SYMPOSIUM REPORT

NHERF family and NHE3 regulation

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The intestinal and renal proximal tubule brush border (BB) Na^+–H^+ exchanger NHE3 binds to members of the NHERF (Na^+–H^+ exchanger regulatory factor) family. These are four proteins (current most used names include NHERF1, NHERF2, PDZK1 and IKEPP) which are related to each other, are present in locations in or close to the BB, and scaffold a variable series of proteins in NHE3-containing complexes in a dynamic manner that is altered by changes in signal transduction which affects NHE3 activity. The specific roles of these proteins in terms of NHE3 regulation as well as interactions with each other and with their many other substrates are only now being defined. Specificity for only one member of the NHERF family in one example of NHE3 regulation, inhibition by elevation in cGMP, is used to describe how NHERF family proteins are involved in NHE3 complex formation and its regulation. In this case, NHERF2 directly binds cGKII in the brush border to form an NHE3 complex, with cGKII also associating with the BB via its myristoylation.

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The NHERF family

The NHERF family consists of four related PDZ domain-containing proteins that are present in the brush border (BB) of the mammalian small intestine, colon and renal proximal tubules (Weinman *et al.* 1995; Yun *et al.* 1997; Custer *et al.* 1997; Scott *et al.* 2002; Kocher *et al.* 2003), as well as in many other cells. These four proteins have two or four PDZ remains with many of their PDZ domains having a high identity with other PDZ domains of this protein family. This report begins to address why four highly homologous and related proteins might be necessary in the same localized domain in polarized epithelial cells. We focus on how each NHERF family protein is different from an evolutionary view and how each uniquely contributes to NHE3 regulation.

PDZ domains are one of the most commonly utilized human protein–protein interacting sequences. In the human genome, there are at least 500 proteins which contain at least 900 PDZ domains (Sheng & Sala, 2001; Hung & Sheng, 2002; Kim & Sheng, 2004; and SMART, a simple modular architecture research tool (http://SMART.embl-heidelberg.de)). There are at least three types of PDZ domains defined by the amino acid identity at the 0 and −2 positions in the C-terminal ligand sequence: Class I, X S/T X I/V/L/M; Class II, X Φ X Φ (Φ hydrophobic amino acid); and Class III, X D/E X V. PDZ domains can also bind to internal sequences, which resemble these C-terminal ligands, but have a B finger configuration (Cuppen *et al.* 1997). Binding of both C-terminal and internal PDZ domain ligands is by entry of the ligand into the linear groove of the PDZ domain (Cuppen *et al.* 1997; Sheng & Sala, 2001; Hung & Sheng, 2002).

Phylogenetics

Epithelial cells contain multiple PDZ proteins, which are localized in specific subcellular domains, including the BB, basolateral membranes, the tight junctions, as well as in the Golgi (CAL) and endosomes (sorting nexin 27) (Cheng *et al.* 2002, 2004; Brone & Eggermont, 2005) (Fig. 1*A*). The major identified class of PDZ domain-containing proteins in the BB is the NHERF family, although the unrelated Shank2 is also present. The NHERF protein family has four members, each of which have between two and four PDZ domains (Fig. 1*B*): NHERF1, also called NHERF or EBP50 (Reczek *et al.* 1997); NHERF2, also called E3KARP, SIP-1 or TKA-1; NHERF3, also called PDZK1, CLAMP, CAP70, DIPHOR-1 or NaPi-CaP1 (Wang *et al.*

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2000); and NHERF4, also called IKEPP, DIPHOR-2 or NaPi-CaP2 (Custer *et al*. 1997). These proteins range in size between 337 and 519 amino acids. NHERF1 and NHERF2 also contain ERM (Ezrin, Radixin, Moesin) binding domains at their C-termini, while NHERF3 and NHERF4 do not.

