Protein Kinase C Phosphorylation Disrupts Na\(^+\)/H\(^+\) Exchanger Regulatory Factor 1 Autoinhibition and Promotes Cystic Fibrosis Transmembrane Conductance Regulator Macromolecular Assembly

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It is becoming clear that cell surface receptors and ion transport proteins function as part of larger macromolecular complexes (1–4). The formation of membrane protein oligomers is necessary for the biosynthesis, signal transduction, and function of membrane receptors and ion transport proteins (5–7). Understanding the mechanisms and functional significance of membrane protein assembly is essential for the identification of molecular targets for therapeutic purposes.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride ion channel in several epithelial tissues, where CFTR is responsible for fluid and electrolyte transport across cell membranes (8–10). Malfunction in CFTR ion transport causes devastating human diseases, such as cystic fibrosis and secretory diarrhea (11, 12). CFTR is controlled by ATP hydrolysis and by protein kinase-dependent phosphorylation (13–17), but recent studies suggest that CFTR is also regulated by a network of protein–protein interactions. The multiple protein complexes that interact with CFTR influence the intracellular trafficking of CFTR and coordinate the ion transport functions of CFTR to maintain cellular ion homeostasis, reviewed in Refs. 1, 18, 19.

The macromolecular interactions of CFTR with other proteins are organized by scaffolding proteins. CFTR contains a DTRL motif in the cytoplasmic C terminus that interacts with a class of postsynaptic density 95/disk-large/zonula occluden-1 (PDZ) scaffolding proteins (20–23). PDZ is a protein–protein interaction modular domain that binds to the cytoplasmic domains of a number of transmembrane receptors and ion transport proteins (24, 25). Scaffolding proteins containing multiple PDZ domains assemble and localize large signaling complexes at specific locations in cells (19, 26–29). In particular, the cytoplasmic domain of CFTR binds to the scaffolding protein Na\(^+\)/H\(^+\) exchanger regulatory factor-1 (NHERF) (20, 21). NHERF contains two PDZ domains, PDZ1 and PDZ2.

An emerging theme in cell signaling is that membrane-bound channels and receptors are organized into supramolecular signaling complexes for optimum function and cross-talk. In this study, we determined how protein kinase C (PKC) phosphorylation influences the scaffolding protein Na\(^+\)/H\(^+\) exchanger regulatory factor 1 (NHERF) to assemble protein complexes of cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel that controls fluid and electrolyte transport across cell membranes. NHERF directs polarized expression of receptors and ion transport proteins in epithelial cells, as well as organizes the homo- and hetero-association of these cell surface proteins. NHERF contains two modular PDZ domains that are modular protein–protein interaction motifs, and a C-terminal domain. Previous studies have shown that NHERF is a phosphoprotein, but how phosphorylation affects NHERF to assemble macromolecular complexes is unknown. We show that PKC phosphorylates two amino acid residues Ser-339 and Ser-340 in the C-terminal domain of NHERF, but a serine 162 of PDZ2 is specifically protected from being phosphorylated by the intact C-terminal domain. PKC phosphorylation-mimicking mutant S339D/S340D of NHERF has increased affinity and stoichiometry when binding to C-CFTR. Moreover, solution small angle x-ray scattering indicates that the PDZ2 and C-terminal domains contact each other in NHERF, but such intramolecular domain-domain interactions are released in the PKC phosphorylation-mimicking mutant indicating that PKC phosphorylation disrupts the autoinhibition interactions in NHERF. The results demonstrate that the C-terminal domain of NHERF functions as an intramolecular switch that regulates the binding capability of PDZ2, and thus controls the stoichiometry of NHERF to assemble protein complexes.

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\(^1\)The on-line version of this article (available at http://www.jbc.org) contains supplemental Equations and Tables A.1–A.3.

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\(^3\)The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; DLS, dynamic light scattering; ERM, ezrin-radixin- moesin; FERM, conserved domain shared by protein 4.1, ezrin, radixin, moesin; IEF, isoelectric focusing; NHERF-1, Na\(^+\)/H\(^+\) exchanger regulatory factor-1; NHERF(P339D/S340D), double mutant of P339D and S340D of NHERF; PDZ, postsynaptic density 95/disk-large/zonula occluden-1; PDZ2-CT, truncated construct of NHERF that includes PDZ2 and the C-terminal domain of NHERF; PDZ2-CT(S339D/S340D), double mutant of (S339D and S340D) of PDZ2-CT; PKA, protein kinase A; PKC, protein kinase C; SAXS, small angle x-ray scattering; SLS, static light scattering; SPR, surface plasmon resonance; PDB, Protein Data Bank.
NHERF-1:  
PDZ1:  
PDZ2:  
PDZ2-CT:  
CT:  

FIGURE 1. Summary of the different NHERF-1 constructs used in this study. NHERF-1 has two PDZ domains, PDZ1 and PDZ2 at the N terminus, as well as a C-terminal domain with the ezrin-binding domain (EBD) that recognizes the ezrin-moesin-radixin family of membrane-cytoskeleton adapter proteins. (shown in Fig. 1), and a C-terminal domain that binds to a class of membrane-cytoskeleton adapter proteins, ezrin-radixin-moesin (30–35). NHERF is thus also called ezrin-binding protein 50 (EBP-50). As a member of a family of proteins that contains two or multiple copies of PDZ domains (36, 37), NHERF participates in directing polarized expression of ion channels and transporters within specific membrane domains of epithelial cells, as well as recruiting cell signaling macromolecular complexes (1, 18, 19, 27–30). Notably, recent studies find that NHERF increases the cell surface expression and rescues the function of ΔF508 CFTR (38, 39), a mutant of CFTR that fails to reach the cell surface and causes cystic fibrosis (11).

NHERF assembles the homotypic CFTR-CFTR association, which is thought to increase the channel activities of CFTR (40, 41). NHERF also organizes the heterotypic interactions of CFTR with other channels, receptors, and with intracellular signaling proteins (42–44). For instance, NHERF forms multiprotein complexes that include CFTR, ezrin, and PKA, which implies that NHERF and ezrin bring PKA and CFTR into a macromolecular complex for PKA-mediated phosphorylation of CFTR (45). In addition, NHERF recruits a multiprotein complex that includes CFTR as well as a β2-adrenergic receptor. This hetero-protein complex transmits signals from the agonists of the β2-adrenergic receptor to stimulate CFTR activities (46). Furthermore, NHERF mediates the macromolecular interactions of CFTR with an inwardly rectifying potassium channel, and such interactions may be responsible for coordinated ion transport activities of these channels (42).

NHERF is a phosphoprotein; it was originally isolated from cells in a phosphorylated state (32). Later studies have identified a number of kinases that phosphorylate NHERF. Hall et al. domain (47) finds that a G protein-coupled receptor kinase phosphorylates NHERF at Ser-289 in the C terminus. NHERF is phosphorylated in a cell cycle-dependent manner by cdc2 kinase at Ser-279 and Ser-301 (48). NHERF is also phosphorylated by PKC in response to extracellular signals such as by the stimulation of G protein-coupled receptors (49). However, attempts to identify the PKC phosphorylation sites in NHERF have generated controversial results (50, 51). More importantly, the effects of phosphorylation on the ability of NHERF to assemble protein complexes, although essential, remain unknown. Phosphorylation is thought to induce oligomerization of NHERF that changes the ability of NHERF to assemble signaling complexes (51, 52).

