Role of a PDZ1 domain of NHERF1 in the binding of airway epithelial RACK1 to NHERF1

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Liedtke, Carole M., Viswanathan Raghuram, C. Chris Yun, and Xiangyun Wang. Role of a PDZ1 domain of NHERF1 in the binding of airway epithelial RACK1 to NHERF1. Am J Physiol Cell Physiol 286: C1037–C1044, 2004.—In past studies, we demonstrated regulation of CFTR Cl channel function by protein kinase C (PKC)-ε through the binding of PKC-ε to RACK1 (a receptor for activated C-kinase) and of RACK1 to human Na+/H+ exchanger regulatory factor (NHERF1). In this study, we investigated the site of RACK1 binding on NHERF1 using solid-phase and solution binding assays and pulldown, immunoprecipitation, and slot blot assay. A GST-tagged PDZ1 domain of NHERF1 was 10-fold higher than its binding to GST-tagged PDZ2 domain of NHERF1. PDZ1 binds to RACK1 in a dose-dependent manner and vice versa, with similar binding constants of 1.67 and 1.26 μg, respectively. Interaction of the PDZ1 domain with RACK1 was not blocked by binding of activated PKC-ε to RACK1. A GST-tagged PDZ1 domain pulled down endogenous RACK1 from Calu-3 cell lysate. An internal 11-amino acid motif of the PDZ2 domain did not bind RACK1. Our results indicate binding of Calu-3 RACK1 predominantly to the PDZ1 domain of NHERF1 at a site encompassing the PDZ1 domain and a site on RACK1 distinct from a PKC-ε binding site. CFTR activation by a PKC-generating agent is not affected by loss of RACK1-NHERF1 interaction.

cystic fibrosis; cystic fibrosis transmembrane conductance regulator; protein-protein interaction; slot blot assay; pulldown; PDZ domain; chloride efflux; immunoprecipitation

CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR (CFTR) is an apical cAMP-dependent Cl channel that is expressed in epithelia of the conducting airways, intestine, and pancreas. Genetic mutations of CFTR lead to the disease cystic fibrosis and cause abnormal Cl secretion as well as perturbed transport of other electrolytes. Regulation of CFTR involves its phosphorylation, principally by protein kinase A (PKA), but also by protein kinase C (PKC), and through direct intramembrane or cytoplasmic protein-protein interactions. PKC-dependent regulation of CFTR is subtle, apparently requiring constitutive activity of a Ca2+-independent PKC isotype PKC-ε (9, 13). In more recent studies, we explored an intracellular signaling mechanism linking PKC-ε to regulation of CFTR. The findings revealed that PKC-ε does not bind to CFTR; rather, PKC-ε directly binds to RACK1, a receptor for activated C-kinase (15). Furthermore, we provided the first observation that RACK1 binds to NHERF1, a Na+/H+ exchange regulatory factor.

RACK1 consists of seven highly conserved repeating units, which usually end in Trp-Asp (WD), and a consensus X 6-94 isotope X 6-94 consistent with WD repeats (19). The RACK1 protein is predicted to have a rigid seven-blade β-propeller structure, formed by the WD repeats, and is recognized as a critical cellular scaffold protein (2, 11, 19). The WD repeat is highly conserved in a wide range of species, including plants, invertebrates, animals, higher mammals, and humans. RACK1 is thought to act as a scaffold for anchoring protein. RACK1 binds to a diversity of signaling molecules, including PKC-βII and -ε isotypes (15, 25), phosphodiesterase PDE4D5 (26, 32), Src tyrosine kinases (2), integrin β-subunit (16), type 1 interferon receptor (28), and recombinant pleckstrin homology (PH) domains (24). Although RACK1 is not a substrate for PKC, it is a substrate for tyrosine kinase Src (3) and is tyrosine phosphorylated in cells expressing platelet-derived growth factor (PDGF)-β (2). The WD repeats are implicated in the binding of RACK1 to its protein partners. However, the specific interaction between RACK1 and its binding partners is complex and may involve multiple points of contact between the two proteins. For example, mapping of the binding PDE4D5 to RACK1 by a combination of yeast two-hybrid and NH2-terminal deletion analyses revealed that binding of PDE4D5 to WD repeats five to seven (WD5–WD7) was necessary for RACK1-PDE4D5 interaction (26). Indeed, mutations (Pro to Ala substitution) within this region indicate that the RACK1-PDE4D5 interaction clusters predominantly on the same face of RACK1. In more recent studies, other proteins that interact with RACK1 have now been mapped to particular WD repeats (15, 19, 25).

The interaction of RACK1 with NHERF1 apparently involves direct binding (15). However, as with other binding partners, the site of interaction is not yet known or well understood.

