPi-Cap1 (35) Rabbit: CAP70 (97) Rat: diphor-1 (22) or PDZ-dc1 (92) or CLAMP (46)</td>
<td>Heterotypic oligomerization to NHERF (34)</td>
<td>(83)</td>
<td>(62, 63)</td>
<td></td>
</tr>
<tr>
<td>IKEPP (99) (NHERF-4) Shank2* (CortBP1) (54) Mouse: NaPi-Cap2 (35) CortBP1* (29) ProSAP1* (8) Shank2E* (77)</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
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<tr>
<td>CAL (12) GOPC (119) PIST (85) FIG (11)</td>
<td>(12)</td>
<td>nd</td>
<td>(119)</td>
<td></td>
</tr>
</tbody>
</table>

*CortBP1, ProSAP1, and Shank2E are splice variants of Shank2; Shank2 described in Ref. 54 is actually CortBP1. †Hogema et al., Dept. of Biochemistry, Erasmus Medical Center, Rotterdam, The Netherlands. PDZ, postsynaptic density-95/discs large/zonula occludens-1; NHERF, Na+/H+ exchanger isoform NHE3 regulatory factor; E3KARP, NHE3 kinase A regulatory protein; PDZK1, PDZ domain protein kidney 1; IKEPP, intestinal and kidney enriched PDZ protein; CAL, CFTR associated ligand; GOPC, Golgi associated PDZ and coiled-coil domain containing; PIST, PDZ domain protein interacting specifically with TC10; FIG, fused in glioblastoma; nd, not done.

been published (35). One recent report (125) demonstrates the binding of IKEPP to NHE3 and Ca2+-dependent stimulation of NHE3 transport activity.

CAL, CFTR-associated ligand (CAL) has two NH2-terminal coiled-coil domains that facilitate homomultimerization and probably also membrane association. On the basis of colocalization studies, CAL is mostly expressed in the Golgi (12). The interaction of the second coiled-coil domain with TC10 (85), a small GTPase, and Syntaxin 6 (11), a SNARE protein, suggests that CAL is involved in vesicle trafficking. Indeed Cheng et al. (12–14) have shown that CAL influences the distribution of CFTR between intracellular membrane compartments and the plasma membrane.

Shank2/CortBP1. SH3 and Ankyrin repeats containing protein 2/Cortactin binding protein 1 (Shank2/CortBP1) belongs to the family of Shank adapter proteins, which includes Shank1–3 (101). Shank2 binds to CFTR, NHE3, and NBC3 in yeast-two-hybrid assays (54). Among eight different PDZ adapter proteins known to be expressed in brain, Shank2/CortBP1 was shown to be specifically expressed in isolated rat pancreatic ducts (96), whereas CFTR, NHE3, and NBC3 are involved in apical Cl– and HCO3– transport (54). Shank2/CortBP1 is also present along the entire crypt-villus axis of rat colon. In addition, a novel splice variant, Shank2E, has recently been described in hepatocytes and cholangiocytes with an apical expression (77). Shank2 counteracts the cAMP-mediated stimulation of CFTR (see further below) (54) as well as the cAMP-mediated inhibition of NHE3 (40), but its role in NBC3 regulation has not been clarified yet.

The Role of PDZ Adapter Proteins in Intestinal Anion Secretion

As mentioned in the introduction, intestinal anion secretion is predominantly mediated by the CFTR channel, possibly in conjunction with one of the SLC26 Cl/HCO3– exchangers in those epithelial cells where both transporters are significantly coexpressed in the same membrane (107). Under those circumstances, the recycling of chloride may increase the transport rates of both CFTR and the anion exchangers (58, 59, 107). PDZ protein-anion transport protein interactions have been implicated in the agonist-mediated regulation of the individual transport proteins, as well as in the cooperation of CFTR with the SLC26 anion exchangers (58, 59). In addition, they have been implicated in trafficking, phosphorylation, homo- and heterodimerization, and membrane retention of CFTR (45, 93, 94, 106, 109–111, 114). IKEPP has also been implicated in the regulation of anion secretion by binding to the GCC (99).

