The N-Terminus of the WD5 Repeat of Human RACK1 Binds to Airway Epithelial NHERF1†

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ABSTRACT: Regulation of the CFTR Cl channel function involves a protein complex of activated protein kinase Cϵ (PKCϵ) bound to RACK1, a receptor for activated C kinase, and RACK1 bound to the human Na^+/H^+ exchanger regulatory factor (NHERF1) in human airway epithelial cells. Binding of NHERF1 to RACK1 is mediated via a NHERF1−PDZ1 domain. The goal of this study was to identify the binding motif for human NHERF1 on RACK1. We examined the site of binding of NHERF1 on RACK1 using peptides encoding the seven WD40 repeat units of human RACK1. One WD repeat peptide, WD5, directly binds NHERF1 and the PDZ1 domain with similar EC_{50} values, blocks binding of recombinant RACK1 and NHERF1, and pulls down endogenous RACK1 from Calu-3 cell lysate in a dose-dependent manner. The remaining WD repeat peptides did not block RACK1−NHERF1 binding. An 11-amino acid peptide encoding a site on the PDZ1 domain blocks binding of the WD5 repeat peptide with the PDZ1 domain. An N-terminal 12-amino acid segment of the WD5 repeat peptide, which comprises the first of four antiparallel β-strands, dose-dependently binds to the PDZ1 domain of NHERF1 and blocks binding of the PDZ1 domain to RACK1. These results suggest that the binding site might form a β-turn with topology sufficient for binding of NHERF1. Our results also demonstrate binding of NHERF to RACK1 at the WD5 repeat, which is distinct from the PKCϵ binding site on the WD6 repeat of RACK1.

Activation of the cystic fibrosis transmembrane regulator (CFTR),[1] an apical Cl channel expressed in epithelia of the conducting airways, intestine, and pancreas, is principally achieved via elevation of cAMP levels with the subsequent activation of protein kinase A (PKA). In the genetic disease cystic fibrosis, mutated CFTR results in abnormal Cl secretion and altered regulation of other ion transporters. Understanding how CFTR is regulated is important in identifying and characterizing signaling mechanisms which may be manipulated to optimize the function of mutated CFTR.

Although activation of CFTR involves its phosphorylation, regulation of CFTR is more complicated. Evidence from our laboratory and others identifies a role for protein kinase C (PKC) and protein−protein interactions involving N- and C-termini of CFTR. Our investigation of PKC-dependent regulation of CFTR led to the PKCϵ isoform being considered necessary for maximal cAMP-dependent activation of CFTR (*1*) and identification of binding of PKCϵ to an apically localized protein called RACK1 (receptor for activated C kinase) (*2*). We also discovered a direct interaction between RACK1 and NHERF1 (*2*), mediated through a binding domain in the PDZ1 domain of NHERF1 (*3*).

In the study presented here, we focus on identifying a site of interaction for NHERF1 on RACK1. RACK1 belongs to an ancient family of WD40 repeat regulatory proteins (*4*−*7*). The proteins characteristically have repeats of up to 40 amino acids with two internal conserved dipeptide sequences, glycine-histidine (GH) and tryptophan-aspartic acid (WD) (*5*, *8*, *9*). RACK1 is a 36 kDa protein consisting of seven conserved WD repeat units and structurally shares a high degree of homology with G-protein Gβ (*10*, *11*). RACK1 and Gβ are predicted to form a rigid seven-blade β-propeller structure, defined by its seven WD40 repeat units. RACK1 binds to a variety of signaling molecules, including PKCβII and -ϵ isotypes (*2*, *12*−*14*), phosphodiesterase PDE4D5 (10), src tyrosine kinases (*15*), integrin β subunit (*16*), type 1 interferon receptor (*17*, *18*), *N*-methyl D-aspartate (NMDA) receptor (*19*), insulin-like growth factor 1 receptor (*20*), inositol 1,4,5-trisphosphate receptors (*21*), PTPμ protein-tyrosine phosphatase (*22*), and dopamine transporter (*23*). Models of the scaffolding function of RACK1 emphasize a role for the WD repeat units as important sites of direct interaction with intracellular proteins. Specific binding of a protein with RACK1 may, however, involve multiple points of contact. For example, binding of human phosphodiesterase isoform PDE4D5 to RACK1 requires sites on WD5−WD7 repeats. PKCβ interacts with WD3 and WD5 repeats, β-integrin with WD5−WD7 repeats, the interferon receptor

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[1] Abbreviations: CFTR, cystic fibrosis transmembrane regulator; EC_{50}, effective concentration, concentration at which a 50% maximal effect occurs; GST, glutathione *S*-transferase; HA, hemataglutinin; His_6, histidine; IC_{50}, inhibitory concentration, concentration at which 50% inhibition occurs; LD, laser densitometry; NHERF1, Na^+/H^+ exchange regulatory factor; PD, pulldown; PDE, phosphodiesterase; PKC, protein kinase C; PBS, phosphate-buffered saline; PDZ, PSD-95/Discs-large/ZO-1 homology; PVDF, polyvinylidene difluoride; RACK1, receptor for activated C kinase; WB, Western blot; WD repeat, repeating units ending in Trp-Asp.

