A Conformational Switch in the Scaffolding Protein NHERF1 Controls Autoinhibition and Complex Formation*

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The mammalian Na+/H+ exchange regulatory factor 1 (NHERF1) is a multidomain scaffolding protein essential for regulating the intracellular trafficking and macromolecular assembly of transmembrane ion channels and receptors. NHERF1 consists of tandem PDZ-1, PDZ-2 domains that interact with the cytoplasmic domains of membrane proteins and a C-terminal (CT) domain that binds the membrane-cytoskeleton linker protein ezrin. NHERF1 is held in an autoinhibited state through intramolecular interactions between PDZ2 and the CT domain that also includes a C-terminal PDZ-binding motif (-SNL). We have determined the structures of the isolated and tandem PDZ2CT domains by high resolution NMR using small angle x-ray scattering as constraints. The PDZ2CT structure shows weak intramolecular interactions between the largely disordered CT domain and the PDZ ligand binding site. The structure reveals a novel helix-turn-helix subdomain that is allosterically coupled to the putative PDZ2 domain by a network of hydrophobic interactions. This helical subdomain increases both the stability and the binding affinity of the extended PDZ structure. Using NMR and small angle neutron scattering for joint structure refinement, we demonstrate the release of intramolecular domain-domain interactions in PDZ2CT upon binding to ezrin. Based on the structural information, we show that human disease-causing mutations in PDZ2, R153Q and E225K, have significantly reduced protein stability. Loss of NHERF1 expressed in cells could result in failure to assemble macromolecular complexes that are important for normal physiological functions.

Dynamic assembly of signaling complexes is, in turn, organized by scaffolding and adapter proteins (1, 2). The mammalian Na+/H+ exchange regulatory factor (NHERF) family of scaffolding proteins assembles macromolecular complexes of transmembrane proteins and regulates receptor signaling and ion transport (3–6). More than 50 biological targets have been identified for NHERF1, and some of these proteins are implicated in human diseases, such as cancer, cystic fibrosis, and chronic kidney disease (7–9). Notable functions of NHERF1 include assembling signaling complexes and regulating the endocytic recycling of the cystic fibrosis transmembrane conductance regulator (CFTR), cell surface adhesion and anti-adhesion proteins, G-protein coupled receptors, and tyrosine kinase receptors (10–14). A recent study that correlates NHERF1 mutations with impaired renal phosphate reabsorption in patient underscores the important physiological role of NHERF1 (7).

NHERF1 consists of two modular protein-protein interaction domains, PDZ1 and PDZ2, which bind to the cytoplasmic domains of transmembrane receptors and channels, and a C-terminal domain that binds ezrin (EB) (Fig. 1). A central feature of NHERF1 is its binding to ezrin and to other ezrin-radixin-moesin (ERM) proteins, forming a communication bridge between plasma membrane proteins and the actin cytoskeleton (15, 16). Ezrin and other ERM proteins are membrane-cytoskeleton linkers that participate in a variety of cytoskeletal-related events, such as cell polarity, intracellular trafficking, cell adhesion, cell motility, and cancer metastasis (15, 17–19). The ERM proteins are autoregulated by intramolecular interactions between the N-terminal 4.1-erzinzadixin/moesin (FERM) domain and the C-terminal actin binding domains (16, 20). In the inactive state, the FERM domain is masked by its own C-terminal domain. Ezrin becomes activated when the intramolecular interactions are disrupted upon phospholipid PIP2 binding and phosphorylation (15). The FERM domain of the activated ezrin binds to target membrane proteins either directly or indirectly through NHERF1 or -2, whereas the C-terminal domain of ezrin interacts with the...
cytoskeletal actin, completing a linkage between cell membranes and the cytoskeletal network.

Remarkably, ezrin modulates the PDZ domains of NHERF1 to assemble multiprotein complexes in a cooperative fashion. A study by Weinman et al. (21) finds that the association of ezrin with NHERF1 is essential for assembling the transmembrane sodium-hydrogen exchanger isofrom 3 (NHE3) complexes and for cAMP-mediated inhibition of NHE3. We have previously shown that when ezrin is bound to the EB domain of NHERF1, the binding affinity of PDZ2 for the C-terminal domain of CFTR increases 24-fold (22). Ezrin binding can also activate NHERF1 to assemble a heterogeneous complex, PTEN, at PDZ1 and β-catenin at PDZ2 (23). A recent study by Mahon (24) finds that ezrin binding to NHERF1 is necessary to promote the co-localization and functional expression of a heterogeneous complex of the transmembrane parathyroid hormone receptor (PTH1R) and the sodium-phosphate cotransporter 2a (NPT2a) at the apical membrane. These studies indicate that ezrin binding to the EB domain enhances the binding of PDZ2 to target proteins and thus positively modulates the full-length NHERF1 to assemble protein complexes. A type I PDZ-binding motif, -SNL$^{358}$, at the C terminus of NHERF1, which overlaps with the EB domain, is hypothesized to interact with PDZ2, thereby impeding PDZ2 from binding to target proteins. The interaction of PDZ2 with the C-terminal (CT) domain of NHERF1 is proposed to function as an autoregulatory molecular switch (25).