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understood. NHERF1 was first identified as a Na+/H+ exchanger 3 (NHE3) binding protein and subsequently characterized as a regulator of NHE3 activity (6, 30, 33). NHERF1 consists of two tandem PDZ (PSD-95/Disc-large/ZO-1 homology) domains and a COOH-terminal sequence that links NHERF1 to the actin cytoskeleton. NHERF1 serves as a scaffold protein to recruit a wide variety of cellular proteins. NHERF1 binds, with high affinity, to the four COOH-terminal residues of CFTR through either of its two PDZ domains (6, 11, 22, 29). The PDZ domains also interact with other signaling proteins, such as PDGF receptor (18), P2Y purinergic receptor (6), β2-adrenergic receptor (6), and the c-yes-associated protein YAP65 (20). NHERF1 associates with the membrane-cytoskeletal adaptor ERM proteins (ezrin, radixin, merlin, and moesin) through its COOH-terminal 30 amino acids (21, 23), suggesting that NHERF1 may link protein complexes of RACK1 and CFTR to the actin cytoskeleton. The NHERF1/CFTR binding is characterized by a consensus sequence of (D/E)(T/S)XV, a sequence also shared by other binding partners, such as the β-adrenergic receptor and the PDGF receptor. The PDZ domains present a peptide binding groove that interacts with the CFTR (D/E)(T/S)XV sequence (5). The binding site consists of conserved positively charged amino acids in a GYGF loop and readily accepts a COOH-terminal carboxylate group. Although the GYGF loop is typically associated with binding of PDZ domains to their target partners, there is growing evidence of PDZ1 domains binding to internal sequences of their binding partners. One example is the binding of neuronal nitric oxide synthase (nNOS) with the PDZ domain of PD-95 or syntrophin, which does not involve the canonical PDZ-peptide interactions (1, 8). Rather, the PDZ1 domain recognizes an internal motif through constrained motifs or head-to-tail oligomers.

In this study, we determined the site on NHERF1 that binds RACK1. We selected a Calu-3 cell line for study because this human serous cell line expresses CFTR and proteins involved in regulating its function, including NHERF1 (15). In addition, Calu-3 cells have been used by our laboratory (12, 13, 15) and others (9) to study PKC-ε dependent regulation of cAMP-dependent activation of CFTR. Using recombinant RACK1, NHERF1, and PDZ1/2 domains of NHERF1, we have demonstrated that recombinant RACK1 binds to PDZ1 domain of recombinant NHERF1 at an 11-amino acid site that includes a GYGF motif. We have also provided evidence that this motif is involved in the binding of endogenous RACK1 to endogenous NHERF1 and that inhibition of this binding does not affect cAMP-dependent CFTR function.

METHODS

Cell isolation and culture. Calu-3 cells were grown in a submerged cell culture on 100-mm² tissue culture plastic, as described previously (15). Cells were used for experiments when confluence was reached, typically 6–8 days after subculture.

Expression of recombinant proteins. NHERF1 in a pGEX4T-1 vector and PDZ1 (amino acid residues 1–139) and PDZ2 (amino residues 132–299), each in a pGEX6P-1 vector (22), were expressed in overnight cultures of DH5α/H9251. Expression of recombinant proteins was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside and 3–6 h of incubation at 37°C, cells were harvested. Recombinant protein was purified by B-PER (Pierce) extraction, followed by affinity chromatography with glutathione-Sepharose B beads. The fusion protein was evaluated by immunoblot analysis for the GST tag with the use of a polyclonal antibody to GST or, for GST-NHERF1, a polyclonal antibody to NHERF1. Recombinant proteins displayed molecular masses of 50 kDa for GST-PDZ domain and 75 kDa for GST-NHERF1. PDZ domain constructs were kindly provided by V. Raghuram (University of Pennsylvania), and the NHERF1 constructs and monoclonal antibody were provided by C. H. C. Yun (Emory University) (7).

Recombinant RACK1 is a 50-kDa protein expressed in Sf9 insect cells. Insect cells were maintained at 27°C in Grace’s insect medium supplemented with 10% fetal bovine serum and 10 μg/ml gentamicin. Viral stocks were provided by Dr. Susan Brady-Kalnay (Case Western Reserve University) and used to transfect Sf9 cells and to express recombinant human RACK1, as described previously (15). The recombinant RACK1 construct consists of a 1,800-bp fragment containing the full-length human RACK1 cDNA plus a polyhistidine tag, a PKA site, a thrombin cleavage site, and a hemagglutinin (HA) tag. Three days after infection, recombinant RACK1 was isolated from cell lysates by using Talon metal affinity resin and was evaluated by immunoblot assay.

Pulldown analysis. For binding assays, aliquots of recombinant RACK1 (2 μg) were mixed with 0.5 μg of GST-PDZ1 or 2.0 μg of GST-PDZ2 in 70 μl of phosphate-buffered saline (PBS) and incubated at room temperature for 15 min. Anti-HA antibody conjugated to protein G-Sepharose beads was added to the protein mixture, and the incubation continued for 1 h at 4°C. Proteins complexed to the agarose beads were recovered by centrifugation, washed extensively with PBS, subjected to gel electrophoresis on 4–15% gel gradient slab gels, and analyzed by immunoblot analysis for the GST tag of the GST-PDZ domain. Protein bands immunoreactive to specific antibodies were detected by using enhanced chemiluminescence. Exposed bands were quantitated by laser densitometry or by a VersaDoc imaging system (Bio-Rad).