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Table 1: Amino Acid Sequence of WD Repeats of Human Tracheal Epithelial RACK1 Expressed in Calu-3 Cells[a]

WD repeat		amino acid sequence			
	1	MTEQMTLRGTLK	12		
WD1	13	GHNGWVTQIATTPQFPDMILSASRDKTIIMWK	44	LTRDETNYGIP	55
WD2	56	QRALRGHSHFVSDVVISSDGQFALSGSWDGTLRLWD	91	LTTGTTTRRFV	102
WD3	103	GHTKDVLSVAFSSDNRQIVSGSRDKTIKLWN	133	TLGVCKYTVQDE	145
WD4	146	SHSEWVSCVRFSPNSSNPIIVSCGWDKLVKVWN	178	LANCKLKTNHI	189
WD5	190	GHTGYLNTVTVSPDGSLCASGGKDGQAMLWD	220	LNEGKHLYTLD	231
WD6	232	GGDIINALCFSPNRYWLCAATGPSIKIWD	260	LEGKIIVDELK	271
WD7	272	QEVISTSSKAEPPQCTSLAWSADGQTLFAGYTDNL	306	VRVWQVTIGTR	317

[a] The sequence of human RACK1 was obtained from total mRNA isolated from the human airway epithelial Calu-3 cell line (*2*). Alignment of WD repeats taken from Chen et al. (*11*). WD repeat peptides were synthesized in the unmodified state or with a His_6 tag on the N-terminus.

with five WD repeats (WD3−WD7), and HIV Nef protein with WD5−WD7 repeats (*10*, *12*, *24*).

RACK1 is clustered at the apical membrane of Calu-3 airway epithelial cells, a feature common to other PDZ ligands (*2*). Crystallographic data indicate the four C-terminal residues of PDZ ligands directly interact with a peptide-binding groove formed between the βB strand and αB helix of the PDZ domain (*25*). However, RACK1 lacks a PDZ binding C-terminal motif, including class I -*X*-S/T-*X*-*Ø*, class II -*X*-*Ø*-*X*-*Ø*, and class III -*X*-D/E-*X*-*Ø* sequences. This suggests recognition of an internal binding site on RACK1 by the PDZ1 domain.

In this study, we examine binding using individual WD repeat units of human RACK1. We also tested the interaction of a peptide encoding a PDZ1−GYGF motif, which we have shown blocks binding of NHERF1 and its PDZ1 domain to RACK1. In previous studies, we found binding of PKCϵ to the WD6 repeat (*2*). Now, we report preferential binding of NHERF1 to the WD5 repeat. The NHERF1 PDZ1 domain mimics binding of NHERF1 to the WD5 repeat, and this binding is competitively inhibited by a PDZ1−GYGF peptide. We also show pulldown of endogenous NHERF1 from Calu-3 cells by the WD5 repeat peptide. Using overlapping peptide segments encoding the WD5 repeat, we demonstrate a 12-amino acid binding segment on the N-terminus of the WD5 repeat as a likely site for binding of the PDZ1 domain. Our results indicate a novel binding site for the PDZ1 domain on RACK1.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture. Calu-3 cells were grown in a submerged cell culture on 100 mm^2 tissue culture plastic, as described previously (*2*). 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) in a pGEX6P-1 vector were expressed in overnight cultures of DH5α cells. Each protein was GST epitope tagged at the N-terminus. After addition of 100 μM isopropyl β-D-thiogalactopyranoside and incubation for 3−6 h at 37 °C, cells were harvested. Recombinant protein was purified using 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 using a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to GST or, for the GST−NHERF1 protein, a monoclonal antibody to NHERF1. The antibody to NHERF1 was kindly provided by C. Yun (Emory University, Atlanta, GA) or purchased from Alpha Diagnostic International (San Antonio, TX). The PDZ1 domain was kindly provided by V. Raghuman (University of Pennsylvania, Philadelphia, PA) and the NHERF1 construct by C. Yun.

Table 2: WD5 Repeat Peptides[a]

[a] Overlapping 8−12-amino acid peptides based on the amino acid sequence of the WD5 repeat were synthesized in an unmodified state.

RACK1 was expressed in Sf9 insect cells, which 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 S. Brady-Kalnay (Case Western Reserve University) and used to transfect Sf9 cells and to express recombinant human RACK1 (rRACK1), as described previously (*2*). The expressed protein encodes the full-length human RACK1 cDNA and a polyhistidine tag, a PKA phosphorylation site, a thrombin cleavage site, and an HA tag. The final purified protein was immunoreactive to antibody to RACK1 and migrated as a single 50 kDa protein band. The molecular mass is consistent with the addition of the various tags.

Solution Binding Assays. Aliquots of GST−NHERF1 and WD repeat protein were mixed and incubated at 30 °C for 20 min. The amino acid sequences of the WD repeat proteins are listed in Table 1. Recombinant RACK1 was added to the mixture and the incubation continued for 20 min followed by addition of anti-GST antibody coupled to agarose beads, prewashed with ice-cold 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Protein complexes were recovered by centrifugation, washed five times to remove unbound material, and analyzed by immunoblot analysis for the HA tag and then reprobed for the GST tag.

