Concerted Roles of SGK1 and the Na⁺/H⁺ Exchanger Regulatory Factor 2 (NHERF2) in Regulation of NHE3

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Key Words

Na⁺/H⁺ exchanger • NHERF • SGK1 • Glucocorticoids

Abstract

Na⁺/H⁺ exchanger regulatory factors, NHERF1 and NHERF2, are structurally related proteins and highly expressed in epithelial cells. These proteins are initially identified as accessory proteins in the regulation of Na⁺/H⁺ exchanger isoform 3, NHE3. In addition to regulation of NHE3, recent studies demonstrate the importance of NHERF1 and NHERF2 in recycling and localization of membrane receptors, ion channels and transporters. Recent studies show that serum- and glucocorticoid-induced kinase 1 (SGK1) specifically interacts with NHERF2 but not with NHERF1, adding to the growing number of differences between the two proteins. The association of SGK1 with NHERF2 is necessary for stimulation of NHE3 activity by glucocorticoids. In addition, SGK1 together with NHERF2 stimulates the K⁺ channel ROMK1, suggesting a broader role of SGK1 in regulation of ion transport.

Introduction

The formation of protein complexes is the basis for efficient propagation in many cellular signaling processes. In this regard, the PDZ domain has emerged as a major mediator of protein sequestration in the plasma membrane. Studies show that Na⁺/H⁺ exchanger regulatory factors (NHERFs) play significant roles in assembly of protein complexes at the apical membrane of epithelial cells. This review focuses on the recent finding that SGK1 in conjunction with NHERF2 is involved in stimulation of NHE3 and other ion transport processes.

Role of SGK1 in Na⁺ transport

Serum and glucocorticoid inducible kinase (SGK) is a serine/threonine kinase, which was originally identified through subtractive cloning of a serum and glucocorticoid-induced mammary tumor cell cDNA library [1]. SGK is an ancient kinase, with homologues

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present in organisms such as *Caenorhabditis elegans* and *Saccharomyces cerevisiae* [2-4]. In mammalian tissues and cells, three isoforms of SGK have been identified: SGK1, SGK2 and SGK3/CISK [1, 5, 6]. SGK1 and SGK3 are ubiquitously expressed whereas SGK2 exhibits more restricted tissue distribution [1, 5]. SGK1 is induced by various stimuli such as serum, glucocorticoids, follicle-stimulating hormone, aldosterone, cell shrinkage, PKA, expression of p53 and injury to the brain [7]. In contrast, SGK2 and SGK3 genes are not induced by serum or glucocorticoids [5]. Although the physiological role of SGK1 in general is not yet clear, studies demonstrate the importance of SGK1 in epithelial ion transport. Significant amount of data support the role of SGK1 in regulation of the epithelial Na⁺ channel (ENaC) in conjunction with the ubiquitin-protein ligase neural precursor cell-expressed, developmentally downregulated isoform Nedd4-2 [8-11]. My laboratory recently observed that SGK1 plays an essential role in regulation of NHE3 by glucocorticoids [12].

NHE3

Mammalian Na⁺/H⁺ exchangers (NHEs) represent a growing family of membrane transporters that drive H⁺ flux in exchange for external Na⁺ in an electroneutral manner. To date, at least seven NHE isoforms have been cloned and partially characterized [13-15]. All the Na⁺/H⁺ exchangers share similar structure with the N-terminal transmembrane domain and the C-terminal cytoplasmic domain. The membrane domain of approximately 450 amino acids performs the ion transport function, whereas cellular signals converge onto the cytoplasmic domain. The C-terminal domain exhibits the largest divergence among the Na⁺/H⁺ exchangers and contains several potential phosphorylation sites and motifs for protein-protein interactions [14, 16, 17]. Works from various laboratories suggest that the interaction between the membrane and cytoplasmic domains is needed in regulation of Na⁺/H⁺ exchangers [18, 19]. The different Na⁺/H⁺ exchangers have distinct pharmacological and physiological characteristics and differ in tissue distribution. Of these, NHE1 is ubiquitously expressed and attributed to the maintenance of intracellular pH. Although functions and expression of other NHE isoforms vary among different tissues and animals, it is widely accepted that NHE3 is the apical Na⁺/H⁺ exchanger in the small intestine, proximal colon and renal proximal tubules [20, 21]. Through osmotic coupling to