In this study, we identified the effects of PKC phosphorylation on NHERF in assembling CFTR complexes, using biochemical and biophysical methods. Our results demonstrate that there are intramolecular-domain-domain interactions between PDZ2 and the C-terminal domain of NHERF. PKC phosphorylation abolishes the autoinhibitory-like interactions and increases the binding affinity of PDZ2 for its peptide ligand. PKC phosphorylation thus regulates the affinity and stoichiometry of NHERF to assemble macromolecular complexes.

MATERIALS AND METHODS

Protein Expression, Site-directed Mutagenesis, and Protein Purification—The human NHERF cDNA encoding PDZ1 (residues 11–99), PDZ2-(150–240), the C-terminal domain termed CT-(242–358), PDZ2 plus the C-terminal domain termed PDZ2-CT-(150–358), and the full-length NHERF-(11–358) were subcloned into the pET151/D-TOPO vectors (Invitrogen), respectively. The pET151/D-TOPO vector encodes a V5 epitope plus hexahistidine fusion tag at the N terminus of an expressed protein. Using the pET151/D-TOPO vector, we have also expressed and purified the last 70-residue peptide of human CFTR, termed C-CFTR-(1411–1480), which includes the PDZ-binding motif DTRL at the very C terminus. Fig. 1 summarizes the different constructs of NHERF used in this study.

Site-directed mutants in the full-length NHERF and the differentially truncated NHERF domains were generated with the QuikChange site-directed mutagenesis kit (Stratagene). All of the expression vectors were subjected to sequence analysis at the DNA Sequencing Facility at Fox Chase Cancer Center. Cell growth and protein purification have been described previously (34).

PKC Phosphorylation Assays and Isoelectric Focusing Two-dimensional Electrophoresis—PKC phosphorylation assays were performed by adding 25 milliunits of PKC (1 μl) to a 20-μl solution containing 1.0 μg of purified protein in 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 1.0 mM dithiothreitol, 1.7 mM CaCl₂, 0.020 mM ATP, and 14 μCi of [γ-32P]ATP. The catalytic domain of PKC from rat brain was purchased from EMD Biosciences, Inc. The solution was incubated at 30 °C for 30 min. The phosphorylation reaction was terminated by adding 20 μl of protein sample buffer. The samples were resolved by SDS-PAGE on 10% Nu/PAGE gels (Invitrogen). The gels were stained with Coomassie Blue, destained, dried, and exposed to chemiluminescence film (Eastman Kodak Co.) to detect 32P incorporation. The chemiluminescence image was quantified by densitometry, using an Epson Perfection V750 PRO digital scanner and a software UN-SCAN-IT Gel from the Silk Scientific.

Isoelectric focusing (IEF) two-dimensional electrophoresis was also used to identify PKC-induced phosphorylation in the full-length NHERF. Before electrophoresis, the PKC phospho-
rylation reaction was performed by adding 25 milliunits of PKC to a 20-μl solution containing 1.0 μg of purified protein, 0.020 mM ATP, 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 1.0 mM dithiothreitol, and 1.7 mM CaCl₂. The phosphorylation reaction was terminated by adding SDS sample buffer. The phosphorylated protein was resolved by IEF electrophoresis, using unphosphorylated NHERF as control.

The isoelectric points (pI) of unphosphorylated and phosphorylated NHERF were estimated based on the amino acid sequence of the protein and the number of phosphorylated Ser/Thr sites. The pI of unphosphorylated NHERF was calculated using an isoelectric point calculator, assuming pKₐ = 4.3 for Glu, pKₐ = 3.7 for Asp, pKₐ = 10.5 for Lys, and pKₐ = 12.5 for Asn. The pI values of NHERF with different number of Ser/Thr phosphorylation sites were calculated, assuming pKₐ = 2.12 for the first ionization and the pKₐ = 7.21 for the second ionization of a phosphorylation group.

Cell Culture and Delivering Proteins in Mammalian Cells—The BioTrek protein delivery reagent (Stratagene, La Jolla, CA) was used to deliver wild-type NHERF, NHERF(S339D), NHERF(S340D), and NHERF(S339D/S340D) in Calu-3 cells. Purified proteins or protein complexes were diluted to 150 μg/ml using phosphate-buffered saline. About 100 μl of the diluted protein solution was transferred to a tube containing the lyophilized BioTrek reagent. The mixture was resuspended and incubated at room temperature for about 5 min. Serum-free medium was then added to the tube to a final volume of 500 μl.

The Calu-3 cell line was purchased from ATCC (Manassas, VA). Calu-3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO₂ atmosphere. The cells were grown in a 6-well plate with 30-mm-diameter wells. When the cell density reached 50–60% confluence, the culture medium was removed by aspiration. The cells were washed once with serum-free medium. After 500 μl of fresh serum-free medium was added to each well, 500 μl of the BioTrek/protein mixture was added dropwise to the cells. The cell culture plate was then incubated at 37 °C and 5% CO₂ in a humidified incubator for 4–5 h before the immunoprecipitation experiments.

Immunoprecipitation and Immunoblotting Experiments—The Calu-3 cell monolayer in a 30-mm-diameter well was washed with phosphate-buffered saline followed by lysis in radioimmunoprecipitation assay (RIPA) buffer at 4 °C. A mouse anti-V5 antibody (Invitrogen) was used to pull down the protein complex. About 1.0 μg of anti-V5 was added to 500 μl of cell lysate and shaken in a cold room for 4 h. About 20 μl of agarose beads (G PLUS; Santa Cruz Biotechnology) were added to the lysate, and the mixture was shaken in a cold room overnight. The beads were then spun and washed three times with 750 μl of RIPA buffer and then resuspended in 50 μl of SDS-PAGE sample buffer. A mouse anti-hemagglutinin antibody (Invitrogen) was used as a mock primary antibody during immunoprecipitation experiments for negative control to verify the specificity of the antibody used to pull down the protein complex.

After electrophoresis, proteins in the SDS-polyacrylamide gel were transferred to nitrocellulose membranes by semi-dry blotting. The membranes were then blocked in 5% fat-free milk in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 3 h at room temperature. Primary rabbit anti-CFTR antibody (Alomone Labs, Jerusalem, Israel) was used to detect CFTR, and primary mouse anti-V5 antibody was used to detect NHERF. The primary antibody was added to the blots and incubated overnight in blocking solution. The following day the blots were washed three times with TBS-T buffer and then incubated with the secondary antibody (anti-mouse horseradish peroxidase for mouse anti-V5 antibody or anti-rabbit horseradish peroxidase for rabbit anti-CFTR primary antibody) for 1 h at room temperature and washed three times with changes of TBS-T buffer. The blots were then developed by enhanced chemiluminescence detection (Pierce) for analysis. The chemiluminescence image was quantified by densitometry, using an Epson Perfection V750 PRO digital scanner and the software UN-SCAN-IT gel.