In the following sections, we review current knowledge on the interaction of these PDZ proteins with the various components necessary for regulated intestinal anion secretion.

CFTR trafficking. Recent studies suggest that CFTR activity is to a large extent regulated by membrane insertion and removal (6). In this context, PDZ interactions have been demonstrated at several points along the biosynthetic and protein secretory pathway of CFTR (Fig. 1). CFTR was among the first proteins shown to require a COOH-terminal PDZ interaction motif for apical expression (80, 81). A more recent study by the same group demonstrated that the COOH terminus of CFTR contains additional apical sorting signals but that the PDZ-binding motif is required for prolonging the CFTR plasma membrane half-life (111). The same study also showed that the PDZ interaction motif of CFTR was required for reinsertion of CFTR from the recycling endosomes into the plasma membrane (111). The endocytosis of CFTR, on the other hand, has been shown to be clathrin mediated and to involve dynamin (reviewed in Ref. 6). Since the above-mentioned experiments were performed by expressing CFTR truncated for its PDZ motif (111), it is presently unknown which of the above-mentioned PDZ proteins, or whether all of them, may serve that function in certain cell types.

Recently, Benharouga et al. (5) studied various truncation mutants of CFTR in epithelial and nonepithelial cells. The authors demonstrated that CFTR sorts to the apical membrane with or without a PDZ-binding motif, but that its presence increased the effect of cAMP on CFTR transport activity. Østedgaard et al. (88) have also studied several mutations of
the CFTR COOH terminus by overexpressing them in CF airway epithelial cells. Again, CFTR was sorted to the apical membrane also in the absence of the PDZ interaction motif, but its biochemical stability was reduced.

The validity of these in vitro studies are underlined by the finding that a patient with the clinical features of cystic fibrosis was identified whose CFTR lacks only the last four COOH-terminal amino acids (The Chromosome 7 Project, http://genet.sickkids.on.ca). Obviously, the lack of the PDZ-binding motif alone causes a serious overall cellular functional defect of CFTR, even if the exact biochemical nature of this defect awaits further clarification.

The Golgi-associated PDZ-protein CAL, also known as PIST (85) or FIG (11), retains CFTR within the cell and enhances its lysosomal degradation (13). TC10, a small rho-GTPase that interacts with CAL and likely inhibits CFTR-CAL binding, resulting in an increased CFTR expression in the plasma membrane (14). When NHERF was coexpressed with CAL, the inhibitory effect of CAL on CFTR surface expression was also overcome (12). This suggests that inhibitors of CFTR-CAL binding, or agonists acting through NHERF and enhancing CFTR-NHERF binding, may promote enhanced apical CFTR expression. Structural studies of the C-terminal domain bound to the PDZ domains of NHERF and other CFTR-associated proteins such as CAL may provide a framework for developing novel drug therapies to enhance CFTR membrane expression and activity. Proof of concept for such an approach has recently been published by Guerra et al. (38), who demonstrated that overexpression of NHERF in Δ508CFTR expressing airway cells results in more mutant CFTR molecules reaching the plasma membrane as well as more functional activity upon PKA stimulation.

**Regulation of CFTR channel gating.** CFTR channel gating is thought to involve three distinct processes, 1) phosphorylation of the regulatory domain, 2) ATP binding and hydrolysis by the nucleotide binding domains, and 3) interactions of CFTR molecules among themselves and with other proteins (reviewed in Refs. 32, 55, and 105). PDZ-domain interactions are important for the first and third process. A model has been developed for PKA activation of membrane resident CFTR (45, 106, 109, 110). PDZ-domain interactions are developed for PKA activation of membrane resident CFTR and results in an increased open probability of CFTR. Rho, phosphorylation by PKC, and other influences, lead to an “open” configuration, in which the ERM domain of NHERF mediates CFTR activation and signaling to CFTR (84).