To evaluate peptide segments of the WD5 repeat, overlapping 8−12-amino acid segments of WD5 were synthesized and used as a competitive inhibitor in solution binding assays using the GST−PDZ1 domain and His_6-HA-RACK1. The WD repeat peptides are listed in Table 2 and designated WD5.A−WD5.E. Specifically, 10 μg of GST−PDZ1 protein and varying amounts of WD5 peptide were mixed and incubated at 30 °C for 20 min. Recombinant RACK1 was added to the mixture and the incubation continued, and proteins were recovered using prewashed and precooled anti-HA antibody conjugated to agarose beads. Protein complexes

were analyzed by immunoblot analysis for the GST tag and then reprobed for the HA tag.

Peptides encoding sequences from WD repeats 1−7 or overlapping 8−12-amino acid segments of the WD5 repeat were synthesized by S. Yatav (Molecular Biotechnology Core Facility, Cleveland Clinic Lerner Research Institute, Cleveland, OH).

In Vitro Solid-Phase Binding Assays. Binding of recombinant proteins was assessed by immobilizing aliquots of 6 μg of His6-WD5 repeat peptide on PVDF membrane paper and then adding varying amounts of the protein of interest, as indicated in the figure legends. Membrane papers were incubated at room temperature for 20 min, and unbound material was removed by extensive washing. Bound recombinant protein was detected by immunoblot analysis using an antibody to the GST tag. To assess binding of the PDZ1 domain to WD5 repeat peptides, 6.4−7.5 μg of peptide was vacuumed onto PVDF paper and overlaid with varying amounts of GST−PDZ1 protein. Membrane papers were incubated and subjected to immunoblot analysis for the GST tag, as described above. The density of exposed bands was quantitated using a VersiDoc instrument.

Immunoprecipitation, Pulldown Assay, and Immunoblot Analysis. Cells were serum-deprived overnight, washed with ice-cold PBS, and lysed in a buffered detergent, as described previously (*2*). Lysates were clarified by pretreatment with agarose beads and then incubated with antibody directed against the protein of interest. To recover tagged recombinant peptide(s) and proteins bound to the peptides, we used antibody to the epitope tag conjugated to agarose beads. Protein complexes were recovered by centrifugation, washed five times in phosphate-buffered saline, and resuspended in Laemmli buffer. Samples were heated for 5 min in a boiling water bath followed by gel electrophoresis on 4 to 15% gradient slab gels. Protein bands were transferred to PVDF membrane paper for immunoblot analysis. Protein bands immunoreactive to specific antibodies were detected using enhanced chemiluminescence.

In pulldown assays, the WD5 repeat peptide was added to 1 mL of Calu-3 total cell lysates. The mixture was incubated for 30 min at room temperature. Talons beads, prewashed five times with PBS and resuspended in lysis buffer, were added to the mixture to bind the His_6-tagged WD5 repeat peptide and bound proteins. Beads were recovered by centrifugation, washed with PBS to remove unbound material, and resuspended in Laemmli buffer. Soluble proteins were subjected to gel electrophoresis on 4 to 15% gradient slab gels, transferred to PVDF membrane paper, and probed by immunoblot analysis for NHERF1.

Data Analysis. Values are reported as means ± the standard error for the number of replicates and/or experiments in parentheses. Immunoreactive exposed protein bands were quantitated using a VersiDoc instrument and a ratio calculated as GST value/HA value. EC_{50} and IC_{50} values were calculated from the ratios using a Prism computer program (GraphPad).

RESULTS

Previously, we reported binding of RACK1, a receptor for C kinase, to NHERF1, a PDZ-containing scaffold protein which also interacts with CFTR at its four terminal amino acids (*2*, *26*−*29*). To determine the binding motif on RACK1 for its interaction with NHERF1, His_6-tagged or untagged peptides encoding each of the seven WD repeats of RACK1 were prepared commercially. The WD repeat peptides were defined as the amino acid sequence with a X^{6-94}-[GH-X^{23-41}-WD] consensus, as shown in Table 1. WD1−WD7 peptides were tested in a solution binding assay for inhibition of binding of recombinant RACK1 to a GST-tagged recombinant NHERF1. Proteins bound to NHERF1 were recovered by pulldown using anti-GST antibody conjugated to agarose beads. The results are illustrated in Figure 1. The WD5 repeat peptide blocked binding at final concentrations of 0.5 and 2.8 μM. The WD1−WD4, WD6, and WD7 repeat peptides did not detectably alter binding of the GST−NHERF1 protein to recombinant RACK1 (Figure 1A). Figure 1B illustrates results from a solution binding assay in which varying concentrations of the WD5 repeat peptide were tested for inhibition of RACK1−NHERF1 binding. The WD5 repeat binding dose-dependently blocked binding; an inhibitory constant (IC_{50}) was calculated as 5.94 μg, equivalent to a final concentration of 21.4 μM.

