Published in final edited form as:

J Mol Biol. 2009 September 11; 392(1): 166–180. doi:10.1016/j.jmb.2009.07.005.

Ezrin induces long-range interdomain allostery in the scaffolding protein NHERF1

Jianquan Li[#], David J.E. Callaway[‡], and Zimei Bu^{#,*}

[#]Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111

[‡] New York University School of Medicine, 550 First Avenue, New York, NY 10016-6402

Abstract

Scaffolding proteins are molecular switches that control diverse signaling events. The scaffolding protein NHERF1 assembles macromolecular signaling complexes and regulates the macromolecular assembly, localization and intracellular trafficking of a number of membrane ion transport proteins, receptors, and adhesion/antiadhesion proteins. NHERF1 begins with two modular protein-protein interaction domains—PDZ1 and PDZ2—and ends with a C-terminal domain. This C-terminal domain binds to ezrin, which in turn interacts with cytoskeletal actin. Remarkably, ezrin binding to NHERF1 increases the binding capabilities of both PDZ domains. Here we use deuterium labeling and contrast variation neutron scattering experiments to determine the conformational changes in NHERF1 when it forms a complex with ezrin. Upon binding to ezrin, NHERF1 undergoes significant conformational changes in the region linking PDZ2 and its C-terminal ezrin-binding domain, as well as in the region linking PDZ1 and PDZ2, involving very long-range interactions over 120 Å. The results provide a structural explanation, at mesoscopic scales, of the allosteric control of NHERF1 by ezrin as it assembles protein complexes. Because of the essential roles of NHERF1 and ezrin in intracellular trafficking in epithelial cells, we hypothesize that this long-range allosteric regulation of NHERF1 by ezrin enables the membrane-cytoskeleton to assemble protein complexes that control cross-talk and regulate the strength and duration of signaling.

Introduction

The transduction of biological signals is tightly controlled by the dynamic assembly and disassembly of protein complexes. Scaffolding proteins play critical roles in regulating of diverse signaling events ^{1; 2}. The scaffolding protein Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1), also known as ezrin binding protein 50 (EBP50), recruits signaling protein partners and directs proteins to specific cellular locations in mammalian epithelial cells. NHERF1 belongs to the Na⁺/H⁺ exchanger regulatory factor family of proteins ^{3; 4}. Members of this protein family contain two or more copies of modular PDZ (PSD-95/Discs-large/ZO-1) domains that are capable of binding to specific amino acid motifs residing in the cytoplasmic portion of a number of transmembrane proteins ^{5; 6}. NHERF1 has two PDZ domains, PDZ1 and PDZ2, and a C-terminal domain, see Figure 1. The NHERF1 PDZ domains bind to a growing list of membrane proteins including ion transport proteins, tyrosine kinase receptors, and the G-protein coupled receptors, for review see ^{7; 8}.

*Address correspondence to: Zimei Bu, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, Phone: (215) 728-7051, Fax: (215) 728-3574, Email: Zimei.Bu@fccc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NHERF1 regulates the intracellular trafficking and signaling of membrane proteins to which it binds. For example, NHERF1 and ezrin anchor the cystic fibrosis transmembrane conductance regulator (**CFTR**) to the actin cytoskeleton, and promote the retention of CFTR in the apical membrane of epithelial cells and the interaction of CFTR with other apical membrane proteins^{9; 10}. Notably, recent studies show that NHERF1 increases the cell surface expression of a disease-causing mutant of CFTR with a deletion at amino acid Phe508 (**ΔF508**)^{11; 12; 13}. The ΔF508 mutant, responsible for 80% of the cases of the genetic disease cystic fibrosis, is trapped in the endoplasmic reticulum after biosynthesis and fails to reach the cell membrane to perform normal functions as a chloride ion channel. NHERF1 also assembles a signaling macromolecular complex comprised of CFTR and the G-protein coupled beta 2 adrenergic receptor. This complex is believed to stimulate the CFTR ion channel by the β₂AR receptor^{14; 15}. Further, NHERF proteins can also function as molecular switches that not only regulate the intracellular trafficking but also alter that the specific pathways the parathyroid hormone receptor 1 (**PTH1R**) signals through^{16; 17}.

An important function of NHERF1 is that its carboxyl-terminal domain binds to ezrin¹⁸. Ezrin is a member of the ezrin/radixin/moesin (**ERM**) family membrane-cytoskeleton linker proteins that plays vital roles in cytoskeletal-related events such as cell polarization, intracellular trafficking, cell adhesion, cell survival, cell motility, as well as tumor metastasis^{19; 20; 21}. Ezrin and other ERM proteins are regulated by head-to-tail like intramolecular interactions between the N-terminal 4.1-ezrin/radixin/moesin (**FERM**) domain and the C-terminal actin binding domains^{22; 23}. In the inactive state, the FERM domain is masked by the actin binding domain. Ezrin becomes activated when these intramolecular interactions are disrupted upon activation by phospholipid phosphatidylinositol-4,5-bisphosphate (**PIP2**) binding and phosphorylation at T567 by PKC, rho kinase, AKT2, and/or at Tyr353 in response to epidermal growth factor treatment^{24; 25; 26}. The activated ezrin binds to target membrane proteins either directly or indirectly through NHERF1 or 2 by the FERM domain while the ezrin C-terminus interacts with the actin filament, forming a communication linkage between the cell membrane and the cytoskeletal network.

It is becoming increasingly clear that NHERF1 and ezrin operate in a cooperative fashion to assemble protein complexes at the cell membrane. A study by Weinman *et al.* shows that expressing NHERF1 without the carboxy-terminal ERM-binding (**EB**) domain leads to the internalization of Na⁺/H⁺ exchanger 3 and abolishes ion transport activities of NHE3²⁷. Truncation of the EB domain results in loss of functional expression of the sodium-potassium-ATPase transporter at the cell membrane²⁸. Similarly, expressing NHERF1 in cells impedes antagonist-induced endocytosis of PTH1R, but deleting the EB domain of NHERF1 results in otherwise inactive ligands to internalize PTH1R²⁹. A recent study by Mahon finds that ezrin binding to NHERF1 is necessary to promote the co-localization and functional expression of a heterogeneous complex of the parathyroid hormone receptor and the sodium-phosphate cotransporter 2a at the apical membrane³⁰. These studies show that ezrin and the ezrin-binding domain in NHERF1 are essential to regulate the intracellular trafficking and function of ion transport proteins and receptors at the cell membrane.

We have previously shown that ezrin positively regulates NHERF1, enabling it to assemble protein complexes cooperatively³¹. In particular, when the FERM domain of ezrin binds to the carboxy-terminal EB domain of NHERF1 with high affinity (with K_d=19nM), the binding affinity of PDZ2 for the 70 amino acid residue C-terminal domain of CFTR (C-CFTR), which contains a type I carboxy-terminal PDZ binding motif -DTRL, increases by 26 fold³¹, see Figure 1. As a result of ezrin binding, the stoichiometry of the full-length NHERF1 binding to C-CFTR is increased from 1:1 to 1:2. Although the binding affinity of PDZ1 for C-CFTR is already high with K_d=298 nM, this interaction is enhanced still further when ezrin is bound to NHERF1. A thermodynamic cycle analysis indicates that ezrin positively modulates the long-

range intramolecular domain-domain interactions in NHERF1, and controls NHERF1 to assemble membrane signaling complexes allosterically, see Figure 1 and legend.

The structures of the isolated PDZ1 and PDZ2 domains of NHERF1, and the interactions of PDZ1 with the carboxy-terminal peptides of membrane receptors and channels have been determined by X-ray crystallography^{32; 33; 34}. X-ray crystallography studies have determined the binding sites in NHERF1 and FERM^{18; 35}. The crystal structure of radixin FERM domain in complex with the EB peptide of NHERF1 reveals that the extreme C-terminal 11 residues (a.a. 348-358) of NHERF1 adopt an amphipathic α -helix with a MDWxxxxx (L/I)Fxx(L/F) motif when bound to the pocket in the FERM subdomain C through hydrophobic interactions. However, the mechanism of long-range allosteric regulation of NHERF1 by ezrin to assemble protein complexes is not known.

