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Interaction of carboxyl-terminal peptides of cytosolic-tail of apactin with PDZ domains of NHERF/EBP50 and PDZK-1/CAP70

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Abstract The C-terminal PDZ-binding motifs are required for polarized apical/basolateral localization of many membrane proteins. Ezrin–radixin–moesin (ERM) proteins regulate the organization and function of specific cortical structures in polarized epithelial cells by connecting filamentous (F)-actin to plasma membrane proteins through EBP50. Previous work showed that the membrane phosphoprotein apactin (an 80-kDa type I membrane protein derived from pro-Muclin) is associated with the acinar cell apical actin cytoskeleton and that this association is modulated by changes in the phosphorylation state of the apactin cytosolic tail. The carboxyl-terminal amino acids of apactin (–STKL–COOH) are predicted to form a type I PDZ-binding domain, similar to that of CFTR (–DTRL–COOH). Pairwise sequence comparison between NHERF/EBP50 and PDZK1/CAP70 PDZ domains reveals significant identity among the 83 amino-acid residues (12–92) of EBP50 and CAP70 (241–323), which are involved in the interaction with the carboxyl-terminal peptides (STKL–COOH and phosphomimetics) of apactin. Hence, the specificity and affinity of interactions are identical between them, which is corroborated with the two hybrid results. Substitution of all the four-carboxyl-terminal amino acids in the wild type to Ala reduces the interaction. Only the carbonyl oxygen and amide nitrogen of Ala are found to be involved in hydrogen bonding. Further, truncation of the wild carboxyl-terminal peptide to RGQPP–COOH, showed very low affinity of interaction with PDZ1 domain. Only the atom $O^{\varepsilon 1}$ of Gln-2 hydrogen bonds with $N^{\varepsilon 2}$ of His72 of PDZ domain. Ser-3 amino acid in wild type apactin protein (STKL–COOH) is not involved in hydrogen bonding with PDZ1 domain. However, substitution of Ser-3 to Asp-3 in PDTKL–COOH peptide increases the affinity of interaction of PDTKL–COOH with PDZ1 domain. Thus, carboxyl-terminal Asp(D) -3, Thr(T) -2, Lys(K) -1 and Leu(L) 0 are involved in numerous interactions with PDZ1 domains of NHERF/EBP50 and PDZK1/CAP70.

Keywords EBP50 · NHERF · PDZ · Apactin · CFTR associated protein 70

Abbreviations

EBP50	ERM (Ezrin, Radixin, Moesin) binding phosphoprotein 50
NHERF	sodium–hydrogen exchange regulatory factor
NHE3	Na-H-exchanger 3
PDZ	PSD-95_Discs-large_ZO-1 homology
CAP70	CFTR associated protein 70

Introduction

EBP50/NHERF [ERM (Ezrin, Radixin, Moesin)-binding phosphoprotein 50/sodium-hydrogen exchange regulatory factor] was independently identified as a regulator factor for Na–H-exchanger 3 (NHE3) [1] and as an ERM-binding phosphoprotein of 50 kDa [2]. EBP50 is a 358-residue adapter molecule that has two PDZ

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(PSD-95_Discs-large_ZO-1 homology) domains and a C-terminal ERM-binding (EB) region. EBP50 can bind to many proteins through its PDZ domains. Most of the ligands, such as cystic fibrosis transmembrane conductance regulator (CFTR) [3] and the type IIa Na_Pi cotransporter Npt2, bind to the PDZ1 domain and only a few proteins, such as β-catenin [4], appear to interact specifically with the PDZ2 domain. Most PDZ domain binding proteins interact with PDZ domains via their C-terminal 3–5 amino-acids. PDZ domains are ~90 residues long and consist of six β-strands (βA–βF) and two α-helices, αA and αB. In canonical PDZ interactions, the PDZ domain binds the C-terminus of the interaction partner in an elongated groove as an antiparallel β-strand between the αB helix and the βB sheet, termed the PDZ binding groove [5, 6]. In general, PDZ proteins function as organizers of protein complexes at the plasma membrane [7]. Through its EB motif, EBP50 binds to the FERM domain of ERM proteins has been involved in a broad array of biological systems.

CFTR associated protein 70 (CAP70) is a scaffolding protein with four PDZ protein interaction domains, which are found in a number of tissues including brain [8, 9]. CAP70 is a scaffolding protein that was purified from the kidney on the basis of its interaction with the carboxyl terminus of CFTR [10]. Like other PDZ domain-containing proteins, such as PSD95 or INAD (inactivation no after potential D), the CAP70 coding sequence is essentially composed of four tandemly positioned PDZ domains. In contrast to other known PDZ domain-containing proteins, CAP70 has the activity to dynamically alter the oligomerization state of the CFTR channel, thereby inducing potentiation of CFTR-induced chloride channel activity [10].

The focus of this study is the PDZ-binding domain of pro-Muclin and its product apactin. Apactin is generated in parallel with Muclin from a common precursor, pro-Muclin. Pro-Muclin is a high-molecular-weight O-glycosylated, sulfated membrane glycoprotein that traffics through the secretory pathway and is proposed to have a role in aggregation/sorting of regulated secretory proteins at the trans Golgi network of the pancreatic acinar cell [11–13]. Pro-Muclin is cleaved after leaving the Golgi, presumably in the immature secretory granule, liberating apactin, which consists of a 16-amino-acid cytosolic domain, a membrane-spanning domain, and a glycosylated luminal domain with a peptide core of about 65 kDa [11]. Muclin remains in the mature zymogen granule associated with the protein content of the granule while apactin is efficiently removed to the apical plasma membrane. The carboxyl-terminal amino acids of apactin (–STKL–COOH) are predicted to form a type I PDZ-binding domain [14], similar to that of CFTR (–DTRL–COOH). The C-terminus of CFTR interacts with a PDZ domain in EBP50/NHERF [ERM (Ezrin, Radixin, Moesin)-binding phosphoprotein 50/sodium–hydrogen exchange regulatory factor] [15]. This association has been reported to be necessary for apical localization of CFTR [16], but other studies indicate that the association of the CFTR cytosolic tail with EBP50/NHERF stabilizes CFTR by increasing recycling to the plasma membrane after endocytosis [17].