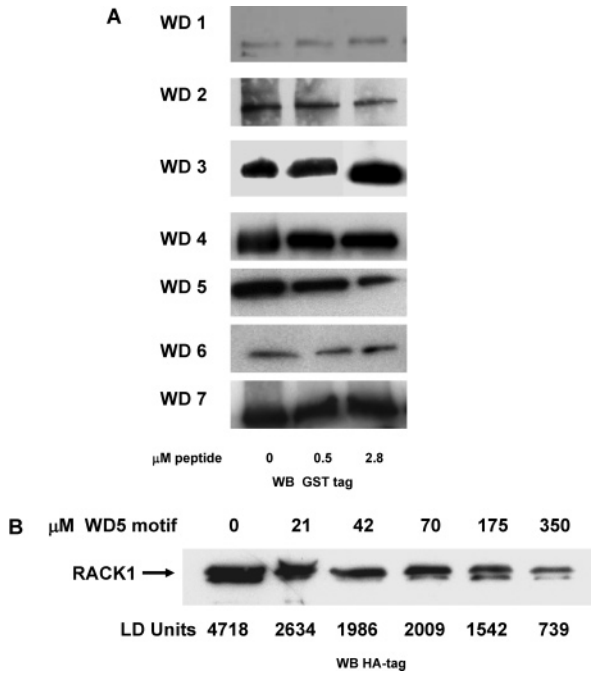

FIGURE 1: In vitro solution binding of recombinant RACK1 to NHERF1. The GST−NHERF1 protein was preincubated with the WD repeat peptide immediately before pulldown with recombinant RACK1. (A) A survey of WD repeat proteins WD1−WD7 indicates inhibition of NHERF1−RACK1 binding by the WD5 repeat peptide. (B) Dose-dependent inhibition of NHERF1−RACK1 binding by the WD5 repeat peptide. A solution binding assay followed by pulldown of His_6-RACK1 protein using Talon beads was performed using 1 μg each of NHERF1 and RACK1 and the indicated concentrations of the WD5 repeat peptide. An inhibitory constant (IC_{50}) of 21.4 ± 3.2 μM ($n = 3$) was calculated. Results are representative of three separate experiments.

On the basis of these results, we predicted the WD5 repeat encodes a binding motif for NHERF1. This was tested using a solid-phase binding assay for which a WD repeat was immobilized on PVDF paper and overlaid with a solution containing varying amounts of the GST−NHERF1 protein. Figure 2 illustrates typical results showing dose-dependent binding of GST−NHERF1 protein to the WD5 repeat peptide. A binding constant (EC_{50}) was calculated from these

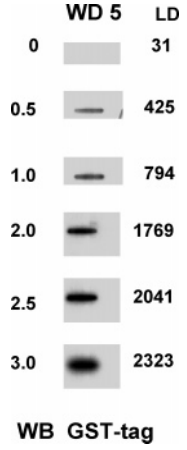

FIGURE 2: In vitro solid-phase binding of the GST−NHERF1 protein to the WD5 repeat. Binding of NHERF1 to the WD5 repeat protein was dose-dependent with an EC_{50} of 1.05 ± 0.14 mg (n = 3), equivalent to 0.28 μM.

data as 0.93 μg of NHERF1, which is equivalent to a final concentration of 0.24 μM.

Binding of RACK1 to NHERF1 involves the PDZ1 domain, as reported recently by this laboratory (*3*). Our data suggest binding of the PDZ1 domain of NHERF1 to RACK1 at the WD5 repeat. To test this hypothesis, we performed direct binding assays of a recombinant GST-tagged protein encoding the PDZ1 domain to the WD5 repeat peptide in a solid-phase binding assay. We found dose-dependent binding of the PDZ1 domain to the WD5 repeat peptide (Figure 3A,B). An EC_{50} for binding of 1.49 ± 0.3 μg (n = 3), equivalent to 0.62 μM, was calculated from the data in Figure 3A. We previously obtained evidence indicating one site for binding of RACK1 to NHERF1 as an 11-amino acid region encoding a GYGF motif on the PDZ1 domain (*3*). A His_6-tagged peptide encoding this motif was designed and synthesized. If the His_6-tagged GYGF−PDZ1 peptide encodes at least a partial binding site for the WD5 repeat of RACK1, we reasoned that the peptide might block the binding of the WD5 repeat with a recombinant GST−PDZ1 domain. Binding of the WD5 repeat protein and GST−PDZ1 domain was assessed with a solid-phase binding assay in the absence or presence of varying amounts of GYGF−PDZ1 peptide. The GYGF−PDZ1 peptide blocked binding of the GST−PDZ1 domain to the WD5 repeat peptide (Figure 3C) in a dose-dependent manner (Figure 3D). An IC_{50} of 6.4 μg was calculated from these data, equivalent to 31 μM.