To determine the mechanisms of ezrin regulating NHERF1 to assemble protein complexes cooperatively, we have used small angle neutron scattering (SANS) to determine domain-domain conformational changes in NHERF1 upon binding to the FERM domain of ezrin that mimics the activated ezrin. Using deuterium labeling and contrast variation small angle neutron scattering, one can determine the conformational changes of a protein component in a protein complex, as well as the composite structure of the complex^{36; 37; 38; 39; 40}. The results show that ezrin binding triggers long-range inter-domain conformational changes in NHERF1, which provide a structural explanation for increased binding affinities of both PDZ domains for their respective target proteins.

Results

NHERF1 adopts an elongated conformation, with three distinct domains having close contacts between the PDZ2 and the C-terminal domains

Previously, we have performed biochemical, light scattering, and solution small angle X-ray scattering (SAXS) experiments to determine domain-domain interactions in NHERF1⁴¹. These results are summarized here for the purpose of comparing with the present SANS studies that determine the conformational changes in NHERF1 upon binding to the FERM domain of ezrin. Static light scattering (SLS) experiments, which determine the absolute molecular mass, show that the full-length NHERF1 is a monomer in solution (Table I and Figure S1). The monomeric full-length NHERF1 is elongated, as shown by the length distribution function $P(r)$ obtained from SAXS experiments (Figure 2B). The radius of gyration of NHERF1 is $R_g=40.9\pm0.6$ Å and the maximum dimension is $D_{max}=140$ Å. The hydrodynamic radius of the full-length NHERF1 is $R_h=41.0\pm0.4$ Å, which is slightly larger than R_g . The 3-D shape of NHERF1 reconstructed *ab initio* from SAXS has three distinct lobes, with two lobes in close contact with each other but the third lobe is well separated from these two lobes (Figure 5A).

When the FERM domain of ezrin is bound to the C-terminal EB domain of NHERF1, the PDZ2 domain experiences a twenty-fold increase in binding affinity for target proteins³¹. To determine if there are intramolecular domain-domain interactions between PDZ2 and the C-terminal (CT) domain, we have generated a truncated construct of PDZ2 plus the intact C-terminal domain (PDZ2CT) (Figure 1B and 1C). PDZ2CT is monomeric in solution, as determined by SLS (Table I). $P(r)$ of PDZ2CT shows a shoulder at 45.8 Å, suggesting that there are two distinct lobes in PDZ2CT (Figure 2C and Figure 4D). The center-of-mass distance between the two domains can be estimated by the position of the shoulder to be 45.8 Å⁴². The *ab initio* reconstructed 3-D shape of PDZ2CT has two lobes (Figure 5C). The center-of-mass distance between the two lobes can also be measured in the 3-D map to be 45.3 Å. In the 3-D shape of PDZ2CT, PDZ2 and CT are in contact with each other. By comparing the 3-D shape of PDZ2CT and that of the full-length NHERF1, we could assign the middle lobe as PDZ2, the lobe at the right side as the CT domain, and the lobe at the left side PDZ1.

Our protein kinase C (**PKC**) phosphorylation experiments also indicate that there domain-domain interactions in PDZ2CT⁴¹. Using site-directed mutagenesis and PKC phosphorylation experiments, we have found that PKC phosphorylates two amino acid residues Ser-339 and Ser-340 in the linker region between PDZ2 and the EB domain. Another Serine 162 in PDZ2, located above the peptide binding pocket, can be phosphorylated by PKC in the truncated PDZ2 domain, but this residue is specifically protected from being phosphorylated by the intact CT domain in PDZ2CT. These scattering and biochemical results show that the PDZ2 and CT domains are in physical contact with each other, but PDZ1 is well separated from PDZ2 and CT.

Contrast match SANS reveals long-range conformational changes in NHERF1 upon binding to the FERM domain of ezrin

The structure of a protein component in a multi-component complex can be determined using selective deuterium labeling and contrast variation SANS, because the neutron scattering lengths of hydrogen and deuterium are quite different. The neutron scattering intensity from a complex composed of the deuterium labeled and the unlabeled components is expressed as³⁶:

$$I(Q) = \Delta\rho_D^2 I_D(Q) + \Delta\rho_H^2 I_H(Q) + \Delta\rho_D \Delta\rho_H I_{DH}(Q) \quad (1)$$

where $\Delta\rho_D = \rho_D - \rho_o$ is the neutron scattering length density contrast between the deuterated component and that of the buffer, and $\Delta\rho_H = \rho_H - \rho_o$ is the neutron scattering length density contrast between the hydrogenated component and that of the buffer. $I_D(Q)$ and $I_H(Q)$ are the scattering from the deuterium labeled and the hydrogenated component, respectively, and $I_{DH}(Q)$ is the term reflecting the interference scattering between the hydrogenated and the deuterated components. The neutron scattering length density of the buffer ρ_o changes with the volume fractions of D₂O, and $\Delta\rho_D$ or $\Delta\rho_H$ changes accordingly. When the neutron scattering length density of the buffer matches that of the unlabeled protein component or that of the deuterium labeled component, Eq. (1) shows that structural information about the deuterium labeled or the unlabeled component can be obtained. Contrast variation neutron scattering experiments have also been applied to determine the structure of protein-nucleic acid and protein-lipid complexes^{43; 44} because the neutron scattering length density of a protein is different from that of a nucleic acid or lipid membranes.

We have produced deuterium labeled NHERF1 and PDZ2CT for the contrast variation SANS experiments. The non-exchangeable deuterium contents for the deuterium labeled NHERF1 and PDZ2CT are 67% and 73%, respectively, as determined by mass spectroscopy. These partially deuterated proteins are designated as **^dNHERF1** and **^dPDZ2CT**. In 42% D₂O solution, the neutron scattering length density of the unlabeled FERM ρ_H matches that of the buffer solution, $\Delta\rho_H \approx 0$. In 100% D₂O solution, the neutron scattering length density of the partially labeled **^dNHERF1** or **^dPDZ2CT** ρ_D matches that of the buffer within experimental error, $\Delta\rho_D \approx 0$ (see Eq. 1).

To determine if deuterium labeling has caused any changes in the oligomer state and conformation of the proteins and complexes, we have performed light scattering and SAXS experiments to compare the molecular mass and the overall conformation of the deuterated proteins and complexes with their respective unlabeled counterparts in H₂O buffer. The labeled **^dNHERF1** and **^dPDZ2CT** are monomeric similar to the unlabeled proteins (Table I and Figure S1). Comparing the hydrodynamic radii, the radii of gyration and P(r) indicates that deuteration does not cause detectable changes in the overall conformations in **^dNHERF1** or **^dPDZ2CT** (Table I and Figure 2A and 2C).

The ^dNHERF1·FERM and ^dPDZ2CT·FERM complexes are formed by mixing the labeled ^dNHERF1 with unlabeled FERM, and ^dPDZ2CT with FERM at 1:1 molar ratio, respectively. The formed complexes are further purified by size-exclusion chromatography. The SLS determined molecular weight indicates that the stoichiometry of the ^dNHERF1·FERM or the ^dPDZ2CT complex is 1:1 (Table I). This result is consistent with our previous surface plasmon resonance binding, static light scattering, and analytical ultracentrifugation results on the unlabeled NHERF1·FERM and PDZ2CT·FERM complexes ³¹.

Figure 2B, 2D and 2E compares the SAXS data I(Q) and P(r) of the deuterated complexes with the unlabeled complexes. Both the light scattering and SAXS results show that deuterium labeling has no apparent effects on the global conformation and the oligomer state of the complexes at the resolution of the scattering experiments, although there is a report from high-resolution crystallography studies that per-deuteration can cause subtle structural changes at the catalytic sites of haloalkane dehalogenase and alters the catalytic functions of this enzyme ⁴⁵.