The structures of several PDZ domain complexes with peptides have helped researchers understand the basis for the various binding preferences, but the manner in which the specificity of binding is achieved may require a broader investigation. There are about 500 different proteins that contain one or more PDZ domains in the human genome, each interacting with one or several peptides, but only ~15 PDZ/ligand complex structures have been solved so far. Consequently, there is a need for a computational procedure to obtain reliable models of the complexes for the cases where the PDZ domain was solved alone or with a different peptide. The procedure for obtaining a model of the complex involves docking a carboxyl-terminal peptide to a PDZ structure, accounting for the structural rearrangement of both backbone and side chains of the peptides.

The apactin C-Tail also has two predicted phosphorylation sites, which potentially provide a means for sophisticated control of protein–protein interactions between the C-Tail and structural or regulatory elements in the cell. Apactin is involved in remodeling of the apical cytoskeleton during regulated exocytosis in a manner controlled by phosphorylation of the apactin C-Tail [18]. Therefore, in this work we have explored mechanisms of interaction for the binding of carboxyl-terminal peptides of C-tail of apactin (STKL–COOH and phosphomimetics) with NHERF/EBP-50 and PDZK1 (NP_067492); aka CAP70.

Materials and methods

BacterioMatch II Two-Hybrid System

To find potential binding partners for the C-Tail of apactin, the BacterioMatch II Two-Hybrid System (#240065, Stratagene, Torrence, CA, USA) was used. The baits were prepared by ligating annealed, phosphorylated overlapping oligonucleotides (Table 1) between the *EcoRI* and *XhoI* sites of the pBT plasmid. After transformation into XL-1 Blue MRF' Kan strain using rich medium and growth at 30°C (to minimize potential toxicity of these plasmids), colonies were screened by PCR using pBT(+) (TCC GTT GTG GGG AAA GTT ATC) and pBT(–) (GGG TAG CCA GCA GCA TCC TGC) primers. The parental plasmid with

Table 1 Overlapping oligonucleotides used to prepare C-Tail bait constructs

Wild type C-Tail
AATTTTACATTGGGAAGGAGGACACACATTGACCGTGGCCAACCTCCAAGTACTAAGTTGTAA
AATGTAACCCTTCCTCCTGTGTGTAACTGGCACCGGTTGGAGGTTCATGATTCAACATTAGCT
Translated protein sequence
N F T L G R R T H I D R G Q P P S T K L Stop
C-Tail-S/D
AATTTTACATTGGGAAGGAGGACACACATTGACCGTGGCCAACCTCCAGACACTAAGTTGTAA
AATGTAACCCTTCCTCCTGTGTGTAACTGGCACCGGTTGGAGGTCTGTGATTCAACATTAGCT
Translated protein sequence
N F T L G R R T H I D R G Q P P D T K L Stop
Δ-4aa-C-Tail (truncate last 4 amino acids to delete PDZ-binding domain)
AATTTTACATTGGGAAGGAGGACACACATTGACCGTGGCCAACCTCCATAA
AATGTAACCCTTCCTCCTGTGTGTAACTGGCACCGGTTGGAGGTATTAGCT
Translated protein sequence
N F T L G R R T H I D R G Q P P Stop

no insert gave a PCR product of 146 bp and the C-Tail inserts gave products of 194 bp (182 bp for Δ-4aa constructs). To verify protein expression of the bait constructs, clones were plated on LB-Cam (chloramphenicol) plates for 24 h at 30°C, and individual clones were picked for protein induction. They were grown overnight at 37°C in 2 ml M9+ His-dropout broth containing 25 μg/ml Cam. In the morning, a fresh 2 ml broth containing 0.1 mM IPTG was inoculated and grown at 37°C until OD600 = 0.5–0.6. The parental plasmid was grown in the same fashion to serve as a negative control. The cells were then pelleted, SDS-PAGE sample buffer was added (250 μl/1.5 OD × ml) followed by sonication to disrupt the bacteria. After pelleting debris, 10 μl of the supernatant was separated on 12.5% acrylamide gel, transferred to PVDF and probed with an antibody to the C-Tail [18] (preadsorbed versus *E.coli*) to visualize fusion proteins, ~29 kDa. As a positive control, GST-C-Tail (2.5 ng purified protein) was used. The GST-C-Tail is slightly smaller than the pBT-C-Tail fusion proteins. The arrow indicates the position of the specific signal (Fig. 1). Next, minipreps of the pBT-C-Tail DNAs were prepared for validation and screening the library. To test for self-activation of the bait constructs, the plasmid pairs in Table 2 below were co-transformed using Validation Reporter Competent Cells. Transformation tests were performed for each of the pBT-C-Tail constructs using minimal medium and culturing at 37°C. Then, 200 μl of cells from adapted outgrowth culture were plated on Selective Screening Medium (5 mM 3-AT); and 100 μl of cells from adapted outgrowth culture were diluted 1:100 in M9+ His dropout broth and plated on Non-selective Screening Medium plates (without 3-AT) followed by incubation for 24 h, 37°C. If no colonies were apparent, the plates were transferred to room temperature in the dark and grown for an additional 16 h. The lower temperature may allow growth of cells containing toxic proteins or weak interactors. The cfu were determined for the Non-selective Screening Medium and the Selective Screening Medium plates and the percentage of co-transformants able to grow on the Selective Screening Medium was calculated. All of the constructs passed these tests and were then used for screening the Target Library. The target library was mouse liver (#982307, Stratagene). The pTRG plasmid cDNA Library was used to prepare the target DNAs. Cells were grown on rich medium (LB-tet) at 30°C for 24 h to amplify the library (about 1×10^6 colonies). The cells were harvested from the plates in SOC medium and the plasmid