The effect of NHERF and E3KARP in facilitating the phosphorylation of CFTR is counteracted by Shank2 (54). It diminishes basal as well as cAMP-induced phosphorylation of CFTR, resulting in a reduction of the open probability of the channel (54). Conversely, knock down of Shank2 in T84 cells by antisense treatment augmented CFTR currents (54). It has been speculated that Shank2 inhibits CFTR by association with a phosphatase or by competition with a PDZ adapter protein that mediates PKA activation and CFTR recycling and degradation.

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![Fig. 1. The role of the postsynaptic density-95/discs large/zonula occludens-1 (PDZ) adapter proteins Na+/H+ exchanger isoform 3 (NHE3) regulatory factor (NHERF) and CFTR-associated ligand (CAL) in membrane traffic of CFTR. The PDZ interaction motif of CFTR is required for reinsertion of CFTR from early endosomes/recycling endosomes back into the plasma membrane; the PDZ adapter protein is NHERF or possibly another PDZ adapter protein such as PDZ domain protein kidney 1 (PDZK1). CAL is predominantly localized in the Golgi. It directs CFTR for lysosomal degradation and thereby decreases total cellular as well as plasma membrane CFTR. NHERF counteracts the effect of CAL. CAL may act as a molecular switch between CFTR recycling and CFTR degradation.](http://ajpgi.physiology.org/)
can interact with ezrin. In the absence of an open ezrin, the COOH terminus of CFTR only binds to the first PDZ domain of NHERF with high affinity. Binding of this complex to the conformational open ezrin molecule increases the affinity of the second PDZ domain for the COOH terminus of CFTR, and a second CFTR molecule is bound to one NHERF molecule (Fig. 2A). Another group showed that only about 2% of CFTR were associated with NHERF, suggesting that CFTR dimerization may occur by other PDZ-protein or PDZ-domain-related events as well (73).

**Coupling of CFTR to SLC26A3 (DRA) and SLC26A6 (PAT1).** Coexpression of CFTR with SLC26A3 (DRA) and SLC26A6 (PAT1) in HEK cells has been shown to result in activation of Cl-/HCO₃⁻ exchange (59, 70, 71), even when chloride transport by CFTR was inhibited using glibenclamide. The activation of SLC26A3 or SLC26A6 activity was further stimulated by cAMP-dependent activation of CFTR. Likewise, cAMP-stimulated CFTR transport rate was higher in CFTR and SLC26A3 (DRA) or SLC26A6 (PAT1) coexpressing cells. A structural interaction of CFTR and the anion exchangers is believed to be the underlying mechanism for this reciprocal activation of transport function. Phosphorylation of the regulatory domain of CFTR facilitates its binding to the so-called STAS domain of the SLC26 anion exchangers (58) (Fig. 2C). The interaction necessitates the binding of CFTR and SLC26A3 (DRA) or SLC26A6 (PAT1) to a common PDZ adapter protein (58). These functional studies are supported by the fact that CFTR and members of the SLC26 family were coprecipitated from heterologous expression systems as well as from mouse pancreatic tissue (59) and are partially colocalized in mouse pancreas and mouse small intestine (58).

In native duodenum or pancreatic ducts, cAMP-mediated activation of CFTR-dependent bicarbonate secretion does not require the activity of an anion exchanger (48, 108). In murine duodenum, the absence of SLC26A6 does not inhibit cAMP-stimulated Cl⁻/HCO₃⁻ secretion, although it significantly reduced basal secretory rate (115). The absence of CFTR, however, has been found to reduce the maximal anion exchange rate in murine duodenum (107). Obviously, further studies in native tissues are needed to resolve these questions.