At various D₂O concentrations, the oligomer state and stoichiometry of the ^dNHERF·FERM and ^dPDZ2CT·FERM complexes can also be determined by contrast variation SANS. Figure 3 is a plot of the normalized square-root of the forward scattering intensity I(0), which is the neutron scattering intensity extrapolated to Q=0 Å⁻¹, against the scattering length density of the buffer ρ₀. Because the reduced SANS intensity are on absolute scales, Figure 3 yields an estimate of the molecular volume or the molecular mass of the measured complex, according to Eq. 2 derived from a formula described by Jacrot and Zaccari ⁴⁶:

$$\left[\frac{I(0)}{N} \right]^{0.5} = [(\rho_D - \rho_0)V_D + (\rho_H - \rho_0)V_H] = \frac{\bar{v}}{N_A} [\rho_D M_D + \rho_H M_H - \rho_0(M_D + M_H)] \quad (2)$$

where N is the number of the complexes in a volume of 1 cm³, $V_H = \frac{M_H \bar{v}_H}{N_A}$ and $V_D = \frac{M_D \bar{v}_D}{N_A}$ are the molecular volume of the hydrogenated component and that of the deuterium labeled component, respectively. M_H and M_D are the molecular masses of the hydrogenated and deuterium labeled components, respectively, and $\bar{v}_H \approx \bar{v}_D \approx \bar{v}$ is the partial specific volume. According to Eq (2), the slope of a linear fit to the normalized I(0)^{0.5} vs. ρ₀ plot gives the molecular volume or the molecular mass, and thus the stoichiometry of the complexes.

Linear fits to the [I(0)/N]^{0.5} vs. ρ₀ plots in Figure 3 give the molecular volume of 75207±2655 Å³ for the ^dPDZ2CT·FERM complex, and 93891±1098 Å³ for the ^dNHERF1·FERM complex. Assuming the partial specific volume of the complexes is 0.75 cm³/g, the slopes of Figure 3 give the molecular mass of ^dPDZ2CT·FERM to be 60.4±8.0 kDa, and that of ^dNHERF1·FERM to be 75.4±9.6 kDa, which are close to the calculated molecular masses of a 1:1 ^dPDZ2CT·FERM and ^dNHERF1·FERM complexes, respectively. Contrast variation SANS thus confirms the 1:1 stoichiometry of the ^dNHERF·FERM and ^dPDZ2·FERM complexes in D₂O buffer solution. Figure 3 also suggests that changing D₂O concentrations does not affect the association state of the complex.

At the 42% D₂O match point, structural information about the ^dNHERF1 or the ^dPDZ2CT component in the complex can be obtained from SANS data (Eq. 1). In 42% D₂O buffer solution, P(r) of ^dPDZ2CT in complex with the FERM domain of ezrin is very different from that of PDZ2CT or ^dPDZ2CT in solution (Figures 4C and 4D). The conformation of PDZ2CT or ^dPDZ2CT in solution is more compact than that of ^dPDZ2CT in the complex. P(r) of ^dPDZ2CT in the complex resembles that of an elongated rod-like object ⁴². The maximum

dimension $D_{\max}$ of $^d\text{PDZ2CT}$ has changed from 85 Å in solution to 125 Å in the complex, (Figure 4D). The R_g of $^d\text{PDZ2CT}$ in the complex is 37.3 ± 1.0 Å, much larger than $R_g=28.0 \pm 0.6$ Å of PDZ2CT or $^d\text{PDZ2CT}$ in solution.

Comparing the 3-D image of $^d\text{PDZ2CT}$ in the complex and that of PDZ2CT in solution reveals changes in domain-domain distance between PDZ2 and the EB binding domain (Figure 5C and 5D). In PDZ2CT , the center-of-mass distance between PDZ2 and CT is 45.3 Å (Fig 5C). When bound to the FERM, the center-of-mass distance between PDZ2 and the EB domain is expanded to 80 Å as measured from the 3-D map (Figure 5D). FERM binding thus induces large conformational changes in $^d\text{PDZ2CT}$. In particular, the region linking PDZ2 and EB domain becomes extended when EB is bound to FERM.

Conformational changes in the full-length $^d\text{NHERF1}$ are significant when in complex with FERM. $P(r)$ of $^d\text{NHERF1}$ in the complex is apparently very different from that of NHERF1 or $^d\text{NHERF1}$ in solution (Figure 4B). The R_g of $^d\text{NHERF1}$ changes from 41.0 ± 1.0 Å in solution to 45.8 ± 0.8 Å in the complex, but the change in $D_{\max}$ is less dramatic from 140 Å to 145 Å. Comparing the 3-D shape of $^d\text{NHERF1}$ in solution and in the complex shows that the region linking PDZ2 and CT becomes more extended in $^d\text{NHERF1}$ (Figure 5A and 5B). The center-of-mass distance between PDZ2 and the EB domain changes from 45.8 Å in solution to 63 Å in the complex, suggesting that domain-domain contacts between PDZ2 and CT are disrupted. In addition, an angle of about 120° is formed between PDZ2 and CT at the location of PDZ2 in the 3-D map of $^d\text{NHERF1}$, which is consistent with the shape changes of $^d\text{NHERF1}$ $P(r)$ in the FERM bound complex.

A comparison of the 3-D image of $^d\text{NHERF1}$ in the complex with that of $^d\text{NHERF1}$ in solution indicates that the region linking PDZ1 and PDZ2 also becomes notably more extended upon binding to FERM (Figure 5A and 5B). The center-of-mass distance between PDZ1 and PDZ2 expanded from 57.1 Å in NHERF1 to 67.0 Å in $^d\text{NHERF1}$ in the complex. FERM binding at the C-terminal EB domain of NHERF1 thus triggers large conformational changes in domain-domain interactions between PDZ2 and EB, as well as between PDZ1 and PDZ2 at a long 120 Å distance away from the FERM binding site.

The overall conformation of FERM in the complex is similar to the crystal structure

In 100% D_2O solution, the neutron scattering length density contrast between $^d\text{NHERF1}$ or $^d\text{PDZ2CT}$ and the buffer becomes zero within experimental error, structural information about the FERM can be obtained. The scattering intensity $I(Q)$ and the overall shapes of $P(r)$ of FERM in both $^d\text{NHERF1}$ -FERM and $^d\text{PDZ2CT}$ -FERM complexes agree well with that calculated from the crystal structure of ezrin FERM domain (Protein Data Bank: 1NI2.pdb)⁴⁷ (Figure 6). $P(r)$ of FERM measured by SANS also overlies well with that calculated from the crystal structure of the homologous radixin FERM domain that is in complex with a 13 amino acid C-terminal tail peptide of NHERF1 reported³⁵. The neutron scattering results suggest that the overall structure of FERM domain is not changed upon forming complex with NHERF1 or with PDZ2CT .

The 3-D envelope of FERM was reconstructed from the SANS data at the contrast match point of $^d\text{NHERF1}$ in 100% D_2O buffer (Figure 6C). Figure 6C also shows the rigid-body docking of the crystal structure of FERM to the 3-D envelope reconstructed from SANS. The SANS experiments thus reveal that, at the 100% D_2O contrast match point, binding to NHERF1 does not cause large scale conformational changes or domain rearrangement in FERM. In the crystal structure of radixin FERM bound to carboxy-terminal peptide NHERF1 , the carboxy-terminal peptide of NHERF1 induces conformational changes in the C subdomain of radixin FERM³⁵. These subtle movements of secondary structures in the subdomain C are not apparent at the resolution of the SANS experiments.