By performing phylogenetic analysis, we found that of all mammalian proteins, the NHERF family members are most highly related to each other, with the next most related protein being sorting nexin 27 (M. Donowitz, A. Sharma and C. Brett, unpublished results; Joubert *et al*. 2004). The 12 PDZ domains of the NHERF family were analysed for their relationships to each other. Based on identity ($\geq$ 20%), they were divided into four groups (A–D) (Fig. 2). The evolution of each of these PDZ domains was traced from worm and fly. They did not originate in bacteria, plants or yeast. While bacteria, plants and yeast have distantly related proteins, these contain circular binding pockets, indicating that they are involved in different types of protein–protein interactions. The worm has two related PDZ domain-containing proteins, CO1F6.6 and 4J893. The PDZ domain of CO1F6.6 and the first PDZ domain of 4J893 are identical and are probably the predecessors of the human NHERF family group C PDZ domains (Fig. 2). The second PDZ domain of 4J893, which is only 5% identical to the first, is most similar to human group B PDZ domains and also to human group A. Group D human NHERF family PDZ domains are not related to PDZ domain proteins in *C. elegans*. *D melanogaster* also has two NHERF family-related protein orthologues which are only ~10% identical to each other. These are CG10939-PA, which shows most homology to human group A PDZ domains, and CG32758-PA, which is most similar to human group D. Thus, group A PDZ domains appear to have separate genetic origins from both worm and fly, whereas the other three human NHERF family PDZ domain groups appear to have potentially originated from single worm or fly PDZ domain-containing genes. Of interest is the 22% identity in the amino acid composition of the second PDZ domain of worm 4J893 and the PDZ domain of fly CC610939.

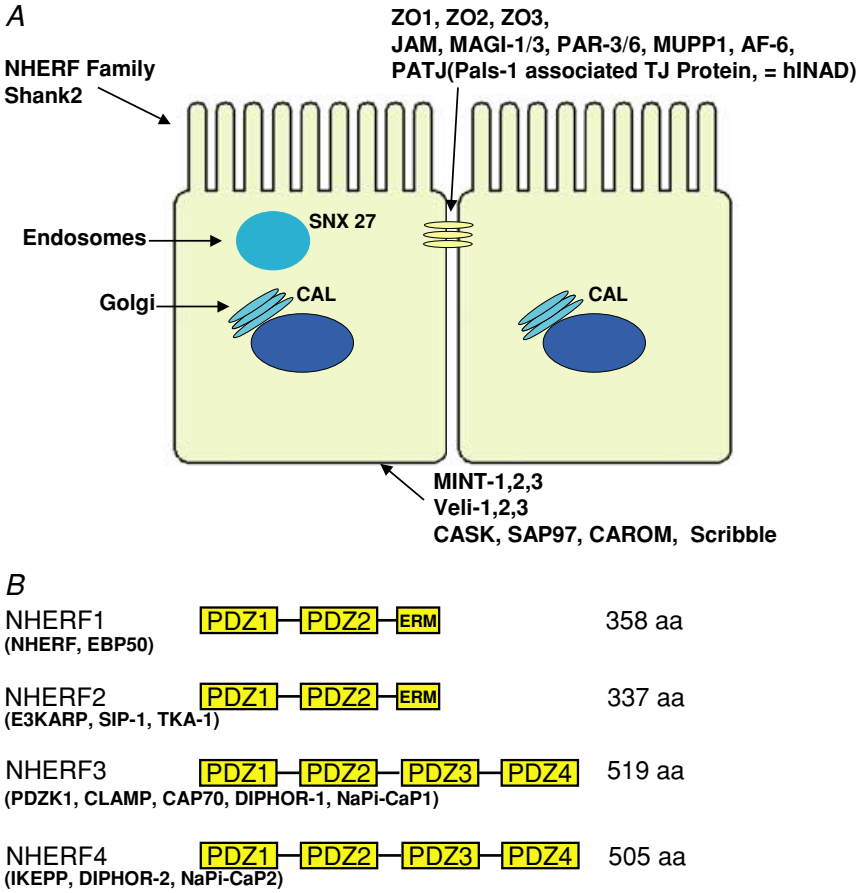

Figure 1. NHERF Family
A, localization of PDZ domain-containing proteins to epithelial cell brush border, basolateral membranes, tight junctions, Golgi or endosomes. *B*, NHERF gene family: shown are the suggested names (NHERF 1–4), multiple previous names of the proteins, protein–protein interacting domains, and number of amino acids. ERM, ezrin, radixin, moesin.

