Signal complex regulation of renal transport proteins: NHERF and regulation of NHE3 by PKA

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Weinman, Edward J., Charles Minkoff, and Shirish Shenolikar. Signal complex regulation of renal transport proteins: NHERF and regulation of NHE3 by PKA. Am J Physiol Renal Physiol 279: F393–F399, 2000.—The activity of the sodium/hydrogen exchanger 3 (NHE3) isoform of the sodium/hydrogen exchanger in the brush-border membrane of the renal proximal tubule is tightly regulated. Recent biochemical and cellular experiments have established the essential requirement for a new class of regulatory factors, sodium/hydrogen exchanger regulatory factor (NHERF) and NHERF-like proteins, in cAMP-mediated inhibition of NHE3 activity. NHERF is the first PSD-95/Dlg/ZO-1 (PDZ) motif-containing protein localized to apical membranes and appears to facilitate cAMP-dependent protein kinase A (PKA) phosphorylation of NHE3 by interacting with the cytoskeleton to target a multiprotein complex to the brush-border membrane. Other recent experiments have indicated that NHERF also regulates the activity of other renal transport proteins, suggesting that the signal complex model of signal transduction in the kidney may be more common than presently appreciated. This article reviews studies on the regulation of NHE3 by NHERF, PKA, and ezrin and introduces the concept of regulation of renal transporters by signal complexes. Although not the primary focus of this review, recent studies have indicated a role for NHERF in membrane targeting, trafficking, and sorting of transporters, receptors, and signaling proteins. Thus NHERF and related PDZ-containing proteins appear to be essential adapters for regulation of renal transporters in the mammalian kidney that maintain salt and water balance.

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of a new family of PSD-95/Dlg/ZO-1 (PDZ) motif proteins called the Na⁺/H⁺ exchanger regulatory factor family (NHERF and NHERF2) (46, 48, 51). Subsequent experiments defined an expanding role for the NHERF family of proteins in systems other than the kidney, including the effect of estrogen in estrogen receptor-positive breast cancer cell lines and testicular differentiation (9, 29). An expanding role for NHERF in the function of epithelial cells has also been elucidated, and at least three interrelated but unique areas of interaction between NHERF and the regulation of electrolyte transport have been suggested. First, NHERF appears to function in PKA regulation of NHE3 by facilitating the formation of a multiprotein complex, a “signal complex,” that is necessary to permit phosphorylation of NHE3 and, thereby, inhibit its activity. Second, there is intriguing new evidence that implicates NHERF in membrane targeting, trafficking, and sorting of not only epithelial transporters but also receptors and signaling proteins (4, 22, 23, 37). NHERF appears to target the cystic fibrosis transmembrane regulator (CFTR) to the plasma membrane. In addition, NHERF regulates the endocytic retrieval of the β2-adrenergic receptor and, possibly, the PY2 purinergic receptor, and regulates the fate of the retrieved proteins by sorting them to either recycling endosomes for reinsertion into the plasma membrane or to lysosomal degradatory pathways. Third, new evidence indicates an important role of the NHERF family of protein in the regulation of certain membrane receptors such as the platelet-derived growth factor receptor. NHERF and NHERF2 function to permit dimerization of subunits of the receptor with the consequent activation of tyrosine kinase activity and downstream signaling events. The present review focuses on PKA regulation of NHE3 and the evidence that this form of regulation involves participation of a preformed complex of proteins including the transporter itself, ezrin, PKA, and NHERF (45, 54). At the present time, PKA regulation of NHE3 is the only well-studied example of regulation by a signal complex in the kidney although it is suspected that this form of signal transduction may be common and that other examples will be elucidated shortly. The role of NHERF in processes related to targeting, trafficking, and sorting of epithelial transporters, receptors, and signaling proteins, as well of the role of this family of protein in receptor signaling pathways, will be reviewed in a later communication.

1 The nomenclature regarding the NHERF family of proteins has become confused. Rabbit Na⁺/H⁺ NHERF was partially sequenced in 1993 and cloned in 1995. In 1997, the Na⁺/H⁺ exchanger 3 protein kinase A regulatory protein (E3KARP) was cloned. E3KARP is identical to a protein named tyrosine kinase activator 1 (TKA1). In 1998, two groups cloned the human homolog of NHERF, one group calling the protein hNHERF, the other ezrin binding protein 50 (EBP50). It has recently been suggested that NHERF be used for the originally described protein and NHERF2 to designate E3KARP/TKA1. When identified, other members of this family of protein would be numbered sequentially and single lower case prefixes used to distinguish homologs. This convention will be used in this review.