Contrast variation SANS reveals the composite structures of the d NHERF1-FERM and d PDZ2CT-FERM complexes

To determine the composite structure of the d NHERF1-FERM and the d PDZ2CT-FERM complexes, we have employed the two phase *ab initio* method to reconstruct the 3-D images of the d NHERF1-FERM and the d PDZ2CT-FERM complexes using the SANS data at various contrasts. The 3-D images of the d NHERF1-FERM and d PDZ2CT-FERM complexes were restored using MONSA by fitting the multiple neutron scattering curves simultaneously at various contrasts^{38; 48} (Figure 7). In the complex composed of deuterium labeled and unlabeled components, the deuterated component represents one phase, and the unlabeled component is designated as the 2nd phase.

Figure 7C is the composite structure of the d NHERF1-FERM complex, which shows the 3-D shape and the spatial orientation of the deuterium labeled d NHERF1 and the unlabeled FERM components. Although the reconstructed 3-D models of the d NHERF1-FERM complex and its two components are somewhat less detailed than the individual components reconstructed at contrast match points, the size and shape of d NHERF1 in the composite structure are very similar to that reconstructed at the 42% D₂O match point, and the compact conformation of FERM in the complex is compact similar to that reconstructed at the 100% D₂O match point.

The composite structure of the d NHERF1-FERM complex reveals the relative orientation and position of the two components, with the FERM domain located at one end of d NHERF1. The PDZ domains and the FERM domain are assigned in the composite structure. The composite 3-D shape of the d PDZ2CT-FERM complex (Figure 7C right panel) has a compact phase that resembles the shape of FERM domain, and an elongated phase that represents the opened d PDZ2CT (Figure 7C). Upon binding to FERM, d PDZ2CT becomes extended. The FERM domain is bound at one end of PDZ2CT, presumably at the EB domain.

Discussion

Using solution small angle X-ray scattering and contrast variation small angle neutron scattering, we show that binding of the FERM domain of ezrin to the C-terminus of scaffolding protein NHERF1 induces large conformational changes in NHERF1. Large conformational changes occur in the region linking PDZ2 and the ezrin-binding domain, suggesting that intramolecular domain-domain interactions between PDZ2 and the EBD are disrupted upon ezrin binding. Significant conformational changes are also apparent in the region that links PDZ1 and PDZ2. The present neutron scattering results thus provide a structural explanation of our binding results and thermodynamic analyses, which demonstrate positive allosteric regulation of NHERF1 by ezrin as it assembles membrane protein complexes. The neutron scattering results reveal that this allosteric control originates from domain-domain interactions.

Our binding and thermodynamic analyses show PDZ1, PDZ2 and EBD are energetically coupled, see Figure 1. FERM binding to the C-terminus of NHERF1 increases the binding capability of both PDZ2 and PDZ1. The SAXS and SANS data show that NHERF1 adopts an elongated conformation, with PDZ1, PDZ2 and the C-terminal domain arranged in a row, see Figure 2. The center-of-mass distance between PDZ1 and EBD are 120 Å apart. Thus, together with the thermodynamic analyses, the results demonstrate that all three distant binding sites, PDZ1, PDZ2, and EBD communicate with each other at a very long distance.

What is the physiological consequence of allosteric regulation of NHERF1 by ezrin as it assembles membrane proteins? Studies of the intracellular dynamics of ezrin and ion transport proteins provided the first clues. In cells, the internalization of membrane of transporters and/or receptors, and the recycling of these membrane proteins back to the cell surface are dynamic processes^{25; 49; 50; 51; 52}. PDZ proteins and possibly the activation of ezrin play a significant

role in the retaining transport proteins or receptors at the membrane. Ion channels and/or receptors proteins complexed with NHERF1 and activated ezrin are populated in the more immobile apical membrane subdomains because of their interactions with actin cytoskeleton. The inactive ezrin is localized in the more mobile endosomes that are exchanging with the immobile apical subpopulation as well as with late endosomes. Allosteric control of NHERF1 by activated ezrin to assemble protein complexes may be an important mechanism for engaging cross-talk among ion transport proteins and/or receptors while they reside in the cell membrane, thus modulating the strength and magnitude of signaling. Allosteric regulation of NHERF may also be an effective means to terminate signaling once ezrin is deactivated and the membrane protein is internalized, thus control the duration of signaling. The actin cytoskeleton thus dictates a control of membrane receptors and/or ion transport proteins via the allosteric regulation of NHERF1 by ezrin. Future studies of how the conformational dynamics of ezrin and NHERF1 and the actin cytoskeleton influence the signaling of ion transport proteins or receptors in cellular context will provide more evidence of the physiological significance of allosteric regulation of NHERF1 by ezrin and the cytoskeleton.

What is the mechanism of FERM induced long-range conformational changes and long-range energetic coupling in NHERF1? A type I PDZ-binding motif -SNL at the carboxy-terminus of NHERF1, which overlaps with the EB sequence, has been proposed to interact with PDZ2 through intramolecular domain-domain interactions and hold PDZ2 in an auto-inhibition state ^{41; 53}. Our NMR studies indeed find that there are dynamic intramolecular interactions between the NHERF carboxy-terminus and the carboxy-binding loop and the peptide binding pocket in PDZ2, although the intramolecular interactions are quite weak (Bhattacharya et al, submitted). The NMR studies also show that the CT domain, including the EB domain and the region linking PDZ2 and the EB domains, is largely disordered. Because FERM interacts with the EB domain strongly, FERM binding can easily overcome the relatively weak intramolecular interactions between EBD and PDZ2. The crystal structure shows that the NHERF1 C-terminal (348-358) adopts α -helical conformation when bound to FERM. Combining the NMR, crystallography and the present SANS studies suggests that FERM binding disrupts the intramolecular interactions between PDZ2 and EB, and uncovers PDZ2 to have increased binding affinity for target proteins.

Moreover, there is also evidence that PDZ1 interacts with the linker region between PDZ1 and PDZ2³². The binding affinity of PDZ1 for its target proteins is increased when including a flank region at the carboxy-terminal end beyond the canonically identified PDZ structural boundary (ref. ⁵⁴ and Li et al in preparation). The conformation of the PDZ1-PDZ2 linker region thus affects the binding affinity of PDZ1. Ezrin binding may trigger conformational changes in PDZ2, and PDZ2 in turn propagates the allosteric signals ^{55; 56} to cause conformational changes in the PDZ1-PDZ2 linker region. As a result, binding affinity of PDZ1 for its target proteins is increased at a site far away from the ezrin binding site. Our SAXS and SANS studies of the conformational changes in NHERF1 complexes are on mesoscopic scales. High resolution structural studies of the conformational changes in the PDZ1 plus the linker region will provide insight into the mechanism of such allosteric domain-domain interactions at atomic resolution.

There is increasing evidence that fast protein dynamics dictate energy propagation and the allosteric behavior in a protein ^{55; 56; 57; 58; 59; 60; 61}. Binding inspires changes in deterministic motion on fast ps to ns time scales, which then facilitates the stochastic motion needed to make large conformational changes longer micro to millisecond time scales. In the case of NHERF1, protein motion may play significant roles in influencing the long-range allosteric behavior not only within a PDZ domain ^{55; 56; 62} but also at the domain level. It would be interesting to determine the time-scale and amplitude of protein motion within a domain as well as long-

range coupled protein domain motion^{63; 64; 65} in NHERF1, and estimate how protein domain motion affects the allosteric binding properties of NHERF1.

Materials and Methods

Protein expression and purification

The pET151/D-TOPO vector (Invitrogen, Inc) was used to express the FERM domain of human ezrin (**FERM**, amino acid residues 1-298), the full-length human NHERF1 (residues 11-358), and PDZ2 plus the C-terminal domain (**PDZ2CT**, residues 150-358). The protein expressed by the pET151/D-TOPO vector contains an N-terminal V5 epitope plus a hexa-histidine fusion tag. All plasmids were subjected to DNA sequencing to verify the DNA sequence.