In the pancreas, models are required to explain the exceptionally high HCO₃⁻ concentration in the distal pancreatic ducts. In this organ, the model of CFTR-anion exchanger coupling was first developed, because early data suggested a low HCO₃⁻ permeability of epithelial Cl⁻ channels (86). It is now clear that the expression and activity of basolateral anion uptake mechanisms may also dramatically change the anion secretion through an anion conductance (25) and that they are likely to play a major role in the anion composition of the pancreatic fluid (30, 47). Methods have been developed to measure secretory activity in murine pancreatic ducts (30, 70). Thus the regulation of pancreatic anion secretion and the role of PDZ proteins will likely be further elucidated in the near future.

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2 The expression of CFTR also induces the expression of SLC26A3 (DRA) and SLC26A6 (PAT1) on the transcriptional level in tracheal epithelia cells and in cultured pancreatic duct cells (36, 118).

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Invited Review

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![Fig. 2. PDZ interaction of CFTR. Several models for the role of PDZ adapter proteins in the activation of CFTR have been developed. A: CFTR channel open probability is increased by the formation of CFTR dimers through interaction with common PDZ adapter proteins like NHERF (93, 94) or PDZK1 (114). NHERF primarily binds with its first PDZ domain to one CFTR molecule. After activation of ezrin (1), NHERF binds with its ERM binding domain to the NH₂-terminal domain of ezrin (2). This increases the affinity of the second PDZ domain for CFTR (3) facilitating the formation of dimers (74). B: similar to Fig. 3, ezrin may function as an A kinase anchoring protein (AKAP), allowing PKA to phosphorylate CFTR in a complex of CFTR, NHE3 kinase A regulatory protein (E3KARP) or NHERF, ezrin, and PKA (45, 106, 109, 110). The models shown in A and B may coexist. C: CFTR and SLC26A3 (DRA, down regulated in adenoma) or SLC26A6 (PAT1, putative anion transporter 1) function is increased by interaction of their R- and STAS domains. This complex is further stabilized by the interaction of both proteins with a common PDZ adapter protein (58, 59).](http://ajpgi.physiology.org/)

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http://ajpgi.physiology.org/
Electroneutral NaCl absorption in the ileum and proximal colon is largely mediated by a coupled action of NHE3 and the anion exchanger SLC26A3 (DRA). Both the absence of NHE3 in a knockout model (98) or that of SLC26A3 (DRA) in the human autosomal recessive disease congenital chloride diarrhea (51) cause severe diarrhea and changes in plasma electrolyte composition. Both NHE3 and SLC26A3 (DRA) have PDZ binding motifs and bind to the same set of PDZ proteins (66, 123). However, to date, most studies on the functional relevance of PDZ protein interaction have only been performed for NHE3. Similar to CFTR, NHE3 is regulated by changes in membrane insertion and probably by changes in the transport activity of membrane resident NHE3 molecules (26). PDZ adapter-NHE3 interactions are likely involved in both modes of regulation. A large number of endogenous substances, as well as bacterial and viral enterotoxins or drugs, have been shown to affect electroneutral salt absorption in the intestine (65). For some of these, a direct effect on NHE3 has been established (21, 27, 52, 121, 122). PDZ adapter proteins are likely involved both in the agonist-mediated stimulation as well as inhibition of NHE3.

**cAMP- and cGMP-mediated inhibition of NHE3.** In PS120/NHE3 cells, a fibroblast cell line that lacks endogenous NHE3 and has been stably transfected with NHE3, either E3KARP or NHERF is required for cAMP-mediated inhibition of NHE3 (Fig. 3, right) (124). Coprecipitation studies revealed that not only NHE3, but also the PKA-anchoring protein ezrin, binds to NHERF and E3KARP (67, 116). This suggested that E3KARP or NHERF serves as an adapter between NHE3 and ezrin. Ezrin is a known PKA anchoring protein. In analogy to CFTR phosphorylation, the close proximity of PKA to NHE3, brought about by the PDZ adapter and ezrin, is currently thought to be a necessary prerequisite for PKA-mediated phosphorylation of NHE3 (67, 123, 127).

