NHERF: targeting and trafficking membrane proteins

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Shenolikar, Shirish, and Edward. J. Weinman. NHERF: targeting and trafficking membrane proteins. Am J Physiol Renal Physiol 280: F389–F395, 2001.—Vectorial ion transport initiated by Na+/H+ exchanger isoform 3 (NHE3) mediates the reabsorption of NaCl and NaHCO3 in renal proximal tubule cells. NHE3 activity is modulated by numerous physiological stimuli. Biochemical and cellular experiments identified Na+/H+ exchanger regulatory factor (NHERF) as a protein cofactor essential for cAMP-mediated inhibition of NHE3 activity. Identification of numerous NHERF targets, including several transmembrane receptors and ion transporters, has broadened the role of this PSD-95/Dlg-1, Drosophila disk large/ZO-1 domain-containing adapter protein in membrane physiology. NHERF also associates with members of the ezrin/radixin/moesin family of actin-binding proteins and thus links NHE3 to the actin cytoskeleton. Formation of this multiprotein complex facilitates NHE3 phosphorylation and hormonal control of Na+/H+ exchange. NHERF also plays a critical role in targeting transport proteins to apical membranes. Moreover, the NHERF signaling complex functions as a regulatory unit to control endocytosis and internal trafficking of membrane proteins. This article reviews the new evidence that implicates NHERF in wider aspects of epithelial membrane biology.

sodium/hydrogen exchanger regulatory factor; apical membrane; ion transport; PSD-95/Dlg/ZO-1 domain; phosphoproteins; actin cytoskeleton

THE POLARIZED EPITHELIUM of the mammalian kidney proximal tubules reabsorbs most of the water, sodium, chloride, bicarbonate, phosphate, and potassium filtered across the glomerular membranes and is responsible for absorbing all the glucose and amino acids that filter through the kidney. Vectorial transport across the proximal tubule also plays a key role in secreting various organic anions and cations into the urine. Physiological factors, such as the hemodynamic forces in the kidney, Starling or intrarenal physical forces, hormones, and neural factors, modify epithelial ion transport and control sodium excretion in the proximal tubule cells.

The kidney also plays a critical role in maintaining the acid-base balance, by removing excess H+ and HCO3-. In the proximal tubule, H+ is secreted into the lumen by Na+/H+ exchanger isoform 3 (NHE3), a specific isoform of the Na+/H+ exchanger found in renal apical membranes. H+ secretion, in turn, results in the generation of HCO3-, which is returned to the systemic circulation. NHE3 activity is regulated by intracellular pH (through a pH-sensitive activator site) as well as various hormones and growth factors. Numerous studies have investigated the physiological mechanisms that regulate NHE3 activity (37). This review focuses on the contributions of Na+/H+ exchanger regulatory factor (NHERF), an apical membrane PSD-95/Dlg-1, Drosophila disk large/ZO-1 (PDZ) domain-containing protein, that participates in multiple cellular mechanisms, including protein phos-
phorylation (36, 52), targeting, and trafficking, that regulate NHE3 function in renal tissues.

HORMONAL REGULATION OF Na+/H+ EXCHANGER ISOFORM 3

NHE3 is the predominant isoform of the Na+/H+ antiporter in apical membranes of renal and gastrointestinal tissues. NHE3 differs from other Na+/H+ exchanger isoforms in its transport properties, drug sensitivity, and subcellular and tissue distribution. The most significant structural difference between the Na+/H+ exchanger isoforms is in their COOH-terminal cytoplasmic tail, suggesting that their unique properties are derived in part through these sequences. Cabado and colleagues (7) showed that cAMP inhibited the Na+/H+ exchange activity of NHE3 but not NHE1 expressed in antiporter-deficient Chinese hamster ovary (AP-1) cells. A chimeric NHE1 containing COOH-terminal sequences from NHE3 was inhibited by cAMP whereas the reverse chimera, NHE3, containing NHE1 COOH-terminal sequences, was not responsive to the second messenger. This suggested that the COOH-terminal sequences in NHE3 were targeted by hormones that regulate Na+/H+ exchange at renal apical membranes.

Fibroblast growth factor increases cellular NHE3 activity by promoting its incorporation in the plasma membrane (32). In contrast, treatment with parathyroid hormone (PTH) downregulates NHE3 and translocates the antiporter to internal membranes (23). Thus the trafficking of NHE3 from the cell surface to internal membranes represents an additional mechanism for the acute regulation of NHE3 by hormones and growth factors (3). D’Souza and co-workers (13) showed that NHE1 is expressed exclusively on the cell surface of AP-1 cells whereas NHE3 is partially internalized and accumulates in recycling endosomes. The studies suggested that recycling of NHE3 is dictated by COOH-terminal endocytic sequence that controls antiporter expression at the cell surface.