The protein expression plasmids were transformed into Rosetta 2(DE3) cells (Novagen). The cells were grown until the optical density at 600 nm reaches 0.8-0.9, and were induced with 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for 2 hours. The harvested cells were resuspended and lysed in buffer containing 20 mM sodium phosphate buffer, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM imidazole, pH=7.5. The protein extracts were first further purified by Ni²⁺ HiTrap chelating column (Amersham Biosciences). The proteins were then purified and analyzed by size-exclusion chromatography, using a Superdex 200 10/30 column (Amersham Biosciences). The N-terminal fusion tag was cleaved using Tobacco Etch Virus protease (Invitrogen) after purification. After fusion tag cleavage, the calculate molecular masses for 38656 g/mol for NHERF1, 23505 g/mol for PDZ2CT, and 35772 g/mol for FERM.

When expressing deuterated full-length NHERF1 and PDZ2CT, Rosetta 2 (DE3) cells were grown at 37°C in sterile M9 medium containing 99.9% D₂O (Cambridge Isotope Laboratories) until O.D._{600nm} reaches 0.8. The cells were induced with 0.25 mM IPTG. Protein purification and fusion tag removal for the deuterated proteins were the same as described above.

Mass spectroscopy experiments for determining deuterium content in labeled proteins

The deuterium content of dNHERF1 and dPDZ2CT was measured by MALDI-TOF using a Reflex IV mass spectrometer (Bruker Daltonics, Billerica, MA) between 5000 and 60,000 *m/z* in linear mode. The protein peaks were internally calibrated using myoglobin as standard. The non-exchangeable deuterium content determined by mass spectroscopy is 67% for dNHERF1, and 73% for dPDZ2CT. The molecular mass of dPDZ2CT is 24691 g/mol, and that of dNHERF is 40017 g/mol.

Static and dynamic light scattering experiments

Dynamic light scattering experiments were performed using a DynaPro Molecular Sizing Instrument (Wyatt Technology Corporation) with a laser of wavelength 824.7 nm at a fixed 90° scattering angle. Before light scattering experiments, the sample was centrifuged at 10,000 rpm for 5-10 minutes. Protein concentrations were varied from 0.5-2 mg/ml during light scattering measurements. Light scattering experiments were performed at 10°C.

Small angle X-ray and neutron scattering experiments

The SAXS instrument and data reduction methodology have been described previously^{31; 41; 66; 67} In the present study, a typical $0.01 < Q < 0.3 \text{ \AA}^{-1}$ range is employed, where

$Q = \frac{4\pi \sin(\theta/2)}{\lambda}$ is the magnitude of the scattering vector, θ is the scattering angle, and $\lambda = 1.54 \text{ \AA}$ is the wavelength of the Cu-K α X-ray.

The protein concentrations used for SAXS experiments are about 1-2 mg/ml, at which, NHERF1, PDZ2CT, and the dNHERF1·ezFERM and dPDZ2CT·ezFERM complexes are monomeric. At these concentrations, the inter-molecular interference effects are negligible because the radii of gyration R_g and the protein concentration normalized forward scattering intensities $I(0)$ are independent of protein concentrations. SAXS experiments were performed at 10°C.

Small angle neutron scattering experiments were performed at the NG7 30m SANS instrument at the NIST Center for Neutron Research, National Institute of Standards and Technology⁶⁸. The neutron wavelength was 6 Å with the neutron wavelength spread ($\Delta\lambda/\lambda$) being 0.11 (full width at half-maximum). The source aperture diameter was 5.08 cm, and the source-to-sample length was 542 cm. The data were collected at two sample-to-detector distances, 265 and 125 cm, and both sample-to-detector distances have a 25 cm detector offset. These settings gave the effective Q range between 0.0122 and 0.4596 Å⁻¹.

For contrast variation SANS experiments, the dPDZ2·ezFERM and dNHERF1·ezFERM complexes were exchanged, respectively, into 0%, 10, 20%, 42%, 80%, 90% and 100% volume fractions of D₂O buffers containing 20 mM Tris-HCl (pH=7.5) and 150 mM NaCl. The concentration of the dPDZ2CT·ezFERM complex was 3.72 mg/ml, and the concentration of dNHERF1·ezFERM was maintained at 2.02 mg/ml in H₂O solution and in all different volume fractions of D₂O solutions. For protein samples dissolved in 100%, 90% and 80% D₂O solution, 2 mm path-length quartz cells were used. For samples dissolved in 0%, 10%, 20% and 42% D₂O solutions, 1 mm path-length quartz cells were used. SANS measurements were performed at 10°C.

SANS data were reduced using the procedures developed at NIST⁶⁹. The two-dimensional scattering data were corrected for nonuniform detector response, and for scattering from the quartz cells and from ambient neutrons in the room. The scattering data were normalized by the incident beam flux on the sample and by sample thickness to obtain absolute scale intensity (scattering cross section per unit volume). The corrected data were then circularly averaged to yield the one-dimensional $I(Q)$. The incoherent background scattering was determined by averaging the intensity of the plateau region of Q between 0.35 to 0.45 Å⁻¹, and subtracted from the scattering data.

The reduced scattering data are plotted as scattering intensity $I(Q)$ vs. Q profiles. Inverse Fourier transformation of $I(Q)$ gives the length distribution function $P(r)$ that is the probability of finding two scattering points at a given distance r from each other in the measured macromolecule. $P(r)$ was generated by the program GNOM⁷⁰. The radius of gyration R_g , which is related to the size and shape of a protein, can be obtained from $P(r)$ or from the Guinier approximation⁴².

The 3-D molecular envelopes were reconstructed from SAXS or SANS using the *ab initio* program DAMMIN developed by Svergun et al⁴⁸ to generate a set of PDB-formatted dummy bead coordinates. For each construct, about 10-15 models were generated by running the program in slow mode or expert mode. The models are averaged using DAMAVER⁷¹. The normalized spatial discrepancy (**NSD**) values, which measure the reproducibility of the models used in averaging, are given in the figure legends. The program package Situs was used to convert the dummy bead coordinates into envelopes⁷². Situs was also used for rigid-body docking of the crystal structure to the generated envelopes. The graphics are generated using UCSF Chimera⁷³.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work utilized facilities supported in part by the National Science Foundation under Agreement No. DMR-0454672. We acknowledge the support of the National Institute of Standards and Technology, U.S. Department of Commerce, in providing the neutron research facilities used in this work. We thank the Biotechnology Facility at the Fox Chase Cancer Center for mass spectrometry support. We thank Paul Butler and Susan Krueger for helpful discussion. This work is supported by the National Institutes of Health Grant R01 HL086496 and W.W. Smith Charitable Trust (to Z. B.).