Light Scattering Experiments—Light scattering experiments were performed using a DynaPro Molecular Sizing Instrument (Wyatt Technology Corp., Santa Barbara, CA) with a laser of wavelength 824.7 nm at a fixed 90° scattering angle. The DynaPro was used to perform both dynamic light scattering (DLS) and static light scattering (SLS). DLS measures the hydrodynamic radius of the proteins. SLS determines the absolute molecular mass of a protein, which is independent of the size and shape of a protein. Before light scattering experiments, the sample was centrifuged at 10,000 rpm for 5–10 min to remove dust. Protein concentrations were varied from 0.5 to 3 mg/ml during light scattering measurements. The scattering intensity of the buffer background was subtracted from that of the sample solution. Light scattering experiments were performed at 10 °C. The data were analyzed with the software Dynamics version 6.

Surface Plasmon Resonance Binding Experiments and Data Analysis—Surface plasmon resonance (SPR) experiments were performed on a Biacore 1000 instrument (Biacore Life Sciences, NJ) at 25.0 °C. Before the binding experiments, the hydrogel matrix of the BIACore CM5 Biosensor chips was activated by N-hydroxysuccinimide and N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (Biacore Life Sciences, NJ). The ligand, which is 3 μl of 5 μg/ml CFTR dissolved in 10 mM sodium acetate, pH 5.2, was injected to coat the activated surface. Uncross-linked ligand was washed away, and uncoated sites were blocked by 1 M ethanolamine, pH 8.5. The analytes (NHERF or NHERF mutants) were prepared in HBS-EP buffer containing 10 mM HEPES buffer, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant polysorbate 20. The analyte was injected over the C-CFTR-coated surfaces at a series of concentrations at 50 μl/min for 3 min. At the end of each injection, the sensor chip was regenerated with 4.0 M MgCl₂, 50 mM triethylenediamine, pH 9.15, and HBS-EP buffer.

The SPR response curves were obtained by subtracting the background signal generated by injecting the analyte over a control cell without ligand coating to remove the bulk refractive index effects. The nonspecific binding was corrected by subtracting the signal generated by HBS-EP buffer alone. The response curve reached an equilibrium plateau in all cases (shown in Fig. 5A), and the average of the plateau region was taken and plotted as a function of analyte concentrations to
obtain the binding curve (shown in Fig. 5B). For monovalent analyte binding, the dissociation constant \(K_d\) was obtained by fitting the binding curve to a monovalent binding model as shown in Equation 1,

\[
RU = \frac{C^1 \times \text{max}}{C^1 + K_d} \quad \text{(Eq. 1)}
\]

where RU is the response unit when the analyte flows over the ligand-coated sensor chip; \(C^1\) is the concentration of the analyte, and max is the maximum response unit. The \(K_d\) and max values were obtained by nonlinear curve fitting to Equation 1 using the commercial software Origin 6.1 (OriginLab Corp., Northampton, MA).

For bivalent analytes, the three flow cells of a sensor chip were coated with C-CFTR at three different densities by injecting the C-CFTR solution twice to each flow cell at 3 \(\mu\)l of 3 \(\mu\)g/ml, 1 \(\mu\)l of 10 \(\mu\)g/ml, and 3 \(\mu\)l of 20 \(\mu\)g/ml. This operation gave three values of response units of 50, 80, and 400 in the three flow cells, respectively, corresponding to three different coating densities. The analyte was then injected at various concentrations to flow over the C-CFTR-coated chip. After background subtraction, the response unit of the plateau region of each response curve was taken and plotted against the analyte concentrations.

The bivalent binding model can be expressed as shown in Equation (see Supplemental Material for the derivation of Equation 2),

\[
RU = [A]\left(m_1\frac{[L]}{K_{d1}} + m_2\frac{[L]^2}{K_{d1}K_{d2}}\right) \quad \text{(Eq. 2)}
\]

where \(K_{d1}\) and \(K_{d2}\) are the dissociation constants of the two PDZ domains, respectively; \(m_1\) and \(m_2\) are the responses of binding to PDZ1 and PDZ2, respectively, \([A]\) is the analyte concentration, and \([L]\) is the unbound ligand concentration as shown in Equation 3,

\[
[L] = \sqrt{\left[\frac{[A]}{K_{d1}} + 1\right]^2 + \frac{8[A]}{K_{d1}K_{d2}}[L] - \left[\frac{[A]}{K_{d1}} + 1\right]} - \frac{K_{d1}K_{d2}}{4[A]} \quad \text{(Eq. 3)}
\]

with \([L_T]\) being the total ligand concentration. A similar bivalent binding model was described by Herr et al. (53).

The binding curves at all three ligand concentrations were fitted simultaneously (or globally) to Equation 2 to obtain \(K_{d1}\) and \(K_{d2}\). During the fitting process, \(m_1\), \(m_2\), and \(L_T\) were local fitting parameters, whereas \(K_{d1}\), \(K_{d2}\), and \([A]\) were global fitting parameters. The global fitting to Equation 2 was performed using the software Origin 6.1.

Solution Small Angle X-ray Scattering (SAXS)—SAXS experiments were performed with an in-house apparatus, utilizing a MicroMax-TM-007 HF Microfocus rotating anode generator as the x-ray source (Rigaku/MSC, Woodlands, TX). In the present study, a 0.014 (Q) 0.32 \(\text{Å}^{-1}\) range is covered, where \(Q = (4\pi \sin(\theta/2))/\lambda\) is the magnitude of the scattering vector; \(\theta\) is the scattering angle, and \(\lambda\) is the wavelength of the x-ray. Before SAXS experiments, the V5 epitope plus a hexahistidine fusion tag at the N terminus of NHERF and NHERF(S339D/S340D) was cleaved off using the tobacco etch virus protease (Invitrogen) after protein purification. SAXS experiments were performed at 10 °C. The protein concentrations used for SAXS experiments are about 1–2 mg/ml. Light scattering experiments show that, at these protein concentrations, NHERF, NHERF(S339D/S340D), PDZ2-CT, and PDZ2-CT(S339D/S340D) are monomeric, and the inter-molecular interference effects are negligible.

Details about SAXS data reduction and analysis have been described previously (54–56). The reduced scattering data are plotted as scattering intensity \(I(Q)\) versus \(Q\) profiles. The radius of gyration \(R_g\), which is related to the size and shape of a protein, can be obtained from the Guinier approximation (57, 58) as follows: \(\ln(I(Q)) = \ln(I(0)) - (1/3)Q^2R_g^2\) by least squares fitting in the \(QR_g < 1.3\) region. The forward scattering intensity, \(I(0)\), is linearly proportional to the molecular weight of the protein complexes. 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 GROM (59). The three-dimensional molecular envelopes were reconstructed using the \(ab\) initio algorithm developed by Svergun (60) to generate a set of PDB-formatted dummy bead coordinates. The three-dimensional molecular envelopes reconstructed from SAXS were generated using the program package Situs, developed by Wriggers and Birmanns (61).