passive water absorption, NHE3 is a major constituent in basal Na⁺ absorption in intestine and a frequent target of inhibition in many diarrheal diseases [22, 23]. In kidney, as much as 70% of the total HCO₃⁻ reabsorption can be attributed to NHE3 [21, 24, 25]. Although no specific disease has been associated to specific defects in NHE3 protein or gene, deficiency in NHE3 expression in mouse results in severe intestinal and renal defects associated with significant perturbation in Na⁺ and HCO₃⁻ reabsorption [20, 21, 25, 26].

Na⁺/H⁺ exchangers display remarkable functional versatility. In addition to exhibiting sensitivity to intracellular pH, they are modulated by changes in cell volume, cellular ATP concentration, growth factors and hormones [13, 24, 27]. Despite of large efforts made in recent years in understanding regulation of NHEs, molecular mechanisms underlying Na⁺/H⁺ exchanger regulation are not fully understood. Regulations of Na⁺/H⁺ exchangers are complex involving phosphorylation-dependent and independent mechanisms, cytoskeletal interaction, and membrane recycling between the plasma membrane and cytoplasmic pools [28-31]. For more details readers should refer to recent reviews on the mammalian Na⁺/H⁺ exchangers [14, 16].

PDZ domain

The PDZ domain was identified as a homologous element present in PSD-95, the Drosophila disc-large tumor suppressor protein DlgA and the tight-junction protein ZO-1. The PDZ domain initially referred to as the Dlg homology region (DHR) or the GLGF repeat, based on the presence of a Gly-Leu-Gly-Phe sequence motif [32]. The PDZ domain is made up of 80-90 amino acid residues that preferentially bind short peptide sequences located at the C-terminus of the interacting proteins [33, 34]. High-resolution structure studies of PSD-95, hDlgA, nNOS, phosphatase hPTP1E and NHERF1 show that PDZ domains consist of six β -sheets and two α -helices forming a hydrophobic pocket to accommodate a C-terminal carboxylate group. [35-38]. Mutational analysis demonstrates that PDZ domains have distinct peptide binding specificity with the consensus sequence -(T/S)-x-V/L [34, 39-41]. In addition to the prototypical C-terminal binding, PDZ domains are also observed to form dimers or interact with internal sequences that mimic a free C-terminus by forming β -hairpin "finger" [37, 42, 43]. In one case, the interaction involves non-C terminal cyclic peptides [44].

Fig. 1. Proposed organization of proteins involved in the PKA-dependent regulation of NHE3.

NHE3 associated regulatory proteins, NHERF1 and NHERF2

NHE regulatory factor 1, NHERF1, was cloned as a protein that can reconstitute protein kinase A (PKA)-dependent inhibition of NHE3 in renal brush border vesicles [45, 46]. Using the cytoplasmic domain of NHE3 as bait in a yeast two-hybrid system, Yun et al. [47] cloned E3KARP (NHE3 kinase A regulatory Protein), which was later renamed NHERF2 to be consistent with the nomenclature of the family. NHERF2 shares 62% similarity with NHERF1 and both proteins contain two tandem PDZ domains. It was shown that NHERF1 and NHERF2 reconstitute PKA-dependent inhibition of NHE3 in a cell line lacking these accessory proteins [47]. To determine the functional roles of NHERF2 in regulation of NHE3 by PKA, we considered two possibilities. An earlier work implicated PKA-dependent phosphorylation of NHERF [48], and the general function of proteins with PDZ domains as scaffold proteins raised the possibility of the direct scaffolding of PKA by NHERFs acting as A kinase anchoring proteins (AKAP) that specifically binds the regulatory subunit of type II PKA. However, neither mode was proven significant in

regulation of NHE3 [49]. Using a pull-down assay, the human homologue of NHERF1, EBP50 (Ezrin Binding Protein 50), was identified as a protein that binds the cytoskeletal protein ezrin with high affinity [50]. It is subsequently demonstrated that NHERF1 and 2 interact with ezrin through the C-terminal 29 amino acids [51, 52]. The essence of this interaction is that ezrin functions as an AKAP [49, 53]. This linkage to ezrin via NHERFs allows compartmentalization of PKA to the vicinity of NHE3 enabling phosphorylation of NHE3 [49, 50, 54] (Figure 1). Through the interaction with ezrin, NHERFs link NHE3 to the actin cytoskeletal network. Although not proven unequivocally, the sensitivity of NHE3 to the actin disrupting agents may result from the association of NHE3 with actin filaments by NHERFs/ezrin [55].