References

1. Pawson T, Scott JD. Signaling through scaffold, anchoring, and adaptor proteins. *Science* 1997;278:2075–80. [PubMed: 9405336]
2. Bhattacharyya RP, Remenyi A, Yeh BJ, Lim WA. Domains, Motifs, and Scaffolds: The Role of Modular Interactions in the Evolution and Wiring of Cell Signaling Circuits. *Annu Rev Biochem*. 2006
3. Donowitz M, Cha B, Zachos NC, Brett CL, Sharma A, Tse CM, Li X. NHERF family and NHE3 regulation. *J Physiol* 2005;567:3–11. [PubMed: 15905209]
4. Thelin WR, Hodson CA, Milgram SL. Beyond the brush border: NHERF4 blazes new NHERF turf. *J Physiol* 2005;567:13–9. [PubMed: 15994182]
5. Harris BZ, Lim WA. Mechanism and role of PDZ domains in signaling complex assembly. *J Cell Sci* 2001;114:3219–31. [PubMed: 11591811]
6. Sheng M, Sala C. PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci* 2001;24:1–29. [PubMed: 11283303]
7. Shenolikar S, Voltz JW, Cunningham R, Weinman EJ. Regulation of ion transport by the NHERF family of PDZ proteins. *Physiology (Bethesda)* 2004;19:362–9. [PubMed: 15546854]
8. Weinman EJ, Hall RA, Friedman PA, Liu-Chen LY, Shenolikar S. The Association of Nherf Adaptor Proteins with G Protein-Coupled Receptors and Receptor Tyrosine Kinases *. *Annu Rev Physiol* 2006;68:491–505. [PubMed: 16460281]
9. Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Stutts MJ, Milgram SL. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J Biol Chem* 1998;273:19797–801. [PubMed: 9677412]
10. Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ, Lefkowitz RJ. A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na⁺/H⁺ exchanger regulatory factor family of PDZ proteins. *Proc Natl Acad Sci U S A* 1998;95:8496–501. [PubMed: 9671706]
11. Bossard F, Robay A, Toumaniantz G, Dahimene S, Becq F, Merot J, Gauthier C. NHE-RF1 protein rescues {Delta}F508-CFTR function. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1085–1094. [PubMed: 17237149]
12. Guerra L, Fanelli T, Favia M, Riccardi SM, Busco G, Cardone RA, Carrabino S, Weinman EJ, Reshkin SJ, Conese M, Casavola V. Na⁺/H⁺ Exchanger Regulatory Factor Isoform 1 Overexpression Modulates Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Expression and Activity in Human Airway 16HBE14o- Cells and Rescues {Delta}F508 CFTR Functional Expression in Cystic Fibrosis Cells. *J Biol Chem* 2005;280:40925–40933. [PubMed: 16203733]
13. Fanelli T, Cardone RA, Favia M, G L, Zaccolo M, Monterisi S, De Santis T, Riccardi SM, Reshkin SJ, Casavola V. Beta-oestradiol rescues DeltaF508CFTR functional expression in human cystic fibrosis airway CFBE41o- cells through the up-regulation of NHERF1. *Biol Cell* 2008;100:399–412. [PubMed: 18184109]
14. Naren AP, Cobb B, Li C, Roy K, Nelson D, Heda GD, Liao J, Kirk KL, Sorscher EJ, Hanrahan J, Clancy JP. A macromolecular complex of beta 2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proc Natl Acad Sci U S A* 2003;100:342–6. [PubMed: 12502786]
15. Li C, Naren AP. Macromolecular complexes of cystic fibrosis transmembrane conductance regulator and its interacting partners. *Pharmacology & Therapeutics* 2005;108:208–223. [PubMed: 15936089]

16. Wheeler D, Garrido JL, Bisello A, Kim YK, Friedman PA, Romero G. Regulation of Parathyroid Hormone Type 1 Receptor Dynamics, Traffic, and Signaling by the Na⁺/H⁺ Exchanger Regulatory Factor-1 in Rat Osteosarcoma ROS 17/2.8. *Cells* 2008;22:1163–1170.
17. Mahon MJ, Donowitz M, Yun CC, Segre GV. Na⁽⁺⁾/H⁽⁺⁾ exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature* 2002;417:858–61. [PubMed: 12075354]
18. Reczek D, Berryman M, Bretscher A. Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. *J Cell Biol* 1997;139:169–79. [PubMed: 9314537]
19. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* 2002;3:586–99. [PubMed: 12154370]
20. Elliott BE, Meens JA, SenGupta SK, Louvard D, Arpin M. The membrane cytoskeletal crosslinker ezrin is required for metastasis of breast carcinoma cells. *Breast Cancer Research* 2005;7:R365–R373. [PubMed: 15987432]
21. Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt SM, Helman LJ. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. *Nat Med* 2004;10:182–6. [PubMed: 14704791]
22. Algrain M, Turunen O, Vaheri A, Louvard D, Arpin M. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J Cell Biol* 1993;120:129–39. [PubMed: 8416983]
23. Gary R, Bretscher A. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Mol Biol Cell* 1995;6:1061–75. [PubMed: 7579708]
24. Bretscher A. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. *J Cell Biol* 1989;108:921–30. [PubMed: 2646308]
25. Shiue H, Musch MW, Wang Y, Chang EB, Turner JR. Akt2 phosphorylates ezrin to trigger NHE3 translocation and activation. *J Biol Chem* 2005;280:1688–95. [PubMed: 15531580]
26. Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Tsukita S. Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol* 1998;140:647–57. [PubMed: 9456324]
27. Weinman EJ, Wang Y, Wang F, Greer C, Steplock D, Shenolikar S. A C-terminal PDZ motif in NHE3 binds NHERF-1 and enhances cAMP inhibition of sodium-hydrogen exchange. *Biochemistry* 2003;42:12662–8. [PubMed: 14580213]
28. Lederer ED, Khundmiri SJ, Weinman EJ. Role of NHERF-1 in Regulation of the Activity of Na-K ATPase and Sodium-Phosphate Co-transport in Epithelial Cells. *J Am Soc Nephrol* 2003;14:1711–1719. [PubMed: 12819230]
29. Sneddon WB, Syme CA, Bisello A, Magyar CE, Rochdi MD, Parent JL, Weinman EJ, Abou-Samra AB, Friedman PA. Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50). *J Biol Chem* 2003;278:43787–96. [PubMed: 12920119]
30. Mahon MJ. Ezrin promotes functional expression and parathyroid hormone-mediated regulation of the sodium-phosphate cotransporter 2a in LLC-PK1 cells. *Am J Physiol Renal Physiol* 2008;294:F667–75. [PubMed: 18184743]
31. Li J, Dai Z, Jana D, Callaway DJ, Bu Z. Ezrin controls the macromolecular complexes formed between an adapter protein Na⁺/H⁺ exchanger regulatory factor and the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 2005;280:37634–43. [PubMed: 16129695]
32. Karthikeyan S, Leung T, Birrane G, Webster G, Ladas JA. Crystal structure of the PDZ1 domain of human Na⁽⁺⁾/H⁽⁺⁾ exchanger regulatory factor provides insights into the mechanism of carboxyl-terminal leucine recognition by class I PDZ domains. *J Mol Biol* 2001;308:963–73. [PubMed: 11352585]
33. Karthikeyan S, Leung T, Ladas JA. Structural basis of the Na⁺/H⁺ exchanger regulatory factor PDZ1 interaction with the carboxyl-terminal region of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* 2001;276:19683–6. [PubMed: 11304524]