EVOLUTION OF THE SIGNAL COMPLEX MODEL OF CELL REGULATION

Physiological processes are determined by the interaction of many proteins and are often represented as biochemical cascades, whereby a single protein is activated by an external stimulus. This activated protein then acts as a catalyst to convert a second protein from an inactive to an active state, and the sequential activation process is continued until the final effect is expressed. Sequential activation cascades were defined for extracellular processes, and when it became possible to measure intracellular biochemical reactions the cascade paradigm was incorporated into the cell context. A growing number of experimental observations, however, are difficult to explain or rationalize by using a sequential linear activation model. For example, the protein concentration in areas immediately adjacent to cell membranes might be high enough to restrict the free movement of proteins required by the cascade model. Moreover, a number of processes such as perception of light and dark, involving the opening and closing of multiple channels, are mediated by specific protein kinases and protein phosphatases (38). Despite the complexity, these processes occur with remarkable rapidity, at rates too rapid to be explained by mere sequential activation and deactivation of individual proteins. Finally, there has been a perplexing issue related to the observations that individual cells may contain multiple hormone receptors that use the same signal pathways. Nonetheless, the cells are able to respond to each hormone in a specific manner. For example, proximal tubule cells contain receptors for dopamine, parathyroid hormone, and β2-adrenergic agonists. Despite the fact that all three of these receptors use cAMP as a second messenger, the cellular response to each hormone is unique. From such considerations, it was suggested that some phenomena were better explained by postulating that there were complexes of proteins preassembled in discrete locations within the cell (27, 33). Moreover, under some circumstances, the reactive proteins were required concurrently rather than sequentially as in the cascade model. Although other names have been used, the term signal complex encompasses the basic elements of this alternate model. The presence of preassembled multihomolog signal complexes within cells provides a reasonable model to explain rapid cellular responses to stimuli and to explain cell specificity in response to hormones and neurotransmitters.

Signal complexes require a mechanism for the individual proteins to bind to one another to maintain the complex. It was soon recognized that members of the ezrin-moesin-radixin family of proteins, and the more distantly related merlin protein, might provide a “scaffold” for signal complexes by virtue of their ability to bind to both the actin core of cells as well as to proteins in the plasma membrane. Soon thereafter, a number of adaptor proteins were recognized that contained protein-protein binding motifs. Within the category of
adaptor proteins, identification of members of the PDZ class of proteins has grown rapidly (17, 28).

IDENTIFICATION OF THE REQUIREMENT FOR REGULATORY PROTEINS FOR PKA REGULATION OF NHE3

Experiments from a number of laboratories have indicated that cAMP inhibits sodium and water transport in the renal proximal tubule (1, 6, 40). The inhibition of sodium transport by cAMP required the presence of bicarbonate in the luminal perfusate and was associated with inhibition of bicarbonate reabsorption (5, 21). Collectively, these experiments suggested that cAMP inhibited the apical brush-border membrane Na+/H+ exchanger. Using isolated rabbit renal brush-border membranes, we demonstrated that activation of membrane-bound PKA or provision of exogenous PKA inhibited Na+/H+ exchange activity in an ATP-dependent manner and that this inhibition was blocked by a specific inhibitor of the protein kinase (16, 44). We reasoned, therefore, that the Na+/H+ exchange transporter, the structure of which was unknown at the time, was phosphorylated and that identification of the membrane proteins phosphorylated by PKA might provide an approach to isolating the exchanger. In later experiments, however, particularly those involving assay of Na+/H+ exchange activity in artificial lipid vesicles, we observed that freezing and thawing, or trypsin treatment of solubilized renal brush-border membrane proteins, resulted in normal or increased Na+/H+ exchange transport activity but loss of regulation of the transporter by PKA (41–44). We subsequently isolated a brush-border membrane protein that, when coreconstituted with trypsinized brush-border membrane proteins, restored the inhibitory response to PKA (46). This protein did not express Na+/H+ exchange activity, and it became clear that it was not the brush-border membrane Na+/H+ exchanger that we had sought but rather a regulatory cofactor required for PKA regulation of the transporter. While our studies were in progress, Sardet et al. (32) reported cloning of cDNA for a housekeeping form of a Na+/H+ exchanger, now called NHE1, and Tse et al. (36) and Orłowski et al. (25) reported cloning of other members of this family of proteins, including NHE3, the epithelial isoform. In 1995, after isolating enough of the regulatory protein for partial amino acid sequencing, we cloned the factor and named it the NHERF (48). PS120 cells are a human fibroblast cell line that has been negatively selected to contain no Na+/H+ exchangers, and they do not express native NHERF. cAMP did not affect Na+/H+ exchange activity in PS120 cells expressing NHE3, but if NHERF was coexpressed with NHE3, cAMP inhibited the activity of the transporter (52). Thus these studies provided in vivo confirmation of the role of NHERF in PKA regulation of NHE3.