Diverse functions of NHERFs

Although NHERF was identified as a protein potentially regulating NHE3, Northern analysis of NHERF1 and NHERF2 on human tissues shows broad expression patterns of both isoforms, suggesting that functions of NHERFs are not limited to regulation of

NHE3 [47]. This assumption is supported by identification of proteins other than NHE3 interacting with NHERFs. These include the G protein-coupled receptors, the cystic fibrosis transmembrane conductance regulator (CFTR), platelet-derived growth factor receptor, the intestinal $\text{Cl}^-/\text{HCO}_3^-$ exchanger encoded by the down regulated in adenoma (DRA) gene, phospholipase $\text{C}\beta$, mammalian Ca^{2+} channel Trp4, transcriptional factors and the ezrin-radixin-moesin (ERM) family proteins [41, 50, 56-64]. Because of their roles in many signaling pathways converging onto membrane proteins, NHERFs represent a prototypical example of a scaffold protein organizing multi-signaling elements on the plasma membrane. Due to the similarities between NHERF1 and NHERF2 in structure and amino acid sequences, it is generally assumed that both proteins interact with the same target proteins, and hence the functions of NHERF1 and 2 are often thought to be redundant. However, increasing data demonstrate that NHERF1 and NHERF2 interact with different sets of proteins [12, 61-68].

The functions of NHERFs are diverse but a few aspects are worthy discussing in this review. One function of NHERFs is to mediate clustering of signaling proteins in micro-domains to facilitate cellular signaling processes. The importance of protein compartmentalization is amply demonstrated in PKA-dependent inhibition of NHE3 in which NHERFs bring PKA in the proximity of NHE3 by acting as a functional AKAP. Hall et al. elegantly demonstrated that the compartmentalization can alter the outcome of a cellular signal by showing that an activation of the β_2 -adrenergic receptor sequesters NHERF1 to the C-terminal end of the receptor rendering it unavailable for interaction with NHE3 and hence NHE3 is not regulated despite an elevation in cAMP level [56]. Similarly, the interaction of NHERF2 with the parathyroid hormone (PTH) receptor has been shown to direct the signaling of the PTH receptor between adenylate cyclase and phospholipase $\text{C}\beta$ [60].

The second major function of NHERFs that is becoming increasingly evident is regulation of endocytosis and endocytic recycling. Deletion of the C-terminal PDZ interacting motif of the β_2 -adrenergic receptor specifically prevents recycling of the β_2 -adrenergic receptor without affecting other endocytic pathways [69]. Similarly, The C-terminal deletion of the cystic fibrosis transmembrane conductance regulator (CFTR), disabling its interaction with PDZ domains, drastically reduces the apical membrane retention of

CFTR by altering its endocytosis [70]. On the other hand, NHERF interaction of the κ opioid receptor promotes entry into the endocytic pathways [71].

In other cases, proper apical targeting of membrane proteins has been related to the PDZ binding of NHERFs. The polarized distribution of CFTR requires the C-terminal PDZ-binding motif although additional motifs are also required for the proper apical targeting [72, 73]. The type IIa Na/P_i -cotransporter (NaPi4) present in the apical membrane of renal proximal tubules interacts with the first PDZ domain of NHERF1 [74]. Overexpression of the first PDZ domain of NHERF1 is thought to disrupt its interaction with the cytoskeleton and decreases the apical expression of NaPi4 [75]. In fact, genetic deletion of NHERF1 results in internalization of NaPi4 and decreased apical targeting NaPi4 in the renal proximal tubules [76]. Takeda et al. demonstrated that coupling of podocalyxin to the actin cytoskeleton via NHERF2 is essential in maintenance of foot-processes in glomerular epithelial cells [77]. It is subsequently shown that the interaction with NHERF2 is necessary for the apical membrane targeting of podocalyxin in MDCK cells [78].