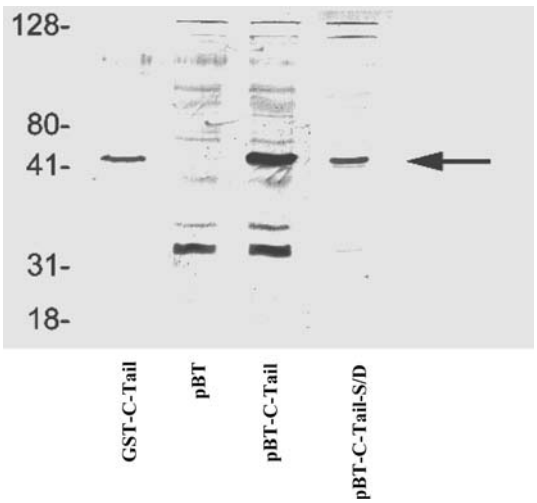


Fig. 1 Transformed *E.coli* were pelleted, run on 12.5% gels, and transferred to PVDF. The blots were probed with the anti-C-Tail antibody we used, after preincubation of the antibody with *E.coli* to reduce non-specific staining. As a positive control, GST-C-Tail (2.5 ng purified protein) was used. The GST-C-Tail is slightly smaller than the pBT-C-Tail fusion proteins. The arrow indicates the position of the specific signal

Table 2 Binding partners for the C-Tail of pro-Muclin/apactin by the BacterioMatch II Two-Hybrid System

Bait protein	Sequence of bait protein	PDZK1 (NP_067492); aka CAP70	NHERF1 (Q28619) aka EBP50
Wild type C-Tail	NFTLGRRTHIDRGQPPSTKL	Yes	Yes
S/D C-Tail	NFTLGRRTHIDRGQPP**D**TKL	Yes	Yes
Δ4aa C-Tail	NFTLGRRTHIDRGQPP	No	No

DNA was isolated using a MaxiPrep kit (Qiagen). The Library was then screened for C-Tail interacting proteins. The Bait and Target DNAs were co-transformed into chemically competent BacterioMatch II Screening Reporter Strain cells according to (Suppliers instructions BacterioMatch® II Two-Hybrid System XR Plasmid cDNA Library). Target clones identified by the library screen were confirmed by transformation of the reporter strain with a purified recombinant pTRG plasmid plus the recombinant pBT plasmids.

Molecular modeling

Molecular modeling was performed using the Molecular Operating Environment (MOE) software package (Chemical Computing Group, Montreal, Canada). The coordinates of the human NHERF PDZ1 domain (residues 11–94) in complex with carboxyl terminal QDTRL of human CFTR complex (PDB ID: 1I92) [19] were taken from the Protein Data Bank (www.rcsb.org). After hydrogen atoms were added, the carboxyl-terminal peptide (QDTRL) and the PDZ1domain were saved separately using MOE. Amber99 force field [20] was used to assign partial charge on protein and all structure minimizations. A structure minimization was performed on the protein using minimization protocol of Steepest Descent (SD), Conjugate Gradient (CG) and Truncated Newton (TN) methods. During the minimization, non-hydrogen atoms were held fixed and the RMSD gradient used in SD was 1,000, in CG were 100 and in the TN step the RMSD was 0.1. The iteration limits in SD and CG are 100, in TN 200. The minimized structure of the protein was used in later docking simulations. All other carboxyl-terminal peptides (Table 3) were built using the QDTRL as a template using MOE. Before docking PEOE charge [21] was used for these peptides and full optimization was performed to minimize each structure using MOE. The minimized structure was used in docking simulations. Docking simulations were done using MOE-Dock. MOE-dock utilizes a Monte Carlo Simulated Annealing (SA) method in docking calculations to search for favorable binding configurations of a small, flexible ligand and a rigid macromolecule in a pre-set box. By generating random changes to the ligand's coordinates, the dock searches in the box and calculates docking energies based on a grid in the box. In all docking calculations, a docking box with a grid consisting of $60 \times 60 \times 60$ points was employed. The spacing of the grid was 0.375 Å. The grid box included the entire binding site and some area just outside of the binding pocket entrance. The iteration limit was set to 20,000, the number of cycles was set to 20 and the number of runs was set to 25 So, a total of 25 docking configurations were determined in each docking calculation. After a preferable binding structure was obtained from docking simulation, the complex was partially minimized by relaxing the ligand and the side chains that were within 7 Å from the ligand while all other atoms were fixed using the above minimization criteria.

For Glide docking studies [22], the oligo peptides were converted to mae format (Maestro, Schrodinger Inc.) and optimized by means of the OPLS-AA force field. The protein and co-crystallized peptides (QDTRL) were combined and hydrogen atoms were added within Maestro. The complexes were stored in the MacroModel dat format. The pprep script shipped by Schrodinger was used to check protonation states and tautomeric forms. The protein-ligand complex was minimized within MacroModel with the OPLS-AA force field by application of the autoref.pl script. Progressively weaker restraints (tethering force constants 3, 1, 0.3, 0.1) were applied to non-hydrogen atoms only. This refinement procedure is recommended by Schrodinger (technical notes for version 1.8), because Glide uses the full OPLS-AA force field at an intermediate docking stage and is claimed to be more sensitive towards geometric details than other docking tools. Minimizations were performed until the average root mean square deviation of the non-hydrogen atoms reached 0.3 A. Glide calculations were performed with Impact version v18007 (Schrodinger, Inc.). Grid calculations was made using Glide from the co-crystallized ligand in the receptor with the default settings for everything and used for flexible docking of ligands. The number of rotatable bonds was set to 32 as some of the ligands have more rotatable bonds then the default value of 15. The final energy evaluation was done with Glide Score, E-model, and van der Waals interactions between the ligand and protein.

