N-terminal PDZ domain is required for NHERF dimerization

Shirish Shenolikar[a,*], Charles M. Minkoff[a], Deborah A. Steplock[b], Christine Evangelista[b], Min-Zhi Liu[b], Edward J. Weinman[b]

[a] *Department of Pharmacology and Cancer Biology, Duke University Medical Center, C315 LSRC, P.O. Box 3813, Research Drive, Durham, NC 27710, USA*

[b] *Department of Medicine, University of Maryland School of Medicine and Medical Service, Department of Veterans Affairs Medical Center, Baltimore, MD 21201, USA*

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Abstract NHERF, a 55 kDa PDZ-containing protein, binds receptors and ion transporters to mediate signal transduction at the plasma membrane. Recombinant NHERF demonstrated an apparent size of 150 kDa on gel filtration, which could be reduced to approximately 55 kDa by protein denaturing agents, consistent with the formation of NHERF dimers. Biosensor studies established the time- and concentration-dependent dimerization of NHERF. Overlays of recombinant NHERF fragments suggested that NHERF dimerization was principally mediated by the N-terminal PDZ-I domain. In PS120 cells, reversible protein phosphorylation modulated NHERF dimerization and suggested a role for NHERF dimers in hormonal signaling. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Na^+/H^+ exchanger-3; PSD-95/Disc large/ZO-1 domain; Protein oligomerization; Okadaic acid; Protein phosphorylation

1. Introduction

The Na^+/H^+ exchanger isoform 3 (NHE3) located in the rabbit renal proximal tubules mediates the reabsorption of sodium, HCO_3^- and water in response to cyclic AMP (cAMP) and parathyroid hormone. Isolated apical membranes demonstrate NHE3 activity that is inhibited by PKA [1]. This led to the isolation of a 45 kDa protein, termed Na^+/H^+ exchanger regulatory factor or NHERF, from detergent-solubilized brush border membranes, that was essential for PKA inhibition of NHE3 reconstituted in liposomes [2]. The rabbit NHERF cDNA encodes a 55 kDa protein [3] that reconstitutes cAMP inhibition of Na^+/H^+ exchange in NHE3-expressing PS120 cells. Recent studies suggest that NHERF links NHE3 to ezrin, a PKA-anchoring protein, to facilitate the cAMP-mediated phosphorylation of NHE3 and inhibition of Na^+/H^+ exchange [4]. NHERF is itself phosphorylated in mammalian cells, most likely by GRK6A, a member of the G-protein-coupled receptor kinase family [5], but the function of this covalent modification remains unknown.

NHERF distribution between cytosol and membranes is modulated by serum factors [6]. As NHERF targets are primarily membrane proteins [7], the role of cytosolic NHERF is currently unclear. Estimates of NHERF in the mammalian kidney exceed those of the known NHERF targets. This also suggests that the majority of NHERF is not bound to target proteins and the physiological signals may dictate NHERF's availability to its membrane targets. We noted a difference in the apparent molecular size of NHERF in the renal cytosol compared to NHERF extracted from brush border membranes. Analysis of recombinant NHERF using several techniques shows that it exists as a dimer both in vitro and in vivo. Our studies suggest that NHERF dimerization requires the first PDZ (PSD-95/Discs large/ZO-1) domain, the binding site for many NHERF targets. Finally, NHERF dimers were dissociated by protein phosphorylation. This suggests that assembly and disassembly of NHERF dimers represents a mechanism for hormonal control of plasma membrane receptors and transporters.

2. Materials and methods

Rabbit NHERF cDNA inserted into pET30A (Stratagene) was expressed in *Escherichia coli* as a hexahistidine-tagged fusion protein and purified on Ni-Sepharose as previously described [8]. The N-terminal PDZ domain (amino acids 11–101) was excised as a *Sac*I fragment and ligated into pET30A digested with BSSH-*Sac*I. The *Sac*I–*Xho*I fragment of the rabbit cDNA encoding a C-terminal fragment of NHERF (amino acids 152–358) was ligated into pET30A digested with the same enzyme. Both NHERF fragments were expressed in *E. coli* and purified on Ni-Sepharose. The NHERF cDNAs were ligated into the following mammalian expression vectors – NHERF cDNA transferred from pET30a into pcDNA3 (provided by Chris Yun, Johns Hopkins University), pBK-CMV (provided by Randy Hall, Emory University) and pFlag-CMV-2 (provided by Maria-Magdalena Georgescu, Rockefeller University) – that yielded hexahistidine-tagged, HA-tagged and FLAG-tagged proteins, respectively. All NHERF constructs were transfected into PS120-NHE3V cells using lipofectamine as described [4]. Immunoprecipitation and Western immunoblotting with anti-hexahistidine (KPL), anti-HA.11 (Babco) and anti-FLAG M2 (Sigma) were carried out essentially as previously described [4].