Regulation of NHE3 by glucocorticoids

Glucocorticoids have been used effectively to achieve rapid symptomatic relief in many clinical situations including inflammatory bowel disease such as ulcerative colitis and Crohn's disease [79]. Despite their wide use, the exact mechanism of action of glucocorticoids remains elusive. The anti-inflammatory effect of corticosteroids arises from the direct and indirect inhibition on cytokines and inflammatory mediators [79]. In addition, it is widely accepted that glucocorticoids have direct effect on intestinal salt and water absorption, and thus alleviate diarrhea [80-82]. Pharmacologic doses of methylprednisolone have been shown to increase Na^+ , Cl^- and water absorption in vivo in both small intestine and colon of rat. It has been shown that 1 to 3 day treatment with methylprednisolone doubled rabbit ileal neutral NaCl absorption by specifically stimulating NHE3 mRNA expression 4-6 fold without affecting NHE1 [83, 84]. Cho et al. found dexamethasone had a stimulatory effect on NHE3 gene expression in rat ileum and proximal colon, but not in jejunum or distal colon [84]. Glucocorticoid excess also increases net acid secretion and HCO_3^- absorption in the kidney by activation of NHE3 gene expression [85-88]. Genomic

Fig.2. Effect of dexamethasone in OK cells. A. OK and OK/NHERF2 cells were incubated in 1 μ M dexamethasone for 24 hr. Northern blot analysis shows an increase in NHE3 mRNA abundance in both cell lines. B. OK, C. OK/NHERF1, D. OK/NHERF2, NHE3 activity is determined fluorometrically. •, control; ▲, dexamethasone treated. Reproduced with permission from ref (14).

cloning of rat NHE3 reveals the presence of glucocorticoid response elements (GRE) in the 5'-flanking promoter region and the glucocorticoid responsiveness of *Nhe3* gene was demonstrated by in vitro transcription assays [89, 90].

Although many of these earlier work suggested genomic stimulation of Na⁺/H⁺ exchange by glucocorticoids, acute effects of glucocorticoids on Na⁺/H⁺ exchange independent of change in NHE3 mRNA abundance were also observed. Incubation of proximal tubules for 3 hr with dexamethasone resulted in significant increase in NHE3 activity without change in NHE3 mRNA abundance [86]. Consistently, incubation

of human colonic carcinoma cell line Caco-2 with dexamethasone for 4 hr led to increased NHE3 transport in the absence of increased NHE3 transcription [12].

Stimulation of NHE3 by SGK1 and NHERF2

Our attempt to study the non-genomic effect of dexamethasone on NHE3 using OK cells, an opposum kidney cell line, led to unexpected findings. Incubation of OK cells with 1 μ M dexamethasone for 24 hr failed to regulate NHE3 activity despite a two-fold increase in NHE3 mRNA abundance (Figure 2). OK cells express

Fig. 3. *In vitro* interaction between SGK1 and NHERF2. (A) SGK1 and SGK3, but not SGK2 interacts with GST-NHERF2. There is no interaction with GST control or GST-NHERF1. (B) Deletion of the C-terminal 4 amino acids completely blocks the interaction with NHERF2. (C) SGK1 interacts with the second PDZ domains of NHERF2. Significantly weaker interaction with the first PDZ domain is also observed. (D) NHERF2 dimerizes via the first PDZ domain. Reproduced with permission from ref [14].

NHERF1 but lack any observable level of NHERF2 expression based on Western immunoblot and Northern analysis. A 24 hr dexamethasone treatment of OK cells stably transfected with NHERF2 led to a two-fold increase in NHE3 activity. In contrast, over-expression of NHERF1 in OK cells had no effect on NHE3 in response to dexamethasone.