The kinetic analysis of cAMP-mediated NHE3 inhibition in OK cells suggested that the amount of transporter in the plasma membrane remains unchanged during the early phase of cAMP-elevation (67). At later time points, a decrease in cell surface NHE3 was also observed (44). Thus phosphorylation of NHE3 both directly inhibits the transport rate and may serve as a signal allowing endocytosis of NHE3 to occur.

Guanylin and its bacterial analog STa (heat-stable enterotoxin) inhibit salt absorption in the small intestine through intracellular cGMP elevation (112). In PS120/NHE3 fibroblasts, E3KARP has been shown to be required for cGMP-mediated inhibition of NHE3 (10). A macromolecular complex is formed that includes NHE3, E3KARP, and membrane bound myristoylated cGK-II (cGMP activated kinase type II), which likely phosphorylates and thus inhibits NHE3.

**Ca/PKC-mediated regulation of NHE3.** NHE3 is inhibited by Ca\(^{2+}\) in mobilizing agents such as carbachol or ionomycin (21) through the action of PKC. In contrast to the inhibition of NHE3 by PKA, PKC-mediated inhibition is caused by a decrease in NHE3 membrane expression (49, 50). The involvement of E3KARP in these processes has been elucidated in four consecutive papers (53, 69, 75, 76).

In PS120/NHE3 fibroblasts, Ca\(^{2+}\)-mediated inhibition of NHE3 has been shown to occur via NHE3 removal from the plasma membrane. This endocytosis specifically requires E3KARP (53, 69) (Fig. 3, left). E3KARP, which is constitutively bound to NHE3, recruits α-actinin-4 and PKCα in a Ca\(^{2+}\)-dependent manner (53, 69). This leads to the clustering of NHE3, E3KARP, α-actinin-4, PKCα, and possibly other proteins (53, 76) and subsequent internalization of the complexes.

Consistent with the findings in heterologous expression systems are data derived from native intestine. Treatment of rabbit ilea with carbachol leads to inhibition of sodium absorption and to a 24% decrease of total brush-border NHE3, which is accompanied by an increase of endosomal NHE3 from 22% to 38% (53, 75, 76). The decrease of brush-border NHE3 protein abundance is reflected by an increase of the NHE3 protein abundance in the detergent-resistant fraction. The for-
mation of these complexes requires the activation of c-src, which is also present in the detergent-resistant fraction (76).

IKEPP has recently been found to bind to NHE3 and to mediate Ca\(^{2+}\)-induced stimulation of NHE3 in PS120/NHE3/ IKEPP cells (125). This was accompanied by a shift of NHE3-and IKEPP-containing complexes to smaller sizes. At present, the physiological relevance of this finding is not clear, especially because Ca\(^{2+}\) is thought to inhibit NHE3 in the intestine.

**Glucocorticoid-mediated stimulation of NHE3.** Glucocorticoids stimulate ileal Na\(^{+}\) absorption, and one of the mechanisms is upregulation of NHE3 expression by transcriptional (122) and posttranslational mechanisms (121). In cell culture, the posttranslational activation of Na\(^{+}/H^{+}\) exchange activity requires E3KARP (i.e., E3KARP cannot be substituted by NHERF) and serum glucocorticoid inducible kinase, isoform 1 (SGK1) (16, 121). Recently, S663 of NHE3 has been identified as the substrate for SGK1 (113). The SGK1-mediated upregulation of NHE3 function also involves phosphatidylinositol 3 (PI3)-kinase, because it can be blocked with LY294002 (121).

Because PI3-kinase is known to influence the surface expression of NHE3 (50), it is tempting to hypothesize that SGK1 upregulates NHE3 via an increase in NHE3 surface expression, but this has not been explored yet.