RESULTS

PKC Phosphorylates Ser-339 and Ser-340 in the C Terminus of NHERF, but the C-terminal Domain Protects Ser-162 in PDZ2 from Being Phosphorylated—To determine whether PKC phosphorylates NHERF, we have performed in vitro kinase assays and IEF two-dimensional electrophoresis. After PKC phosphorylation reactions, the protein samples were resolved by IEF two-dimensional electrophoresis. The IEF two-dimensional gel of the unphosphorylated NHERF is shown in the upper panel of Fig. 2B. The two dots on the left side of the most intense spot indicate that one or two of the amino acid residues in NHERF are deamidated. The lower panel of Fig. 2B is the IEF two-dimensional gel of the phosphorylated NHERF. Compared with unphosphorylated NHERF, the most intense spot of the PKC phosphorylated NHERF has shifted leftward by about 0.2 pH unit (Fig. 2B).

The number of phosphorylation sites in NHERF can first be estimated by examining a relative shift in pH unit of the phosphorylated NHERF to that of the unphosphorylated proteins in the IEF two-dimensional gel. Based on the amino acid sequence of NHERF, the calculated pl of the unphosphorylated NHERF is pl = 5.69, see “Materials and Methods” for PI calculation. When one Ser or Thr is phosphorylated in NHERF, pl of the phosphorylated protein becomes 5.6, which has a 0.09 pH unit difference from the unphosphorylated NHERF in the two-dimensional IEF gel. When two residues in NHERF are phosphorylated, the calculated pl is about 5.51, which gives a 0.19 pH unit difference in the two-dimensional gel. If three Ser and/or Thr groups are phosphorylated, the calculated pl = 5.42, which will be a 0.27 pH unit shift on a two-dimensional gel. Because in
Solution Structure of NHERF-1 by SAXS

FIGURE 2. Identification of PKC phosphorylation sites in NHERF. A, amino acid residues that can be phosphorylated by PKC. The arrow at Ser-162 indicates that this residue can only be phosphorylated in the truncated PDZ2, but not in PDZ2-CT or in the full-length NHERF. B, IEF two-dimensional gel electrophoresis used to detect PKC phosphorylation in the full-length NHERF. The vertical direction represents molecular weight differences, and the horizontal direction signifies pH gradient. Upper panel, unphosphorylated NHERF used as a control. The two dots to the left of the most intense spot indicate that one or two of the amino acid residues in NHERF are likely de-amidated. Lower panel, PKC-phosphorylated NHERF. The phosphorylated spot has shifted leftward by about 0.2 pH unit, suggesting that two amino acid residues are phosphorylated, see text. C, γ-32P incorporation to identify PKC phosphorylation in NHERF and in differently truncated domains of NHERF. Upper panel, γ-32P incorporation by different NHERF mutants after PKC phosphorylation. Lower panel, Coomassie Blue-stained SDS-PAGE. D, upper panel, γ-32P incorporation by different PDZ2 mutants after PKC phosphorylation. Lower panel, Coomassie Blue-stained SDS-PAGE. WT, wild type. E, upper panel, γ-32P incorporation by different CT mutants after PKC phosphorylation. Lower panel, Coomassie Blue-stained SDS-PAGE. F, upper panel, γ-32P incorporation by different PDZ2-CT mutants after PKC phosphorylation. Lower panel, Coomassie Blue-stained SDS-PAGE. G, upper panel, γ-32P incorporation by different NHERF mutants after PKC phosphorylation. Lower panel, Coomassie Blue-stained SDS-PAGE.

Fig. 2B, the pI of PKC-phosphorylated NHERF shifts about 0.2 pH unit leftward, two residues in NHERF are likely to be phosphorylated. The phosphorylation sites are further determined by site-directed mutagenesis and [γ-32P]ATP incorporation experiments after PKC phosphorylation, see below.

To identify which domains in NHERF are phosphorylated by PKC, we performed PKC phosphorylation reactions on the full-length NHERF and on different truncated forms of NHERF in the presence of [γ-32P]ATP. Fig. 2C shows that after PKC and [γ-32P]ATP treatment, the full-length NHERF, PDZ2-CT, and PDZ2 and the C-terminal domain CT incorporate γ-32P, suggesting that these domains are phosphorylated by PKC. Although there is a weak phosphorylation band in PDZ1, the intensity of this band is only about 7.2% that of PDZ2. The probability of PDZ1 to be phosphorylated by PKC is thus very small.

To determine which amino acid residues in PDZ2 and CT are phosphorylated by PKC, we have performed site-directed mutagenesis on possible consensus PKC phosphorylation sites, (S/T)X(K/R), and have examined γ-32P incorporation of these constructs after PKC phosphorylation. In the truncated PDZ2, there are two possible PKC phosphorylation consensus sites, Thr-156 and Ser-170. However, Fig. 2D shows that mutating Thr-156 or Ser-170 to Ala does not reduce the ability of PDZ2 to incorporate γ-32P after PKC treatment. The normalized band intensity of T156A or S170A is nearly the same as that of wild-type PDZ2. T156D or S170D shows a slight decrease in intensity (see supplemental Table A.1 for a quantitative comparison of normalized band intensity). Although another amino acid residue Ser-162 is not a consensus site, we find that S162D or S162A can no longer be phosphorylated by PKC, indicating that Ser-162 is the phosphorylation site. Thus, in the truncated PDZ2 domain, Ser-162 is the primary PKC phosphorylation site. Thr-156 and Ser-170 are not phosphorylation sites, although introducing a charged bulky side chain by an Asp mutation at either Thr-156 or Ser-170 results in PDZ2 having a decreased ability to be phosphorylated, possibly because of long range electrostatic interactions that reduce the probability for Ser-162 to be phosphorylated. An explanation of why Ser-162 but not Thr-156 or Ser-170 is phosphorylated is given under “Discussion.”

In the truncated CT domain, there are four possible consensus PKC phosphorylation sites, Ser-280, Ser-339, Ser-340, and
Ser-349. Fig. 2E summarizes the PKC phosphorylation results of these CT mutants. Mutating Ser-280 or Ser-349 to either Ala or Asp does not apparently decrease the ability for these mutants to be phosphorylated by PKC as compared with the wild-type CT, suggesting that Ser-280 and Ser-349 are not PKC phosphorylation sites.

Fig. 2E shows that intensity of CT(S339A) phosphorylation is about half that of the wild-type CT and that of CT(S339D) is more dramatically decreased (see supplemental Table A.2 for a quantitative comparison of normalized band intensity). Although the CT(S339A) and CT(S339D) mutants have different abilities of being phosphorylated, the phosphorylation intensities of both mutants are reduced as compared with that of the wild-type CT. This result indicates that Ser-339 is one but not the only PKC phosphorylation site in the CT domain. In addition, both CT(S340A) and CT(S340D) show significantly reduced phosphorylation, indicating that Ser-340 is another phosphorylation site. However, the residual band intensity of CT(S340A) is higher than that of CT(S340D), suggesting that introducing a bulky charged side chain at Ser-340 reduces the possibility of phosphorylating the other site in CT. Furthermore, the double mutant CT(S339D/S340D) or CT(S339A/S340A) essentially eliminates the phosphorylation sites in CT. These results thus indicate that, although Ala and Asp mutants have different abilities to reduce PKC phosphorylation, using these mutants can clearly identify that both Ser-339 and Ser-340 are the primary PKC phosphorylation sites in the CT domain.