In previous studies, full-length RACK1 pulled down endogenous NHERF1 from Calu-3 total cell lysates, indicating a specific interaction of RACK1 with NHERF1. In the next experiments, we examined pulldown of endogenous NHERF1 by His_6-tagged WD repeat peptides. Figure 4A illustrates typical results of pulldown of NHERF1, predominantly by the WD5 repeat peptide, which accounts for 28% of total densitometer values. We detected NHERF1 in pulldown assays using WD1 and WD2 repeat peptides, which likely represents nonspecific binding as these two WD repeat peptides do not display dose-dependent binding to GST-tagged NHERF1 (data not shown). WD5 repeat peptide dose-dependently pulled down endogenous NHERF1, as shown in Figure 4B, with an EC_{50} of 5.1 μg, equivalent to 1.3 μM.

We next wanted to clarify which region of the WD5 repeat is essential for binding of NHERF1 at the PDZ1 domain. Our strategy was to construct a series of peptides of 8−12 overlapping amino acids based on the amino acid sequence of the WD5 repeat, as shown in Table 2, and test the peptides for direct binding to the GST-tagged PDZ1 domain of NHERF1. The results for peptides WD5.A−WD5.C are shown in Figure 5A. Peptides WD5.A and WD5.B displayed dose-dependent binding to the PDZ1 domain with EC_{50} values of 1.08 (0.44 μM) and 0.95 μg (0.38 μM), respectively. WD5.C (shown in Figure 5A), WD5.D, and WD5.E peptides displayed nonspecific binding. Because the WD5.A and WD5.B peptides dose-dependently bound to the PDZ1 domain, we selected these two peptides for the next series of experiments on competitive inhibition of binding of recombinant RACK1 with the GST−PDZ1 domain. We conducted solution binding assays using mixtures of the GST−PDZ1 domain, His_6-HA-RACK1, and varying amounts of either WD5.A or WD5.B peptide. Peptides WD5.A and WD5.B dose-dependently blocked binding of RACK1 and the PDZ1 domain (Figure 5B). IC_{50} values of 0.68 ± 0.04 μg (n = 3) for the WD5.A peptide and 1.42 ± 0.4 μg (n = 4) for the WD5.B peptide were calculated. Equivalent molarities are 4.8 and 11.9 μM, respectively.

DISCUSSION

Regulation of CFTR in airway epithelial cells is complicated, involving cAMP-activated PKA, a PKCϵ isoform, and protein−protein interactions. Our previous studies using a Calu-3 human airway epithelial cell line link cAMP-dependent activation of CFTR with PKCϵ through the binding of PKCϵ to a protein designated RACK1, a receptor for activated C kinase. We performed confocal microscopy on Calu-3 cells and found the subcellular localization of RACK1 is confined to the apical region of Calu-3 cells, colocalized with CFTR. Our studies also revealed a direct interaction between RACK1 and NHERF1, a PDZ-expressing protein thought to be localized to the subapical portion of epithelial cells (*2*). The proximity of interacting proteins involved in the regulation of CFTR suggests an important role for binding domains on the proteins as potential sites for development of therapeutics for modulation of CFTR function and, thus, Cl secretion. We used recombinant proteins and pulldown of endogenous proteins to define binding sites for PKCϵ on the WD6 repeat of RACK1 and for RACK1 on the PDZ1 domain of NHERF1 (*2*, *3*).

In this study, we continue these inquires by screening peptides encoding individual RACK1 WD repeat units for binding of recombinant NHERF1 or its PDZ1 domain. The goal of this study was to determine which WD repeat(s) interacts with NHERF1. In solution binding assays, we observe binding of recombinant proteins RACK1 and NHERF1 and a marked, selective inhibition by the WD5 repeat of RACK1 (Figure 1). WD1−WD4, WD6, and WD7 repeat peptides failed to block binding of RACK1 and NHERF1. Direct binding experiments using a solid-phase binding assay demonstrate a lack of specific binding of the WD1 and WD2 repeat peptides to NHERF1 (data not shown) and dose-dependent binding of the WD5 repeat (Figure 2). Taken together, these data indicate preferential binding to the WD5 repeat, which, from a solid-phase binding assay, binds with an EC_{50} of 0.9 μg, equivalent to 0.24 μM. For