Differing effects of hormones on the cellular machinery that controls NHE3 function and trafficking may account for the varied physiological outcome of PTH, calcitonin, vasopressin, and β-adrenergic agonists, all of which increase intracellular cAMP in the proximal tubule (49). Deletion of NHE3 COOH-terminal sequences that render the antiporter constitutively active or incapable of being regulated by hormones points to the cytoplasmic tail as the primary target of cellular mechanisms that regulate NHE3 function in mammalian tissues (63).

NHE3 AND THE CYTOSKELETON

The actin cytoskeleton plays an important role in the sorting and localizing of membrane proteins in polarized epithelial cells (9). Apical and basolateral localization of ion transporters is, in part, determined by their association with the underlying cytoskeleton. The actin cytoskeleton also defines the intrinsic transport activity of NHE3 (29). Disruption of the actin cytoskeleton that suppresses NHE3 activity has little effect on NHE1 function. A chimeric NHE1 containing the COOH terminus from NHE3 acquires the sensitivity to cytochalasin B, an inhibitor of actin polymerization whereas the NHE3 chimera containing the NHE1 COOH-terminal sequences no longer responds to alterations in cytoskeletal structure. Structure-function studies have mapped cytochalasin B sensitivity to a region of NHE3 between amino acids 650 and 684 that contains a putative binding site for ezrin, an apical actin-binding protein. This led to the speculation that ezrin tethers NHE3 to the actin cytoskeleton and thereby defines its localization and function. Thus the inhibition of NHE3 activity after osmotic cell shrinkage may result from the reorganization of the actin cytoskeleton (27). As reported for the cystic fibrosis transmembrane regulator (CFTR), another apical membrane transporter (46), cAMP may induce changes in the actin cytoskeleton to modulate NHE3 activity. However, the insensitivity of NHE3 to cAMP analogs suggests that factors other than the actin cytoskeleton are also necessary for inhibition of NHE3 expressed in the antiporter-deficient PS120 cells (62).

NHERF: A MEMBRANE-CYTOSKELETAL ADAPTER

Studies of Na+/H+ exchange by detergent-solubilized renal apical membrane proteins reconstituted into artificial liposomes identified NHERF, a protein cofactor essential for protein kinase A (PKA)-mediated inhibition of NHE3 (56). NHERF, a 55-kDa phosphoprotein, contains two tandem PDZ (PSD-95/Disc large/ZO-1) domains (57) that are hallmarks of adapters that link membrane proteins to the underlying actin cytoskeleton (14). Subsequently, a yeast two-hybrid protein interaction screen was used to identify potential regulators that associate with the COOH terminus of NHE3 (64). This yielded a structural homolog of NHERF, termed NHE3 kinase A-regulated protein (E3KARP). E3KARP or NHERF2 was also characterized as an activator of the platelet-derived growth factor receptor tyrosine kinase and termed tyrosine kinase activator-1. Consistent with the apical distribution of their target NHE3, NHERF and NHERF2 are predominantly found in brush-border membranes, although some NHERF is present in basolateral membranes (54) where it may transduce PKA signals that regulate other transporters, such as the Na+-HCO3- cotransporter (2). NHERF may also be required to localize the H+-ATPase in both apical and basolateral membranes in renal intercalated cells (5). A small amount of NHERF exists in the cytosol, prompting the speculation that NHERF cycles on and off the membranes in response to physiological stimuli to regulate ion transport in mammalian kidney (54).

NHERF2 mRNA is alternately spliced to produce SRY-interacting protein-1 (SIP-1), a nuclear protein. SIP-1 associates with SRY, a nuclear transcription factor, and controls the expression of testes-specific genes (45). This suggests that protein products of the NHERF2 gene possessing distinct targeting motifs reg-
ulate physiological events in multiple subcellular compartments.

**NHERF LINKS NHE3 TO THE ACTIN CYTOSKELETON**

Human NHERF was isolated as ezrin-binding protein-50 (47). NHERF associates with an NH2-terminal ezrin/radixin/moesin domain (ERMAD) conserved in the family of actin-binding proteins known ERM proteins (48). As a result, all ERM proteins bind NHERF (40) near the COOH terminus, distal to the NHE3 binding site. In this manner, NHERF can assemble a tripartite complex that contains both NHE3 and ezrin (31, 64), which facilitates PKA phosphorylation of NHE3 and inhibits Na+\(\rightarrow\)H+ exchange (55). Earlier studies had characterized ezrin as an anchoring protein for type II PKA (12), which is uniquely associated with apical membranes (50). Thus the NHE3-NHERF-ezrin-PKA complex may relay the cAMP signals that inhibit NHE3 (52). It is tempting to speculate that this complex, which also binds actin, defines the basal NHE3 activity that responds to changes in the actin cytoskeleton.