**Coupling of NHE3 with SLC26A3 (DRA).** Two decades ago, studies (56, 57) in isolated brush-border membrane vesicles provided evidence that the coupling of intestinal brush-border membrane Na\(^{+}/H^{+}\) and Cl\(^{-}/HCO_{3}^{-}\} exchange may occur through the intracellular pH. Due to lack of appropriate techniques to influence and measure intracellular pH in the appropriate fashion in the native intestinal epithelium, proof for this concept has not yet been provided. Furthermore, a physical interaction of both transporters may augment pH-mediated regulation or may be a prerequisite for a regulatory interaction, as has been described for CFTR and the SLC26A3 and SLC26A6 anion exchangers (58, 59). This has prompted us to study whether SLC26A3 (DRA) also has a PDZ interaction motif and whether it interacts with the same PDZ domain protein(s) as NHE3. On the basis of the interaction of both NHE3 and SLC26A3 (DRA) with the second PDZ domain of E3KARP (66), and on the basis of the ability of E3KARP to form dimers (31, 103), we have proposed a model (Fig. 4), in which NHE3 and SLC26A3 (DRA) are physically coupled to each other by a NHERF or E3KARP dimer (66). Since then, it has become clear that SLC26A3 (DRA) in vitro not only interacts with E3KARP but also with NHERF (D. Natour and G. Lamprecht, unpublished observation) and PDZK1 (97), both of which are expressed at much higher levels than E3KARP in the native intestine. Thus the exact composition of SLC26A3 (DRA) (and NHE3)-containing complexes requires further study.

**Interaction of NHE3 and CFTR.** Agonist-stimulated intestinal anion secretion occurs concomitantly with inhibition of electroneutral salt absorption, and in the intestinal tract of CFTR-deficient mice, cAMP-mediated inhibition of electroneutral NaCl absorption is absent (20). This finding is surprising, because cAMP inhibits NHE3 in cells that do not express CFTR (124).

Two recent studies may shed some light on this puzzle. In PS120/NHE3 fibroblasts, the expression and the cAMP-mediated activation of CFTR results in a markedly stronger inhibition of NHE3 by forskolin than found in its absence (1). Luminal NHE3 activity in mouse pancreatic ducts was more strongly inhibited by forskolin perfusion than in ducts from CFTR mutant mice (1). The inhibition of NHE3 by CFTR in PS120 fibroblasts required the COOH-terminal PDZ binding motif of CFTR. Likewise, NHE3 could only be immunoprecipitated from mouse pancreas that expressed CFTR containing the COOH-terminal PDZ binding motif (1). Bagorda et al. (4) recently demonstrated that in a renal cell line, which endogenously expresses CFTR, the heterologous expression of NHE3 results in a decrease in the maximal activation of CFTR by PKA. Likewise, antisense-mediated suppression of CFTR resulted in a decrease in the NHE3 inhibition by cAMP. Again, this reciprocal regulation appeared to occur in a multiprotein complex, likely consisting of CFTR, NHE3, ezrin, E3KARP, and possibly other proteins (4). The former study may explain the findings in the CFTR knockout mice and suggests that in cells that coexpress CFTR and NHE3 endogenously, a PDZ-dependent interaction of the two transporters augments the PKA-mediated switch from an absorbing to a secreting cell.

However, the question remains where such coexpression takes place. Overall expression levels for CFTR in the intestinal mucosa are much lower than for NHE3, and CFTR is predominantly located in the crypts and basal parts of the villi, whereas NHE3 is most strongly expressed in the surface cells. Especially in colonic surface cells, where cAMP-induced inhibition of NHE3 is also absent in CFTR-deficient mice (3), the theory of a structural interaction of NHE3 and CFTR as the basis for this inhibition is, at present, difficult to reconcile with the assumed number of both proteins in the brush-border membrane.

In the jejunal midvillus, where CFTR and NHE3 are coexpressed within the same cell, cAMP activation of CFTR has...
been shown to mediate anion secretion, resulting in cell shrinkage (33). NHE3 inhibition by CFTR-activation-induced cell shrinkage was suggested as an explanation for the observed lack of NHE3 inhibition in CFTR-deficient intestine. Thus alternative explanations have been brought forth that require the functional activity of CFTR in the membrane rather than a physical interaction of CFTR and NHE3 (33).