The structures of the isolated PDZ1, PDZ2 domains of NHERF1 and the structures of the NHERF1 PDZ1 domain in complex with the C-terminal peptides of membrane receptors and channels have been determined by x-ray crystallography and NMR (26, 27). Crystal structures of the C-terminal EB peptide of NHERF1 bound to the FERM domains of radixin and moesin have also been reported (28, 29). Recently, a NMR study has characterized the interactions between a PDZ2 domain and the C-terminal fragment of NHERF1 (30), supporting the hypothesis that there are intramolecular interactions between PDZ2 and the C terminus of NHERF1 (23, 25). However, these studies did not reveal the structural mechanism of PDZ2 “inhibition” and “activation” of the intact protein. In particular, the apparent weak binding affinity of the putative PDZ2 for target proteins, as reported in many biochemical studies (22, 31), remains unexplained. The free energy of PDZ2 stability and binding cannot be explained from these previous structural studies. To gain insight into the dynamic control of NHERF1 by ezrin, we have determined the solution structure of the combined PDZ2 and the intact C-terminal domains (PDZ2CT) of NHERF1 as well as the conformational changes in PDZ2CT upon forming a large complex with ezrin, using high resolution NMR spectroscopy.

**MATERIALS AND METHODS**

**Protein Expression and NMR Sample Preparation**—The genes for PDZ2$^{240}$ (residues 150–240), CT (residues 242–358), PDZ2$^{270}$ (residues 150–270), PDZ2CT (residues 150–358), and the FERM domain of ezrin (residues 1–300) were inserted in pET151/D-TOPO vector (Invitrogen). The recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells (Novagen, Inc.) and purified according to published methods (22). The epitope with a His$_6$ tag at the N terminus of the protein was cleaved by tobacco etch virus protease. After tobacco etch virus cleavage, all proteins have an extra sequence GIDPFT into the dynamic control of structural studies. To gain insight into the dynamic control of NHERF1 by ezrin, we have determined the solution structure of the combined PDZ2 and the intact C-terminal domains (PDZ2CT) of NHERF1 as well as the conformational changes in PDZ2CT upon forming a large complex with ezrin, using high resolution NMR spectroscopy.

**FIGURE 1.** A, schematic representation of the domains of human NHERF1. The C-terminal end of the EB domain contains a canonical PDZ-binding motif. The graph shows the amino acid positions of the differently truncated domains, which include the putative PDZ2$^{240}$ (residues 150–240), PDZ2$^{270}$ (residues 150–270) with the extra C-terminal helical subdomain, and PDZ2CT (residues 150–358). B, sequence alignment of the PDZ domains of human NHERF1 and NHERF2 proteins, annotated with secondary structure elements. CBL, carboxylate binding loop. The residues involved in ligand binding are highlighted in gray and boldface type. The alignment indicates that the sequence in the extra subdomain formed by α3 and α4 is conserved. The alignment was produced in the ClustalX program (66).
Automatic NOE assignment for all three structures spin 1.3 from Bruker Biospin and analyzed using CARA1.5 structure calculations were obtained from 100-ms mixing time was carried out on a 600

ried out using the program CYANA 2.1 (35, 36). The final

15N-edited and 13C-edited three-dimensional NOESY-HSQC
tion coefficient at 280 nM and typically ranged from 200 to 600

M. Chemical shift assignments and structure determination

For analyzing PDZ2CT in complex with the FERM domain of

270, PDZ2CTcoil PDZ2CThelix PDZ2CTFERM

Angular

13C/15N-labeled Leu, Phe, and Val) PDZ2CT samples were

assigned for PDZ2CT. To alleviate severe overlap of resonances

Solution

The backbone r.m.s. deviation and Ramachandran plot calculated for regular secondary structure elements,

Statistical

PDZ2CTcoil PDZ2CThelix PDZ2CTFERM

Energies

CNS/ARIA PDZ2+270

NMR Spectroscopy—The NMR data were acquired on Bruker AVANCE spectrometers equipped with z-axis gradient triple axis CryoProbes at 30 °C. The data were collected at three different field strengths, 700, 800, and 900 MHz, to maximize resolution and sensitivity. Due to the lower stability of isolated PDZ2CT and CT domains, the NMR data for these two samples were acquired at lower temperature (15 °C).

A standard suite of backbone and side-chain experiments were employed for chemical shift assignment of PDZ2CT, PDZ2+270, and CT domains (32, 33). In summary, 94% of resonances for PDZ2+270 and CT and 87% of resonances were assigned for PDZ2CT. To alleviate severe overlap of resonances from the unstructured CT domain linker, selectively labeled (13C/15N-labeled Leu, Phe, and Val) PDZ2CT samples were used to confirm NMR assignments. Distance restraints for structure calculations were obtained from 100-ms mixing time

15N-edited and 13C-edited three-dimensional NOESY-HSQC (aliphatic and aromatic) spectra.

Structure Calculations—NMR data were processed in Topspin 1.3 from Bruker Biospin and analyzed using CARA1.5 (34). Automatic NOE assignment for all three structures (PDZ2+270, PDZ2CT, and PDZ2CT-FERM complex) were carried out using the program CYANA 2.1 (35, 36). The final

ensemble of PDZ2+270 structures with water refinement was calculated in ARIA 2.2 (37).

The joint NOE refinement of PDZ2CT and PDZ2CT-FERM complex structures against small angle x-ray scattering (SAXS) and small angle neutron scattering (SANS) data was carried out in XPLOR-NIH 2.24. Details of the protocol have been described by Lee et al. (38). The collection and processing of the SAXS and SANS data has been described in detail elsewhere (40). A total of 500 structures were calculated, and the 20 structures with the lowest total energy and NOE violations were analyzed in PROCHECK_NMR by inspecting the Ramachandran plots (39). The structural statistics of the ensemble of the 20 best structures are reported in Table 1. The PDZ2CT structure was calculated with two different models (random coil and helix) for the 13-residue (Met546–Leu558) C-terminal EB peptide. For the helix model, the coordinates of the peptide were extracted from the radixin FERM domain complex (Protein Data Bank code 2D10) structure (29) and docked to the PDZ2CT structure in the program Haddock (69) (details of docking calculations are given in the supplemental material). The restraints between the peptide and protein from the Hadock model were combined with experimental NOE data in a full refinement of PDZ2CT domains. The structure of PDZ2CT bound to the ezrin FERM domain includes helical restraints derived from the x-ray structure of the EB domain peptide complexed with the moesin FERM domain (Protein Data Bank code 1SGH) in the calculations (28). The SAXS and SANS data were from our previous publications (25, 40).