Results

We initially screened the target library using the pBT-C-Tail-S/D construct, as this serine was shown to be phosphorylated during stimulated secretion [18] during which

Table 3 Geometry of hydrogen bonds and hydrophobic interaction in the NHERF PDZ1-apactin carboxyl-terminal peptide complexes

Hydrogen bonding			Hydrophobic interaction		
Hydrogen donor groups (HD)	Hydrogen acceptor groups (A)	Distance (Å) (H-A)	Target Protein	Bait protein	Distance (Å)
i. *1I92-Apactin-STKL–COOH*			1I92	Apactin-PSTKL–COOH	
HIS A 72 NE2	THR B-2 OG1	2.74	ILE A 79 D1	LEU B 0 CD1	3.83
GLY A 30 N	PRO B-4 O	3.08	HIS A 72 CE1	PRO B-4 C	3.82
THR B-2 N	LEU A 28 O	3.03	HIS A 27 CE1	LYS B-1 CB	3.77
LEU A 28 N	THR B-2 O	2.85	PHE A 26 CZ	LEU B 0 CD1	3.68
LEU B 0 N	PHE A 26 O	2.78	PHE A 26 CE2	LEU B 0 CD1	3.76
PHE A 26 N	LEU B 0 OXT	3.23			
GLY A 25 N	LEU B 0 OXT	2.80			
TYR A 24 N	LEU B 0 O	2.69			
LYS B-1 NZ	ASN A 22 O	3.06			
ii. *1I92-Apactin-PDTRL–COOH*			1I92	Apactin-PDTRL–COOH	
HIS A 72 NE2	THR B-2 OG1	2.73	ILE A 79 CD1	LEU B 0 CD1	3.83
ARG B-1 NH2	GLU A 43 OE2	2.94	HIS A 72 CE1	PRO B-4 C	3.82
ARG B-1 NE	GLU A 43 OE2	2.87	HIS A 27 CE1	ARG B-1 CB	3.77
ARG A 40 NH1	ASP B-3 OD2	2.93	PHE A 26 CZ	LEU B 0 CD1	3.68
GLY A 30 N	PRO B-4 O	3.08	PHE A 26 CE2	LEU B 0 CD1	3.76
THR B-2 N	LEU A 28 O	3.03			
LEU A 28 N	THR B-2 O	2.86			
HIS A 27 ND1	ASP B-3 OD1	2.55			
LEU B 0 N	PHE A 26 O	2.78			
PHE A 26 N	LEU B 0 OXT	3.23			
GLY A 25 N	LEU B 0 OXT	2.80			
TYR A 24 N	LEU B 0 O	2.69			
ARG B-1 NH2	ASN A 22 O	2.49			
ARG B-1 NH1	ASN A 22 O	2.96			
iii. *1I92-Apactin-PDTKL–COOH*			1I92	Apactin-PDTKL–COOH	
HIS A 72 NE2	THR B-2 OG1	2.73	ILE A 79 CD1	LEU B 0 CD1	3.83
ARG A 40 NH1	ASP B-3 OD2	2.93	HIS A 72 CE1	PRO B-4 C	3.82
GLY A 30 N	PRO B-4 O	3.08	HIS A 27 CE1	LYS B-1 CB	3.77
THR B-2 N	LEU A 28 O	3.03	PHE A 26 CZ	LEU B 0 CD1	3.68
LEU A 28 N	THR B-2 O	2.86	PHE A 26 CE2	LEU B 0 CD1	3.76
HIS A 27 ND1	ASP B-3 OD1	2.55			
LEU B 0 N	PHE A 26 O	2.78			
PHE A 26 N	LEU B 0 OXT	3.23			
GLY A 25 N	LEU B 0 OXT	2.80			
TYR A 24 N	LEU B 0 O	2.69			
LYS B-1 NZ	ASN A 22 O	3.06			
iv. *1I92-Apactin-PAAA–COOH*			1I92	Apactin-PAAAA–COOH	
GLY A 30 N	PRO B-4 O	3.08	HIS A 72 CE1	PRO B-4 C	3.82
ALA B-2 N	LEU A 28 O	3.03	HIS A 27 CE1	ALA B-1 CB	3.77
LEU A 28 N	ALA B-2 O	2.85			
ALA B 0 N	PHE A 26 O	2.78			
PHE A 26 N	ALA B 0 OXT	3.25			
GLY A 25 N	ALA B 0 OXT	2.85			
TYR A 24 N	ALA B 0 O	2.69			