In experiments testing the effect of PKC-ε on PDZ1 binding to RACK1, recombinant RACK1 (2 μg) was incubated with 0.5 μg of GST-PDZ1 alone, in combination with PKC-ε, or with PKC-ε alone. Recombinant human PKC-ε (25 μg) was preactivated by incubation with 30 μg/ml phosphatidyserine and 2 μg/ml diocylglycerol for 15 min at 30°C. After incubation for 30 min at 30°C, anti-HA antibody conjugated to agarose beads was added to pulldown recombinant RACK1, and associated proteins were identified by immunoblot analysis for the GST-tag on PDZ1.

For competition experiments shown in Fig. 5A, 3 μg of recombinant RACK1 coupled to Talon beads were preincubated with a GYGF-PDZ1 peptide, and then 3 μg of NHERF1 were added. Beads were collected by centrifugation, washed, and subjected to 4–15% SDS-PAGE and immunoblot analysis for NHERF1. As a positive control, blots were stripped and reprobed for RACK1.

Pulldown of endogenous RACK1 as shown in Figs. 1B and 5B was performed by using GST-PDZ1 domain or His₆-tagged GYGF-PDZ1 peptide. Total cell lysates were prepared and incubated at room temperature for 30 min with 1–2 μg of recombinant protein. Anti-GST conjugated to agarose beads was used to pulldown GST-PDZ1, and Talon beads were used to pulldown His₆-tagged GYGF-PDZ1 peptide. Beads were recovered by centrifugation, washed extensively with PBS, and resuspended in Laemmli buffer. Samples were heated for 5 min in a boiling water bath, cooled, and then subjected to 4–15% SDS-PAGE and immunoblot analysis for RACK1 by using a monoclonal antibody to RACK1. Endogenous RACK1 was detected as a 37-kDa protein band.

NHERF1 was immunoprecipitated from Calu-3 cells as previously described (15). In brief, cells were grown to confluence, serum deprived overnight, and washed with ice-cold (PBS). Cells were lysed in 1 ml of lysis buffer consisting of 100 mM NaCl, 50 mM NaF, 50 mM Na2HPO4, 0.1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 1 mM Na- vanadate, and protease inhibitor cocktail set III (Calbiochem). Lysates were clarified by pretreatment with agarose beads and then incubated with a polyclonal antibody.
directed against NHERF1. Immune complexes were recovered by using protein A beads. Immune complexes attached to washed beads were heated to boiling for 5 min in Laemmli buffer and then subjected to SDS-PAGE on 4–15% gradient slab gels. Protein bands were transferred to polyvinylidene difluoride (PVDF) membrane paper for immunoblot analysis for RACK1. Protein bands immunoreactive to specific antibodies were detected by using enhanced chemiluminescence.

In vitro binding assays. Binding of recombinant RACK1 to GST-tagged PDZ1 domain and vice versa was achieved by immobilizing 12 μg of one protein onto PVDF membrane paper in a slot blot apparatus and then adding varying concentrations of the second protein. Membrane papers were incubated at room temperature for 20 min, and unbound material was removed by extensive washing. Bound protein was detected by immunoblot analysis, using polyclonal antibodies directed to the HA tag or a monoclonal antibody to RACK1.

To evaluate a putative binding motif in the PDZ domains, we examined peptides encoding an internal GYGF motif of the NHERF1 PDZ domains (5). The sequence was based on human NHERF1 (GenBank accession no. AF036241; Ref. 21). The motif for PDZ1 was KGPGNYGFHLH, and that for PDZ2 was KGPSGYFNLH; each was synthesized with no tag or with a His6, epitope tag at the NH2 terminus. Aliquots of 12 μg of peptide were immobilized on PVDF paper and then overlaid with 0–3 μg of recombinant RACK1. Membrane papers were incubated at room temperature for 20 min, washed extensively to remove unbound recombinant RACK1, and subjected to immunoblot analysis for the HA tag of recombinant RACK1.