2.1. Gel filtration of NHERF

Recombinant rabbit His-NHERF was subjected to gel filtration on Superose 6 FPLC in 50 mM Tris–HCl, pH 7.5 containing 0.1 mM EDTA, 15 mM β-mercaptoethanol and 150 mM NaCl at a flow rate of 0.5 ml/min. NHERF, denatured in 6 M guanidine hydrochloride (GuHCl) at 25°C for 30 min, was also subjected to gel filtration in 50 mM Tris–HCl, pH 7.5 containing 0.1 mM EDTA, 15 mM β-mercaptoethanol and 6 M GuHCl. Molecular weight protein markers (vitamin B12, ovalbumin, γ-globulin and thyroglobulin) were obtained from Bio-Rad. Protein elution was monitored as absorbance at 280 nm

*Corresponding author. Fax: (1)-919-681 9567.
E-mail: sheno001@mc.duke.edu

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using a buffer blank. Western immunoblotting with anti-NHERF was also used to monitor NHERF elution.

2.2. Biosensor analysis of NHERF binding

Purified recombinant His-NHERF (100 ng) was coupled to dextran-coated cuvettes using EDC/NHS as recommended by the manufacturer (IAsys Application Note 2.1). The cuvette was washed extensively with 50 mM Tris–HCl, pH 7.5 containing 0.1 mM EDTA, 15 mM β-mercaptoethanol and 150 mM NaCl to remove unbound protein. Soluble His-NHERF was added in the same buffer with rapid stirring and protein–protein binding monitored as a time-dependent change in polarization of the laser in Arc-secs.

2.3. Overlays with NHERF

Recombinant full-length His-NHERF and its fragments were subjected to SDS–PAGE on 10% (w/v) acrylamide gels. The proteins were electrophoretically transferred to PVDF membranes, blocked with 1% non-fat milk and incubated with 1 μM phosphorylated NHERF, ^{32}P-labelled at a serine in the linker region using PKA and detected by autoradiography or unphosphorylated His-NHERF monitored by Western blotting with anti-S-tag monoclonal antibody [9].

3. Results and discussion

NHERF (M_r 45 000 on SDS–PAGE) obtained from detergent-solubilized rabbit brush border membranes migrated on gel filtration with an apparent M_r of approximately 60 000 [3]. In contrast, the 100 000 × *g* supernatant of rabbit kidney cortex contained NHERF (M_r 55 000 on SDS–PAGE) that migrated with an apparent M_r of 150 000 (data not shown). To examine the basis for this size difference, we fractionated highly purified recombinant His-NHERF by Superose 6 FPLC (Fig. 1, solid line). Bulk of the protein migrated as a peak of M_r 150 000 with less than a third showing an apparent M_r 50 000 consistent with monomeric His-NHERF. Incubation of His-NHERF in 6 M GuHCl (Fig. 1, dotted line) reduced its apparent molecular size. The denatured His-NHERF migrated as a broad peak ranging from M_r 75 000 to 45 000. This demonstrated that soluble His-NHERF formed oligomeric complexes that could be disrupted by GuHCl.

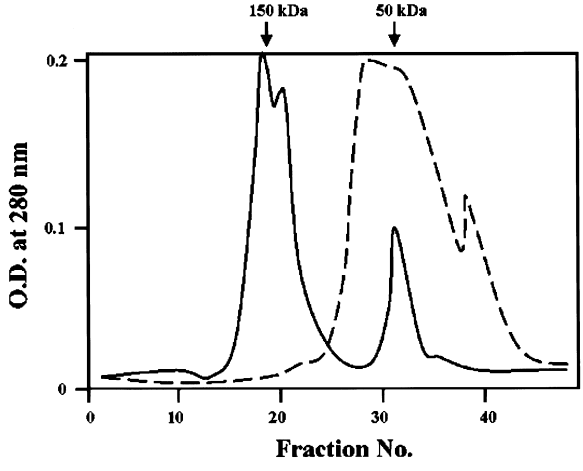

Fig. 1. Gel exclusion chromatography of recombinant NHERF. Purified recombinant rabbit His-NHERF was subjected to gel filtration on Superose 6 FPLC (solid line) as described in Section 2. Apparent M_r values of two major protein peaks were calculated using standard molecular weight protein markers and are indicated with arrows. Recombinant NHERF, denatured with 6 M GuHCl, was also fractionated following in buffer containing 6 M GuHCl (dotted line).