RECOGNITION OF NHERF AS PART OF A SIGNAL COMPLEX OF PROTEINS

It was appreciated immediately that NHERF contained an ~100-amino acid internal repeat that we postulated to be protein binding domains (48). This was a reasonable conclusion given that NHERF did not contain putative lipid membrane-spanning domains but was, nonetheless, isolated from a brush-border membrane fraction of renal proximal tubule cells. Shortly thereafter, these regions of NHERF were identified as PDZ domains (52). After the demonstration that NHERF bound ezrin, models were developed that hypothesized that NHERF, ezrin, and PKA formed a signal complex that mediated the acute regulation of NHE3 activity (30). An example of such a model is shown in Fig. 1. The predictions of the model proved to be accurate, and the remaining discussion focuses on the biochemical properties of NHERF as they pertain to its physiological role as a regulator of the action of PKA on NHE3 activity.

NHERF Binds to NHE3 and Mediates Its Phosphorylation

The model predicts a physical association between NHERF and NHE3. The relationship among NHE3, NHERF, and ezrin was studied by using PS120 cells. As summarized in Table 1, in PS120 cells that are cotransfected and express NHE3 and NHERF, immunoprecipitation of NHERF resulted in the coimmunoprecipitation of NHE3 (54). Immunoprecipitation of NHE3 resulted in the coimmunoprecipitation of NHERF. Of interest, the binding of these two proteins was not affected by cAMP, suggesting a continuing association even in the absence of stimulation by PKA and that PKA-mediated phosphorylation of either NHE3 or NHERF was not required for binding. The interaction between NHE3 and NHERF differs from the relationship between NHERF and the β2-adrenergic receptor, which only interact during agonist occupancy of the receptor (12). Kurashima et al. (19) and Zhao et al. (53) provided evidence that, in a cell line that expresses endogenous NHERF, PKA phosphorylated serine 605 in the intracellular COOH-terminal tail of NHE3 and that phosphorylation of this residue was associated with inhibition of activity. To determine whether NHERF was a necessary cofactor in PKA-mediated phosphorylation of NHE3, the effect of cAMP
was studied by examining two-dimensional phosphopeptide maps of NHE3 immunoprecipitated from PS120 cells metabolically labeled with $^{32}$P (54). In separate experiments using the same cell lines, NHE3 was immunoprecipitated and the immunoprecipitated NHE3 was back phosphorylated in vitro by using PKA and [$\gamma$-$^{32}$P]ATP (54). Both assays indicated that NHERF was required for PKA to phosphorylate NHE3. We interpreted our findings to indicate that PKA-mediated phosphorylation of NHE3 was required to inhibit Na$^{+}$/H$^{+}$ exchange activity and that NHERF was absolutely required for PKA to phosphorylate the transporter (19, 54).

**NHERF Binding to Ezrin Is Required for PKA-Mediated Inhibition of NHE3**

Using a pull-down assay, ezrin was demonstrated to bind with high affinity to a previously unknown protein that was ultimately demonstrated to be the human homolog of NHERF (30). At the same time, another group identified a binding ligand of merlin, which was also identified as human NHERF (24). Ezrin binding to the COOH terminus of NHERF involves a region of NHERF distinct from the PDZ domains (31). To study the in vivo relationship between NHERF binding to ezrin as it relates to PKA regulation of NHE3, PS120 cells expressing NHE3 were cotransfected with either wild-type NHERF or a truncated form of NHERF lacking the ezrin binding domain. In contrast to wild-type, truncated NHERF did not coimmunoprecipitate ezrin, did not result in cAMP-associated phosphorylation of NHE3, and, most importantly, did not support cAMP-mediated inhibition of NHE3 activity (45). Thus NHERF binding to ezrin is critical to its function in mediating PKA inhibition of NHE3 activity. The above evidence demonstrates the in vivo interaction between NHERF and NHE3 and the requirement for NHERF binding to ezrin. Lamprecht et al. (20) and others (7) demonstrated that NHERF was not an A kinase anchoring protein (AKAP), but earlier studies indicated that ezrin itself could function as an AKAP (7, 20). Accordingly, the model proposed PKA binding to ezrin although the possible role of another AKAP linked to the complex is not excluded. It would appear that NHERF functions in this complex to facilitate the physical approximation of PKA to NHE3 and the subsequent phosphorylation of the tail of the transporter. In turn, the phosphorylation of serine 605 results in decreased NHE3 activity.