CD Experiments—CD experiments were performed with an Aviv 400 Spectropolarimeter (Aviv Biomedical, Inc., Lakewood, NJ). The protein samples were dissolved at 0.1–0.2
NMR Study of NHERF1 and Ezrin Complex

mg/ml in buffer containing 20 mM phosphate, pH 7.5, 150 mM NaCl, and 1 mM dithiothreitol. Urea-induced unfolding curves were measured by recording the ellipticity at 222 nm on protein samples dissolved at 0.1 mg/ml in urea solutions at different concentrations containing 20 mM potassium phosphate and 150 mM NaCl, pH 7.5, and 1 mM dithiothreitol. The unfolding curves were fit to a two-state model to obtain the C-terminal domain of CFTR (C-CFTR) or the C-terminal domain of NHERF1 (CT). About 3 μl of 10 μg/ml of C-CFTR or 5 μl of 10 μg/ml NHERF1-CT domain dissolved in 10 mM sodium acetate, pH 5.2, was injected to coat the activated surface. The unbound ligand was washed away, and uncoated sites were blocked by 1M ethanolamine at pH 8.5. The analyte concentration to obtain the ΔG values was 0.14 ppm (41). The experiments were performed at 20 °C.

Surface Plasmon Resonance Binding Experiments—Binding experiments were performed using a Biacore 1000 (Biacore Life Sciences, NJ) at 25.0 °C. The Biacore CM5 Biosensor chips were activated by N-hydroxysuccinimide and N-ethyl-N’-(3-dimethylaminopropyl) carbodimide. The ligand is the 70-amino acid residue C-terminal domain of CFTR (C-CFTR) or the C-terminal domain of NHERF1 (CT). About 3 μl of 10 μg/ml of C-CFTR or 5 μl of 10 μg/ml NHERF1-CT domain dissolved in 10 mM sodium acetate, pH 5.2, was injected to coat the activated surface. The unbound ligand was washed away, and uncoated sites were blocked by 1M ethanolamine at pH 8.5. The analyte concentration to obtain the ΔG values was 0.14 ppm (41). The experiments were performed at 20 °C.

RESULTS

Overall, the 1H-15N HSQC spectrum of PDZ2CT (amino acid residues 150–358) (Fig. 2A) is characteristic of a heterogeneous conformation of a structured PDZ domain, coexisting with a largely disordered C-terminal region (Fig. 2B) that typically yields a high density of sharp peaks between 8 and 8.5 ppm in the proton dimension (supplemental Fig. S3). This observation is supported by our CD results of the isolated domains, which show that the PDZ2 domains are structured, but the CT domain is largely disordered (see Fig. 4). The overlay of NMR spectra offers a glimpse into the extent of the perturbation introduced in the structure by intramolecular domain-domain interactions (Fig. 2, A and B). The shifted resonances of the PDZ domain relative to that of the intact PDZ2CT is characteristic of significant changes of intramolecular interactions between domains that are not structurally independent modules (Fig. 2A).

Redefining the Structural Boundary of a PDZ Domain—Primary sequence analysis, using Blast and pfam (42), predicts the “conventional” PDZ2 domain of NHERF1 starts at Leu154 and ends at Val231. However, by analyzing the backbone chemical shifts of PDZ2CT, we find that the structured C-terminal region extends to Asn252, well beyond Val231 at the conventionally predicted boundary for a putative PDZ domain and as reported in the x-ray or the NMR structures of PDZ2 (Protein Data Bank codes 2OZF and 2JXO). We find that the structured C-terminal extension is an integral part of the PDZ2 domain as discussed below. This unexpected finding prompted us to clone and to determine the structure of a longer construct (residues 150–270), henceforth referred to as PDZ2 +270.

A representative NMR structure of PDZ2 +270 is shown in Fig. 3A. The core structure is the conventionally defined PDZ2 fold (residues 150–231) (Fig. 3B), and the backbone superimposes (Cα r.m.s. deviation = 0.9 Å) with that of the putative PDZ2240 structure (Protein Data Bank code 2OZF). The char-
characteristic β-sandwich structure is composed of six strands stacked in an anti-parallel fashion into two β-sheets (Fig. 3A). Strands β1, β6, and β5 are grouped into a single sheet that shares the β4 strand with a second sheet formed by β2 and β3 connected by the two helices α1 and α2. The partially open hydrophobic cavity, enclosed by the β-sandwich, serves as a robust scaffold to recruit peptide-based ligands (Fig. 3B). The conformation of the carboxylate binding (CB) loop (-GYGF-) capable of forming hydrogen bond pairs with the peptide ligand is unaltered in the PDZ2 domain (43). In this example, we see the formation of an entirely different hydrophobic core that involves the distal side of the β4 and β6 strands of PDZ2 by hydrophobic interactions. B, top view of the ligand binding site in PDZ2 with important side chains labeled. C, hydrophobic cluster in the C-terminal helical extension of PDZ2 with important side chains labeled. D, x-ray structure of the third PDZ domain from PSD-95 complexed with the CRIP1 peptide (Protein Data Bank code 1BE9). E, hydrophobic cluster from the C-terminal extension of PDZ3 of PSD-95. The graphics were generated using the University of California San Francisco Chimera package (67).