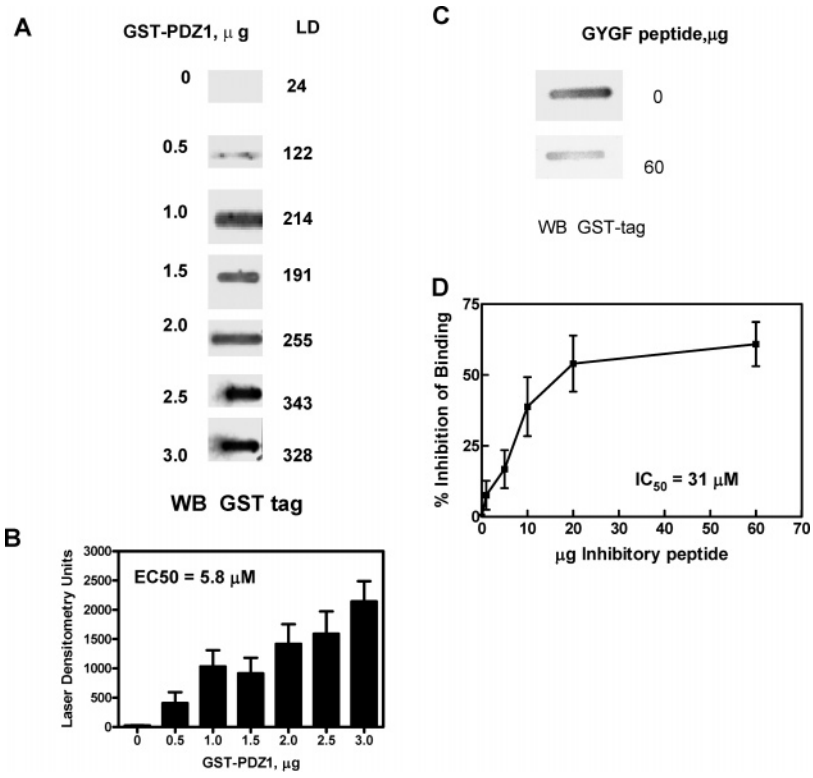

FIGURE 3: Binding of the WD5 repeat peptide to the PDZ1 domain of NHERF1. Aliquots containing 6 μg of WD5 repeat peptide were immobilized on PVDF paper and overlaid with varying amounts of recombinant GST−PDZ1 domain (A and B) or a mixture of 3 μg of GST−PDZ1 domain and varying amounts of a PDZ1−GYGF peptide (C and D) for 20 min at room temperature. The results are representative of three independent experiments. (A) PDZ1 domain binding was detected as dose-dependent binding. (B) Bar graph of data from panel A. An EC_{50} for binding was calculated as 14.3 ± 0.3 μg (n = 3), equivalent to 5.8 μM. (C) Binding of the PDZ1 domain to the WD5 repeat was blocked by a His_6-GYGF−PDZ1 peptide. (D) A GYGF−PDZ1 inhibitory peptide dose-dependently blocked binding. An IC_{50} for inhibition of 6.4 μg was calculated, equivalent to 31 μM.

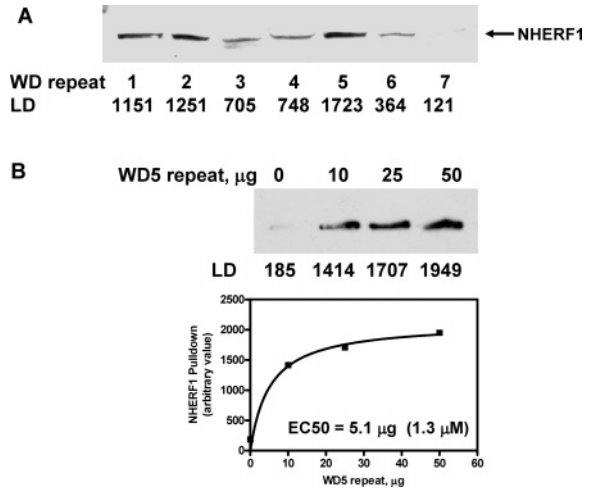

FIGURE 4: Pulldown of endogenous NHERF1 by the WD repeats. (A) To test pulldown of endogenous NHERF1, 50 μg of the His_6-tagged WD repeat peptide was added to a 1 mL aliquot of Calu-3 total cell lysate. Pulldown of NHERF1 was performed as described in Experimental Procedures. Exposed protein bands from immunoblot analysis for NHERF1 were quantitated by densitometry. (B) Varying amounts of the His_6-tagged WD5 repeat peptide was added to 1 mL aliquots of Calu-3 total cell lysate. The WD5 repeat peptide pulled down endogenous NHERF1 in a dose-dependent manner with an EC_{50} value of 1.3 μM, or a nominal amount of 5.1 μg. The results are representative of four independent experiments.

comparison, the EC_{50} value for binding of RACK1 to NHERF1 was 3.1 μg, or a nominal concentration of 1.2 μM, as determined by solid-phase binding assays (*2*).

To determine whether the PDZ1 domain is involved in binding of NHERF1 to the WD5 repeat, we assessed direct binding of a recombinant GST-tagged PDZ1 domain to the WD5 repeat. Our data show dose-dependent binding of the GST-tagged PDZ1 domain to the WD5 repeat peptide with an EC_{50} of 1.49 μg, equivalent to 0.6 μM. For comparison, a calculated EC_{50} for binding of the PDZ1 domain to RACK1 was 1.7 μg, or a nominal concentration of 0.7 μM (*3*). The comparable EC_{50} values indicate binding at a similar site. Further support for a role for the WD5 repeat in the binding of NHERF1 comes from the results of pulldown experiments. The WD5 repeat peptide pulled down endogenous NHERF1 from Calu-3 total cell lysates in a dose-dependent manner with an EC_{50} value of 1.3 μM, or a nominal amount of 5.1 μg (Figure 4).