Table 3 continued

Hydrogen bonding			Hydrophobic interaction		
Hydrogen donor groups (HD)	Hydrogen acceptor groups (A)	Distance (Å) (H-A)	Target Protein	Bait protein	Distance (Å)
v. *1I92-Apactin-RGQPP–COOH*			1I92	Apactin-RGQPP–COOH	
HIS A 72 NE2	GLN B-2 OE1	2.73	HIS A 72 CE1	ARG B-4 C	3.82
GLY A 30 N	ARG B-4 O	3.08	HIS A 27 CE1	PRO B-1 CB	3.74
GLN B-2 N	LEU A 28 O	3.03	PHE A 26 CZ	PRO B 0 CG	3.50
LEU A 28 N	GLN B-2 O	2.86	PHE A 26 CE2	PRO B 0 CG	3.55
PHE A 26 N	PRO B 0 OXT	3.19	PHE A 26 CE1	PRO B 0 CG	3.71
GLY A 25 N	PRO B 0 OXT	2.81	PHE A 26 CD2	PRO B 0 CG	3.82
TYR A 24 N	PRO B 0 O	2.69	PHE A 26 C	PRO B 0 CD	3.69
			PHE A 26 C	PRO B 0 CG	3.86
vi. *1I92-Apactin-QDTRL–COOH*			1I92	Apactin-QDTRL–COOH	
HIS A 72 NE2	THR B-2 OG1	2.73	ILE A 79 CD1	LEU B 0 CD1	3.83
ARG B-1 NH2	GLU A 43 OE2	2.94	HIS A 72 CE1	GLN B-4 C	3.82
ARG B-1 NE	GLU A 43 OE2	2.87	HIS A 27 CE1	ARG B-1 CB	3.77
ARG A 40 NH1	ASP B-1 OD2	2.93	PHE A 26 CZ	LEU B 0 CD1	3.68
GLY A 30 N	GLN B-4 O	3.08	PHE A 26 CE2	LEU B 0 CD1	3.76
THR B-2 N	LEU A 28 O	3.03			
LEU A 28 N	THR B-2 O	2.86			
HIS A 27 ND1	ASP B-1 OD1	2.55			
LEU B 0 N	PHE A 26 O	2.78			
PHE A 26 N	LEU B 0 OXT	3.23			
GLY A 25 N	LEU B 0 OXT	2.80			
TYR A 24 N	LEU B 0 O	2.69			
ARG B-1 NH2	ASN A 22 O	2.49			
ARG B-1 NH1	ASN A 22 O	2.96			

time the actin cytoskeleton undergoes rearrangement. From the initial interaction screen, 67 positive clones were obtained. PCR with the pTRG+/– primers was used to identify by insert size unique clones, of which there were 14. Rescreening was performed according to the supplier's instructions which reduced the number of verified interactors to four: NHERF1 (Q28619; also known as EBP50); PDZK1 (NP_067492; also known as CAP70); PEX5 (CAB09694); and Golli-interacting protein (AY028804; also known as LIM interactor-interacting factor, NLI-IF); The pTRG clones of these genes were then used to test interactions with the wild type C-Tail of pro-Muclin/apactin and the various phosphomimetics (Table 1). Also tested were two bait constructs with the terminal 4 amino acids deleted (Δ-4aa C-Tail, Table 1), since the C-terminus contains the PDZ-binding domain (Table 2).

To determine which subdomains of CAP70 (PDZK1) are sufficient to bind the apactin carboxyl-terminal peptide, we did pairwise sequence comparison between EBP50 (Q28619) and CAP70 (NP067492) based on previous analyses of conserved regions of EBP50 [19] which was shown to be an interacting partner for apactin [18]. As shown in Fig. 2a, significant identity exists among the 83 residues (12–92) of EBP50 and CAP70 (241–323). Shading indicates the amino acids of the PDZ domain that are most highly conserved among both the proteins revealing similar topology of the PDZ domain, including the amino acids (Lys19, Asn22, Gly23, Tyr24, Gly25, Phe26, His27, Leu28, His72, Val76, Ile79, Arg80, Ala82 and Leu83) involved in interaction with carboxyl terminal residues of interacting protein (bait protein). All the peptide ligands of bait protein insert into the NHERF PDZ1 binding pocket antiparallel to the $\beta 2$ strand and extends the β-sheet of PDZ1 (Fig. 2b). In this arrangement, the invading pentapeptide (carboxyl-terminal peptides of apactin PSTKL and its mutants) is highly ordered. The binding pattern of carboxyl-terminal peptides of apactin is similar to that of CFTR carboxyl-terminal peptide (Fig. 3a–f). The carbonyl oxygen of Pro-4 of the ligand hydrogen bonds with the amide nitrogen of Gly30 of the PDZK1 protein (Fig. 3a, b, d, e; Table 3), indicating that Pro-4 does not contribute to the specificity of the interaction. By contrast, Asp(D) -3, Thr(T) -2, Lys(K) -1 and Leu(L) 0 are engaged in numerous interactions with PDZ1, consistent with

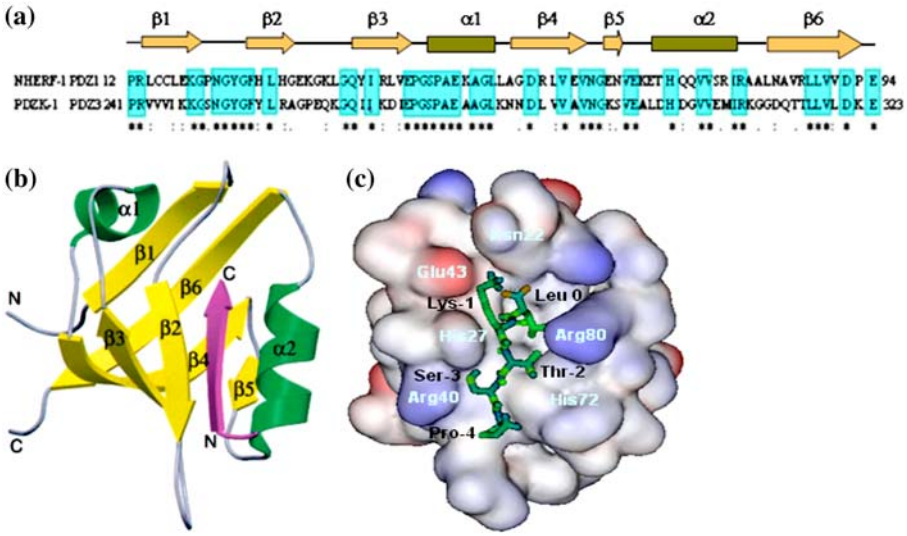


Fig. 2 Structure of the NHERF PDZ1 domain bound to the Apactin carboxyl terminal sequence PSTKL. (**a**) sequence comparison of PDZ domains that bind to Apactin. The indicated PDZ domains from human NHERF 1[23] and murine PDZK1/CAP70 [9] were aligned using ClustalX. Absolutely conserved residues are shown on *blue* background. The secondary structure of NHERF PDZ1 is indicated at the *top*. (**b**) ribbon diagram of the NHERF PDZ1 domain bound to the Apactin-PSTKL-COOH peptide. The strands $\beta1$–$\beta6$ are shown in *yellow*, and the helices $\alpha1$ and $\alpha2$ are shown in *green*. The peptide ligand PSTKL is shown in *green*. The figure was made using MOLSCRIPT [24] and Raster3D [25]. (**c**) surface topology of the NHERF PDZ1 bound to the peptide PSTKL. The figure was generated using DS Visualizer