**THE SIGNIFICANCE OF THE PHOSPHORYLATION OF NHERF**

NHERF was demonstrated to be an in vitro substrate for PKA when it was isolated initially by using sequential column chromatography (41). This finding was confirmed when recombinant NHERF proteins became available (47). By using site-directed mutagenesis, phosphorylation was localized to an array of serine residues at positions 287, 289, and 290 of the NHERF protein (47). Mutation of these three residues resulted in a loss of activity of the protein when assayed in vitro. Accordingly, we believed that PKA phosphorylated not only NHE3 but also NHERF. However, when NHERF was expressed in metabolically labeled HEK-293 cells, we found that NHERF was phosphorylated in unstimulated cells and that cAMP had little effect on its phosphorylation (47). This observation was confirmed subsequently when NHERF was expressed in opossum kidney cells and in PS120 cells (20, 54). Lamprecht et al. (20) ruled out the possibility that the apparent lack of effect of cAMP on the phosphorylation of NHERF was the result of increased phosphorylation of one residue of the protein and a decrease in the phosphorylation of another by phosphopeptide mapping of NHERF immunoprecipitated from $[^{32}$P]ATP-labeled cells (20). These studies suggested that NHERF was phosphorylated on a single residue; findings were consistent with preliminary in vitro data suggesting a 1:1 molar ratio of phosphate to NHERF protein. Recently, it was demonstrated that serine 289 of NHERF is phosphorylated in vivo and that the endogenous "NHERF kinase" is G protein receptor kinase (GRK) 6A (13). This finding is striking given that this is only the second example of this class of protein kinases showing preference for a substrate other than a seven-membrane-spanning receptor. The physiological significance of this observation is as yet unknown, but it may provide an explanation of why cAMP did not appreciably increase the in vivo phosphorylation of NHERF given that the basal phosphor-
ylation of serine 289 of NHERF by GRK 6A would have blocked the site from subsequent phosphorylation by PKA (20, 47, 54).

In the above studies, NHERF was overexpressed, and it remained possible that there was a small pool of NHERF within the NHE3 signal complex that was specifically phosphorylated by PKA but that this phosphorylation was obscured by the large amount of non-complexed NHERF phosphorylated by GRK 6A. To test this possibility, NHERF containing serine-to-alanine mutations of residues 287, 289, and 290 was transfected in PS120 cells expressing NHE3 (54). The mutant NHERF was not phosphorylated in vivo in the basal state or after cAMP but did communoprecipitate NHE3, did mediate the phosphorylation of NHE3, and was effective as wild-type NHERF in mediating cAMP inhibition of NHE3 activity. These results indicate that the phosphorylation of NHERF is not required for it to function in PKA regulation of NHE3. In this regard, NHERF is like a closely related PDZ protein, NHERF2, which also supports cAMP inhibition of NHE3 but is not a phosphoprotein in the basal state or after stimulation with cAMP (51, 52).

THE NATURE OF THE BINDING OF NHERF TO NHE3

To determine the biologically relevant portion of NHERF that interacts with NHE3, recombinant proteins representing full-length rabbit NHERF, PDZ I, or PDZ II (including the COOH terminus) were assayed by using a reconstitution assay of renal brush-border membrane proteins (47). Full-length NHERF and PDZ II supported PKA-associated inhibition of Na⁺/H⁺ exchange activity, but PDZ I did not. In recent experiments, we expressed rabbit NHE3 and either full-length mouse NHERF, PDZ I, or PDZ II (including the COOH terminus) in PS120 cells (45, see Table 1). We found that full-length NHERF and the PDZ II domain of NHERF communoprecipitated with NHE3, were associated with cAMP-mediated phosphorylation of NHE3 and cAMP-mediated inhibition of Na⁺/H⁺ exchange activity. PDZ I did not communoprecipitate with NHE3, did not support the phosphorylation of the transporter, and was not effective in mediating inhibition of transporter activity by cAMP. Moreover, the efficacy of full-length NHERF and PDZ II was approximately equal in both the in vitro and in vivo experiments. These results suggested that NHE3 binds to NHERF in the PDZ II region of the protein and that some portion of the COOH terminus of NHERF is required for interaction with NHE3. In a similar fashion, NHERF2 binds NHE3 using PDZ II and also requires the COOH terminus of the protein to demonstrate binding, at least in vitro (51).