CFTR Cl channel function. To measure CFTR function, we measured 36Cl efflux from Calu-3 cells in serum-starved cells untreated or treated with either a BioPORTER peptide delivery system (14) or myristoylated GYGF-PDZ1 peptide (myr-PDZ1). The myr-PDZ1 was dissolved in DMSO and diluted 1:10 in Hank’s balanced salt solution buffered with 10 mM HEPES, pH 7.0 (HPSS). myr-PDZ1 (50 μg) and 36Cl (1.5 μCi) were added to cell monolayers grown on 12-well tissue culture dishes or 24-mm Transwell inserts, followed by a 1-h incubation at 32°C. For BioPORTER delivery of peptide, the BioPORTER reagent was dissolved in methanol, and a 10-μl aliquot per cell monolayer was transferred to a polyethylene vial and dried under a stream of N2. The dried reagent was reconstituted in 50 μl of HPSS per cell monolayer containing an aliquot of peptide in HPSS. The solution was mixed by repeated pipetting, followed by incubation at room temperature for 5 min. The total volume of the peptide-BioPORTER complex was increased to 500 μl in HPSS. For cell monolayers grown on filter inserts, the filters were inverted and basolateral surface flooded with the peptide-BioPORTER complex or BioPORTER reagent alone or with HPSS alone. Cells were incubated for 3 h at 32°C at atmospheric CO2. During the last hour of incubation, 1.5 μCi 36Cl in HBSS was added to the basolateral solution. The solution with radioactive tracer was removed from cultures treated with myr-peptide or with peptide-BioPORTER complex, and cells were washed twice with HPSS, pretreated to 32°C, to remove excess 36Cl. 36Cl efflux was measured as previously described (13). In brief, cell monolayers were incubated for 60 s in 0.5 ml of HBSS, and HBSS was collected for radioactive counts. Sequential aliquots of HPSS were added and removed every 60 s for up to 11 min. The first three aliquots were used to establish a stable baseline in efflux buffer only. Agonists were added after the third aliquot was removed. Inhibitors were present in the bathing medium for the last 15 min of the 36Cl loading period and during the efflux period. Radioactive counts remaining in the cells were extracted with 0.1 N NaOH. The fraction of intracellular 36Cl remaining in the cell layer during each time point was calculated from the sample and extract counts. Time-dependent rates of 36Cl efflux were calculated as ln (36Clinc/36Clmax)÷(t1 – t2), where 36Cl is the percent intracellular Cl at time t and t1 and t2 are successive time points.

Data analysis. Immunoreactive protein bands were quantitated by laser densitometry or by a VersaDoc Imaging System (Bio-Rad). Values are reported as means ± SE. Values are representative of at

Fig. 1. RACK1 (a receptor for activated C-kinase) preferentially binds to PDZ1 domain of NHERF1 (a Na+/H+ exchanger regulatory factor). A: recombinant RACK1 (2.0 μg) was mixed with 0.5 μg of glutathione S-transferase (GST)-PDZ1 (lane 1), 2.0 μg of GST-PDZ2 (lane 2), or no recombinant peptide (lane 3) and incubated at room temperature for 15 min, as described in Methods. Proteins complexed to RACK1 were pulled down (PD) using anti-hemagglutinin (HA) antibody agarose beads. The agarose beads were pelleted by centrifugation, washed extensively with PBS, subjected to gel electrophoresis on 4–15% gradient slab gels, and analyzed by immunoblot analysis for the GST tag of the GST-PDZ domain. Bands were quantitated by laser densitometry (LD). Results are representative of 5 separate experiments with at least duplicate experimental conditions. Analysis by LD, when normalized for the amount of PDZ domain, reveals that recombinant RACK1 pulled down at least 10 times more PDZ1 compared with PDZ2 domain. Controls for nonspecific binding show that GST-tagged fusion proteins were not pulled down with anti-HA-antibody conjugated to agarose beads. WB, Western blot. B: pulldown of endogenous RACK1 by GST-PDZ1 domain. Calu-3 cells were grown to confluence and serum deprived overnight. Total cell lysate (TCL) was prepared and incubated with 3 μg of GST-PDZ1 or GST-PDZ2 domain. Proteins bound to the PDZ domains were recovered by pulldown of GST-tagged protein, using 40 μl of anti-GST antibody conjugated to agarose beads, and analyzed by immunoblot analysis. Duplicate pulldowns are shown. Results are representative of 4 separate experiments, each performed in quadruplicate. RACK1 was detected as a 37-kDa band in a control of TCL (20 μg) and in pulldowns from GST-PDZ1 domain but not PDZ2 domain. In control experiments for nonspecific binding, RACK1 was not pulled down with anti-GST agarose beads.
least three or more experiments, unless otherwise stated, and treatment effects were evaluated with a two-sided Student’s t-test.

Materials. Peptides encoding sequences of partial PDZ domains were synthesized by the Molecular Biotechnology Core Facility of the Cleveland Clinic Institute (Cleveland, OH). Purity and structural integrity of the peptides were confirmed by HPLC, amino acid analysis, and mass spectrometry. Polyclonal anti-PKC-ε, anti-GST, and anti-HA antibodies, anti-GST and anti-HA agarose beads, horseradish peroxidase-coupled secondary antibodies (anti-mouse IgG and anti-rabbit IgG), and protein A-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Baculovirus-expressed recombinant PKC-ε was obtained from Pan Vera (Madison, WI), and anti-mouse RACK1 monoclonal antibody was from Transduction Laboratories (Lexington, KY). Sepharose and agarose beads and tissue culture supplies were purchased from Invitrogen–GIBCO (Gaithersburg, MD), and precast 4–15% gradient slab gels were from Bio-Rad. An enhanced chemiluminescence reagent was purchased from Amersham (Piscataway, NJ). All other chemicals were reagent grade.