We speculated that a protein interacting with NHERF2 is necessary to mediate the dexamethasone effect in OK cells and searched GenBank for proteins interacting with NHERF2 and potentially mediate the dexamethasone effect. A potential candidate for this role was SGK1 with the C-terminal sequence of -DSFL and its known response to glucocorticoids. Specific interaction between SGK1 and NHERF2 was subsequently demonstrated. SGK1 specifically interacts with NHERF2 but not with NHERF1, consistent with the inability of NHERF1 to reconstitute the glucocorticoid-dependent stimulation of NHE3 in OK cells (Figure 3). Interestingly, SGK3 exhibits interaction

Fig.4. SGK1 is necessary for the dexamethasone-dependent activation of NHE3. Hemagglutinin (HA) tagged SGK1/K127Q is expressed in OK cells. The stimulatory dexamethasone effect is markedly blocked by the presence of HA-SGK/K127Q. Reproduced with permission from ref (14).

with NHERF2 though at a lower affinity than SGK1. A recent study shows that SGK3 is targeted to endosomal compartments via a Phox homology (PX) domain, and the physiological significance of SGK3 interaction with NHERF2 remains unclear [91]. One possibility is the sequestration of SGK3 and 3-phosphoinositide-

Fig. 5. A model of glucocorticoid-dependent stimulation of NHE3.

dependent protein kinase I (PKA) (see below). Consistent with the typical PDZ interaction, the interaction between SGK1 and NHERF2 is blunted when the C-terminal 4 amino acids of SGK1 is deleted.

Co-expression of GST-SGK1 and NHERF2 in fibroblasts previously transfected with NHE3 resulted in stimulation of NHE3. But expression of NHERF2 or GST-SGK1 alone had no effect on NHE3 activity [12]. More importantly, co-expression of "kinase-dead" SGK1 mutant with K127Q mutation markedly blocked the effect of dexamethasone demonstrating the necessity of SGK1 (Figure 4). Signaling pathways leading to stimulation of SGK1 is not fully resolved, but the activation of NHE3 by dexamethasone is phosphatidylinositol 3-kinase (PI3K) dependent since glucocorticoid-stimulation of NHE3 is blocked by LY294002, a PI3K blocker. However, because PI3K is essential for recycling of NHE3 between the plasma membrane and endosomal compartments, the direct role of PI3K requires further study [31, 92].

The molecular nature of glucocorticoids-mediated stimulation of NHE3 is not known. Based on previously studies, it appears that glucocorticoid treatment increases the plasma membrane abundance of NHE3 [93]. The optimal consensus sequences for phosphorylation by SGK are R-x-R-x-x-S/T where S/T is the site of

phosphorylation [94, 95]. Within NHE3, there is a single RxRxxS motif at amino acid 663, which is conserved in all NHE3 cloned from rabbit, rat, human and opossum. Our initial study suggests that NHE3 can be phosphorylated by SGK1 in vitro. However, it remains speculative that SGK1 phosphorylates NHE3 at this site in vivo to either directly increase its transport activity or to increase the plasma membrane distribution of NHE3.

The glucocorticoid-activation of NHE3 appears to be bi-phasic (Figure 5). The initial acute phase does not require synthesis of NHE3 but most likely involves post-translational modification of pre-existing NHE3 proteins by SGK1. During the second chronic phase, newly synthesized NHE3 proteins are translocated into the plasma membrane and SGK1 and NHERF2 are required for this activation.

The interaction between NHERF2 and NHE3 occurs via the second PDZ domain of NHERF2 and an internal region (aa 585 and 660) located within the cytoplasmic domain of NHE3 [52]. The second PDZ domain of NHERF2 also binds SGK1 [12]. If both the target protein and the kinase interact with the same motif of NHERF2, how the proteins involved are assembled? This is probably achieved by dimerization of NHERF2 [12, 43]. A homodimer of NHERF2 should be able to bind NHE3 on one end and SGK1 on the other end.

Does NHERF2 interact with PDK1?

SGK1 differs from the proto-oncogene akt/PKB since it lacks the Pleckstrin homology domain (PH) domain for membrane anchoring and its activation is independent of phosphatidylinositol-3,4,5-triphosphate [94]. Because NHERF2 interacts with a large number of membrane spanning proteins, the interaction with NHERF2 may present a membrane anchoring mechanism for SGK1. This is the probable case for NHE3 regulation by SGK1, although further studies are needed to substantiate this speculation. Brickley et al. recently reported that the N-terminal domain of SGK1 mediates ubiquitin-mediated degradation in addition to facilitating the plasma membrane association of SGK1 in a breast cancer cell line [96]. It is plausible that the N-terminal region of SGK1 is required for general membrane targeting as suggested by Brickley and coworkers, but the association with NHERF2 is necessary for specific targeting of SGK1 to potential substrates.