Data from Knockout Animals

Two different knockout mice of NHERF (78, 104) and one type of knockout mice of PDZK1 (62), E3KARP, and CAL (120) have been generated.

Female NHERF knockout mice generated by Shenolikar et al. (104) have a lower body weight, reduced lifespan, and decreased bone mineral density compared with their heterozygous or wild-type littermates. Both male and female knockout animals show increased urinary phosphate excretion and altered NaPi-IIa expression in the proximal tubule (104). The expression of NHE3 in the proximal tubule (82) as well as the enterocyte (18) is unaltered, but Na\(^+\)/H\(^+\) exchange is not inhibited by cAMP any more (82). In the enterocyte (18) is unaltered, but Na\(^+\)/H\(^+\) exchange is not inhibited by cAMP any more (82). In the enterocyte, cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange is still present both in the ileum (82) and colon (18), but in the colon to a reduced extent. Interestingly, cAMP-mediated activation of CFTR-dependent anion current is also reduced (18).

As a consequence, ileal fluid production is decreased in NHERF knockout mice (B. Hogema et al., unpublished observations). All in all, these mice should be less susceptible to enterotoxin-mediated diarrheal disease, but this has not been tested yet.

The NHERF knockout mice generated by Morales et al. (78), on the other hand, do not show increased mortality of the females, although they also have decreased serum phosphate levels. The kidneys do not show macroscopic or microscopic alterations. There is strong downregulation of ezrin and activated ERM proteins, which is more pronounced in the intestine than in the kidney. These animals have disorganized intestinal microvilli and dispersed actin-rich terminal webs. The number of goblet cells is increased. Intestinal ion transport studies in these animals have not been reported yet.

E3KARP knockout mice have also recently been generated, and various organ systems are in the process of being studied. In the intestinal tract, cAMP-dependent activation of anion secretion, cAMP-mediated inhibition of NHE3, and forskolin-inhibition of fluid absorption in isolated small intestinal loops is not altered (B. Hogema et al., unpublished observations, but differences in Ca\(^{2+}\) mediated inhibition of NHE3 are similar to those described in heterologous expression systems (A. Cinar et al., unpublished observations), i.e., the inhibitory effect of increased intracellular Ca\(^{2+}\) is lost in the knockout animals.

PDZK1 knockout mice do not have a gross anatomical phenotype (62) but have elevated levels of cholesterol due to a defect in membrane expression of the scavenger receptor (63). When these mice are challenged with a high phosphate diet, they show decreased NaPi-IIa expression and increased renal phosphate excretion compared with the wild-type animals (9).

Intestinal ion transport has recently been studied in PDZK1 knockout mice (19). They show slightly diminished forskolin-stimulated duodenal anion secretion. In the small intestinal mucosa, basal Na\(^+\) absorption is reduced by about 50%, the immediate inhibitory effect of forskolin is lost, but some inhibition does occur at later stages. In colonic surface cells, basal NHE3 activity was reduced by about 50%, and the immediate inhibitory effect of forskolin was lost. NHE3 abundance in the brush-border membrane was decreased by 30% compared with wild-type littermates, whereas NHE3 mRNA expression was significantly increased and CFTR expression was not significantly altered. The data suggest that a complex regulatory interaction of PDZK1 and NHE3 takes place in the intestine, affecting agonist-mediated NHE3 regulation and BBM protein abundance in an, as yet, incompletely understood fashion.