As initially defined, PDZ domains bind to the COOH terminus four amino acids of their respective ligands, and the amino acid sequences that bind to a specific PDZ class are narrowly defined (17, 28). Using a peptide display library, Wang et al. (39) determined the optimal amino acid sequence for binding to each of the two PDZ domains of NHERF. Although PDZ I and PDZ II share >80% identity with one another, the predicted substrates for each domain are unique. Nonetheless, the preferred binding sequences for either of the PDZ domains of NHERF are not present in the COOH terminus of NHE3, indicating that NHERF-NHE3 binding must involve a different paradigm. There is precedent for nonclassic interactions between PDZ domains and other proteins, and PDZ-mimetic sequences located in internal portions of proteins have been described (10, 14, 26, 34). It is worth noting that it has not been established that NHE3 binding to NHERF involves the PDZ domain per se. NHERF has been demonstrated to interact with several substrates by using sequences in the NHERF protein distinct from the PDZ domains. The best-studied example of such an interaction is NHERF binding to ezrin (31). It is possible that NHERF binding to NHE3 does not involve the PDZ domains directly. Indeed, if neither ezrin nor NHE3 utilizes PDZ regions for binding, the NHERF protein could potentially recruit additional proteins by using its PDZ protein-protein interactive domains. These possibilities remain to be explored.

NHERF AND THE REGULATION OF OTHER RENAL TRANSPORTERS AND CHANNELS

The potential role of NHERF and NHERF-like proteins to organize signal complexes to facilitate the regulation of other renal transporters and channels is just beginning to be explored. On the basis of sequence specificity of the terminal four amino acids, it was predicted that the CFTR would bind to NHERF. Several recent papers have provided in vitro confirmation of the high affinity of NHERF for CFTR (11, 35, 39). To date, however, no direct physiological correlate of the in vitro binding has been defined although a recent report indicates that NHERF targets CFTR to the plasma membrane (23). There appears to be an interaction between the renal outer medullary potassium channel (ROMK) and NHERF. Of considerable interest is the suggestion that NHERF may function to link and/or to assemble an ion channel complex consisting of CFTR and ROMK (50). The significance of these observations is also under active investigation. Finally, both in vitro and in vivo experiments by Bernardo et al. (2) have indicated that the renal Na⁺-HCO₃⁻ cotransporter (NBC) is regulated by PKA and that this regulation has an absolute requirement for NHERF in a manner analogous to the relationship between NHERF and NHE3 (2). By using a reconstitution assay to determine the activity of NBC, it was found that PKA did not inhibit the transporter unless recombinant NHERF was present. In addition, PKA did not inhibit native NBC activity in type 1 bumetanide-sensitive cotransporter cells that lack endogenous NHERF. When these cells were transfected with NHERF, however, activation of PKA inhibited NBC activity. The relationship between PKA inhibition of NHE3 and PKA inhibition of NBC, and the requirement for NHERF in both these processes, raises the possibility that NHERF is part of a signal complex that regulates
NBC activity. This question is under investigation. It is also of interest that, in the renal proximal tubule, there is coordinated regulation of NHE3 in the apical membrane and NBC in the basolateral membrane. It remains possible that NHERF is involved in the parallel regulation of these two transporters.

CONCLUDING REMARKS

Adaptor proteins, including those proteins containing PDZ domains, were initially ascribed a scaffold function that facilitated the function of other regulatory proteins such as protein kinases and protein phosphatases. It is our present view that NHERF functions in the PKA regulation of NHE3 in such a manner. Recent studies suggest, however, the possibility that NHERF plays a more dynamic role in the physiology of some cells. For example, treatment of estrogen receptor-positive cells with estrogen rapidly increases NHERF mRNA and protein concentrations whereas treatment of serum-starved opossum kidney cells with serum decreases NHERF mRNA and protein (9, 49). The specificity and physiological relevance of these observations are under study. Nonetheless, it is curious that some cells express mechanisms for rapid regulation of cellular NHERF, a property not usually ascribed to adaptor proteins. Hall et al. (12) demonstrated that agonist occupation of the β2-adrenergic receptor resulted in the movement of NHERF from a submembrane location to the plasma membrane where it colocalized with the receptor. Such a dynamic response is not typical of this class of proteins. Moreover, the shift of NHERF from other sites in the cell to the β2-adrenergic receptor is associated with a functional change in the behavior of the response of NHE3 to activation of PKA. The model that ensues these observations suggests an internal competition between proteins with affinity for either of the PDZ domains of NHERF. It is far too early to generalize, but the implications of such experiments are far reaching for understanding the potential involvement of NHERF and NHERF-like proteins in cellular processes and signal transduction.

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