**PHOSPHORYLATION OF NHE3-NHERF-EZRIN COMPLEX**

NHE3 is a phosphoprotein that shows a complex mode of regulation by hormones (37). NHE3 is phosphorylated in cells by PKA (65) and protein kinase C (PKC) (60), with the major phosphorylation sites residing in the cytoplasmic tail. Mutagenesis of multiple serines has failed to clarify the precise role of NHE3 phosphorylation in ion transport, and it has been suggested that NHE3 phosphorylation combines with the recruitment of cellular factors to control antiporter function (37).

NHERF is also a phosphoprotein in vivo (21, 58). Mutations of serines near the COOH terminus that abolish NHERF phosphorylation both in vitro (58) and in vivo (21) have little effect on its ability to transduce cAMP signals that inhibit NHE3 activity in PS120 cells (66). Despite its initial isolation as an in vitro substrate of PKA, NHERF phosphorylation in vivo is not significantly modulated by cAMP (21, 59). Finally, NHERF2 is not a phosphoprotein in vivo but is equally effective in inhibiting NHE3 activity after cAMP elevation (31). Thus the NHERF phosphorylation does not appear to be essential for cAMP regulation of NHE3, and its functional significance remains unknown.

Recent studies indicate that NHERF is constitutively phosphorylated in HEK-293 cells by GRK6A, a member of the G protein-coupled receptor kinase family (21). Interestingly, GRK6A associates with the NH2-terminal PDZ domain of NHERF, which facilitates NHERF phosphorylation and most likely defines the remarkable specificity of GRK6A as a NHERF kinase. It has been suggested that NHERF phosphorylation by GRK6A may modify NHERF localization and/or association with cellular targets, but to date no experimental evidence has been obtained to support this speculation.

The third component of the NHERF complex, ezrin, is present in cells in both active and inactive states (48). Inactivation of ezrin results from an intermolecular head-to-tail interaction between a COOH-terminal sequence, the C-ERMAD, and the NH2-terminal domain, or N-ERMAD, containing the NHERF-binding site. The inactive state of ERM proteins precludes ERM binding to NHERF and the actin cytoskeleton (18, 48). Hormones and growth factors, which result in the phosphorylation of a COOH-terminal threonine, by the Rho-associated protein kinase (42) or PKC-\(\tau\) (43) activate the ERM proteins. Whether the COOH-terminal phosphorylation of ERM proteins is the cause or effect of their activation is still unclear. Inactive radixin is poorly phosphorylated in vitro by Rho-kinase (33). This suggests that an additional mechanism, such as the binding of the phosphatidylinositol, phosphatidyl 4,5-bisphosphate, activates ERM proteins (34) with their subsequent COOH-terminal phosphorylation functioning to preserve the activated state.

Phosphorylated ERM proteins are concentrated in microvilli (42). Anoxic injury to kidney proximal tubule cells causes rapid dephosphorylation of ezrin and the breakdown of microvillar architecture (10). This is thought to reflect the unopposed action of an ERM phosphatase after ATP depletion by anoxia. ERM proteins directly associate with a protein phosphatase that regulates their function (16). The type-1 protein serine/threonine phosphatase containing the myosin-binding subunit (MBS) associates with moesin and accumulates at moesin-containing sites in cells treated with phorbol esters. The C3 botulinum toxin, which inhibits the Rho GTPase, prevents MBS and moesin accumulation at the membranes. How the Rho-kinase phosphorylates ERM proteins bound to the MBS-bound phosphatase, which can reverse the phosphorylation, was resolved by the finding that Rho-kinase also phosphorylates MBS and inhibits the ERM phosphatase (28). The coordination of the ERM kinase and phosphatase activities allows effective phosphorylation and activation of the actin-binding proteins, which in turn dictates the ability of ezrin and the actin cytoskeleton to regulate NHE3.