34. Karthikeyan S, Leung T, Ladas JA. Structural determinants of the Na⁺/H⁺ exchanger regulatory factor interaction with the beta 2 adrenergic and platelet-derived growth factor receptors. *J Biol Chem* 2002;277:18973–8. [PubMed: 11882663]
35. Terawaki, Si; Maesaki, R.; Hakoshima, T. Structural Basis for NHERF Recognition by ERM Proteins. *Structure* 2006;14:777–789. [PubMed: 16615918]
36. Engelman DM, Moore PB. Determination of quaternary structure by small angle neutron scattering. *Annu Rev Biophys Bioeng* 1975;4:219–41. [PubMed: 1098555]
37. Zhao J, Hoyer E, Boylan S, Walsh DA, Trewella J. Quaternary structures of a catalytic subunit-regulatory subunit dimeric complex and the holoenzyme of the cAMP-dependent protein kinase by neutron contrast variation. *J Biol Chem* 1998;273:30448–59. [PubMed: 9804812]
38. Petoukhov MV, Svergun DI. Joint use of small-angle X-ray and neutron scattering to study biological macromolecules in solution. *Eur Biophys J* 2006;35:567–76. [PubMed: 16636827]
39. Trewella J. Structural themes and variations in protein kinase A as seen by small-angle scattering and neutron contrast variation. *Eur Biophys J* 2006;35:585–9. [PubMed: 16625363]
40. Stuhmann HB, Haas J, Ibel K, Koch MHJ, Crichton RR. Low-Angle Neutron-Scattering of Ferritin Studied by Contrast Variation. *Journal of Molecular Biology* 1976;100:399–413. [PubMed: 1255716]
41. Li J, Poulikakos PI, Dai Z, Testa JR, Callaway DJ, Bu Z. Protein kinase C phosphorylation disrupts Na⁺/H⁺ exchanger regulatory factor 1 autoinhibition and promotes cystic fibrosis transmembrane conductance regulator macromolecular assembly. *J Biol Chem* 2007;282:27086–99. [PubMed: 17613530]
42. Glatter, O.; Kratky, O. *Small Angle X-ray Scattering*. Academic Press; New York: 1982.
43. Ho DL, Byrnes WM, Ma WP, Shi Y, Callaway DJ, Bu Z. Structure-specific DNA-induced conformational changes in Taq polymerase revealed by small angle neutron scattering. *J Biol Chem* 2004;279:39146–54. [PubMed: 15247286]
44. Bu Z, Wang L, Kendall DA. Nucleotide binding induces changes in the oligomeric state and conformation of Sec A in a lipid environment: a small-angle neutron-scattering study. *J Mol Biol* 2003;332:23–30. [PubMed: 12946344]
45. Liu X, Hanson BL, Langan P, Viola RE. The effect of deuteration on protein structure: a high-resolution comparison of hydrogenous and perdeuterated haloalkane dehalogenase. *Acta Crystallographica Section D* 2007;63:1000–1008.
46. Jacrot B, Zaccari G. Determination of Molecular Weight by Neutron Scattering. *Biopolymers* 1981;20:2413–2426.
47. Smith WJ, Nassar N, Bretscher A, Cerione RA, Karplus PA. Structure of the active N-terminal domain of Ezrin. Conformational and mobility changes identify keystone interactions. *J Biol Chem* 2003;278:4949–56. [PubMed: 12429733]
48. Svergun DI. Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys J* 1999;76:2879–86. [PubMed: 10354416]
49. Bertrand CA, Frizzell RA. The role of regulated CFTR trafficking in epithelial secretion. *Am J Physiol Cell Physiol* 2003;285:C1–18. [PubMed: 12777252]
50. Alexander RT, Furuya W, Szaszi K, Orłowski J, Grinstein S. Rho GTPases dictate the mobility of the Na/H exchanger NHE3 in epithelia: Role in apical retention and targeting. *PNAS* 2005;102:12253–12258. [PubMed: 16103375]
51. Haggie PM, Stanton BA, Verkman AS. Increased Diffusional Mobility of CFTR at the Plasma Membrane after Deletion of Its C-terminal PDZ Binding Motif. *J Biol Chem* 2004;279:5494–5500. [PubMed: 14660592]
52. Puthenveedu MA, von Zastrow M. Cargo Regulates Clathrin-Coated Pit Dynamics 2006;127:113–124.
53. Morales FC, Takahashi Y, Momin S, Adams H, Chen X, Georgescu MM. NHERF1/EBP50 head-to-tail intramolecular interaction masks association with PDZ domain ligands. *Mol Cell Biol* 2007;27:2527–37. [PubMed: 17242191]
54. Cushing PR, Fellows A, Villone D, Boissguerin P, Madden DR. The relative binding affinities of PDZ partners for CFTR: a biochemical basis for efficient endocytic recycling. *Biochemistry* 2008;47:10084–98. [PubMed: 18754678]

55. Fuentes EJ, Der CJ, Lee AL. Ligand-dependent dynamics and intramolecular signaling in a PDZ domain. *J Mol Biol* 2004;335:1105–15. [PubMed: 14698303]
56. Fuentes EJ, Gilmore SA, Mauldin RV, Lee AL. Evaluation of energetic and dynamic coupling networks in a PDZ domain protein. *J Mol Biol* 2006;364:337–51. [PubMed: 17011581]
57. Cooper A, Dryden DTF. Allostery without conformational change, a plausible model. *Eur Biophys J* 1984;11:103–109. [PubMed: 6544679]
58. Igumenova TI, Frederick KK, Wand AJ. Characterization of the fast dynamics of protein amino acid side chains using NMR relaxation in solution. *Chemical Reviews* 2006;106:1672–1699. [PubMed: 16683749]
59. Hilser VJ, Thompson EB. Intrinsic disorder as a mechanism to optimize allosteric coupling in proteins. *Proc Natl Acad Sci U S A* 2007;104:8311–5. [PubMed: 17494761]
60. Ishikawa H, Kwak K, Chung JK, Kim S, Fayer MD. Direct observation of fast protein conformational switching. *Proceedings of the National Academy of Sciences of the United States of America* 2008;105:8619–8624. [PubMed: 18562286]
61. Swain JF, Gierasch LM. The changing landscape of protein allostery. *Current Opinion in Structural Biology* 2006;16:102–108. [PubMed: 16423525]
62. Lockless SW, Ranganathan R. Evolutionarily conserved pathways of energetic connectivity in protein families. *Science* 1999;286:295–9. [PubMed: 10514373]
63. Bu Z, Neumann DA, Lee SH, Brown CM, Engelman DM, Han CC. A view of dynamics changes in the molten globule-native folding step by quasielastic neutron scattering. *J Mol Biol* 2000;301:525–36. [PubMed: 10926525]
64. Bu Z, Cook J, Callaway DJ. Dynamic regimes and correlated structural dynamics in native and denatured alpha-lactalbumin. *J Mol Biol* 2001;312:865–73. [PubMed: 11575938]
65. Bu Z, Biehl R, Monkenbusch M, Richter D, Callaway DJ. Coupled protein domain motion in Taq polymerase revealed by neutron spin-echo spectroscopy. *Proc Natl Acad Sci U S A* 2005;102:17646–17651. [PubMed: 16306270]
66. Bu ZM, Perlo A, Johnson GE, Olack G, Engelman DM, Wyckoff HW. A small-angle X-ray scattering apparatus for studying biological macromolecules in solution. *Journal of Applied Crystallography* 1998;31:533–543.
67. Bu Z, Engelman DM. A method for determining transmembrane helix association and orientation in detergent micelles using small angle x-ray scattering. *Biophys J* 1999;77:1064–73. [PubMed: 10423450]
68. Glinka C, Barker J, Hammouda B, Krueger S, Moyer J, Orts W. The 30m SANS instruments at NIST. *J Appl Cryst* 1998;31:430–445.
69. Kline SR. Reduction and analysis of SANS and USANS data using IGOR Pro. *Journal of Applied Crystallography* 2006;39:895–900.
70. Semenyuk AV, Svergun DI. GNOM - A program Package for small-angle scattering data-processing. *J Appl Cryst* 1991;24:537–540.
71. Volkov VV, Svergun DI. Uniqueness of ab initio shape determination in small-angle scattering. *Journal of Applied Crystallography* 2003;36:860–864.
72. Wriggers W, Birmanns S. Using situs for flexible and rigid-body fitting of multiresolution single-molecule data. *J Struct Biol* 2001;133:193–202. [PubMed: 11472090]
73. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera - A Visualization System for Exploratory Research and Analysis. *J Comput Chem* 2004;25:1605–1612. [PubMed: 15264254]
74. Fersht, AR. *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*. W.H. Freeman and Company; New York: 1998.
75. Svergun DI, Richard S, Koch MH, Sayers Z, Kuprin S, Zaccai G. Protein hydration in solution: experimental observation by x-ray and neutron scattering. *Proc Natl Acad Sci U S A* 1998;95:2267–72. [PubMed: 9482874]

Figure 1.

Thermodynamic cycle analysis reveals FERM induced long-range inter-domain allostery in the scaffolding protein NHERF1. FERM binding to NHERF1 increases the binding affinities of both PDZ1 and PDZ2 for C-CFTR. (A) The “coupling” energy⁷⁴ for FERM binding to the NHERF1 carboxy-terminus and for NHERF binding to C-CFTR is $\Delta \Delta G = \Delta G_3 - \Delta G_1 = \Delta G_4 - \Delta G_2 = -3.64 \text{ kcal/mol}$. K_d values, taken from our previous publication, are measured by surface plasmon resonance³¹. (B) The coupling energy for FERM binding to PDZ2CT and for PDZ2CT binding to C-CFTR is $\Delta \Delta G = -1.93 \text{ kcal/mol}$. Comparing the coupling energy of C-CFTR binding to the full-length NHERF1 with that of binding to PDZ2CT suggests that FERM binding induces long-range allosteric binding behavior in NHERF. (C) Schematic

representation of the different domains in NHERF1 and in the PDZ2CT construct. The **CT** domain of NHERF includes the EB domain and the region linking PDZ2 and EB.