Location and unique binding partners of NHERF proteins

We now return to the question of why there are four related and highly homologous PDZ proteins from the NHERF family in the BB of enterocytes or the renal proximal tubule in polarized Na^+ absorptive epithelial cells. We suggest that they may have different locations and different binding partners both of which may relate to the fact that they evolved from different PDZ domains in worm or fly. Immunofluorescent localization studies were performed using confocal microscopy of the NHERF family members in polarized Caco-2 cells (a human colon cancer Na^+ absorptive cell line), which contain NHE3, and in mouse ileum (N. C. Zachos, O. Kovbasnjuk & M. Donowitz, unpublished observation). In Caco-2 cells, NHERF1 and NHERF3 (PDZK1) are predominantly in the BB with marked colocalization with NHE3 under basal conditions (Table 1). NHERF2, as was initially reported by Wade *et al.* (2003), is present in the BB but predominantly localizes to the area just below the BB in the area of the intermicrovillar clefts. It has some colocalization with NHE3. NHERF4 (IKEPP) has minimal BB location and is primarily cytosolic. It overlaps with NHE3 in the area below the BB. Similar observations have been made in the mouse ileum. NHERF1 and NHERF3 (PDZK1) are predominantly in the ileal BB. NHERF2 localizes to the BB and just below the BB as well as more diffusely in the cytosol. NHERF4 (IKEPP) has minimal or no BB localization and is distributed throughout the cytosol. The localizations of NHERF1-3 appear similar to reports in the mouse proximal tubule (Wade *et al.* 2003; Madjdpour *et al.* 2004).

While NHERF1 and NHERF2 were initially recognized as NHE3 binding partners (they were named for that interaction), they have subsequently been identified as binding to many proteins, now approaching 60 (Shenolikar *et al.* 2004). While members of the NHERF family share protein binding partners, specific protein–protein interactions also have been discovered. As reviewed by Shenolikar *et al.* (2004), NHERF2 has N, Ne binding partners that do not bind to NHERF1, which include TAZ (transcriptional co-activator with PD*Z*-binding motif) (Kanai *et al.* 2000), α-actinin-4 (Kim *et al.* 2002), phospholipase C B_3 (Hwang *et al.* 2000), cGMP kinase I and II (Cha *et al.* 2005), DRA (down-regulated in adenoma) (Lamprecht

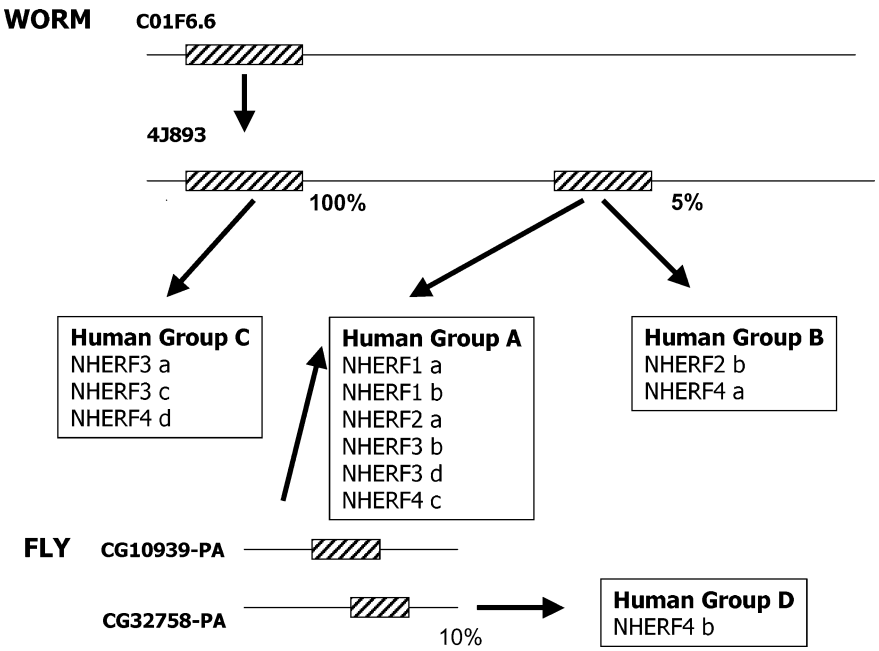

Figure 2. Evolution of the PDZ domains of the mammalian NHERF family from *C. elegans* and *D. melanogaster*