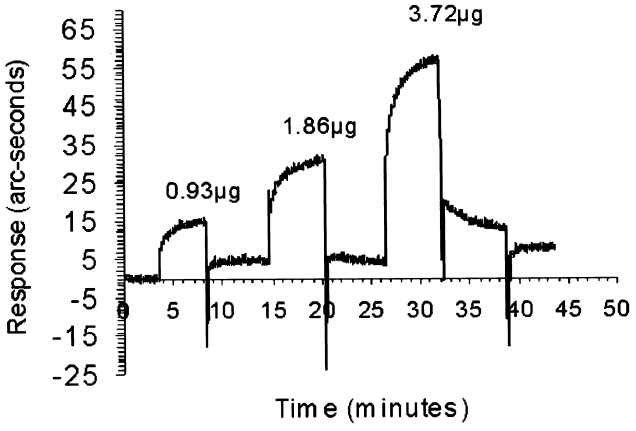

Fig. 2. Biosensor analysis of NHERF–NHERF binding. Recombinant His-NHERF was immobilized to a dextran-coated cuvette as described in Section 2. Following extensive washing, increasing amounts of soluble His-NHERF were added. NHERF binding was observed as time- and concentration-dependent increase in rotation of the polarized laser in Arc-secs.

To further analyze the oligomerization of NHERF, we utilized a biosensor (Fig. 2). Soluble His-NHERF bound to His-NHERF immobilized on the dextran surface of the biosensor cuvette in a time- and concentration-dependent manner. The data were most consistent with the formation of NHERF homodimers. NHERF was immobilized at low protein concentration and washed extensively to favor the coupling of monomeric NHERF to the biosensor cuvette. However, as discussed above, the soluble ligand was largely dimeric His-NHERF. Thus, NHERF–NHERF association seen with the biosensor represents an exchange of NHERF from the soluble dimers to the NHERF monomers immobilized in the cuvette. High concentrations of soluble His-NHERF showed non-specific, hydrophobic binding to the dextran surface. Thus, standard algorithms for analyzing protein–protein interactions could not be applied and the affinity for NHERF dimerization could not be accurately determined. However, we estimated from these studies that that NHERF self-associates with low affinity with K_d between 1 and 10 μM.

An overlay assay [9] was used to define the regions of NHERF that mediate its dimerization. Full-length NHERF and the N-terminal PDZ-I domain (amino acids 11–101) bound with low affinity (requiring between 10 and 20 μg of protein), to both phosphorylated (Fig. 3) and unphosphory-

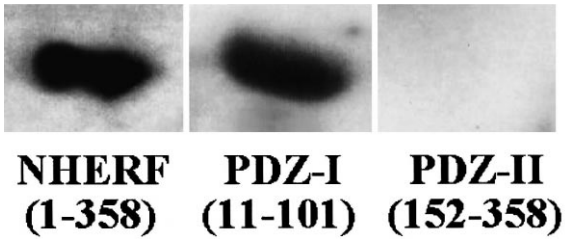

Fig. 3. Overlays with recombinant NHERF. Full-length His-NHERF (amino acids 1–358), the fragments termed PDZ-I (amino acids 11–101), and PDZ-II (amino acids 152–358) were subjected to SDS–PAGE. The proteins were electrophoretically transferred to PVDF membrane, renatured in buffer lacking SDS and incubated with ^{32}P-labelled NHERF, whose binding was visualized by autoradiography.