The subfamily of protein kinases that includes cAMP and cGMP-dependent protein kinases and protein kinase C (AGC subfamily) requires phosphorylation in T-loop for activation. Similarly, SGK1 requires phosphorylation at Thr²⁵⁶ located within the T-loop by 3-phosphoinositide-dependent kinase I (PDK1) [5, 94, 95]. Biondi et al. showed that interaction between PDK1 and some of its substrates is mediated via the PIF (PDK1-interacting fragment) motif [97]. PIF interacts with the PIF binding pocket of PDK1 that acts as a docking site and enhance the phosphorylation of its substrates. The PIF motif comprising the consensus amino acid sequences of F-x-x-F-S/T-Y is present in a number of kinases including SGK, p70 ribosomal S6 kinase (S6K), PKA and akt [97, 98].

Recently, Chun et al. identified a potential PIF motif at the C-terminus of NHERF2, F-S-N-F. By pull-down assay, Chun et al. demonstrated that PDK1 interacts with the C-terminal PIF motif of NHERF2, and NHERF2 co-immunoprecipitates with ectopically expressed SGK1 and PDK1 in COS-1 cells [99]. This interaction mediates activation of SGK1 by PDK1 by phosphorylation at Thr²⁵⁶ of SGK1. Although intriguing, the activation of SGK1 via the ternary complex formed by NHERF2, SGK1 and PDK1 cannot fully account for the enhanced phosphorylation at Thr²⁵⁶ by phosphorylation at Ser⁴²² or the mutation of Ser⁴²² to Asp [94, 95, 100].

Activation of ROMK1

In the distal regions of the kidney and colon, Na⁺ retention by epithelial Na⁺ channel (ENaC) is balanced by efflux of K⁺ by K⁺ channel (ROMK) [101]. Coexpression of SGK1 and ENaC in *X. laevis* oocytes leads to stimulation of a Na⁺ current but no effect on ROMK-mediated K⁺ current was observed [8]. This is in contrast to the stimulative effect on voltage-gate K⁺ channel by SGK1 [102, 103]. However, colocalization of ROMK1 and NHERF2 in the collecting duct principal cells raises the possibility of concerted effect of SGK1 and NHERF2 [67]. In addition, ROMK1 lacks a PY motif that mediates interaction with the WW domain of Nedd4-2, but contains a PDZ binding motif at the C-terminal end further suggesting potential interaction with NHERF2. The coexpression of ROMK1 together with SGK1 and NHERF2 in *X. laevis* oocytes led to a significant increase in the K⁺ current [104]. Coexpression of SGK1 and NHERF2 did not affect the I/V relation of ROMK1, although there was a small acidic shift of pK_a of ROMK1 upon coexpression of SGK1 and NHERF2 compared to each protein alone.

It has recently been shown that SGK1 increases ENaC abundance and activity by altering the interaction between ENaC and the ubiquitin-protein ligase Nedd4-2 [9, 10]. Quantification of surface ROMK1 by surface biotinylation showed that ROMK1 abundance was increased by more than 200% when coexpressed with SGK1 and NHERF2 compared to SGK1 or NHERF2 alone [104]. Subsequent treatment with brefeldin A, which disrupts the budding process at the Golgi apparatus by inhibiting the essential assembly of coat proteins, suggested a stabilizing effect of SGK1/NHERF2 on ROMK1 protein in the plasma membrane [104]. However, whether NHERF2 stabilizes ROMK1 expression in the plasma membrane via the cytoskeletal linkage or impede the endocytic retrieval process is not known.

Perspectives

Considerable progress has been made in recent years in understating of the physiological roles of SGK1. In addition to acting as a pro-survival kinase similar to akt, it is evident that SGK1 plays a significant role in

regulation of Na transport. The role of SGK1 in regulation of ENaC is now well accepted. Recent studies demonstrate that SGK1 is a major protein in regulation of ion transport and in some cases the presence of a scaffold protein NHERF2 is a prerequisite for stimulation. Future challenges entail identifying other ion channels and transporters regulated by SGK1 and NHERF2. But more importantly, physiological significances of such regulation need to be realized.

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