PDZ domains of the NHERF family were defined using ScanSite MotifScan. The 12 human NHERF family PDZ domains were individually analysed for sequence relationship by the MegAlign program (DynaStar, Inc.). The domains were grouped by closest relationships. Relationship with *C. elegans* and *D. melanogaster* proteins were also analysed by MegAlign. All protein names and sequences were obtained from NCB1. *C. elegans* has two PDZ domain-containing proteins that are related to the NHERF family. CO1F6.6 contains one PDZ domain while 4J893 contains two PDZ domains, one identical to that in CO1F6.6 and the other 5% identical. This suggests that the second PDZ domain of 4J893 may have evolved by gene duplication. Each of the two worm PDZ domains is a precursor to certain groups of PDZ domains of the human NHERF family. The *D. melanogaste*r proteins CG10939-PA and CG32758-PA each contain one PDZ domain (10% identical to each other) and are precursors to the human NHERF family.

Table 1. The NHERF family

NHERF members	Localization	NHE3 colocalization
NHERF (NHERF1)	BB>>Sub-Ap, IC	++
E3KARP (NHERF2)	BB<Sub-Ap, IC	+
PDZK1 (NHERF3)	BB>>Sub-Ap	+++
IKEPP (NHERF4)	BB<<Sub-Ap	+

BB, brush border; IC, intracellular.

et al. 2001), podocalyxin (Takeda *et al.* 2001), and SRY-1 (Poulat *et al.* 1997) and SGK1 (Yun *et al.* 2002). Thus given the scaffolding role recognized for PDZ domain proteins in general, this specificity of binding suggests that the organization of different proteins into unique complexes associating with the different NHERF family members may be one of the consequences of having multiple NHERF family members in a similar part of the cell. In this review, emphasis will be on how NHERF family members and related complex formation are involved in NHE3 regulation, as an example of exploration of the epithelial function of the NHERF family.

The NHE3 protein is estimated to have a size of ∼87 kDa. However, sucrose density gradient separation of multiple cell lines that express NHE3, as well as intact ileum, have demonstrated that NHE3 exists simultaneously in many large multiprotein complexes ranging in size from 200 to 1200 kDa under basal conditions (Li *et al.* 2004) (Fig. 3). These complexes probably indicate that NHE3 is binding to multiple proteins simultaneously, although multimerization of NHE3 may contribute. By comparing surface and total NHE3 size, it was observed that plasma membrane NHE3 is in larger complexes than intracellular NHE3. This indicates that not all complexes are formed in the ER/Golgi and it can be assumed that as NHE3 trafficks between the BB and recycling endosomes, it associates with some different proteins. Thus the NHE3-containing complexes are dynamic. One example of the dynamic nature of NHE3 complexes is illustrated in Fig. 4 and relates to acute inhibition of NHE3 activity by carbachol activation of M3 muscarinic receptors (Li *et al.* 2004). As part of this regulation, the amount of plasma membrane NHE3 is decreased in parallel with inhibition of NHE3 activity and as illustrated, NHE3 partially shifts into larger size complexes. α-Actinin-4, which binds to NHE3 by scaffolding to NHERF2, also shifted into larger complexes in parallel with NHE3 after carbachol treatment (Kim *et al.* 2002; Li *et al.* 2004). A role for the NHERF family proteins in forming these large NHE3-containing complexes has been assumed but not established as yet.

NHERF family members and NHE3 regulation

Experimental models used to understand the role of the NHERF family in NHE3 regulation have been developed using several cell and animal models. (1) The NHERF family members have been individually expressed in NHERF family null fibroblasts (PS120 cells), which also stably express exogenous NHE3. The cells contain small amounts of NHERF1 endogenously but do not express the other NHERF family members (Yun *et al.* 1997; Ahn *et al.* 2001). Studies of how regulation of NHE3 is affected by expressing single or multiple members of the NHERF family give insight into the role of NHERF family members in terms of scaffolding NHE3-containing complexes. (2) In epithelial cell models, including Caco-2 cells that endogenously express NHE3 and all four NHERF family members, RNA_i has been successfully used to knock down members of the NHERF family individually and should be useful in reducing them in combinations. Thus eliminating single or multiple NHERF family members and then evaluating NHE3 activity should allow a better understanding of NHERF family effects on NHE3 regulation. However, there is the limitation that remaining NHERF family members might compensate for the knock down by changing expression levels or changing intracellular location. Thus, while knockout or knockdown models might reveal the importance of redundancy, this may not necessarily indicate the role of the NHERF family members under normal conditions in which all four members are present.