Thus the first data that emerge from studies in the native intestine underline the importance of PDZ domain-interaction in the regulation of intestinal electrolyte transport. They also demonstrate, however, that the situation is more complex than in heterologous coexpression systems, in which one or two components of a potential multiprotein complex are strongly overexpressed, but others may be underrepresented. Presently, only relatively little experimental knowledge has been obtained from knockout studies, and methods have varied between different groups. Yet, it appears that NHERF, E3KARP, and PDZK1 have more cell- and signal-specific functions than could have been anticipated from heterologous expression systems. It follows that the signaling complexes for CFTR and NHE3 are likely different in different organs and under different circumstances. New and exciting findings on the role of this family of proteins in native tissues are likely to come up in the near future.

In summary, since the cloning of NHERF by Weinman et al. (117) in 1995 and the discovery that it is a necessary cofactor for cAMP-mediated inhibition of NHE3 in renal brush-border membranes, much progress has been made in the field. E3KARP, PDZK1, IKEPP, CAL, and Shank2 have been identified as additional PDZ adapter proteins that interact with some or all major ion transporting proteins in the gastrointestinal tract, i.e., CFTR, NHE3, and the SLC26 transporters SLC26A3 (DRA) and SLC26A6 (PAT1). Functional characterization has expanded from a quite simple model of a stationary complex that facilitates phosphorylation of a single transporter protein in response to a single signal, to much more complex models such as signal complex formation affecting several different signals, clustering of one or several transport proteins, and regulation of membrane traffic of various transporter proteins.

The coordinated action of different transport proteins in the same membrane, as well as in the contralateral membrane, is of particular interest during vectorial ion transport in intestinal epithelia. Therefore, several questions are of particular interest for future studies in the intestine. First, despite the existence of the models presented in this review, the actual components of the PDZ-adaptor mediated multiprotein complexes in the intestinal brush-border membrane during regulated transport are

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5 Different from the mouse, PDZK1 expression is minimal in human and rabbit colon (97, 114).
unknown and likely contain more proteins than our current models predict. The isolation of such complexes from native intestine is extremely difficult and fraught with many uncertainties. Unfortunately, intestinal cell lines, unless genetically manipulated, uniformly express NHE3 and SLC26A3 (DRA) at low levels. However, ingenious methods have been developed to search for protein interaction partners and to study the interaction and its functional consequences in heterologous expression systems. The recent advance of transgenic mouse generation with cell-specific or inducible expression/knockout of a protein, of multiple-knockout mice, and of small-interfering RNA-based approaches in vivo, allows to test the applicability of the derived models to the situation in native organs. Second, in the past, absorptive and secretory functions within the intestinal epithelium were thought to be separated along the crypt-villus axis (100). Current models on complex formation of intestinal ion transporters require CFTR, NHE3, and the SLC26 anion exchangers in the same cellular membrane. At least in some small intestinal villus cells, there is evidence that this may be so (33). According to current models, the switch from the absorptive to the secretory state has to be accompanied by a dramatic reorganization of the multiprotein complexes within an enterocyte. How is, in this situation, the binding affinity between PDZ adapters and transporters determined and how is it regulated? First reports have been published on the second-messenger-dependent regulation of the affinity between PDZ-adaptor and ligand (15, 17, 72, 93). Crystallographic studies have revealed the structural basis for PDZ-domain-ligand interactions and the extension of such studies to ligand-PDZ adapter interactions during regulation of intestinal transport processes is urgently needed (64a). Third, drug targeting of PDZ-domain-ligand interactions holds great therapeutic promise (24). Secretory diarrhea, the number-one killer among gastrointestinal disease worldwide, is mediated by stimulation of anion secretion and inhibition of salt absorption, and, as detailed above, both processes are thought to be mediated by multiprotein complex formation and to involve a common set of adapter and anchor proteins. Targeting of specific PDZ-domain-ligand interactions may theoretically interfere with both processes and thus prevent electrolyte and fluid loss. But even much simpler approaches, such as an interruption of PDZ-domain-ligand interactions that retain transport proteins in the cell interior, or in the membrane, may be of great benefit in cystic fibrosis or diarrheal diseases.

REFERENCES


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