The novel C-terminal extension (Arg<sup>233</sup>–Asn<sup>252</sup>) consists of two helices α3 and α4, forming a closed hydrophobic cluster at the stem of the putative PDZ fold. The pairwise side-chain interactions include residues Phe<sup>238</sup>, Phe<sup>239</sup>, Val<sup>244</sup>, Ile<sup>245</sup> (α3), and Leu<sup>251</sup> (α4) and those in strands β1 (Leu<sup>154</sup>), β4 (Val<sup>200</sup>) and β6 (Leu<sup>239</sup> and Val<sup>231</sup>) (Fig. 3, A and C). Consequently, an exposed hydrophobic patch on the surface of the β-barrel is effectively buried by the packing of the extra helices in the PDZ2 domain.

To evaluate the effects of this novel helical extension on the overall thermodynamic stability of the extended PDZ2<sup>240</sup>, we have compared the urea unfolding using the CD signals at 222 nm (Fig. 4C and Table 2). The extended PDZ2<sup>240</sup> domain is more stable (ΔΔG<sub>u</sub> = 2.4 kcal mol<sup>-1</sup>) than the canonical structure PDZ2<sup>240</sup>, but only marginally less stable than PDZ2CT (ΔΔG<sub>u</sub> = 3.3 kcal mol<sup>-1</sup>).

Our surface plasmon resonance experiments show that, compared with the putative PDZ2<sup>240</sup>, the extended PDZ2<sup>240</sup> has a 10-fold increase in binding affinities for either the C-terminal domain of CFTR or the C-terminal domain of NHERF1 (Fig. 4D and Table 2). C-CFTR and the CT domain of NHERF1 contain the type I PDZ-binding motifs -DTRL and -FSNL, respectively. By contrast, the affinity of the less stable PDZ2<sup>240</sup> for these PDZ-binding motifs is much weaker and similar to that of the autoinhibited PDZ2CT, where the target binding site is obstructed (Table 2).

These results corroborate with mutational studies of the PDZ domain of PTB-BL, where a correlation between domain stability and target affinity has been identified (45). Because our results show that the C-terminal subdomain extension in PDZ2<sup>240</sup> is at a distance from the active binding site, the increased binding capability is facilitated through a distal allosteric communication mechanism between the two hydrophobic cores instead of direct engagement.

Side-by-side we have compared the αβ type C-terminal extension observed in the third PDZ domain structure from PSD-95 complexed with the CRIP1 peptide (Protein Data Bank code 1BE9) (43). In this example, we see the formation of an entirely different hydrophobic core that involves the distal side of the β2 and β3 strands from the active site (Fig. 3, D and E). The other well known example of an extended PDZ domain is that of the neuronal nitric-oxide synthase with a β-hairpin finger at the C-terminal end. The β-hairpin finger engages the syntrophin PDZ domain directly in an unusual head-to-tail arrangement of the two proteins to form a heterodimer (46, 47). The presence of such extensions adds to the structural diversity of an otherwise homologous family of PDZ domains serving unique roles in each example (43, 46).

The C-terminal helical extension of a PDZ fold appears to be a feature that is shared by many PDZ domains. Based on multiple sequence alignment alone, the C-terminal hydrophobic residues are conserved across all the PDZ domains within the
NHERF family of proteins, suggesting similarly functional roles for this extended helical fold (Fig. 1 and supplemental Fig. S1). With this C-terminal extension, the binding affinity of the extended PDZ1 domain of NHERF1 for C-CFTR is also increased 3.7-fold (Table 2).

Our extensive data base searching and multiple alignments suggest that, outside the NHERF family proteins, the amino acid sequence at the C-terminal sections of the PDZ domains is not well conserved (supplemental Fig. S1). Nevertheless, secondary structure analysis predicts α-helical propensity at the C-terminal end in a majority of the PDZ domains analyzed in supplemental Fig. S1. These include many of the PDZ domains that are important in cell signaling, such as those of the human harmonin, protein-tyrosine phosphatase, tamalin, and PARR3, and PDZ1 of PSD-95, as well as the PDZ domains of Drosophila melanogaster INAD (supplemental Fig. S1). We hypothesize that the helical extension at the C-terminal section of these PDZ domains may tune the stability and/or binding affinity of PDZ-mediated interactions. In addition to regulating the target affinity, the C-terminal extensions to PDZ domains are also known to mediate multimerization in harmonin (48).

We point out that there are exceptions in the subset of PDZ domains that we have analyzed. For instance, the PDZ1, PDZ2, and PDZ5 domains of the multiple PDZ domain protein (MUPP1) and PDZ2 of inactivation no afterpotential D lack the helical structure at their respective C-terminal ends (supplemental Fig. S1). Detailed proteomic (49) and mutational analysis (50) can be interpreted as indicating a large distribution through selectivity space, and these additional structural elements are probably of significance.