Another polarized Na^+ absorptive cell that endogenously expresses NHE3 is the OK (opossum kidney) renal proximal tubule cell line. Most OK cell lines express NHERF1, NHERF3 and NHERF4 but no or minimal amounts of NHERF2 (Yun *et al.* 2002). Thus it is possible to ask what the effect of NHERF2 in a polarized epithelial cell is by studying OK cells ectopically expressing NHERF2. (3) Knockout mouse models have been developed for NHERF1 and NHERF2 and preliminary study of their effects on NHE3 regulation in proximal tubule and small intestine have begun, providing initial insights in the roles of these two proteins in NHE3 regulation in an intact epithelial tissue (Shenolikar *et al.* 2002; Weinman *et al.* 2003; Cunningham *et al.* 2004; Morales *et al.* 2004). The long-term goal of these studies is to understand the role of the NHERF family members in human ileum and renal proximal tubule. At this time no studies have been carried out to define the roles of NHERF family members in human epithelial tissue.

NHE3 in the small intestine is part of electroneutral NaCl absorption in which NHE3 is linked to a brush border Cl^-–HCO^-_3 exchanger thought to either be DRA (down-regulated in adenoma) or PAT-1 (putative anion transporter-1) (Petrovic *et al.* 2002; Jacob *et al.* 2002). NHE3 is up- and down-regulated as part of digestion, initially being inhibited and then stimulated later by changes in the intestinal neurohumoral milieu. In diarrheal diseases there is exaggeration of this inhibition of NHE3, which is more prolonged. Acute regulation of small intestinal neutral NaCl absorption and specifically of NHE3, occurs within minutes to hours, whereas in

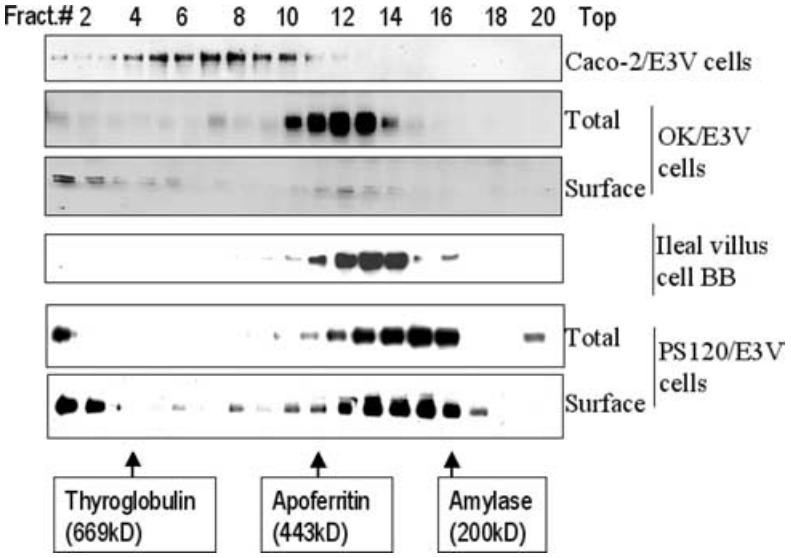

Figure 3. NHE3 is present in large, multiprotein complexes in the membranes of multiple cell types, including the ileal Na^+ absorptive cell brush border
NHE3 expressed in multiple cell types was solubilized in 1% Triton X-100 and placed on 5–35% discontinuous sucrose density gradients and separated by size, as described previously (Poulat *et al*. 1997). In cells expressing the largest amount of NHE3 on the plasma membrane (Caco-2 cell) and when the plasma membrane NHE3 was compared to total NHE3, there were generally larger NHE3 complexes on the plasma membrane (Li *et al*. 2004).

the kidney this occurs within many hours to a few days. In understanding acute NHE3 regulation in intact epithelial tissues, consideration of NHE3 complexes are required. When considering NHE3 complexes and the role of PDZ proteins, it is important to remember that NHERF family members hetero- and homodimerize or heteromultimerize using their PDZ domains (Lau & Hall, 2001). In addition, NHERF1, NHERF2 and NHERF3 have C-terminal PDZ binding ligands. Thus, BB PDZ proteins are likely to be in the same or related complexes binding to each other as well as assembling scaffolding proteins with other protein ligands, including NHE3.