biochemical evidence on the central role of these residues in the specificity and affinity of the NHERF PDZ1-apactin interaction (as mentioned above by bacterial 2-hybrid assay). Specifically, the $O^{\delta1}$ atom of Asp-3 hydrogen bonds with $N^{\delta1}$ of His27, and the $O^{\delta2}$ atom of Asp-3 form a salt bridge with $N^{\eta1}$ of Arg40 (Fig. 3b, c, d, Table 3). Similarly, the amide nitrogen and carbonyl oxygen of Thr-2 hydrogen bond with the carbonyl oxygen and amide nitrogen of Leu28, respectively, while the $O^{\gamma1}$ atom of Thr-2 hydrogen bonds with the $N^{\varepsilon2}$ atom of the conserved His72 (Fig. 3a–d, Table 3). The side chain and carboxylate group of Leu 0 enter into a deep cavity formed by Tyr24, Gly25, Phe26, Leu28, Val76, and Ile79 residues (Fig. 3a–d). The $C^{\delta1}$ atom of Leu 0 makes hydrophobic contacts with the atoms $C^{\varepsilon2}$ and C^{ζ} of Phe26 and $C^{\delta1}$ of Ile79. In addition, the carboxyl oxygen of Leu 0 hydrogen bonds with the amide nitrogens of Gly25 and Phe26, whereas the carbonyl oxygen of Leu 0 hydrogen bonds directly with the amide nitrogen of Tyr24. The side chain amide nitrogen N^{ζ} of Lys-1 hydrogen bonds with carbonyl carbon of Asn22 (Fig. 3a, d). The C^{β} atom of Lys-1 makes hydrophobic contacts with the $C^{\varepsilon1}$ atom of His27. In contrast, Ser-3 amino acid in wild type apactin protein (STKL–COOH) is not involved in hydrogen bonding with PDZ1 domain. However, substitution of Ser-3 to Asp-3 in PDTKL–COOH peptide increases the affinity of interaction of PDTKL–COOH with PDZ1 domain. This is evident from the docking study of the carboxyl-terminal peptides of C-tail of apactin (STKL–COOH and phosphomimetics) with NHERF/EBP-50 and PDZK1 (NP_067492); aka CAP70. The glide score, E-model and the MOE docking energy are all found to be more negative in QDTRL and it followed in the order of PDTRL, PDTKL, PSTKL, PAAAA and RGQPP (Table 4). This reveals that the peptide QDTRL has the highest interaction than that of other peptides. Substitution of all the four carboxyl-terminal amino acids in the wild type to Alanine further reduces the interaction. Only the carbonyl oxygen and amide nitrogen of Alanine are found to be involved in hydrogen bonding. Truncation of the wild carboxyl-terminal peptide to RGQPP–COOH, showed very low affinity of interaction with PDZ1 domain (Table 4). Only the atom $O^{\varepsilon1}$ of Gln-2 hydrogen bonds with $N^{\varepsilon2}$ of His72 of PDZ domain.

Discussion

The isobutyl side chain of Leu 0 in the ligand fits tightly in the hydrophobic cavity of PDZ1, suggesting that this stereochemical complementarity may underlie the strict requirement for carboxyl-terminal leucine in all the high affinity ligands of NHERF PDZ1 [3, 27, 28]. Conceivably, smaller side chains would leave vacated spaces within this hydrophobic cavity that would be energetically unfavorable [29], whereas bulkier side chains would not readily fit within this pocket. Moreover, the hydrophobic character of the cavity would likely exclude polar and charged side