There is also a cAMP-mediated mechanism for suppressing the activity of a renal protein phosphatase. Dopamine D1-receptor agonists that inhibit NHE3 activity (15) also generate a cAMP-activated phosphatase inhibitor, a dopamine- and cAMP-regulated phosphoprotein of apparent relative molecular weight of 32,000 termed DARPP-32. DARPP-32 inhibits the protein phosphatase that dephosphorylates Na+\(\rightarrow\)K+\(\rightarrow\)ATPase and thus amplifies the cAMP signals that regulate the sodium pump in renal basolateral membranes (1). Interestingly, DARPP-32 specifically targets the type-1 phosphatase. Thus Rho-mediated signals may cooperate with cAMP signals to inhibit renal type-1 phosphatases and promote the phosphorylation and activation of ERM proteins.
hance their competition for NHERF. On other hand, NHE3 function in apical membranes must be closely coordinated with the basolateral Na\(^+\)-HCO\(_3\) cotransporter to facilitate transcellular sodium transport in proximal tubules. The role of NHERF in coordinating the hormonal control of these two transporters in mammalian kidney requires further investigation.

The majority of NHERF targets thus far identified, including CFTR (51), P2Y1 purinergic receptor, platelet-derived growth factor receptor (19), and H\(^+\)-ATPase (5), associate with the NH\(_2\)-terminal PDZ domain (PDZ-I) of NHERF and thus, like the \(\beta_2\)-AR, may impinge on NHE3 activity. Nonmembrane proteins, such as GRK6A, also associate with PDZ-I and are recruited to the plasma membrane where they may regulate NHERF and other membrane proteins (21). The COOH-terminal half of NHERF that must bind NHE3 and ezrin to regulate NHE3 activity (55) associates with yet other NHERF targets. The c-Yes tyrosine kinase-associated protein YAP65, which through its association with NHERF PDZ-II localizes the c-Yes tyrosine kinase to apical membranes (38), and phospholipase C-\(\beta\)3 (24), which binds PDZ-II and COOH-terminal sequences of NHERF2, may directly compete with NHE3 for NHERF or NHERF2 binding. Thus PDZ-II targets may be even more effective regulators of antiporter function. Phospholipase C-\(\beta\)3 appears to be unique among proteins that bind NHERF and NHERF2 and is the only known target that is specific for NHERF2. The mechanism by which NHERF2 transmits G protein-coupled receptor signals to activate phospholipase C-\(\beta\)3 and promote phosphoinositol metabolism remains unknown.

**NHERF TARGETS AND NHE3 REGULATION**

The growing number of NHERF targets (Fig. 1) raises the possibility that these proteins also participate in NHE3 regulation. In this regard, the hormonal activation of the \(\beta_2\)-adrenergic receptor (AR) promotes its association with the NHERF PDZ-I domain (20), which plays no apparent role in NHE3 binding and regulation (55). Yet, agonist occupation of \(\beta_2\)-AR prevents the PKA-mediated inhibition of NHE3. Paradoxically, hormonal activation of \(\beta_2\)-AR, which elevates cAMP levels, increases the antiporter activity. Deletion of a COOH-terminal sequence in \(\beta_2\)-AR, on the other hand, prevents NHERF binding and results in the well-characterized cAMP-mediated inhibition of NHE3. Bimodal regulation of NHE3 by \(\beta_2\)-AR may explain a long-standing puzzle in renal physiology, whereby some cAMP-generating hormones were shown to inhibit NHE3 activity and others activated the antiporter (53). Several aspects of this bidirectional regulatory mechanism are still unclear. For example, \(\beta_2\)-AR is primarily basolateral whereas NHE3 is essentially apical. Separation of the two NHERF targets in distinct membrane compartments may further en...
to NHERF misroutes the hormone-occupied receptor to lysosomes and promotes its degradation. Mutations in NHERF that prevent its binding to ezrin as well as pharmacological disruption of the actin cytoskeleton have similar effects on the fate of the internalized β2-AR. This suggests that the three components of the signaling complex that regulates NHE3, namely, NHERF, ezrin, and the actin cytoskeleton, also control the trafficking and sorting of NHERF targets, such as the β2-AR. The phosphorylation of a serine within the PDZ motif of β2-AR by GRK5 prevents its association with NHERF (8) and represents a cellular mechanism for regulating endocytic sorting of the receptor. Recent studies show that ezrin binds p85 regulatory subunit of the PI3-kinase (17) implicated in the regulation of NHE3 recycling. Thus NHERF and associated proteins may be actively involved in controlling the internalization and trafficking of membrane targets, such as the β2-AR and NHE3.