**Disease Mutations Affect PDZ2**

—A recent study by Karim et al. (7) identifies the correlation of three NHERF1 mutations, L110V, R153Q, and E225K, with impaired renal phosphate reabsorption in patients with chronic kidney disease. In the proximal tubule of the kidney, the transmembrane sodium-phosphate cotransporter 2a (NPT2a) is responsible for the reabsorption of phosphate from urine. Impaired renal phosphate reabsorption leads to kidney stone formation and bone demineralization. The ability of NPT2a to transport phosphate ions depends on the correct localization of NPT2a at the apical membrane of polarized epithelial cells, which is modulated by the parathyroid hormone (PTH). PTH binding to receptor PTH1R triggers a cascade of cellular signaling events that regulate NPT2a endocytosis and thus the capacity of NTP2a to uptake phosphate. NHERF1 interacts with both NPT2a and PTH1R by binding to the PDZ motifs in their respective cytoplasmic tails. NHERF1 is required for correct apical localization of NPT2a and PTH1R (51, 52). The disease mutations of NHERF1, L110V, R153Q, and E225K, are found to inhibit the renal phosphate transport by NPT2a, in a similar fashion as in NHERF1+/−/− kidney cells (7, 53).

The R153Q and E225K mutations are located in PDZ2 outside the ligand binding sites (Figs. 1 and 5). We have analyzed the effects of R153Q and E225K mutations on the structure and stability of PDZ2.270

### FIGURE 4

PDZ2 shows increased helicity, stability, and binding affinity for ligands compared with the putative PDZ2. A, overlay of CD spectra of PDZ2, PDZ2, and PDZ2CT; B, CD spectrum of isolated CT; C, the urea denaturation curves of PDZ2, PDZ2, and PDZ2CT measured by CD; D, comparison of the binding affinities of C-CFTR for PDZ2, PDZ2, and PDZ2CT. The binding curves are from equilibrium SPR measurements.

### TABLE 2

Comparison of the stability and binding affinity of PDZ2, PDZ2, and PDZ2CT

| PDZ2 variant | Kd binding to C-CFTR | ΔΔGbinding | Kd binding to NHERF1-CT | ΔΔGbinding | M (urea denaturation) | Cm | ΔG | ΔΔGm
|--------------|----------------------|-------------|--------------------------|-------------|----------------------|-----|-----|-------|
| PDZ2.240     | 4.800 ± 0.300         | 0           | 803 ± 64                 | 0           | 0.42 ± 0.20          | 1.9 ± 0.2 | 1.0 ± 0.1 |
| PDZ2.270     | 0.267 ± 0.011         | −1.71       | 93.4 ± 5.9               | −1.28       | 0.83 ± 0.05          | 3.9 ± 0.1 | 3.4 ± 0.1 |
| PDZ2CT       | 5.300 ± 0.200         | 0.06        | 1230 ± 186              | 0.25        | 0.91 ± 0.13          | 4.2 ± 0.1 | 4.3 ± 0.2 |
| PDZ2CT-FERM  | 0.202 ± 0.003         | −1.88       |                         |             |                      |       |     |       |
| PDZ2CT(R153Q)| 0.930 ± 0.045         |            |                         |             |                      |       |     |       |

* Cm is the urea concentration at which the protein is 50% unfolded.
* The free energies have been extrapolated to 0 M urea.
using unfolding studies and NMR. The CD unfolding experiments show that R153Q is considerably less stable ($T_{m}$) than the wild-type protein ($T_{m}$) (Fig. 5D). The higher peak count in the NMR HSQC spectrum of R153Q than that of wild-type PDZ2 reflects the small fraction (7%) of unfolded protein in R153Q at 30 °C with characteristic random coil amide proton shifts (Fig. 5C). Notwithstanding the minor population of the unfolded state, the chemical shift mapping and CD spectra of the predominantly folded conformation of R153Q are structurally similar to those of PDZ2 (Fig. 5C and supplemental Fig. S4, A and C). The deleterious effects of the R153Q mutation can be attributed to the loss of a potential hydrogen bond/salt bridge between the positively charged bidentate Arg$^{153}$ N$^\alpha$ group in strand β1 and the negative charge of the COO$^-$ groups of Asp$^{197}$ (β4) and Asp$^{232}$ at the N terminus of the α2 helix (Fig. 5A). In the wild-type PDZ2, Arg$^{153}$ N$^\alpha$ is involved in hydrogen bond formation, and the attached protons are protected from fast exchange at neutral pH at even 30 °C.

The binding affinity of R153Q for the C-CFTR ligand decreased about 3.5-fold compared with the wild-type PDZ2 (Table 2 and supplemental Fig. S4B). Because the critical residues of the PDZ-binding motif (-ATRL) at the NPT2a C terminus are similar to those of CFTR (-DTRL), we expect that the R153Q mutant also has lower binding affinity for NPT2a than does the wild-type PDZ2. Due to the significant difference in the sequence of PTH1R PDZ-binding motifs (-ETVM) from that in CFTR, we cannot draw the same conclusion, and this is a subject for future investigation.

The E225K mutation has a dramatic effect on the conformational stability of PDZ2 domain, and this mutant fails to express as an intact protein in E. coli at either 37 or 20 °C. In the wild-type protein, the negatively charged Glu$^{225}$ is complemented by the positive charge of Lys$^{158}$ and Lys$^{227}$ on the exposed surface of the α2 helix (Fig. 5B). The unfavorable electrostatic energy of the E225K mutant would destabilize the native protein.
Although these mutations are located outside the ligand binding site of the PDZ2 domain, the mutations R153Q and E225K evidently destabilize the native state and would therefore tend to degrade in a cellular context. Such turnover effects may contribute to pathophysiological effects independent of any changes of ligand affinity. Reduced protein stability could translate into the loss of functional NHERF1 expressed in cells and diminish the available number of NHERF1 molecules to assemble transmembrane protein complexes of NPT2a at the cell membrane. Interestingly, based on sequence alignment, the third NHERF1 mutation L110V is located in the α4 helix (Figs. 1 and 3C) of the C-terminal helix-turn-helix subdomain of PDZ1. Homology modeling (54) using the newly determined structure of PDZ2+270 as a template suggests that L110 is involved in forming the extended hydrophobic network with the putative PDZ1. The mutation to a smaller hydrophobic side chain in L110V could potentially disrupt the packing interactions and reduce PDZ1 stability and/or the affinity of PDZ1 binding.