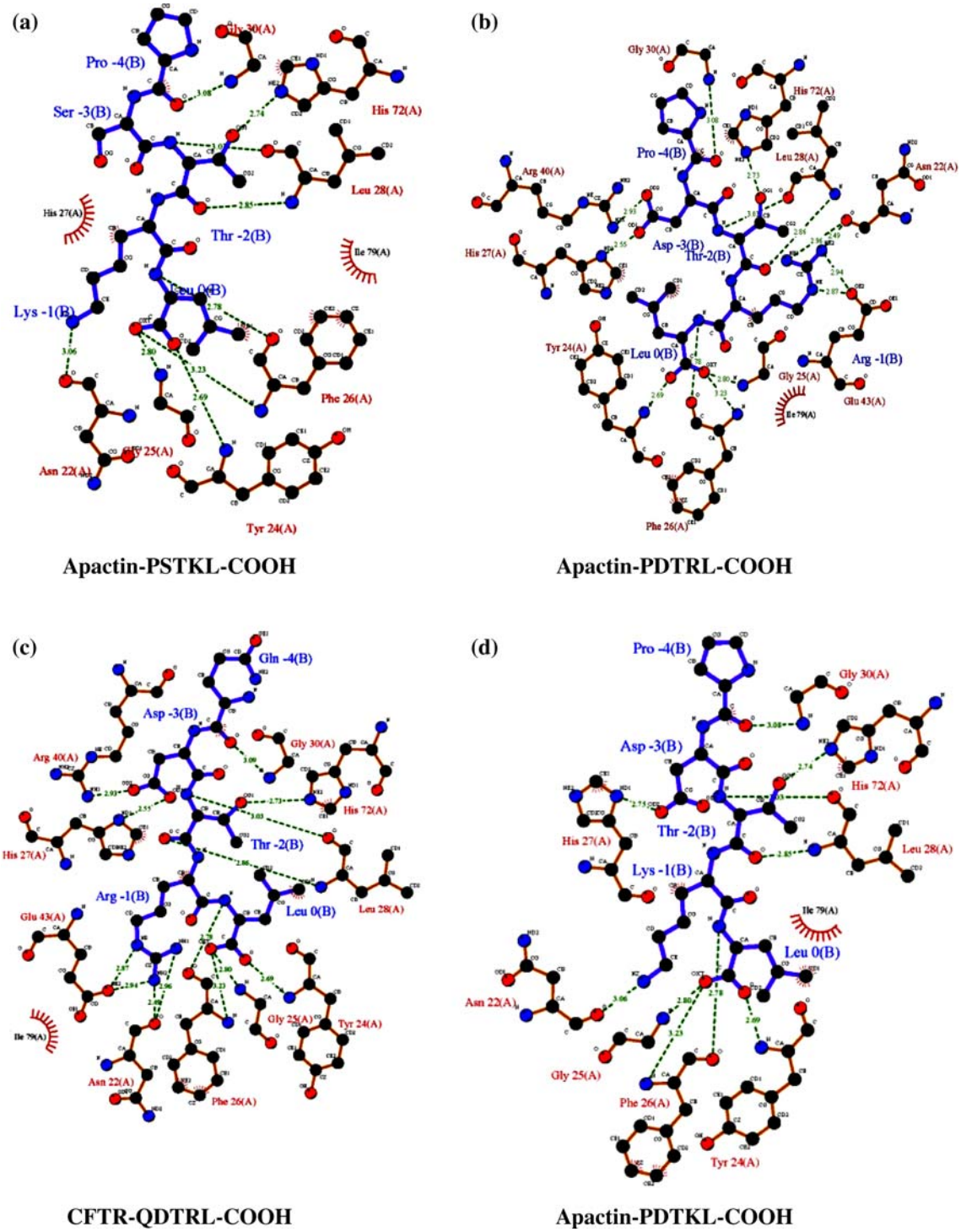
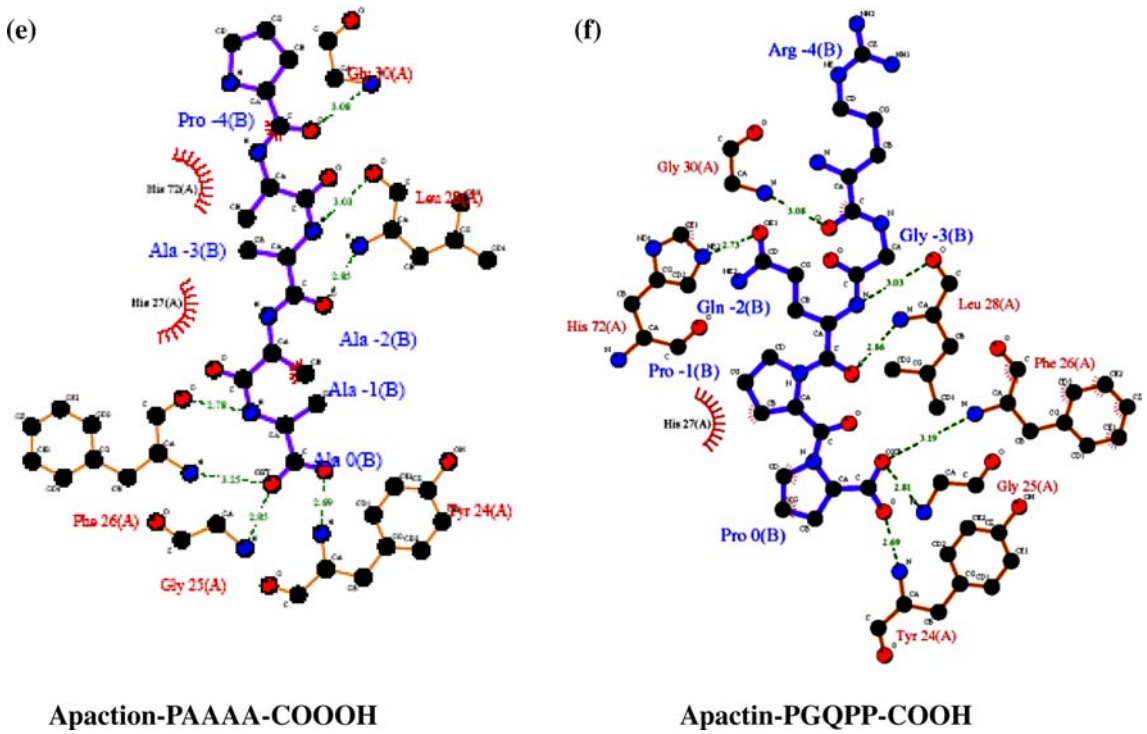


Fig. 3 (**a**–**f**) Two-dimensional representation of the interactions observed between the NHERF PDZ1 residues (orange) and the peptide ligand (purple). *Dashed lines* denote hydrogen bonds, and *numbers* indicate hydrogen bond lengths in Å. Hydrophobic interactions are shown as *arcs* with *radial spokes*. The figure was made using LIGPLOT [26]

chains. It therefore appears that the volume, shape, and hydrophobicity of the PDZ pocket provide the structural determinants for the selection of stereochemically complementary hydrophobic carboxyl-terminal side chains for high affinity binding.