cGMP inhibition of NHE3 requires NHERF2

Here we draw on the example of the recently characterized process of NHERF2-mediated cGMP inhibition of NHE3 to illustrate the role of NHERF proteins in NHE3 regulation involving BB NHE3-containing complexes (see Fig. 5). NHERF2 is needed as a scaffold to allow cyclic GMP to inhibit NHE3 by a cyclic GMP kinase II (cGKII)-dependent mechanism within a large multiprotein complex (Hwang *et al.* 2000). To understand the mechanism, it is important to realize that most cell culture models lack cGKII (Jarchau *et al.* 1994; Vaandrager *et al.* 1998), including both PS120 cells and OK cells, which were used to develop this model. Adding cGMP to PS120 cells containing NHERF2 and NHE3 in the presence of cGKII (but not in its absence) acutely inhibited NHE3 activity. In contrast, when NHERF2 was replaced with NHERF1, cGMP failed to alter NHE3 activity. Thus specificity for NHERF2 compared with NHERF1 was demonstrated. Moreover, in the absence of either cGKII or NHERF2, there was no change in NHE3 activity. The possibility that this cGMP effect was mediated by PKA instead of PKG was eliminated since a cGMP kinase-specific ligand similarly inhibited NHE3, and a specific cGMP kinase inhibitor blocked the effect.

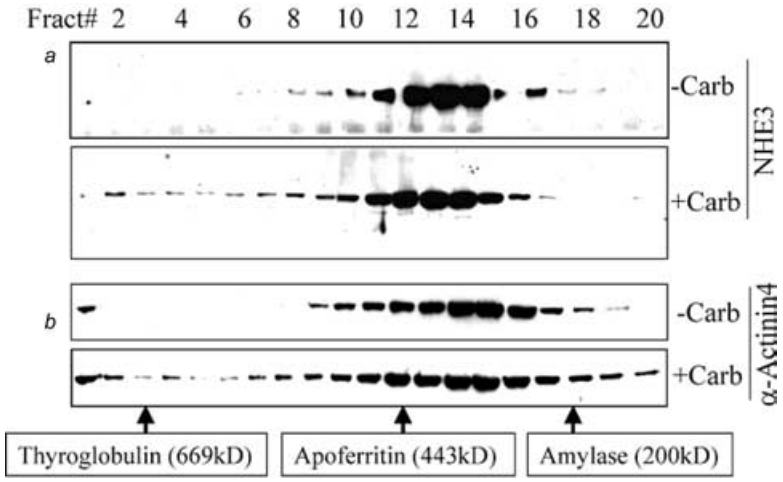

Figure 4. NHE3 complexes in the ileal brush border are dynamic, increasing in size with regulation by carbachol/Ca^{2+} elevation
Sucrose density gradient separation similar to that in Fig. 3 (Poulat *et al*. 1997) was carried out with ileal Na^+ absorptive cell BB prepared after 10 min exposure of ileum to carbachol *in vitro*. Western analysis demonstrated that the size of NHE3 complexes were larger after carbachol exposure. Similar to the increase in size of NHE3 after carbachol addition, α-actinin-4 was distributed in multiple, large complexes which increased in size after carbachol, in parallel with the changes in size of NHE3 complexes (Li *et al*. 2004).

This mechanism was separable from cAMP-mediated inhibition of NHE3 in these cells, as demonstrated by showing that maximal concentrations of cGMP and cAMP caused an additive inhibition of NHE3 (Cha *et al.* 2005).

The specificity for NHERF2 in this process was conserved in OK cells (Cha *et al.* 2005). OK cells endogenously express NHERF1, NHERF3 and NHERF4 and no or small amounts of NHERF2 (Yun *et al.* 2002). In these cells, cGMP failed to inhibit NHE3 in the presence or absence of transiently expressed cGKII. In contrast, in cells stably transfected with NHERF2, cGMP inhibited NHE3, but only in cells expressing cGKII. This effect requires NHERF2 binding to NHE3 because when NHE3 is mutated not to bind NHERF2, cyclic GMP no longer inhibits NHE3. Thus cGMP inhibits NHE3 both in fibroblasts and in polarized epithelial cells expressing NHE3 on the apical surface by a process that requires both NHERF2 and cGKII.