NHERF AND APICAL MEMBRANE TARGETING

Once internalized, NHE3 must be recycled to the apical membrane compartment to maintain cell polarity and vectorial transport. A number of mechanisms have been proposed for membrane sorting in polarized cells (35). These include the protein and lipid content of vesicles and the coding of vesicular proteins that targets them to the appropriate membrane compartments. Although molecular determinants have been identified for basolateral targeting of membrane proteins, such as the tyrosine-based signals and dileucine motifs (6), the molecular determinants of apical targeting remain unknown. This has fostered the view that apical localization is a default mechanism with specific signals required for the basolateral targeting of membrane proteins. However, recent studies show that PDZ adapter proteins direct the polarization or targeting of apical membrane proteins (4).

Evidence pointing to PDZ interaction as a mechanism for apical targeting came from studies of Kv3 K+ channel subunits. Expression of channel isoform with distinct COOH-terminal sequences produced by alternative splicing showed that isoforms containing PDZ COOH-terminal motifs were localized to apical membranes of polarized cells (44). In contrast, splice variants lacking PDZ motifs were targeted basolaterally. Studies of GABA transporter isoforms also showed that the GAT-3 isoform was apically targeted whereas GAT-2 was targeted to basolateral membranes (41). Chimeric transporters containing sequences from GAT-2 and GAT-3 established the critical importance of the COOH terminus in membrane sorting. Deletion of the three COOH-terminal amino acids mislocalized GAT-3 and suggested that PDZ interaction dictates its localization at the apical surface.

Changes in CFTR trafficking are seen in ~90% of humans with cystic fibrosis. CFTR mutations that delete the COOH-terminal sequences are mislocalized. Moyer and colleagues (39) showed that the disease mutation, S1455X, which results in the loss of the COOH-terminal 26 amino acids, targets virtually all of the CFTR protein to basolateral membranes. These studies have implicated the interaction of CFTR with NHERF (and/or NHERF2) in both correct apical localization and the intracellular trafficking of CFTR. This suggests that NHERF controls many different facets of apical membrane biology.

MEMBRANE POLARITY AND RENAL DISEASE

In proximal tubule cells, the microvilli of brush-border membranes form an early site of ischemic damage. Ezrin binding to the actin cytoskeleton is disrupted after anoxia in vivo and results in dramatic changes in microvillar morphology (10). Similar changes in the renal epithelial morphology accompany acute renal failure. In both cases, the loss of actin cytoskeleton integrity precedes the loss of microvilli in brush-border membranes. The resulting redistribution of membrane proteins leads to a loss in cell polarity and deficits in vectorial ion transport. The disruption of the actin cytoskeleton may thus be considered an important determinant of the early morphological changes associated with ischemic injury and kidney dysfunction. A key feature of the renal stress pathway is the loss or downregulation of proteins like NHE3. This suggests a potential link between trafficking of NHE3 and renal injury during anoxia. A better understanding of the physiological roles of the NHERF complex could conceivably lead to novel therapeutic strategies to limit cell injury and/or hasten tissue repair after acute renal failure and ischemia.

The best understood disease of epithelial polarity is the autosomal dominant polycystic kidney disease (ADPKD), the most common lethal genetic disease in humans. Mutations in the PKD1 gene have been linked to 85% of the individuals with ADPKD. Loss of function of polycystin, the product of the PKD1 gene, is in part associated with abnormal localization of Na+/K+-ATPase to apical membranes, leading to abnormal sodium and fluid secretion (61). EGF receptors are also apically mislocalized and thus appear in the cyst epithelia, where they may contribute to increased epithelial cell proliferation in ADPKD. The PKD1 gene is located on chromosome 16 close to the NHERF2 gene (25). Chromosomal deletions in a subset of ADPKD patients may result in the loss of both genes and could be associated with the increased severity of ADPKD (22). In this regard, NHE3 and CFTR, both targets of NHERF2, although showing normal apical distribution in ADPKD epithelia, are found intracellularly in a subset of cysts, potentially indicating the absence or impairment of NHERF2 function. Thus it is tempting to suggest that diseases like ADPKD and cystic fibrosis, which involve altered membrane polarity, may in part reflect aberrant NHERF and/or NHERF2 functions.

In conclusion, we are just beginning to understand the cellular mechanisms that localize NHE3 and other proteins to renal apical membranes and control their recycling and function in epithelial cells. Emerging
evidence suggests that the PDZ proteins, NHERF and NHERF2, play a critical role not only in cell signaling but also in membrane targeting and trafficking of ion transporters and receptors. An understanding of NHERF function and regulation should provide new insights into the physiological and pathophysiological events that regulate ion transport in mammalian kidney.

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