Structural Model of PDZ2CT—In the isolated PDZ2+270, almost all of the NMR resonances could be assigned, and PDZ2+270 has a defined structure. In contrast, the isolated CT is largely disordered with the exception of Phe^232-Ala^334 (supplemental Fig. S3). In particular, the EB region (Met^346–Leu^358) possessing the C-terminal type I PDZ-binding motif is a random coil (supplemental Fig. S3).

For the tandem PDZ2CT domains, complete backbone and side-chain chemical shift assignments are challenging. About 87% of the resonances are assigned in the isolated CT construct. This is largely due to the unusual relaxation properties at the putative target binding sites of PDZ2 and at the C-terminal end of the CT that presumably interacts with PDZ2. Specifically in PDZ2, the amide resonances along the carboxylate binding loop (Tyr^160 and Gly^165), the β2 strand (Phe^166, Asn^167, Leu^168, His^169, and Ser^170), Glu^171 in the β3 strand, and the α2 helix (His^217) (Figs. 2A and 6A) are lost due to exchange broadening in the intermediate time scale regime. Partial side-chain information was recovered from 13C-edited multidimensional experiments. These results are a strong indication that these residues in PDZ2 could be involved in interacting with CT.

In the CT region of PDZ2CT, residues Phe^323–Ala^334 in the N-terminal end of the EB domain form a short helix (α5 in Fig. 7C) based on chemical shift and NOE analysis, whereas line broadening obliterates resonances from the C-terminal residues (Met^346–Leu^358). Exceptions are the methyl resonances of Leu^358 and Leu^359 that can be assigned. The disordered EB region in the isolated CT domain does not adopt the well formed α-helical conformation when EB peptide is bound to the FERM domain of radixin, as seen in the crystal structure (29). Collectively, these observations suggest that, through weak intramolecular interactions with PDZ2, helical structures are induced and stabilized in the otherwise largely disordered CT domain.

In PDZ2CT, our experiments show that NMR perturbation exists in the extended C-terminal segment Met^346–Leu^358 of 13 residues, suggesting interactions well beyond the short type 1 PDZ-binding motif (~LFSNL^358) in the EB region. The nominal length of the canonical PDZ-binding peptide motifs is only 3–5 residues aligned as an anti-parallel strand with the PDZ β2 strand (Fig. 3B) (26, 43), although longer contacts have been reported (55). By contrast, the binding surface mapped on the PDZ2+270 domain does not increase proportionately in area (Fig. 6A).

Previous biochemical experiments and an NMR study have provided low resolution information about the nature of interactions between the PDZ2 and CT fragments (23, 30). Morales et al. (23) have identified residues Leu^358 and Ser^356 in the type 1 PDZ-binding motif as crucial to interact with PDZ2 because deletion mutant ΔSNL^358 releases autoinhibition in NHERF1. Based on biochemical binding results, Morales et al. (23) have assumed that the length of interaction presented by a helical peptide could match the existing binding pocket in the PDZ2 domain without the necessity of adopting an extended mode of interaction with a β strand. Although direct structural evidence is lacking, helix-breaking mutations (E353P and F355P) are known to release autoinhibitory interactions (23, 30). Additional evidence in favor of a possible helical binding model is the periodic change in the NMR transverse relaxation rates along the length of the putative binding surface of the amphipathic helix measured by Cheng et al. (30).

Because of the largely disordered CT domain, a single "high resolution" structure of the entire PDZ2CT is not an accurate representation of the conformational ensemble. Thus, we have built a suitable model for the coupled domains of PDZ2CT by refining the NMR structure using solution SAXS data as constraints. Several studies have proposed that combining SAXS data with NMR restraints can yield significantly improved accuracy in NMR solution structure determination of multidomain proteins, multimeric assemblies, and tight macromolecular complexes (56–59).

The SAXS data used here are from our recent publication (25). During refinement, the ensemble of PDZ2CT structures was initially calculated assuming the exchange-broadened EB region (Met^346–Leu^358) to be unstructured because this peptide structure is marginally stable (30). The NOEs between the aromatic ring of Phe^166 in PDZ2 and methyl protons of Leu^358 in CT provided the sole restraint for docking the C-terminal residue Leu^358 to the PDZ2+270 domain. The NMR and SAXS jointly refined structures of PDZ2CT are shown in Fig. 7A. Fig. 7B shows the fit of the calculated scattering curve generated from a representative NMR structure to the experimental SAXS data. The ensemble averaged χ value of the SAXS fits is quite small (0.09 ± 0.01), indicating that the joint refinement process was successful. After refinement, the conformational space sampled by the flexible CT domain is restricted to a more limited conformational space than the unrefined ensemble.

The refined model has an average radius of gyration R_g = 26.7 Å, in agreement with the globular PDZ2+270 domain tethered to the extended loop-like structure from the CT domain (Fig. 7A), which also rules out intermolecular interactions between two different PDZ2CT molecules. In the refined structure, the N-terminal end (residues 323–334) of the EB region is disengaged from the PDZ2+270 domain (Fig. 7C). The superposition of the backbone residues (positions 151–257) improves slightly (1.6 Å) when the binding site residues (β2, β3,
α2, and CB loop) are excluded from the r.m.s. deviation calculation (1.1 Å). Hence the β-barrel structure of the PDZ scaffold in the autoinhibited state is intact and well determined despite the conformational flexibility within the binding site.