RESULTS

Binding of RACK1 to PDZ domains of NHERF1. Direct binding of RACK1 and NHERF1 in Calu-3 cells implies a specific site of interaction on NHERF1. NHERF1 has two PDZ domains, which interact with CFTR (6, 10, 20, 22). To determine whether RACK1 interacts with a PDZ domain, we used recombinant RACK1 and PDZ1 and PDZ2 domains of NHERF1 in a solution binding assay, followed by pulldown of RACK1 (Fig. 1). PDZ domains were detected by immunoblot analysis for a GST tag on the NH2 terminus of the recombinant PDZ domains. As shown in Fig. 1A, PDZ1 and PDZ2 domains were detected in immunoblots, indicating binding to recombinant RACK1. Protein bands were quantitated by laser densitometry and normalized for the amount of recombinant PDZ domain used in the experiment. This analysis revealed that recombinant RACK1 pulled down at least 10 times more PDZ1 domain compared with the PDZ2 domain.

The finding that recombinant RACK1 and PDZ domains of NHERF1 interact in an in vitro assay suggests an interaction with endogenous RACK1. To test this, we used recombinant GST-PDZ1 or GST-PDZ2 domains to pulldown endogenous RACK1 from total cell lysates prepared from Calu-3 cells. A 37-kDa protein band immunoreactive with a monoclonal antibody to RACK1 was detected in total cell lysates (Fig. 1B, top and middle) and in pulldowns with GST-PDZ1 domain (Fig. 1B, top) but not GST-PDZ2 domain (Fig. 1B, middle). Positive controls were run to confirm the presence of the GST-PDZ domain in the pulldown assays (Fig. 1B, bottom). The apparent binding of a GST-PDZ2 domain to recombinant RACK1, but not to endogenous RACK1, could be due to several factors, such as conformation of the recombinant proteins, a low-affinity binding site, association of endogenous proteins with endogenous RACK1, or a combination of several of these factors. On the basis of these results, we focused the next series of experiments on the binding of RACK1 to the PDZ1 domain.

Independent binding of PDZ1 domain and PKC-ε to RACK1. RACK1 preferentially binds activated PKC-ε in Calu-3 cells at the WD6 repeat (15). We next wanted to determine whether the interaction between PKC-ε and RACK1 would modify the binding of the PDZ1 domain to RACK1. Binding studies were performed by solution assay, followed by pulldown of RACK1 and immunoblot analysis for the GST tag on the recombinant PDZ1 domain. We detected binding of PDZ1 to RACK1 in the absence or presence of activated PKC-ε (Fig. 2, top, lanes 1 and 2). Varying activated PKC-ε from 5 to 80 ng (nominal 0.9 to 15 μM) gave the same results (data not shown). To analyze the data, laser densitometry values were obtained and normalized as a ratio of no PKC-ε to PKC-ε. The average value of 1.20 ± 0.24 (n = 8) was not significantly different from 1.0, indicating no significant difference between the two experimental conditions. This finding indicates that the PDZ1 domain binds to RACK1 at a site other than the WD6 repeat. We also ran positive controls to show binding of activated PKC-ε to RACK1 in the absence or presence of GST-PDZ1 domain (Fig. 2, bottom, lanes 2 and 3). Binding properties of RACK1 and PDZ1 domain were next examined with the use of recombinant proteins in a slot blot binding assay. Figure 3 shows binding of the two proteins in a dose-dependent manner. EC50 values of 1.26 μg for recombinant RACK1 and 1.67 μg for GST-PDZ1 suggest a 1:1 binding stoichiometry.

RACK1 binding motif in PDZ1 domain. To determine a binding motif for RACK1, which lacks a PDZ-binding COOH-terminal sequence, we designed an 11-amino acid peptide containing a PDZ-binding COOH-terminal sequence, which lacks a PDZ-binding COOH-terminal sequence, we designed an 11-amino acid peptide
based on the sequence of human NHERF1 PDZ1 domain. The peptide sequence KGPNGYGFHLH contains an NH$_2$-terminal His$_6$ tag and a GYGF motif, which replaces a GLGF sequence found in the earliest recognized PDZ domains (6). As a control, we also tested a peptide based on the sequence of human NHERF1 PDZ2 domain, KGSPGYGFNLH, which also embeds a GYGF motif. The peptides were immobilized onto membrane paper, and direct binding of RACK1 was detected by immunoblot assay for the HA tag on recombinant RACK1. As shown in Fig. 4, top, RACK1 bound to the PDZ1 peptide but not to the PDZ2 peptide. The PDZ peptides differ in the amino acids flanking the GYGF motif, which may influence tertiary structure and could explain the absence of RACK1 binding to the PDZ2 peptide. We also examined binding of RACK1 at amounts ranging from 0.5 to 3 $\mu$g. RACK1 bound to the PDZ1 peptide in a dose-dependent manner (Fig. 4, bottom) with an EC$_{50}$ of 2.4 $\mu$g.