Further studies examined the mechanisms by which NHERF2 allowed cGMP to inhibit NHE3. Based on overlay and pull-down assays of protein–protein interactions, NHERF2 bound cGKII, while NHERF1 failed to bind with similar affinity. *In vivo* studies used a previously described chimera of cGKI and the first N-terminal 29 amino acids from cGKII, as well as intact cGKI, and a cGKII mutant (G2A) that lacks its myristoylation site (which is required for plasma membrane association) (Vaandrager *et al.* 1998; Vaandrager *et al.* 1996). These cGMP kinase mutants were studied in PS120 cells containing NHERF2. Neither cyclic cGKI nor cGKII lacking the myristoylation site reconstituted cGMP inhibition of NHE3. In contrast, a chimera of wild-type cGKI and the first 29 amino acids of cGKII which was myristolyated and thus bound to the plasma membrane did reconstitute cGMP inhibition of NHE3. Thus cGKII must be myristoylated and attached to the plasma membrane and bound to NHERF2 (binds to PDZ domain 2) to allow cyclic GMP inhibition of NHE3, and this effect involves a complex of NHE3–NHERF2–cGKII. Why cGKI when myristoylated could also reconstitute cGMP inhibition of NHE3 is probably because NHERF2 is a broad specificity cGMP kinase anchoring protein (GKAP) and binds GKI as well as cGKII (Cha *et al.* 2005).

Current models describing NHERF family reconstitution of cAMP inhibition of NHE3 include the concept that NHERF1 or NHERF2 using their ERM binding domain, bind to ezrin, which serves as a low affinity cAMP kinase anchoring protein (AKAP) (Yun *et al.* 1998; Dransfield *et al.* 1997) to place PKAII (and consequently the locally liberated catalytic subunit of PKA) in a location to phosphorylate NHE3 on at least two Ser residues, which affects NHE3 activity. It was thus asked whether NHERF2 binding to ezrin was similarly required for cyclic GMP inhibition of NHE3. In these studies the NHERF2 C-terminal 30 amino acids (NHERF2Δ30) were truncated and this truncation mutant was expressed in PS120/NHE3 cells. NHERF2Δ30 failed to bind ezrin but continued to bind cGMP kinase II (Cha *et al.* 2005). Furthermore, NHERF2Δ30 reconstituted cGMP inhibition of NHE3 but failed to reconstitute cAMP inhibition of NHE3. Thus ezrin binding is not necessary for the cGMP inhibition of NHE3. This difference provides part of the explanation for the additivity of the cAMP and cGMP inhibition of NHE3.

Thus our current model for cGMP inhibition of NHE3 involves NHE3, NHERF2 and cGKII forming a complex at the plasma membrane, with cGKII having to bind to the plasma membrane by two mechanisms, one involving its myristoylation and the other binding to the PDZ domain 2 of NHERF2. Activation of guanylate cyclase-C, which is present in the BB, by guanylin or *E. coli* stable toxin A, increases guanylate cyclase activity and cGMP content near the BB to locally activate cGKII (Fig. 5). While in this model NHERF2 is attached to