To further elucidate details of side-chain interactions within the binding site, we made use of ambiguous restraints generated from chemical shift perturbation (Fig. 6A) to dock a well folded helical peptide (Met346–Leu358) using the program Haddock (see supplemental material for details). The C-terminal helical peptide is taken from the crystal structure of the EB peptide in complex with the radixin FERM domain (29). Long range restraints between the domains from the docked structure are used to generate a final ensemble of intact PDZ2CT structures and are refined against the SAXS and NOE data. By modeling the C-terminal peptide (Met346–Leu358) as a helix in the NMR calculations, there is no significant change in the χ value from the SAXS fits (0.11 ± 0.03) whereas the Rg value (26.70 Å) is unchanged. The ensemble of the structures, with the C-terminal end adopting a helical conformation, is shown in the lower panel of Fig. 7A. Fig. 7D shows a representative snapshot of the PDZ2CT structure with the C-terminal EB adopting a helical conformation.

The models so derived suggest that the principal driving force for the interaction is the insertion of the C-terminal leucine (Leu358) within a hydrophobic groove enclosed by the aromatic side chain of Tyr164 and Phe166 (Fig. 6B). The anchor-
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The contribution of other residues in the type 1 PDZ-binding motif, Ser\textsuperscript{356} (−1) and Phe\textsuperscript{355} (−3), can be evaluated by comparing our structural models with the published biochemical data. The structure shows a hydrogen bond pairing between Ser\textsuperscript{356} backbone O and Asn\textsuperscript{167} side-chain N\textsuperscript{62} in the β2 strand whose NMR resonances are selectively broadened in PDZ2CT but are recovered in the PDZ2\textsuperscript{+270} domain. This particular interaction could explain the much higher specificity of the NHERF1 C terminus for the PDZ2 and not the PDZ1 domain of NHERF1 (23). The amino acid sequence in the PDZ1 binding site is nearly identical to that of PDZ2 domain except for His\textsuperscript{27} in PDZ1 and Asn\textsuperscript{167} in PDZ2 (Fig. 1). The imidazole side chain of His\textsuperscript{27} in PDZ1 could be unfavorable for the specific interaction with the backbone oxygen of Ser\textsuperscript{356} (Fig. 1). In addition, mutating Ser\textsuperscript{356} to Ala has a minor impact on binding (23), suggesting that at the −1-position, the interactions of backbone atoms with PDZ2 are more important than the side chain.

The effect of Phe\textsuperscript{355} on binding to PDZ2 is not well understood, although mutating Phe\textsuperscript{355} to a Pro or Arg abrogates auto-

FIGURE 7. Structural model of the intact PDZ2CT from NMR refined against SAXS. A, stereoview of the ensemble NMR structures of PDZ2CT after refinement against SAXS. The backbone of the PDZ2\textsuperscript{+270} domain is shown in black, and the CT domain is shown in blue. Top, the C-terminal EB region is shown in random coil conformation. Bottom, the C-terminal EB region is shown in helix conformation. The images were generated in MOLMOL 2.1 (68). B, length distribution functions $P(r)$ calculated from the NMR structure of PDZ2CT with the EB region adopting helix conformation (red) or random coil conformation (black). The inset shows the fits of the calculated scattering profiles from the NMR structures to the experimental SAXS data (filled squares), with the EB region adopting a helix (red) or random coil (black) conformation. C, a representative ribbon structure of the intact NMR structure of PDZ2CT from A. The C-terminal domain includes the single N-terminal helix (residues 323–334) and the EB region that adopts a random coil conformation. D, a representative ribbon structure of PDZ2CT with the C-terminal EB region adopting a helical conformation and docked into the PDZ2 domain via Leu\textsuperscript{358}.
inhibition of NHERF1 (23). In our structural model, the backbone Phe355 carboxylate O is within hydrogen bond distance of Leu348 amide NH, whereas the aromatic ring is partly buried in a hydrophobic cleft between helix α2 and strand β2, contributing to the overall affinity (supplemental Fig. S6B).

To distinguish the role of the side chain from the backbone, we made a third mutation, F355A, assuming that Ala would stabilize the helix but reduce the contribution of the hydrophobic side chain. The mutation failed to eliminate the interaction with the C-terminal peptide as judged by similarities in the 15N HSQC spectra overlay (Fig. 6C) and three-dimensional NOESY data (not shown) of coupled PDZ2CT domains. Relatively small backbone perturbation of residues in strand β2 and helix α2 is in agreement with limited structural change in the mutant (supplemental Fig. S7B). In contrast, the control spectrum of F355P revealed a dramatic shift of resonances with the release of intramolecular interactions (Fig. 6D and supplemental Fig. S7C). The much smaller methyl group of Ala leads to some loss of binding surface and stability of the helix but is not sufficient to disrupt the EB structure in the same manner as the proline. As a consequence, we recover the intensity of several resonances in the EB region that are lost in the FERM fragment (29) and the F355P mutant, suggesting that residues 353–358 are critical for disrupting the EB structure in the same manner as the proline.

The 15N-labeled PDZ2CT in complex with the unlabeled FERM domain is much smaller than Leu358.

Using the NOE data for residues Arg351–Thr314 and the 15N HSQC data (not shown) of coupled PDZ2CT domains, we estimated that the PDZ2 and FERM domains are separated by a distance of 80 Å and hence not in contact with each other (40).