If the GYGF-PDZ1 peptide binds to RACK1, then we predict that the GYGF-PDZ1 peptide should prevent binding of NHERF1 and RACK1. To test this hypothesis, we used a solution binding assay in which RACK1 coupled to Talon metal affinity resin was first incubated with varying concentrations of the GYGF-PDZ1 peptide. GST-tagged NHERF1 was added to the protein mixture, and the incubation continued. RACK1 and proteins associated with it were pulled down by centrifugation. Binding of NHERF1 to RACK1 was detected by immunoblot analysis for the GST tag (Fig. 5A, top). Maximal binding of NHERF1 occurred in the absence of PDZ1 peptide (Fig. 5A, top, lane 1). Increasing concentrations of the PDZ1 peptide blocked binding of NHERF1 to RACK1. The IC$_{50}$ (inhibitory concentration) for the peptide was 0.11 $\mu$M. A positive control for the recombinant RACK1 confirmed the presence of RACK1 in the solution binding assays (Fig. 5A, bottom).

If the PDZ1 peptide encodes a binding site for RACK1, the peptide should bind to endogenous RACK1. We examined this by pulldown assay using a GST-tagged GYGF-PDZ1 peptide and antibodies to GST coupled to agarose beads to recover protein complexes. Figure 5B shows that PDZ1 peptide pulled down endogenous RACK1 from Calu-3 total cell lysates. If RACK1 binds to NHERF1 at the PDZ1 domain, we next predicted that a GYGF-PDZ1 peptide would block the pull-down of RACK1 with immunoprecipitated NHERF1 from Calu-3 cells. This was examined by using untagged GYGF-PDZ1 peptide and, as a control, GYGF-PDZ2 peptide. As shown in Fig. 5C, in the absence of a PDZ peptide, RACK1 was detected as a 37-kDa band in immunoprecipitates of NHERF1 from Calu-3 cells. This was examined by using untagged GYGF-PDZ1 peptide and, as a control, GYGF-PDZ2 peptide. As shown in Fig. 5C, in the absence of a PDZ peptide, RACK1 was detected as a 37-kDa band in immunoprecipitates of NHERF1. Preincubation of Calu-3 total cell lysate with 40 $\mu$g of GYGF-PDZ1 reduced the amount of RACK1 detected in NHERF1 immunoprecipitates by 43.0%. GYGF-PDZ2 peptide did not alter the recovery of RACK1 or affect the GYGF-PDZ1 peptide.

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We next wanted to gain more information on the function of the RACK1-NHERF1 interaction. One possibility is that the interaction between RACK1 and NHERF1 contributes to PKC-\(\epsilon\) regulation of cAMP-dependent CFTR function. This
possibility was investigated by using the GYGF-motif of PDZ1 or PDZ2 domain (12µM) and immobi- 

lized to PVDF membrane paper and overlaid with 0–3µg of recombinant RACK1 in a 50-µl total volume. Unbound material was removed by washing, and bound recom- 

binant RACK1 was detected by immunoblot analysis using an antibody to the HA tag of recombinant RACK1 (top). Results are representative of 2 experi- 

ments; each testing 5–6 concentrations of recombinant RACK1 in triplicate. Exposed bands were quantitated by LD from which an EC50 value was calculated (bottom). Top: binding of triplicate samples of 3µg of recombinant RACK1 and, as a control, 0µg of recombinant RACK1, to PDZ peptides. Recombinant RACK1 was detected in slot blots with PDZ1 peptide but not PDZ2 peptide. Bottom: graphs represent LD values for binding of recombinant RACK1 to PDZ1 peptide. Data are means ± SE of LD values. An EC50 value of 2.4µg was calculated from Hill plots of the LD values.

Fig. 4. Binding of RACK1 to PDZ1 peptide. His6-tagged peptide encoding a GYGF motif of PDZ1 or PDZ2 domain (12µg) was immobilized to PVDF membrane paper and overlaid with 0–3µg of recombinant RACK1 in a 50-µl total volume. Unbound material was removed by washing, and bound recombinant RACK1 was detected by immuno- 

Fig. 5. A: competitive inhibition of recombinant RACK1 and GST-NHERF1 binding by a PDZ1 inhibitory peptide. A mixture of 3µg of recombinant RACK1 coupled to Talon beads and the indicated amounts of His6-KG- 

PONGYGFHLH (PDZ1) peptide was incubated for 20 min at room temperature to allow binding of PDZ1 peptide to recombinant RACK1 binding sites. GST-NHERF1 (3µg) was immediately added to the reaction mixture, and the incubation continued for 20 min at room temperature. Talon beads were collected by centrifugation and washed 5 times in PBS to remove unbound material. Samples were solubilized in Laemmli buffer, heated to boiling for 5 min, and subjected to 4–15% slab gel PAGE and immunoblot analysis for NHERF1 by using a polyclonal antibody to NHERF1 (top). Reprobe of the immunoblots for RACK1 indicates the presence of the same amount of recombinant RACK1 in each pulldown complex. Results are representative of 5 experiments, each testing 4–6 concentrations of PDZ1 inhibitory peptide in duplicate. Top: in the absence of inhibitory peptide, GST-NHERF1 was detected as a 75-kDa protein band (lane 1). Increasing amounts of PDZ1 inhibitory peptide reduced the amount of NHERF1 recovered (lanes 2–4), indicating competitive inhibition. Percent inhibition was calculated from OD values obtained by a VersaDoc imaging system. An IC50 for the inhibitory peptide was calculated as 0.11µM. Bottom: RACK1 migrated as a 50-kDa protein band, as predicted from the molecular mass and additional tags on the recombinant protein. B: pulldown of endogenous RACK1 by GST-GYGF- 