We designed overlapping 8−12-amino acid peptides encoding segments of the RACK1 WD5 repeat and used these to narrow the region of interaction between the PDZ1 domain and RACK1. Our data demonstrate a unique 10-amino acid segment immediately after the initial loop region and immediately adjacent to the GH of the WD5 repeat binds PDZ1 and blocks binding of RACK1 to a GST-tagged PDZ1 domain (Figure 5). Comparing overlapping peptide segments led us to predict a six-amino acid segment (LNTVTV, amino acids 194−200) as a likely minimum binding motif. The six-amino acid WD5 binding segment represents amino acids forming the first of four antiparallel β-strands comprising the WD5 repeat immediately distal to a loop linking the WD4 repeat to the WD5 repeat (*7*, *11*). This WD5 β-strand is

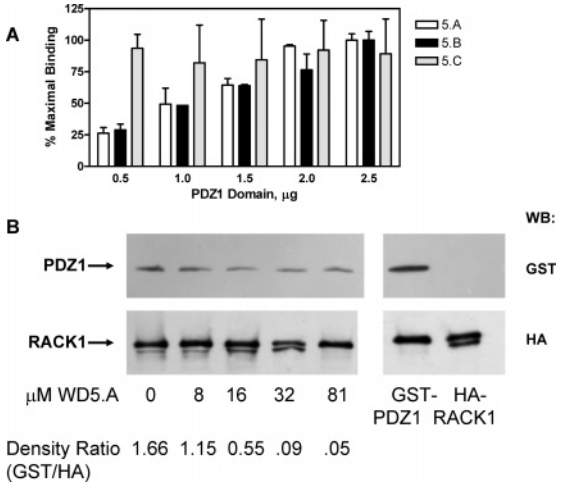

FIGURE 5: Interaction between WD5 repeat peptides and the NHERF1−PDZ1 domain. (A) Direct binding of the WD5 repeat peptide to the GST-tagged PDZ1 domain. Aliquots containing 30 μM WD5 repeat peptide 5.A (white bar), 5.B (black bar), or 5.C (gray bar) were immobilized on PVDF paper and flooded with 0−3 μg (12 μM) of GST-tagged PDZ1 domain. The EC_{50} for binding of peptide 5.A was 1.08 μg (0.44 μM) and for peptide 5.B was 0.95 μg (0.38 μM). Peptide 5.C did not exhibit concentration-dependent binding. Similar results were obtained with peptides 5.D and 5.E. The results are representative of three independent experiments. (B) Competitive inhibition of binding of the PDZ1 domain to RACK1 by WD5 repeat peptides. Solution binding was performed with 10 μg (2 μM) of the GST−PDZ1 domain, 30 μg (6 μM) of His_6-HA-RACK1, and 0−5 μg (40 μM) of peptide 5.A. Protein complexes were recovered using anti-HA antibody conjugated to agarose beads and immunoblots probed for the GST tag. Peptide 5.A, shown here, and peptide 5.B (not shown) blocked solution binding of RACK1 and the PDZ1 domain. The IC_{50} for peptide 5.A was 0.59 ± 0.1 μg ($n = 4$) and for peptide 5.B was 1.42 ± 0.4 μg ($n = 4$), equivalent to 4.8 and 11.9 μM, respectively.

located at an external flat surface of RACK1 and, thus, is accessible for an interaction with NHERF1 (Figure 6 and ref *5*).

In a previous study, we found inhibition of binding of NHERF1 to RACK1 by an 11-amino acid peptide encoding a GYGF motif from the PDZ1 domain, KGPNGYGFHLH (*3*). We examined a role for the PDZ1−GYGF peptide in the binding of GST-tagged PDZ1 to the WD5 repeat peptide via a solid-phase binding assay. The PDZ1−GYGF peptide blocked binding in a dose-dependent manner with an inhibitory constant (IC_{50}) of 6.4 μg of peptide, equivalent to 31 μM (Figure 3C,D). How the PDZ1−GYGF motif binds to the WD5 repeat of RACK1 is still not clear. The GYGF motif resides in a carboxylate binding loop typically associated with binding of PDZ domains to a four-residue C-terminal sequence, (D/E)(T/S)XV (*30*). However, some PDZ domains have now been found to recognize other specific internal peptide motifs in their binding partners. This is exemplified by interactions of the PDZ domain with other distinct protein-binding motifs, including ankyrin repeats (*31*), spectrin repeats (*32*), and LIM domains (*33*). One well-studied example is the binding of neuronal nitric oxide synthase (nNOS) with the PDZ domain of PSD-95 or syntrophin, which does not involve the canonical PDZ−peptide interactions (*34*−*37*). Rather, the PDZ1 domain of syntrophin recognizes a specific β-hairpin internal motif in the PDZ domain within nNOS. The two PDZ domains interact in a head-to-tail fashion. Structurally, these internal