Nonetheless, Ser-339 and Ser-340 are not identical phosphorylation sites in CT. PKC phosphorylation results of the Ser→Asp mutants of Ser-339 and Ser-340 indicate that if Ser-339 is phosphorylated first, PKC can still access and further phosphorylate Ser-340 (see Fig. 2E). But if Ser-340 is phosphorylated first, the probability for Ser-339 to be phosphorylated is relatively small. This result is also confirmed by the Ser→Ala mutants. The probability of phosphorylating Ser-339 in CT(S340A) is smaller than that of phosphorylating Ser-340 in the CT(S339A) mutant. Our results thus suggest that PKC can phosphorylate both Ser-339 and Ser-340, but Ser-340 has larger probability of being phosphorylated than Ser-339.

The site-directed mutagenesis and phosphorylation experiments on the truncated PDZ2 and CT domains show that there are three PKC phosphorylation sites in NHERF, one at Ser-162 of PDZ2 and the other two at Ser-339 and Ser-340 in the CT domain of NHERF. Curiously, the results from PKC-γ.32P phosphorylation of the truncated individual NHERF domains seem not to agree with the IEF two-dimensional electrophoresis experiments on the full-length NHERF, which have identified two PKC phosphorylation sites in the full-length NHERF. We thus compared the PKC phosphorylation of PDZ2 with that of a PDZ2-CT construct that includes PDZ2 and the intact C terminus of NHERF. Fig. 2F shows that the wild-type PDZ2-CT and PDZ2-CT(S162D) can be phosphorylated by PKC, but neither PDZ2-CT(S339D/S340D) nor PDZ2-CT(S162D/S339D/S340D) is phosphorylated by PKC. These results indicate that, in PDZ2-CT, the CT domain of NHERF protects Ser-162 in PDZ2 from being phosphorylated by PKC, but Ser-339 and Ser-340 in CT are not protected from being phosphorylated by PDZ2.

In Fig. 2F and supplemental Table A.3, the normalized intensity of PDZ2-CT(S339D) is 14%, whereas that of PDZ2-CT(S340D) is 1%, implying that phosphorylation at Ser-340 can reduce the access of Ser-339 to PKC phosphorylation. In addition, the normalized intensity of PDZ2-CT(S340A) or PDZ2-CT(S339A) only has a smaller decrease in phosphorylation intensity than CT(S339A) or CT(S340A), suggesting that the presence of the PDZ2 domain may even enhance the ability of Ser-339 or Ser-340 to be phosphorylated by PKC. It is also interesting to note that both the S339A/S340A double mutant of CT and that of PDZ2-CT show a small portion of phosphorylation, 5 and 23% relative to their respective wild-type constructs. This is likely because of the conformational changes caused by the Ala mutations that enable PKC to access to other sites specifically or nonspecifically. The higher intensity of PDZ2-CT(S339S/S340A) phosphorylation than that of CT(S339A/S340A) may suggest a possible partial exposure of Ser-162 in PDZ2 induced by these Ala mutations.

In the full-length NHERF, the double mutant S339D/S340D completely loses its ability to be phosphorylated by PKC, suggesting either Ser-339 or Ser-340 or both are the phosphorylation sites in NHERF (see Fig. 2G). Moreover, the normalized intensities of the single mutants NHERF(S339D) and NHERF(S340D) are significantly reduced, 14 and 1%, respectively, indicating that both Ser-339 and Ser-340 in NHERF can be phosphorylated. However, because the normalized intensity is not 50% for either NHERF(S339D) or NHERF(S340D) phosphorylation, the phosphorylation of Ser-339 and Ser-340 is not independent but negatively regulates each other. NHERF(S340D) is more effective in obstructing Ser-339 from being phosphorylated than NHERF(S339D) in hampering Ser-340 from being phosphorylated. In addition, we also noticed that the normalized intensities of the NHERF mutants are the same as those of the PDZ2-CT mutants, suggesting that the presence of the PDZ1 domain does not change the environment of Ser-162, Ser-339 or Ser-339 for PKC to access the phosphorylation sites.

To summarize the PKC phosphorylation and mutagenesis experiments, we have shown that PKC can phosphorylate two amino acid residues, Ser-339 and Ser-340, in the CT domain of NHERF. An intact C-terminal domain of NHERF prevents Ser-162 of PDZ2 from being phosphorylated. These results demonstrate that there are intramolecular domain-domain interactions between PDZ2 and CT. This result also supports our previous hypothesis that intramolecular domain-domain interactions between PDZ2 and CT prohibit PDZ2 from binding to C-CFTR (34).

**Wild-type NHERF and the PKC Phosphorylation-mimicking Mutant Adopt Monomeric States**—It is controversial whether phosphorylation induces oligomerization of NHERF that changes its ability to assemble signaling complexes. To determine how PKC phosphorylation affects the self-association of NHERF, we have generated the NHERF(S339D/S340D), NHERF(S339D), and NHERF(S340D) mutants, using the negative charges of the Asp side chain to mimic the negatively charged phosphate group because of phosphorylation. Static
light scattering experiments show that in the concentration range from 0.5 to 3 mg/ml (corresponding to 12–70 μM), the molecular masses of the NHERF and those of the mutants are each about 42.3 kDa, which approximates the theoretical molecular mass of an NHERF monomer (Fig. 3). Dynamic light scattering experiments indicate that both the NHERF and the NHERF(S339D/S340D) mutant are monodispersed. Thus, PKC phosphorylation-mimicking mutants of NHERF adopt a monomeric state, as does the wild-type NHERF. At protein concentrations above 120 μM, NHERF shows a weak association, as suggested by a slightly higher apparent molecular mass, but NHERF(S339D/S340D) shows less tendency to associate than NHERF.

Additionally, the DLS measured hydrodynamic radius of NHERF, \( R_h = 44.3 \pm 0.4 \) Å, is slightly smaller than that of NHERF(S339D/S340D) with \( R_h = 45.1 \pm 0.6 \) Å. Because both dynamic light scattering and gel filtration chromatography indicate that the size of NHERF is smaller than that of NHERF(S339D/S340D) (Fig. 3, lower panel), it is tempting to hypothesize that the PKC phosphorylation-mimicking mutation has caused a conformational change in NHERF. This hypothesis is confirmed by SAXS experiments (see Fig. 7 and Fig. 8).

The PKC Phosphorylation-mimicking Mutant NHERF(S339D/S340D) Displays Increased Stoichiometry of Binding to C-CFTR—Previously, we have shown that binding of the full-length NHERF to C-CFTR can best be fitted with a monovalent model with \( K_d = 288 \pm 32 \) nM, suggesting a 1:1 binding stoichiometry (34). Using truncated constructs, we have also shown that the PDZ1 domain of NHERF binds strongly to C-CFTR, with \( K_d = 298 \pm 10 \) nM, but the binding affinity of PDZ2-CT for C-CFTR is rather weak, with \( K_d = 5300 \pm 200 \) nM (34). These results are consistent with the findings by others who have shown that the PDZ1 domain of NHERF binds to C-CFTR and other ligands more strongly than PDZ2 (20, 21, 44). Here we compare the affinity and stoichiometry of C-CFTR binding to NHERF and to the PKC phosphorylation-mimicking mutant NHERF(S339D/S340D) mutant.