lated His-NHERF (data not shown). No NHERF was bound to the C-terminal PDZ-II domain (amino acids 152–385). In other experiments, His-tagged PDZ-I bound the full-length NHERF and PDZ-I while PDZ-II failed to bind full-length NHERF, PDZ-I or PDZ-II (data not shown). These results contrasted sharply with a recent report [10], in which recombinant rat GST-NHERF (termed EBP50 or ezrin binding protein of apparent M_r 50 000) bound GST-NHERF in an overlay (K_d 100 nM). Moreover, both rat NHERF PDZ domains bound each other, with PDZ-I showing a preference for PDZ-1 and PDZ-II preferring PDZ-II. The basis for these very different results is still unclear but as indicated above, NHERF isolated from rabbit renal apical membranes contains PDZ-II and C-terminus that is essential for NHE3 regulation and is phosphorylated at C-terminal serines by PKA [4]. This suggests that the 45 kDa rabbit NHERF was proteolyzed at its N-terminus. Inability of the truncated NHERF to form higher molecular weight complexes supports our suggestion that N-terminal sequences within PDZ-I are required for NHERF dimerization. Chemical cross-linking of rat NHERF primarily yielded dimers [10], consistent with the biosensor data discussed above.

To demonstrate NHERF dimerization in vivo, we expressed mouse NHERF with two distinct tags, HA and FLAG, in PS120 cells. Immunoprecipitation with either anti-HA or anti-FLAG monoclonal antibodies followed by immunoblotting with the converse antibody established the association of HA-NHERF to FLAG-NHERF in vivo (Fig. 4A). To overcome the inefficiency of co-immunoprecipitations, we also expressed mouse NHERF as a hexahistidine-tagged protein in PS120 cells. This enhanced the extraction of NHERF and NHERF-bound proteins from cell extracts. Affinity isolation of His-NHERF on Ni-Sepharose also removed a significant portion of HA-NHERF co-expressed in these cells (Fig. 4A). HA-NHERF did not bind to Ni-Sepharose when it was expressed in cells by itself (data not shown), establishing that the binding of HA-NHERF to the Ni-affinity matrix required its dimerization with His-NHERF.

Next, we analyzed potential cellular mechanisms that regulate NHERF dimerization. Treatment of PS120 cells with 10% serum, 0.1 mM cell-permeable cAMP analogs, the calcium ionophore, A23187, and phorbol esters for periods ranging from 15 min to 3 h had no effect on NHERF dimerization. However, 1 μM okadaic acid, which inhibits the two major protein serine/threonine phosphatases, PP1 and PP2A, resulted in significant reduction in NHERF dimers in the PS120 cell extracts. The process was slow with only 17 ± 1% reduction in NHERF dimerization after 15 min exposure to okadaic acid. NHERF dimerization was, however, greatly reduced (65 ± 7%) when cells were treated for 3 h with okadaic acid. These effects were not due to the cytotoxicity of okadaic acid and could be reversed following the removal of the phosphatase inhibitor. Okadaic acid also did not reduce the expression of His-NHERF. If anything, equal or greater amounts of His-NHERF were extracted by Ni-Sepharose from okadaic acid-treated PS120 cells. This provides the first evidence that protein phosphorylation regulates NHERF dimerization in cells. It is unclear at this time whether phosphorylation of NHERF itself or its cellular targets modulates NHERF dimerization in PS120 cells. The apparent lack of difference in binding of phosphorylated and unphosphorylated NHERF to immobilized NHERF and NHERF peptides