By determining the functionally important interactions between PDZ2 and the largely disordered CT domain of ezrin. Previous biochemical and biophysical studies have demonstrated that FERM binds to the EB region of PDZ2 (270) and in the PDZ2CT-FERM complex (Fig. 8C) suggests that the PDZ domain is uncoupled in the modular structure once it is released from autoinhibitory interactions. These results are in agreement with the recent SANS studies that have estimated that the PDZ2 and FERM domains are separated by a distance of 80 Å and hence not in contact with each other (40).

Using high resolution NMR, we have determined the structure of tandem PDZ2 plus the C-terminal domains of NHERF1. With joint refinement against SAXS data, the NMR structure provides a more complete view of the interactions between PDZ2 and the largely disordered CT domain than any previous studies on the PDZ fragments of NHERF1. By determining the functionally important PDZ2CT construct, our NMR study reveals new structural features associated with the PDZ fold. Our study thus expands the understanding of the relationship between PDZ stability and ligand binding affinity. Further, we have deter-
mined the structural transition in PDZ2CT upon forming a complex with the FERM domain of ezrin.

The α3 helix-turn-α4 helix subdomain at the C-terminal end of PDZ2 is an integral part of the extended PDZ2 structure of NHERF1. This subdomain is allosterically connected to the putative PDZ fold through hydrophobic side-chain interactions and increases both the thermodynamic stability and the binding affinity of the extended PDZ structure for target proteins. From sequence and secondary structure analyses, we find that the helical C-terminal extension of the PDZ fold is conserved in the PDZ domains of NHERF family proteins (Fig. 1 and supplemental Fig. S1) and is probably a general feature frequently shared by many PDZ domains that are important in cell signaling.

So far, this important structural feature of PDZ domains may have been overlooked due to canonical definitions. The PDZ domains were discovered based on the identity of the conserved GLGF repeat. The region outside the assumed PDZ fold has been generally ignored in structural studies, because the linker region lacks homology in amino acid sequence and is usually assumed to be of low complexity or disordered. This oversight can lead to loss of crucial insight into the source of weak binding affinities, such as those reported for the abridged PDZ2240 domain of NHERF1 (22, 31) and the dependence of PDZ binding affinities on the lengths of truncation (61, 62). Our results suggest that amino acid sequence variation within a PDZ domain or in the PDZ-binding motif is not the only means to render specificity for PDZ-target protein interactions. The structure flanking the core PDZ fold can also influence target peptide binding. The roles of the extended structure(s) may include modification of “dynamic allostery” as suggested for the PDZ3 domain of PSD-95/SAP90 (63).

The difference in the stability and binding affinities between PDZ2+270 and the putative PDZ2240 supports the view that increased stability of PDZ domains may also translate into higher binding affinity for their cellular target proteins (45). The origin of the higher binding affinity of the extended PDZ+270 can be explained by invoking an alloste-
ric mechanism where the energetically coupled hydrophobic network (64), which extends beyond the putative PDZ fold, provides a more structurally sound framework for PDZ to anchor a ligand. The marginally stable PDZ2CT domain pays an unfavorable entropic cost for any structural reorganization during ligand binding and hence has much lower affinity for the target.

We show that the dynamic intramolecular domain-domain interactions in PDZ2CT regulate the binding capability of the PDZ2 domain. Our NMR results indicate that the relatively weak association is characterized by transient “on-off” contacts at the binding sites. Our results do not exclude the possibility that part of the PDZ-binding motif has a propensity for helix formation as postulated in previous studies (23, 30), but strongly suggest that the FSNL PDZ-binding motif at the C terminus is only weakly tethered to the carboxylate binding loop in PDZ2. Any induced structures are transient and marginally stable. These structural results are supported by our thermodynamic data that PDZ2CT is only slightly more stable than PDZ2CT (Fig. 4 and Table 2). The origin of the weak affinity can be traced to equilibrium between ordered and disordered states of the EB region that destabilizes the contacts at the interface.

Further, we provide a structural framework for understanding the mechanism that regulates the binding affinity of PDZ2 by the distal interactions between EB and the FERM domain of ezrin. The FERM domain of ezrin binds with high affinity for the C-terminal EB domain of NHERF1 (22, 29, 60). Upon FERM binding to the EB domain, the affinity of PDZ2 for the target proteins increases dramatically, indicating long range domain-domain couplings (22, 23). The published structural studies of the isolated FERM and PDZ domains offer no explanation for such cooperative binding behaviors in assembling protein complexes.

By combining NMR and small angle scattering experiments, we reveal the details of the conformational transition in PDZ2CT and the corresponding release of the intramolecular domain-domain couplings between the PDZ2 and CT domains upon binding to FERM. The nanomolar binding affinity of the FERM domain drives the competitive displacement of the weak intramolecular PDZ2-EB interactions. The strong binding between FERM and EB also induces a disorder-to-ordered helix conformational transition in the EB region (22, 29, 60). We expect that the large conformational changes in PDZ2CT associated with the release of autoinhibition are compensated for or overcome by the enthalpic effect of the binding reaction.

Both NMR and SANS indicate that PDZ2CT and the FERM domain are far apart in the complex. The largely disordered linker region between PDZ2CT and the EB region thus serves as a flexible spacer between PDZ2CT and FERM in the complex. The exposed PDZ binding site is poised for docking other targets to form a ternary complex.

Our studies provide mechanistic insight into the allosteric regulation of the scaffolding protein NHERF1 by the membrane-cytoskeleton adapter protein ezrin to assemble large macromolecular complexes. Future efforts will determine how the long range forces are propagated (65) across the full-length NHERF, driving the assembly of the multimeric complexes.

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