Figure 2.

Comparing the conformation of NHERF1 with ^dNHERF1, PDZ2CT with ^dPDZ2CT, NHERF1-FERM with ^dNHERF1-FERM, and PDZ2CT-FERM with ^dPDZ2CT-FERM by SAXS. **(A)** $I(Q)$ of NHERF1 (open square) and ^dNHERF1 (filled square). $I(Q)$ of PDZ2CT (open circle) and ^dPDZ2CT (filled circle). **(B)** $I(Q)$ of NHERF1-FERM (open triangle) and ^dNHERF1-FERM (solid triangle), and PDZ2CT-FERM (open diamond) with ^dPDZ2CT-FERM (solid diamond). $I(Q)$ data are scaled by arbitrary factors in order to compare the superimposition of $I(Q)$ of the unlabeled proteins and their deuterium labeled counterparts. **(C)** $P(r)$ functions of NHERF1 and ^dNHERF1, PDZ2CT and ^dPDZ2CT. The symbols are the same as in (A). **(D)** $P(r)$ functions of NHERF1 and ^dNHERF1. **(E)** $P(r)$ functions of PDZ2CT and ^dPDZ2CT. In (D) and (E), the symbols are the same as in (B). $P(r)$ functions are normalized by the forward scattering intensity $I(0)$. The Guinier plots are shown in Figure S2.

Figure 3.

Using contrast variation SANS to determine the stoichiometry of the complexes. $[I(0)/N]^{0.5}$ vs. ρ_o plots of the $^d\text{NHERF1} \cdot \text{FERM}$ (filled square) and $^d\text{PDZ2CT} \cdot \text{FERM}$ (filled circle) complexes. The forward neutron scattering intensity $I(0)$ is obtained from $P(r)$ function fittings. The reduced SANS intensities are on absolute scales.

Figure 4.

FERM binding induces significant conformational changes in $^d\text{NHERF1}$ and $^d\text{PDZ2CT}$. **(A)** Comparing the SAXS data of NHERF1 in solution (open square) with the SANS data of $^d\text{NHERF1}$ in the complex in 42% D_2O (filled square). The insets are the Guinier plots. The lines are fits to the experimental SAXS and SANS data when reconstructing the 3-D shapes *ab initio*, with χ^2 being 1.097 and 1.040 for NHERF1 and $^d\text{NHERF1}$, respectively (see Figure 5). **(B)** $P(r)$ functions of NHERF1 in solution and $^d\text{NHERF1}$ in the complex at the contrast match point. **(C)** SAXS data of PDZ2CT in solution (open circles) and SANS data of $^d\text{PDZ2CT}$ in complex with FERM in 42% D_2O (filled circles). The insets are the Guinier plots. The lines are fits to the experimental SAXS and SANS data when reconstructing the 3-D shapes *ab initio*, with χ^2 being 1.105 and 1.004 for PDZ2CT and $^d\text{PDZ2CT}$, respectively (see Figure 5). The SANS scattering intensity was scaled in order to show both the SAXS and SANS data in the same window. **(D)** $P(r)$ of PDZ2CT in solution from SAXS and that of $^d\text{PDZ2CT}$ in complex with FERM from SANS in 42% D_2O .

Figure 5.

Comparing the 3-D shape of NHERF1 in solution with that of $dNHERF1$ in complex with FERM (upper panel); comparing the 3-D shape of PDZ2CT in solution with that of $dPDZ2CT$ in complex with FERM (lower panel). The crystal structures of PDZ1 (PDB code: 1G9O) and PDZ2 (PDB code: 2OZF) of NHERF1 are docked in the envelopes. The 3-D shape is an average of 10 to 15 models. The NSD values of the models used in generating the averaged shapes of NHERF1 and PDZ2CT range from 0.748 to 1.00 and from 0.564 to 0.642, respectively. The NSD values of the models used in generating the averaged shapes of $dNHERF1$ and $dPDZ2CT$ range from 0.646 to 0.851, and from 0.609 to 0.764, respectively.

Figure 6.

Comparing the conformation of FERM in the complexes with the crystal structure. **(A)** SANS data of the FERM domain of ezrin in complex with dPDZ2CT (filled circle) and with dNHERF1 (filled square) at the 100% D_2O contrast match point. The line is the fit to the experimental SANS data when reconstructing the 3-D shape *ab initio* with χ^2 being 0.9989.

(B) Comparing $P(r)$ functions of the SANS data shown in (A) with those calculated from the crystal structures of FERM domains. Symbols are the same as those shown in (A). The blue solid line is the $P(r)$ calculated from the crystal structures of the FERM domain of ezrin (pdb code: 1NI2), and the red dashed line is the $P(r)$ calculated from the crystal structure of radixin FERM domain in complex with the C-terminal tail of NHERF1 (PDB code: 2D10). The program CRYSON was used to calculate the SANS scattering curve from the crystal structure⁷⁵. **(C)** Rigid-body docking of the crystal structure of the FERM domain of ezrin to the molecular envelope of FERM generated from SANS at the 100% D_2O contrast match point. The 3-D shape of FERM was generated from the SANS data of dNHERF1-FERM in 100% D_2O . The NSD values for 10 models used in generating the averaged shape range from 0.566 to 0.731.

Figure 7. Contrast variation SANS reveals the composite structure of the dNHERF1-FERM complex. (A) SANS data of dNHERF1-FERM (left panel) and dPDZ2CT-FERM (right panel) at various contrasts. The lines are fits to the scattering curves when reconstructing the 3-D composite structure of the complex. The χ^2 values of fitting are shown in the plots. (B) The 3-D models of the dNHERF1-FERM (left panel) and dPDZ2CT-FERM (right panel) reconstructed *ab initio* from contrast variation SANS. The crystal structures of PDZ1 (1G9O), PDZ2 (2OZF), and ezrin FERM domain (PDB code: 2D10) are docked into their respective 3-D envelopes. About 10-15 models generated from the program MONSA are averaged using the program DAMAVER. The averaged models are presented, with $\langle \text{NSD} \rangle = 0.751$ for dNHERF-FERM, and $\langle \text{NSD} \rangle = 0.459$ for dPDZ2CT-FERM

Table I
Size and molecular mass of NHERF1, PDZ2CT, NHERF1-FERM, and PDZ2CT-FERM with their deuterium labeled counterparts

	R_g (SAXS) (Å)	D_{max} (SAXS) (Å)	R_h (DLS) (Å)	M_w Theoretical (kDa)	M_w^* SLS (kDa)	M_w^* contrast variation SANS (kDa)
PDZ2CT	27.3±1.3	85±5	30.8±0.5	23.5	24.5±1.5	
^d PDZ2CT	27.4±0.5	85±5	30.0±0.4	24.8	25.3±2.2	
NHERF1	40.9±0.6	140±5	41.9±0.4	38.6	36.6±1.5	
^d NHERF1	41.0±1.0	140±5	41.4±0.3	40.4	38.0±3.6	
NHERF1-FERM	51.6±0.5	175±5	53.0±0.8	74.4	76.5±9.5	
^d NHERF1-FERM	52.0±0.6	175±5	52.6±0.4	76.2	74.7±8.0	75.4±9.6
PDZ2CT-FERM	44.0±0.3	150±5	45.0±0.5	59.3	58.4±6.5	
^d PDZ2CT-FERM	43.6±0.3	150±5	44.5±1.0	60.6	58.9±8.2	60.4±6.0

* An uncertainty of about 10% in protein concentration is taken into account when estimating the errors in molecular mass.