PDZ1 peptide. Calu-3 cells were grown to confluence and serum deprived overnight. TCL was prepared and incubated with 15µg of GST-GYGF-PDZ1 peptide for 40 min at room temperature. Anti-GST antibody conjugated to agarose beads (40µl) was added and incubated with TCL for 1 h at 4°C to pull down the peptide and associated proteins. Unbound material was removed by washing with PBS. Immunoblot analysis for RACK1 was performed on duplicate pulldowns by using a monoclonal antibody to RACK1 and, as a control, 20µg of TCL. Results are representative of 2 separate experiments, each performed in quadruplicate. RACK1 was detected as a 37-kDa band in pulldowns (lanes 1 and 2) and in TCL (lane 3). C: inhibition of communoprecipitation of endogenous RACK1 with NHERF1 by GYGF-PDZ1 peptide. Calu-3 TCL was prepared, as described above, and incubated with 50µg of GYGF-PDZ1 or GYGF-PDZ2 peptide, or both, for 60 min at room temperature. NHERF1 was immunoprecipitated from TCL and subjected to gel electrophoresis on 4–15% gradient slab gels and immunoblot analysis for RACK1. RACK1 was detected in immunoprecipi- 

tates of NHERF1 untreated with GYGF-PDZ1 peptide (lane 1) and in 20µg of TCL (lane 5), as previously reported (15). Pretreatment with GYGF-PDZ1 peptide alone (lane 2) or in combination with GYGF-PDZ2 (lane 4) reduced recovery of RACK1. GYGF-PDZ2 did not affect the recovery of RACK1 (lane 3) or, in combination, prevent the loss of RACK1 with GYGF-PDZ1 (lane 4). Results are representative of 3 separate experiments, each performed in duplicate.
PDZ domain proteins have been shown to mediate protein-protein interactions during receptor and ion channel clustering and to recruit kinases and phosphatases to their membrane-associated substrates. A four-residue COOH-terminal sequence (X-T/S-X-V-COO-) engages the PDZ1 domain through anti-parallel interactions with the β-strand of the domain (5). The peptide binds in a groove formed between βB strand and αB helix. The βA-βB connecting loop contains the GLGF (GYGF in human NHERF1) motif, which has an important role in hydrogen bond coordination with the COOH-terminal carboxylate group of the ligand. CFTR is thought to interact with NHERF1 through its COOH-terminal DTRL sequence (6, 22, 29); this is less likely for RACK1, which lacks a PDZ-binding COOH-terminal motif. There is now considerable evidence that PDZ domains can recognize internal peptides, which are independent of a COOH terminus, and that these internal sites of interaction are important in the assembly of signaling networks. The best-studied internal PDZ ligand is nNOS. The PDZ domain nNOS specifically heterodimerizes with the PDZ domains of PSD-95/98 or α- syntrophin in neurons and muscle cells (1, 8, 27). In the crystal structure of the nNOS-syntrophin PDZ domain complex, the domains interact in a head-to-tail configuration. The nNOS PDZ domain contains a β-hairpin “finger” that docks in the syntrophin peptide-binding groove, thus mimicking a peptide ligand. The sharp β turn apparently replaces the carboxyl terminus. This interaction is one example of an alternative class of PDZ domain interactions that promote participation of PDZ domains in diverse protein networks. PDZ domains are also reported to interact with other distinct protein-binding motifs, including ankyrin repeats (17), spectrin repeats (31), and LIM domains (4). Our finding that PDZ1 domains interact with RACK1, a WD repeat protein, suggests the interaction of NHERF1 and its PDZ1 domain with a new class of binding motifs that may or may not require a negatively charged carboxylate ion. Further structural studies of the GYGF motif should elucidate the determinants that promote binding to RACK1.

Functional studies were also performed to ascertain the physiological significance of the RACK1-NHERF1 interaction. Measurement of CFTR channel activity by 36Cl efflux showed that delivery of GYGF-PDZ1 peptide into Calu-3 cells with the use of two different approaches did not block or enhance cAMP-dependent Cl efflux (Table 1). Our data suggest a model in which GYGF-PDZ1 peptide delivered into Calu-3 cells binds to endogenous RACK1 and, thus, prevents its binding to NHERF1. The data also indicate that binding of RACK1 to NHERF1 is not necessary for PKC-ε-mediated regulation of cAMP-dependent CFTR function. A model derived from earlier studies depicts regulation of CFTR function by constitutive activity of PKC-ε through its binding to RACK1 (9, 13, 15). Because CFTR function is unaffected by GYGF-PDZ1 peptide, we speculate that the physiological function of a PKC-ε-RACK1 complex is uninterrupted or, indeed, may already be completed. In addition, the binding of RACK1 to NHERF1 in Calu-3 cells is likely to have a function different from the regulation of cAMP-dependent CFTR function.