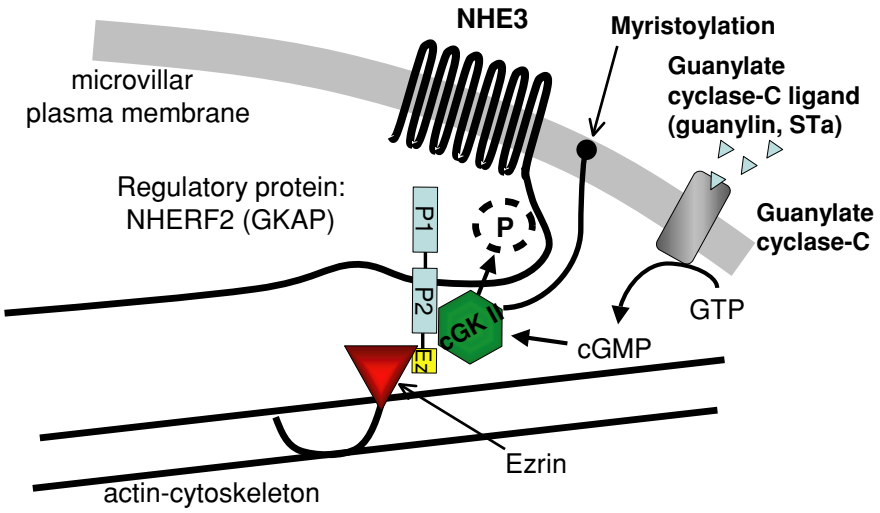

Figure 5. Model of cGMP inhibition of NHE3 in OK cell brush border and PS120 cells
NHERF2 is required for the cGMP inhibition of NHE3. NHERF2 acts as a cGMP kinase anchoring protein (GKAP), forming a complex of NHE3, NHERF2 and cGKII. cGKII is required for cGMP to inhibit NHE3. To carry out this effect, cGKII must be fixed in two locations, by its N-terminus via myristoylation to the plasma membrane and to the second PDZ domain of NHERF2 (Cha *et al*. 2005). Given the BB localization of guanylate cyclase C, cGMP is generated at the BB where it probably acts on the cGKII, which is part of the BB NHE3 complex.

the cytoskeleton via ezrin binding to the NHERF2 ERM domain, this is not necessary for the cGMP inhibition of NHE3. Further questions that must be answered to understand cGMP inhibition of NHE3 include whether the substrate cGMP/cGKII directly phosphorylates is NHE3, whether cGMP inhibition of NHE3 is associated with increased trafficking of NHE3 off the plasma membrane, and what are all the mechanisms which explain the additively of cGMP plus cAMP inhibition of NHE3. Moreover, since guanylate cyclase C binds NHERF4, and even larger complex in the BB may be involved in this signaling complex related to cGMP inhibition of NHE3.

Not presented in this review in detail is that NHERF2 has also been demonstrated to be involved with NHE3 inhibition by elevated intracellular calcium ($[Ca^{2+}]_i$) in a mechanism that involves both complex formation and changes in trafficking (Kim *et al.* 2002; Lee-Kwon *et al.* 2003; Li *et al.* 2004). NHERF2 but not NHERF1 reconstitutes Ca^{2+} inhibition of NHE3 (Kim *et al.* 2002; Lee-Kwon *et al.* 2003; Li *et al.* 2004). In this effect, NHERF2 scaffolds a complex involving NHE3, α-actinin-4 and PKCα. The α-actinin-4 comes from the focal adhesions in fibroblasts and probably from the tight junctions in epithelial cells (Li *et al.* 2004), while PKCα joins the complexes from the cytosol (Lee-Kwon *et al.* 2003). The specificity of the NHERF2 effect comes from the fact that NHERF2 but not NHERF1 binds α-actinin-4 (Kim *et al.* 2000). The mechanism involves initial forming of complexes which become larger over time and are followed by endocytosis at least of NHE3 (Kim *et al.* 2002; Li *et al.* 2004). Actinin-4 is involved in the complex formation as well as endocytosis since dominant-negative actinin-4 acts as an inhibitor of Ca^{2+}-induced inhibition of NHE3 (Kim *et al.* 2000). PKCα binds to NHERF2 but also binds to NHERF1 (Lee-Kwon *et al.* 2003). The role of PKC is not in forming the complexes but in allowing endocytosis, and it does not appear to be involved with NHE3 phosphorylation, probably instead phosphorylating components of the endocytic machinery (Lee-Kwon *et al.* 2003).

In summary, NHE3 exists simultaneously in multiple large multiprotein complexes. These are dynamic and change as part of acute NHE3 regulation. Proteins that are part of these complexes include the NHERF family of PDZ domain proteins. Certain NHERF family members are required for specific examples of acute NHE3 regulation. Identified mechanisms of specificity involve binding partners brought into the NHE3 complexes and different locations in or near the BB of each of the members of the NHERF family. Roles identified for the NHERF family members in acute NHE3 regulation include scaffolding to allow complex formation and also in changing rates of regulated endocytosis and/or exocytosis. While the amount of plasma membrane NHE3 does not appear to be dependent on the NHERF family (implying lack of involvement in either delivery from the Golgi or plasma membrane retention), other processes which are regulated by other PDZ domain proteins or the NHERF family with different ligands, such as mechanisms of recycling under basal or regulated conditions and phosphorylation-dependent complex formation, have yet to be evaluated for NHE3.

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