First, to examine the interactions of the full-length CFTR with NHERF(S339D/S340D) in mammalian Calu-3 cells, we have performed immunoprecipitation and immunoblotting experiments. Calu-3 is a human airway epithelial cell line that expresses CFTR endogenously. The wild-type NHERF,
NHERF(S339D), NHERF(S340D), and NHERF(S339D/S340D) were delivered into Calu-3 cells, respectively, as described under “Materials and Methods.” Because NHERF contains an N-terminal V5 epitope tag, an anti-V5 antibody was used to pull down the protein complexes that co-immunoprecipitate with NHERF, NHERF(S339D), NHERF(S340D), and NHERF(S339D/S340D) mutants. Fig. 4 presents an immunoblot of the co-immunoprecipitated CFTR complexes. Before immunoblotting, the same amount of differently immunoprecipitated complexes was loaded for electrophoresis. Comparing the band intensities suggests that CFTR interacts more strongly with NHERF(S339D/S340D) than with the wild-type NHERF.

SPR experiments were performed to compare the binding of C-CFTR to NHERF and to NHERF(S339D/S340D). Fig. 5A is a typical SPR response sensorgram, after injecting the wild-type NHERF of different concentrations on the C-CFTR-coated sensor chips. The average of the plateau region in Fig. 5A was taken to plot the binding curves shown in Fig. 5, B–D. Fig. 5B is the wild-type NHERF to C-CFTR binding curve, which is best fitted by the monovalent binding model of Equation 1 with a dissociation constant $K_d = 288 \pm 32$ nM. Fig. 5C shows the NHERF(S339D/S340D) to C-CFTR binding curves at three C-CFTR coating densities on the sensor chip (described under “Materials and Methods”). A monovalent binding model cannot fit well the binding curves (see Fig. 5C). Using the bivalent model of Equation 2, a global fit to all three binding curves simultaneously gives two $K_d$ values, $K_d1 = 104 \pm 22$ nM and $K_d2 = 302 \pm 33$ nM. Thus, compared with the wild-type NHERF, NHERF(S339D/S340D) exhibits both affinity and stoichiometry change when binding to C-CFTR (see also Table 1).

The truncated construct PDZ2-CT(S339D/S340D) also has increased binding affinity for C-CFTR when compared with the wild-type PDZ2-CT (Fig. 5D), but the magnitude of increase is not as significant as that of the full-length NHERF (Table 1). This is likely due to that in the full-length NHERF, as both the CT domain and PDZ1 influence the binding capability of the PDZ2 domain. In addition, Table 1 demonstrates that mutation in the CT domain increases the binding affinities of both PDZ1 and PDZ2 in the full-length NHERF, suggesting that PDZ1, PDZ2, and the CT domain influence each other in the full-length NHERF. Because SAXS experiments indicate that both NHERF and NHERF(S339D/S340D) adopt an elongated conformation, with PDZ1 and the CT domain being 125 Å apart (Figs. 7 and 8), communications among different domains of NHERF are over a long range.

SPR experiments were also performed to determine the binding of C-CFTR to the single mutants NHERF(S339D) and NHERF(S340D). The results are listed in Table 1. When compared with the double mutant NHERF(S339D/S340D) binding to C-CFTR, we found that the effects of single mutations on binding to C-CFTR are not additive.

Light scattering experiments were performed to determine the molecular masses and the stoichiometry of the C-CFTR-NHERF(S339D/S340D) and the C-CFTR-NHERF complexes. Before light scattering experiments, 54.25 μM C-CFTR was incubated with 9.05 μM NHERF(S339D/S340D) or with 9.05 μM NHERF, corresponding to 6:1 CFTR:NHERF(S339D/S340D) or 6:1 CFTR:NHERF (Table 1). This is likely due to that in the full-length NHERF, as both the CT domain and PDZ1 influence the binding capability of the PDZ2 domain. In addition, Table 1 demonstrates that mutation in the CT domain increases the binding affinities of both PDZ1 and PDZ2 in the full-length NHERF, suggesting that PDZ1, PDZ2, and the CT domain influence each other in the full-length NHERF. Because SAXS experiments indicate that both NHERF and NHERF(S339D/S340D) adopt an elongated conformation, with PDZ1 and the CT domain being 125 Å apart (Figs. 7 and 8), communications among different domains of NHERF are over a long range.

Solution Structure of NHERF-1 by SAXS

FIGURE 5. SPR analysis indicates that NHERF(S339D/S340D) has increased binding affinity for C-CFTR, and the stoichiometry of NHERF(S339D/S340D) binding to C-CFTR is 1:2 as compared with a 1:1 stoichiometry of NHERF to C-CFTR binding. A, representative SPR sensogram (response unit (RU) versus time) for NHERF binding to C-CFTR. At each analyte concentration, the average of the response in the plateau region is taken to obtain the binding curve. B, SPR NHERF to C-CFTR binding curve (response unit (RU) versus concentration) is best fitted by a 1:1 monovalent model (solid line), see Equation 1. C, NHERF(S339D/S340D) to C-CFTR binding curves at three C-CFTR coating densities on the sensor chip. The solid lines are global fit to the bivalent binding model (Equation 2). The dashed lines are fits to the monovalent model Equation 1, which does not describe the binding curve well. D, PDZ2-CT(S339D/S340D) (open triangle) mutant shows higher binding affinity for C-CFTR than does PDZ2-CT (solid triangle). The solid lines are fits to the monovalent model Equation 1.

TABLE 1

| Binding affinity of C-CFTR for different NHERF mutants | $K_{d1}$ | $K_{d2}$ |
|---|---|---|
| NHERF | 288 ± 32 | |
| NHERF(S339D/S340D) | 104 ± 22 | 302 ± 33 |
| NHERF(S339D) | 73 ± 16 | 894 ± 100 |
| NHERF(S340D) | 64 ± 10 | 791 ± 95 |
| PDZ2-CT | 5300 ± 200 | |
| PDZ2-CT(S339D/S340D) | 1600 ± 100 | |
**Solution Structure of NHERF-1 by SAXS**

**FIGURE 6.** Gel filtration and SLS experiments show that NHERF(S339D/S340D) and C-CFTR form a complex with a molecular mass of 65.5 ± 1.0 kDa (solid line), suggesting that the stoichiometry of the complex is 1:2. C-CFTR and NHERF form a complex with a molecular mass of 54.1 ± 0.1 kDa (dashed line), indicating a 1:1 C-CFTR-NHERF complex. Before gel filtration, NHERF and NHERF(S339D/S340D) were incubated with C-CFTR at a molar ratio of 1:6. The peak at 15.8-ml elution volume is from unbound C-CFTR.

SAXS experiments, and have compared the conformational changes in NHERF and in NHERF(S339D/S340D). SAXS determines the overall conformation of proteins or macromolecular complexes (57, 58). Recent advances in ab initio computational reconstruction of real space images of proteins make it possible to determine low resolution molecular envelopes of proteins or protein complexes (62, 63). Here we use SAXS to provide information about the changes in protein domain spatial arrangements in a multidomain scaffolding protein NHERF.