Strikingly, the guanido group of Arg-1 of the ligand forms two salt bridges with $O^{\varepsilon 2}$ of Glu43 and two hydrogen bonds with the carbonyl oxygen of Asn22 of the PDZ protein (Fig. 3b, c). Indeed, the side chain of Lys-1 of the ligand forms only one hydrogen bond with the carbonyl



Apaction-PAAAA-COOOH **Apactin-PGQPP-COOH**

Fig. 3 continued

oxygen of Asn22 of the PDZ protein. This reveals that the mutant type PDTRL and QDTRL of apactin binds with the PDZ1 domain with high affinity. Nevertheless, previous biochemical studies demonstrated that arginine is the preferred residue at position-1 of the ligand for optimal binding to NHERF PDZ1 [27, 28]. Affinity selection experiments showed that NHERF PDZ1 selected almost exclusively ligands with arginine at position-1 from random peptides [28]. Furthermore, point mutagenesis of the penultimate arginine to alanine, phenylalanine, leucine, or glutamic acid decreased the affinity of the PDZ1-ligand interaction by 2–10-fold [28]. The multiple interactions between the Arg-1 guanido group and PDZ1 residues Glu43 and Asn22 observed explain the remarkable preference for a penultimate arginine by NHERF PDZ1. By contrast, the hydrogen bonding and salt bridge of Asp-3 with the PDZ domain increase the affinity of mutant PDTRL by PDZ domain than that of wild type STKL. The point mutagenesis of Thr-2 to Ala reduces the affinity further. Consequently, PDZ domains have a preference for specific side chains at position-1 and -3 interact optimally with peptide ligands having the corresponding penultimate residues.

Interestingly, the NHERF and PDZK1/CAP70 PDZ domains that bind to the apactin tail [18] have almost identical amino acids, which are involved in the interaction. Hence, the specificity and affinity of interactions were found to be identical between them. Further the two-hybird result also reveals similar results (Table 2). The interactions of pro-Muclin/apactin C-Tail constructs with PDZK1 and EBP50 are not unexpected, as these PDZ proteins should recognize the PDZ-binding domain at the C-terminus of the C-Tail. The native CAP70 protein was found in actin-rich domains, such as the apical surfaces of epithelial cells in the small intestine and kidney as well as flopodia-like structures in cultured epithelial cells [10]. Together, the evidence suggests that CAP70 may interact with actin filaments and/or other cytoskeletal components via an intermediate adaptor protein that specifically links CAP70 to cytoskeletal components, thereby connecting the CAP70 interacting cell surface receptors and ion channels to intracellular cytoskeleton structures.

The interaction with PEX5 found in the two-hybird screen is likely based on the similarity of the C-terminus of pro-Muclin/apactin to the peroxisomal caroboxyl-terminus import signal peptide on peroxisomal proteins synthesized on free ribosomes [Ser-Lys-Leu] [28]. However, since pro-Muclin/apactin is a membrane protein and not free in the cystosol, this signal is probably not of biological significance. It is interesting that one protein was identified that still interacted with the C-Tail bait lacking the 4 C-terminal amino acids. This was Golli-interacting protein, and its interaction was abolished when the Thr was mutated to Asp. This may be significant as Golli-interacting protein has a CTD-phosphatase domain, and the results suggest that the Thr site may be able to bind this phosphatase.

Table 4 Result of the docking of ligands (carboxyl-terminal peptide complexes) with PDZ1 NHERF

Rank	Ligand.	Glide Score (Kcal/mol)	Emodel**	CvdW*	Hydrogen Bonds	MOE Docking Energy(Kcal\mol)
1	QDTRL	–11.06	–181.9	–78.4	14	–3.0772
2	PDTRL	–10.94	–184.1	–79.4	14	–2.2064
3	PDTKL	–10.56	–190.9	–78.1	11	–2.1118
4	PSTKL	–7.52	–160.5	–64.3	9	–2.1058
5	PAAAA	–6.85	–128.3	–55.8	7	–1.2579
6	RGQPP	–6.85	–117.6	–49.2	7	–1.08

Glide Score (GScore): GScore = $a \times$ vdW + $b \times$ Coul + Lipo + Hbond + Metal + BuryP + RotB + Site; where vdW = van der Waals energy, Coul = Coulomb energy, Lipo = Lipophilic contact term HBond = Hydrogen-bonding term, Metal = Metal-binding term, BuryP = Penalty for buried polar groups, RotB = Penalty for freezing rotatable bonds, Site = Polar interactions in the active site, and the coefficients of vdW and Coul are: $a = 0.065$, $b = 0.130$

* CvdW = Coul + vdW is the non-bonded interaction energy between the ligand and the receptor

** Emodel is a specific combination of GScore, CvdW, and the internal torsional energy of the ligand conformer

We have demonstrated that carboxyl-terminal Asp(D) -3, Thr(T) -2, Lys(K) -1 and Leu(L) 0 are involved in numerous interactions with PDZ1 domains of NHERF and PDZK1/CAP70. Substitution of all the four carboxyl-terminal amino acids in the wild type to Ala reduces the interaction. Further, truncation of the wild-type carboxyl-terminal peptide to RGQPP–COOH, showed very low affinity of interaction with PDZ1 domain. The scores, E-model and MOE docking energy reveal that the peptide QDTRL has the highest interaction with NHERF/EBP-50 and PDZK1 (NP_067492); aka CAP70 than other carboxyl-terminal peptides (Table 4).

However, substitution of Ser-3 to Asp-3 in wild type apactin (PSTKL–COOH) increases the affinity of interaction of PDTKL–COOH with PDZ1 domain. This is in conformity with our earlier studies that stimulation-mediated Ser phosphorylation increases apactin association with the actin cytoskeleton thereby, maintaining tight bundling of actin microfilaments at the apical surface. [18].

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