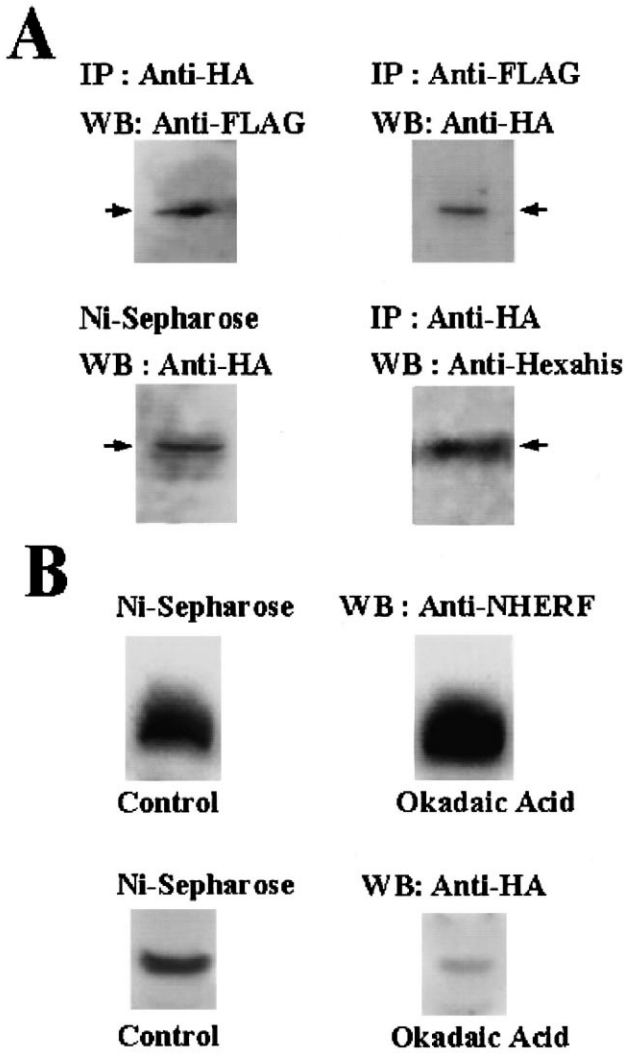

Fig. 4. NHERF dimerization in PS120 cells. A shows the immunoprecipitations (IP) with anti-HA (top left) and anti-FLAG (top right) from PS120 cells expressing HA- and FLAG-NHERF. Western immunoblotting (WB) showed that HA-NHERF bound FLAG-NHERF in PS120 cell extracts. Nickel chelate chromatography was used to isolate His-NHERF from PS120 extracts. Immunoblotting with anti-HA antibody established the presence of HA-NHERF in eluates from the Ni-column (bottom left). Immunoblotting of anti-HA immunoprecipitates with an anti-hexahistidine antibody also established the association of His-NHERF with HA-NHERF (bottom right). B shows that Ni-Sepharose adsorbed His-NHERF from extracts of control (top left) and PS120 cells treated for 3 h with 1 μM okadaic acid (top right), readily recognized by immunoblotting with an anti-NHERF antibody. Association of HA-NHERF with His-NHERF was seen in control cells (bottom left) but was significantly reduced in cells treated with okadaic acid (bottom right).

in the overlay assay would argue against the phosphorylation of NHERF as the primary mechanism of dissociating NHERF dimers. On the other hand, insensitivity of the overlays may miss small changes in the affinity of the phosphorylated and unphosphorylated NHERF dimers. Clearly, further work is required to elucidate the mechanisms that regulate NHERF complexes in mammalian cells and in turn control its physiological functions.

The NHERF PDZ-I binds β2-adrenergic receptor [11], P2Y and PDGF receptors [9], cystic fibrosis transmembrane regulator [12], and GRK6A protein kinase [5]. Dimerization of

NHERF via PDZ-I is likely to preclude its binding to these targets and perhaps represents an inactive or latent pool of NHERF. On the other hand, ligand binding to β2-adrenergic receptors recruits NHERF [11] and has been implicated in the desensitization of hormone signals [13]. In this context, NHERF dimerization may facilitate the clustering of receptors at clathrin-coated pits and promote their internalization and degradation [13]. Signaling by the PDGF receptor tyrosine kinase (PDGF-R), another NHERF target, mandates PDGF-R dimerization. Here, dimerization of NHERF and its homologue, NHERF2, facilitates PDGF signaling [14], accounting for the identification of NHERF2 as TKA-1, an activator of PDGF-R [9].

PDZ-I is dispensable for the assembly of NHE3/NHERF/ezrin complex that mediates cAMP inhibition of Na^+/H^+ exchange in PS120 cells [4] and NHERF dimerization via PDZ-I may not prevent NHE3 regulation. However, association of β2-adrenergic receptor to NHERF PDZ-I prevents NHE3 inhibition by cAMP [11]. While the precise mechanism remains unclear, NHERF dimerization via PDZ-1 could also preclude its interaction with NHE3 and the dissociation of NHERF dimers by protein phosphorylation may be necessary for its function as an NHE3 regulator. In conclusion, we have shown that NHERF dimers exist in vivo and in vitro and comprise a pool of NHERF whose dissociation is regulated by reversible protein phosphorylation. Future studies will address the physiological importance of NHERF dimerization and define the phosphorylation events that regulate NHERF dimerization and function in mammalian cells.

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