Our data do not allow us, as yet, to discern whether CFTR binds to NHERF1 in vivo and whether RACK1 participates in the regulation of CFTR-NHERF1 binding. One outcome of our study is the identification of the PDZ1 domain of NHERF1 as

### Table 1. cAMP-dependent CFTR function is unaffected by GYGF-PDZ1 peptide

<table>
<thead>
<tr>
<th>Rate of 36Cl efflux, min⁻¹</th>
<th>Untreated</th>
<th>GYGF-PDZ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.138±0.008 (10)</td>
<td>0.121±0.012 (9)</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.277±0.023* (10)</td>
<td>0.260±0.015* (19)</td>
</tr>
<tr>
<td>Epinephrine + chelerythrine</td>
<td>0.119±0.013* (7)</td>
<td>0.127±0.015 (6)</td>
</tr>
</tbody>
</table>

Values are reported as means ± SE for the number of separate cell monolayers analyzed (shown in parentheses). Cell monolayers were preincubated with 50 μg of myristoylated GYGF-PDZ1 peptide and 1.5 μCi 36Cl at 32°C for 1 h. After 45 min of incubation, chelerythrine was added to indicated cell monolayers to a final concentration of 10 μM. CFTR function was measured as the rate of 36Cl efflux, as described in Ref. 13. Rates in epinephrine-stimulated cells represent peak flux, usually 3 or 4 min after addition of epinephrine to a final concentration of 10 μM. *P < 0.0001 compared with vehicle. †P < 0.002 compared with epinephrine-stimulated cells.

**DISCUSSION**

The association of two scaffold proteins, RACK1 and NHERF1, in airway epithelial cells is thought to regulate cAMP-dependent CFTR function (15). The goal of this study was to discern the site of interaction of RACK1 on NHERF1. The results of this study provide evidence for the interaction of RACK1 with the PDZ1 domain of NHERF1 in airway epithelial cells. In a pulldown assay, using lysates from Calu-3 airway cells, endogenous RACK1 was pulled down by recombinant GST-PDZ1 domain but not by recombinant GST-PDZ2 domain (Fig. 1B). However, recombinant RACK1 associated with both recombinant PDZ domains, although with a higher affinity to the GST-PDZ1 domain (Fig. 1A). The binding of RACK1 to GST-PDZ1 and vice versa were dose dependent with similar effective binding constants, suggesting a 1:1 stoichiometry (Fig. 2). RACK1 also binds activated PKC-ε in an in vitro assay and is coimmunoprecipitated with PKC-ε from Calu-3 cells (15). The binding of activated PKC-ε to RACK1 does not prevent the association of the PDZ1 domain with RACK1 (Fig. 3). Indeed, because PKC-ε binds to RACK1 at the WD6 repeat, these results imply that the PDZ1 domain binds at a different region on RACK1.

PDZ domains are composed of six β sheets capped by two α helices, which form a peptide-binding groove that interacts with at least the last four COOH-terminal amino acids of interacting proteins, such as CFTR (6, 22, 29). To determine whether RACK1 interacts with the peptide-binding groove, we obtained an 11-amino acid peptide that embedded the GYGF carboxylate-binding loop of PDZ1. The GYGF motif was obtained from NHERF1 cloned from human lung DNA (6). Whether RACK1 interacts with the peptide-binding groove, we speculate that the physiological function of a PKC-ε-RACK1 complex is uninterrupted or, indeed, may already be completed. In addition, the binding of RACK1 to NHERF1 is not necessary for PKC-ε-mediated regulation of cAMP-dependent CFTR function. A model derived from earlier studies depicts regulation of CFTR function by constitutive activity of PKC-ε through its binding to RACK1 (9, 13, 15). Because CFTR function is unaffected by GYGF-PDZ1 peptide, we speculate that the physiological function of a PKC-ε-RACK1 complex is uninterrupted or, indeed, may already be completed. In addition, the binding of RACK1 to NHERF1 in Calu-3 cells is likely to have a function different from the regulation of cAMP-dependent CFTR function.

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Our data do not allow us, as yet, to discern whether CFTR binds to NHERF1 in vivo and whether RACK1 participates in the regulation of CFTR-NHERF1 binding. One outcome of our study is the identification of the PDZ1 domain of NHERF1 as
a target for drug design. One possible target is a unique internal conformational site that interacts with RACK1. Learning the determinants on RACK1 that are necessary for its interaction with the PDZ1 domain of NHERF1 may allow for the development of potent and useful inhibitors of distinct PDZ domain-mediated interactions.

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