The SAXS data of NHERF and NHERF(S339D/S340D) are presented in the left column of Fig. 7 and in Table 2. SAXS indicates that NHERF is smaller than the double mutant NHERF(S339D/S340D). The larger size of NHERF(S339D/S340D) is evident by comparing the radii of gyration of NHERF(S339D/S340D) with \( R_g = 43.4 ± 0.2 \) Å and NHERF with \( R_g = 40.3 ± 0.3 \) Å. Although the asymmetric \( P(r) \) functions, shown in the left panel of Fig. 7C, indicate that NHERF and NHERF(S339D/S340D) are both elongated molecules, NHERF(S339D/S340D) is more extended than NHERF. The maximum dimension of NHERF (S339D/S340D) is \( D_{\text{max}} = 160 \) Å, whereas that of NHERF is 140 Å.

PKC phosphorylation-mimicking mutation also causes apparent conformational changes in the PDZ2-CT construct. The right column of Fig. 7 shows the SAXS data on the wild-type PDZ2-CT and PDZ2-CT(S339D/S340D). Both Guinier and \( P(r) \) analyses indicate that PDZ2-CT(S339D/S340D) is larger than PDZ2-CT, with \( R_g = 25.9 ± 0.2 \) Å and \( D_{\text{max}} = 85 \) for PDZ2-CT and \( R_g = 28.4 ± 0.4 \) Å and \( D_{\text{max}} = 100 \) Å for PDZ2-CT(S339D/S340D).

Fig. 8 illustrates the ab initio reconstructed three-dimensional molecular envelopes of NHERF and PDZ2-CT from SAXS data. In Fig. 8A, the three-dimensional envelope of NHERF has three lobes, with one lobe at the right end of the molecule to be in contact with the middle lobe. In Fig. 8C, the three-dimensional envelope of PDZ2-CT reveals two lobes of different sizes. The known crystal structure of the PDZ2 domain of NHERF (PDB code 2OZF) can be digitally docked in the small lobe of the PDZ2-CT envelope, and we can assign that the larger lobe as the CT domain of PDZ2-CT. By comparing the shapes of the molecular envelopes of NHERF and PDZ2-CT, we can assign the domains of the full-length NHERF shown in Fig. 8A, with the left lobe as PDZ1, the middle lobe as PDZ2, and the right lobe as the CT domain of NHERF. The crystal structure of the PDZ1 domain of NHERF (PDB code 1G9O) can also be docked in the left lobe of NHERF envelope. It is of interest to note that in both Fig. 8A of wild-type NHERF and Fig. 8C of PDZ2-CT, the assigned CT domain is folded back to be in contact with PDZ2.

The above domain assignment can further be verified by determining the distance between the center-of-mass of the PDZ1 domain and that of PDZ2-CT. Assuming NHERF is composed of PDZ1 and PDZ2-CT, the radius of gyration of the full-length NHERF is given by the parallel axis theorem (64) as shown in Equation 4,

\[
R_g^2 = \omega_1 R_{g1}^2 + \omega_2 R_{g2}^2 + \omega_1 \omega_2 L_{12}^2
\]

where \( R_g \) is the radius of gyration of the full-length NHERF; \( R_{g1} \) and \( R_{g2} \) are those of PDZ1 and PDZ2-CT, respectively. The parameter \( L_{12} \) is the distance between the centers of mass of PDZ1 and PDZ2-CT; \( \omega_1 \) and \( \omega_2 \) are the weight fractions of PDZ1 and PDZ2-CT that can be computed from the molecular mass of the respective fragments. In Equation 4, we ignored the linker connecting PDZ1 and PDZ2-CT. Because \( R_{g1} \) can be calculated from the crystal structure, and \( R_g \) and \( R_{g2} \) are obtained

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from SAXS experiments, Equation 4 gives $L_{12} = 72.3$ Å. The parameter $L_{12}$ can also be measured in the *ab initio* reconstructed envelope of NHERF, which is $L_{12} = 73.8$ Å and agrees with the $L_{12}$ computed from Equation 4. Both the $R_g$ analysis and the reconstructed envelopes thus indicate that PDZ1 is about 72–74 Å away from the PDZ2-CT domain. The parallel axis theorem analysis of the $R_g$ confirms the domain assignment in the full-length NHERF shown in Fig. 8A.

Similarly, the different domains in NHERF(S339D/S340D) can be assigned (see Fig. 8B). The three-dimensional envelope of NHERF (S339D/S340D) also has three lobes, but the assigned CT lobe at the right end is released from being in contact with the middle lobe. Fig. 8D is the molecular envelope of PDZ2-CT(S339D/S340D), which is also more extended than that of PDZ2-CT. Particularly, the extended shape of PDZ2-CT(S339D/S340D) in Fig. 8D is quite similar to the portion assigned as the PDZ2 plus CT domains of NHERF(S339D/S340D) in Fig. 8B.

By comparing the radii of gyration, $P(r)$ functions, the $D_{max}$ values, and the reconstructed three-dimensional envelopes, SAXS shows that the PKC phosphorylation-mimicking mutations have caused a domain rearrangement of the CT domain in NHERF. Together with the SPR, the light scattering, and the gel filtration results, our study indicates that there are intramolecular domain-domain interactions between PDZ2 and the C-terminal domain of NHERF. PKC phosphorylation-mimicking mutations at S339D and S340D disrupt such autoinhibitory interactions.

**DISCUSSION**

We find that PKC phosphorylates NHERF at Ser-339 and Ser-340 in the C-terminal domain. The residue Ser-162 of PDZ2 is only phosphorylated in the truncated PDZ2 domain, but an intact CT domain specifically prevents Ser-162 from being phosphorylated. We further show that introducing phosphorylation-mimicking mutations at Ser-339 and Ser-340 disrupts the intramolecular domain-domain interactions between PDZ2 and the C-terminal domain of NHERF. PKC phosphorylation-mimicking mutations at S339D and S340D disrupt such autoinhibitory interactions.

**TABLE 2**

|                               | $R_g$     | $D_{max}$ |
|-------------------------------|-----------|-----------|
| NHERF                         | 40.3 ± 0.3| 140       |
| NHERF(S339/340D)              | 43.4 ± 0.2| 160       |
| PDZ2-CT                      | 25.9 ± 0.2| 85        |
| PDZ2-CT(S339/340D)           | 28.4 ± 0.4| 100       |

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NHERF without C-terminal domain leads to internalization of NHE3 and abolishes ion transport activities of NHE3. Likewise, truncating the C-terminal domain of NHERF also results in loss of function of the sodium-potassium-ATPase transporter (65). In addition, Sneddon et al. (66) have shown that expressing NHERF in cells inhibits antagonist-induced endocytosis of parathyroid hormone 1 receptor, but truncating the ERM binding domain of NHERF results in otherwise inactive ligands to internalize the parathyroid hormone 1 receptor. These studies thus suggest that the C-terminal domain of NHERF is a critical link that couples membrane proteins to ezrin and to the actin cytoskeletal network.