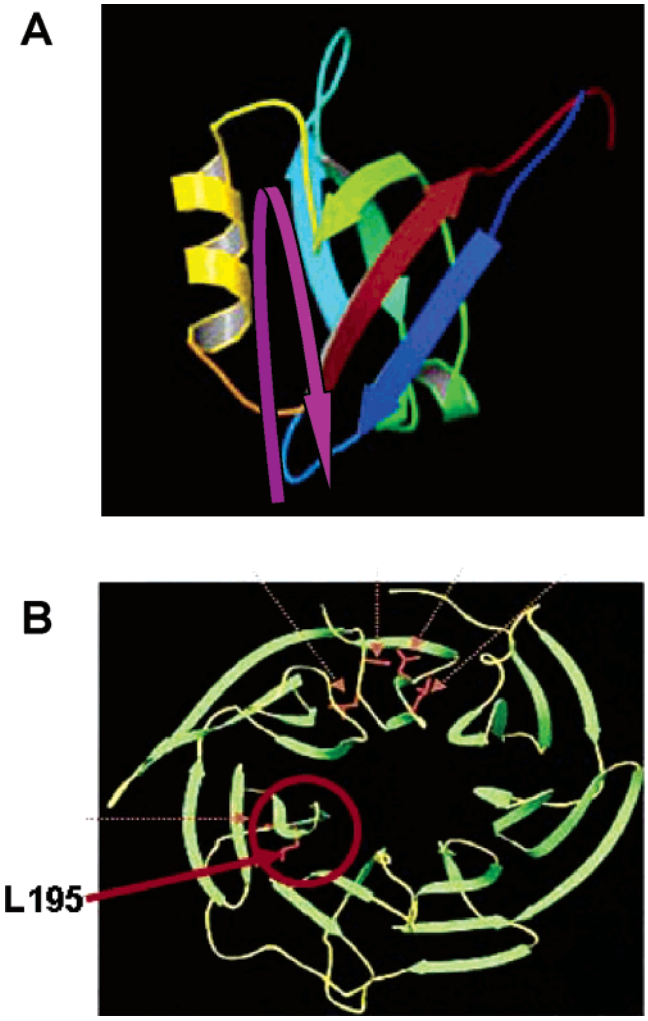

FIGURE 6: Structural relationship between the RACK1 WD5 repeat and the PDZ1 domain of NHERF1. (A) Ribbon diagram of the PDZ1 domain with strand βB in light blue (PDB entry 1G90) and WD5 interaction domain with a putative β-turn (purple). (B) Ribbon diagram of the top view of RACK1 based on the structure of Gβ1 (PDB entry 1TGB). The location of the L195 residue on the WD5 β-strand is marked with a red arrow at the junction of a loop (red circle) joining the WD4 and WD5 repeats (*10*, with permission from Elsevier).

binding motifs lack the (D/E)(T/S)XV terminal negatively charged carboxylate recognition motif yet bind with comparable binding affinities. General rules for PDZ-mediated protein−protein interactions have emerged from these studies (*36*). A key feature for internal motifs is a stabilized β-turn, which may occur through many different types of amino acid sequences. The WD5 β-strand may comprise a β-turn of sufficient topology and accessibility for an interaction with the PDZ1 domain of NHERF1, as illustrated in Figure 6. More detailed studies must be carried out to determine whether other stabilizing forces, such as salt bridge interactions, contribute to the interaction.

Our results on binding of the PDZ1−GYGF peptide to the WD5 repeat of human RACK1 suggest a class of binding motif with structural requirements different from those of the three recognized classes of PDZ domains (*38*). There may, however, be other considerations with regard to the relative contribution of the PDZ1−GYGF motif to the binding of RACK1. A predicted loss of cAMP-dependent activation of CFTR by an 11-amino acid peptide encoding

this motif delivered into Calu-3 cells was not observed. One possible explanation for this finding is in situ low-affinity binding to RACK1 due to conformational constraints, sequestration into intracellular vesicles, rapid metabolism through a catabolic pathway, or inaccessibility of the RACK1 binding motif.

Other proteins interacting with RACK1 bind at multiple sites, each with a different affinity (*10*). Thus, our data may indicate one site for binding of NHERF1 to RACK1. In analogy with PDE4D5 binding, NHERF1 binding may involve WD repeats in addition to the WD5 repeat. Regions connecting WD4 to WD5 and WD5 to WD6 might also present interacting surfaces. Our results reported here indicate a prominent role for the WD5 repeat in NHERF1 binding. We have yet to completely map the NHERF1 binding sites on RACK1, but on the basis of our studies, we suspect there are multiple contact regions.

These initial studies have provided a foundation for further investigations into the dynamics of protein interactions in cellular signaling involving RACK1 and functional consequences of binding to RACK1. The results clearly differentiate binding of PKCϵ and NHERF1 to RACK1 on the basis of preferential binding to distinct WD repeats. This is particularly important because, in airway epithelial cells, PKCϵ and NHERF1 may simultaneously bind to RACK1. In analogy to the association of PKCβII, neuronal $GABA_A$ receptor β3 subunit, and RACK1, PKCϵ may phosphorylate NHERF1 and thus facilitate its physiological function (*39*). Alternatively, PKCϵ may phosphorylate a protein proximal to NHERF1 or PKCϵ itself. Thus, the proximity of WD5 and WD6 repeats and proteins bound to each may be important factors in the regulation of cAMP-dependent CFTR function.

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