Previously, we have reported that when ezrin binds to the C-terminal domain of NHERF, PDZ2 is activated to interact with C-CFTR (34), implying that there are intramolecular domain-domain interactions between PDZ2 and the CT domains. In this study, we find that Ser-162 in PDZ2 can only be phosphorylated in the truncated PDZ2, but the presence of an intact C-terminal domain prevents Ser-162 from being phosphorylated. Our results corroborate the study by Fouassier et al. (51) who find that Ser-337/Ser-338 in rat NHERF-1 are phosphorylated by PKC, because the sequence of the last 29 amino acid residues (330–358) of human NHERF-1 and that of the last 29 amino acid residues (328–356) of rat NHERF-1 are identical. Phosphorylation at Ser-339/Ser-340 in the C-terminal domain of NHERF leads to increased binding affinity of PDZ2 for CFTR, possibly because of conformational changes in the flexible linker region between PDZ2 and CT, which causes disruption of the interactions between PDZ2 and CT domain because of long range electrostatic effects. Our results demonstrate that, besides being a critical link between the cell membrane, ezrin, and the actin cytoskeletal network, the C-terminal domain of NHERF also functions as an intramolecular switch that regulates the binding capability of PDZ2, and thus controls the stoichiometry of NHERF to assemble protein complexes.

A recent crystallography study by Terawaki et al. (67) shows that the last 11 amino acid residues at the C terminus of NHERF adopts an α-helical conformation when bound to the FERM domain of radixin (where FERM is the conserved domain shared by protein 4.1, ezrin, radixin, moesin). Upon the completion of this work, the crystal structure of PDZ2 becomes available (PDB code 2OZF). Using the available coordinates of PDZ2 and the C-terminal peptide of NHERF (amino acid residues 339–358), we have performed computational docking of these two structures using the program ZDOCK (68). The docked complex, shown in Fig. 9A, suggests that C-terminal end of the peptide, in particular the hydrophobic side chain of Leu-358, is inserted into the hydrophobic pocket formed by the GYGF loop (amino acid residues 163–166). This GYGF loop is also known as the carboxylate-binding loop and plays a critical role in recognizing the C-terminal amino acid residue of a target peptide (69–71). In the docked structure, the side chain of Ser-162 is masked by the C-terminal peptide. The docked structure thus provides an explanation of how the C-terminal
that the side chain of Thr-156, located on the surface of phosphorylated by PKC. The crystal structure of PDZ2 indicates experiments indicate that Thr-156 and Ser-170 are not phosphorylated by PKC. In the truncated PDZ2, PKC is capable of phosphorylating Ser-162 even though Ser-162 is not a consensus phosphorylation site. Analyzing the crystal structure of truncated PDZ2 reveals that Ser-162 is located in the loop formed between β1 and β2 strands and is exposed to PKC phosphorylation. The program ZDOCK was used for docking the complex. The program Pymol was used to generate the graphics. In addition to the autoinhibition interactions between PDZ2 and the C-terminal domain of NHERF, the hydrogen bond cluster formed among Ser-170, Gln-177, and His-212 in PDZ2 may also impede PDZ2 from binding to target peptides, which contributes to the weak binding capabilities of PDZ2. In particular, PKC phosphorylation is believed to be a very C terminus that interacts with PDZ2, other factors in the CT domain also contribute to regulating the conformation of PDZ2-CT construct have different conformational states and ligand binding capabilities. We hypothesize that besides the phosphorylation-mimicking mutant NHERF(S339D/S340D). It is thus reasonable to contemplate that Ser-162 in the truncated PDZ2 is phosphorylated, but not that in the PDZ2-CT(S339D/S340D) construct. These intriguing results indicate that the truncated PDZ2 and the PDZ2 domain in the PDZ2-CT construct have different conformational states and ligand binding capabilities. We hypothesize that besides the very C terminus that interacts with PDZ2, other factors in the CT domain also contribute to regulating the conformation of PDZ2 for activation.

CFTR is regulated by PKA and PKC. PKC is known to stimulate CFTR channel activities and to enhance CFTR activation (73–76). In particular, PKC phosphorylation is believed to be a prerequisite for the subsequent channel activation by PKA (77) and increases the response of CFTR channel activities to PKA phosphorylation (73–76). PKC can phosphorylate CFTR directly at multiple sites, particularly in the regulatory R

domain of NHERF prevents PDZ2 from binding to its target peptides, and it protects Ser-162 from being phosphorylated by PKC.
Solution Structure of NHERF-1 by SAXS

domain. The findings presented in this study implicate that PKC may also influence CFTR functions indirectly by phosphorylating scaffolding proteins and changing the affinity and stoichiometry of these scaffolding proteins to assemble CFTR complexes.

Although this study reveals the effects of PKC phosphorylation on NHERF binding to CFTR, our results are not limited to understanding the regulation of CFTR by NHERF. Besides binding to CFTR, NHERF interacts with the cytoplasmic domains of a number of receptors, such as the β2-adrenergic receptor, the parathyroid hormone 1 receptor, and the platelet-derived growth factor receptor. In addition, NHERF interacts with a number of other ion transport proteins. PKC phosphorylation of NHERF may also increase the affinity for these PDZ targets, and change the stoichiometry of NHERF binding to these membrane receptors or ion transporters. In the case of β2-adrenergic receptor, a study by Hall et al. (78) has shown that there is association between NHERF and β2-adrenergic receptor under basal cellular conditions. But the interaction between NHERF and β2-adrenergic receptor is enhanced following β2-adrenergic receptor agonist stimulation, which can also activate a variety of signaling cascades, including the PKC signaling pathway (78). This study thus provided early evidence that PKC phosphorylation increases the binding affinity of NHERF to its target proteins, enhancing its ability to assemble macromolecular complexes (78).

Interestingly, NHERF has been shown to interact with merlin, an ERM-related protein and the product of the neurofibromatosis 2 tumor suppressor gene, NF2 (81). PKA has been shown to phosphorylate merlin and promote merlin-ezrin heterodimerization, an event that is suggested to convert merlin from a growth-suppressive to a growth-permissive state (82). It is noteworthy that occasional mutations of the NHERF gene accompanied by loss of heterozygosity have been reported in human breast cancers (83). Mutations occurred at the conserved PDZ domains at the NHERF N terminus or at its C-terminal motif that binds to merlin. NHERF tumorigenic mutations decreased or abolished its interaction with merlin or with the metastasis suppressor, SYK. Taken together, these findings suggest that NHERF may act as a tumor suppressor in human breast carcinoma that may be interconnected to the SYK and merlin suppressors.

PKC can phosphorylate ezrin at Thr-567 in the C-terminal domain, which is believed to contribute to the activation of ezrin (84). The activated ezrin then interacts with the cytoskeletal actin and NHERF, causing NHERF to bind to CFTR (34). A recent x-ray crystallography study by Terawaki et al. (67) of the FERM domain of radixin in complex with the last 20 C-terminal amino acid residues (339–358) of NHERF revealed that residues 339–346 of NHERF form an unstructured loop in the protein complex. SPR-binding studies by the same group show that truncation of residues 331–343 of NHERF decreases its binding affinity for the FERM domain of radixin by a factor of 55. The PKC phosphorylation sites Ser-339 and Ser-340 thus may influence the binding of NHERF to the FERM domain of ERM protein. Further study is needed to determine how PKC phosphorylation influences the interaction between NHERF and ezrin.

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