Systematic Analysis of a Simple Adaptor Protein PDZK1: Ligand Identification, Interaction and Functional Prediction of Complex

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Key Words

Adaptor protein • Literature mining • PDZ • PDZK1 • Protein interaction

the study of other adaptor proteins that contain peptide-binding domains.

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Abstract

PDZK1 is a simple adaptor protein with four protein interaction PDZ domains, but without any other known functional domains. Here, we used yeast two-hybrid screening of a random peptide library and high-throughput validation screening of a specialized PDZ ligand candidate library to systematically and comprehensively identify PDZK1 ligands. The potential functional associations of the ligands were predicted by functional annotations from a MILANO literature search and subcellular localizations. The ligands were considered more likely to be functionally associated if they had similar patterns of functions or closely related functions. For some functionally associated ligand pairs, interaction with one ligand was found to be influenced by another ligand in a yeast three-hybrid system. Many G-protein signaling pathway-related proteins were found to interact with PDZK1, and they were likely to be functionally associated with transporters based on their closely related functions. This strategy can be extended to

Introduction

The PDZ domain is involved in a variety of important cellular functions, such as intracellular routing of proteins, regulation of neurotransmitter transporters, and the formation of multi-protein complexes [1, 2]. A general function of PDZ domains is to promote the clustering of proteins into complexes at the plasma membrane [3]. Typically, the PDZ domain is specialized for binding to the extreme carboxyl termini of its target proteins [4, 5]. Some PDZ domains can create further functional diversities through other interaction modes, such as dimerization of PDZ-PDZ domains [6, 7] and recognition of internal sequences [8, 9].

A large number of adaptor proteins contain several PDZ domains. Ilya Bezprozvanny (2001) divided the multi-PDZ domain proteins into three general groups [10]: MAGUKs (contain GUK domains) simple adaptors (contain PDZ domains only) and complex adaptors (contain other functional and/or protein-protein interaction

domains in addition to PDZ domains). All simple PDZ adaptors contain PDZ domains from different groups, probably because different specificities of PDZ domains in these proteins are needed to provide the flexibility required to assemble signal transduction complexes. Simple adaptor proteins provide no information about their own functions, but their ligands with known functions can provide clues to the functions of the complex.

PDZK1 contains four tandem PDZ domains [11]. Each PDZ domain interacts with many multifunctional proteins, and this affects subcellular localization, as summarized in Table 1. In northern blot analysis, PDZK1 mRNA was detected in the mouse liver, kidney, small intestine and testis [12].

Several powerful methods, including oriented peptide library, SPOT synthesis, phage display, and yeast two-hybrid (Y2H) methods, have been used for the characterization of the peptide recognition specificities of individual domain families. Here, we used yeast two-hybrid screening of a random peptide library and high-throughput validation screening of a specialized candidate ligand library to systematically and comprehensively identify the ligands of PDZK1. The advantages are that affinity competition from different ligands can be avoided. Transient interactions can be captured, and actual binding sequences can be isolated [13-15]. A random peptide library allows the identification of specific peptides to deduce the consensus-binding sequences in a single round of screening [16].

Many of the known ligands that interact with PDZ proteins are functionally associated [17-20]. We assumed that the ligands of an adaptor protein are more likely to be associated functionally if they meet one of the following criteria. Firstly, if two ligands of same protein have similar patterns of functions, they are likely to be functionally associated. Since the number of publications with co-occurrences of biological process terms roughly reflects the likelihood of their functional association in biological processes two ligands were considered more likely to be associated functionally when both had similar functional patterns in their corresponding functional search for biological process terms. Secondly, if the ligands all have a closely related function, they are more likely to be functionally associated. The closely related functions could be defined by the ratio of co-occurrences of two biological processes in relevant publications.

The effect of one ligand's binding to PDZK1 on another ligand's presence in predicted ligands pairs with functional associations was studied using a three-hybrid system.

PDZ domains	Proteins	Sequences	Reference
PDZ1	BCR	STEV	[46]
	SR-BI	EAKL	[47]
	SLC22A12	STQF	[48]
	MRP2	STKF	[49]
	MAP17	STPM	[49]
	SLC9A3	STHM	[50]
	CFTR	DTRL	[11]
	SLC22A11	STSL	[51]
PDZ2	CLC-3B	STTL	[52]
	SLC22A4	ITAF	[50]
	SLC22A5	STAF	[53]
	SLC22A12	STQF	[48]
	SLC15A2	KTKL	[29]
	UBC9	FAPS	[50]
PDZ3	SLC26A3	ETKF	[50,54]
	SLC17A1	HTRL	[50]
	SLC34A1	ATRL	[12,50,55]
	SLC15A2	KTKL	[29]
	SLC21A13	KTKL	[50]
	SLC26A6	VTRL	[50]
	SLC26A3	ETKF	[50,54]
	CFTR	DTRL	[11]
PDZ4	SLK	STGS	[50]
	SSTR5	TSKL/TSRL	[28]
	SLC22A12	STQF	[48,50]
	SLC22A4	ITAF	[53]
	SLC22A5	STAF	[53]
	MAP17	STPM	[49,50]
	SLC9A3R1	FSNL	[50]
	SLC9A3R2	FSNF	[50]
	CFTR	DTRL	[11]
	D-AKAP2/AKAP10	STKL	[50]
	SLC22A11	STSL	[51]

Table 1. Interaction of each PDZ domain in PDZK1 with C-terminal domain sequences of several membrane proteins from other independent experiments.

Materials and Methods

Preparation of bait plasmids

cDNAs encoding PDZK1's four PDZ domains were obtained by PCR from PDZK1_mouse ORF (Swiss-Prot accession number Q9JIL4). PDZK1 PDZ1 cDNA was amplified by PCR with sense primer 5'-tcc ccc ggg tat gcc ctc cac ctt caa ccc cag a-3' and antisense primer 5'-cgc gga tcc tta ctg att ttt cac agc ctt ctc ata gga-3'. The PCR product was digested with SmaI and BamHI and cloned into the GAL4 BD vector, pBridge, which carries *Trp1* as a selection marker. PDZK1 PDZ2 cDNA was amplified by PCR with sense primer 5'-ccg gaa ttc ggg atg aac gga gc ggt g-3' and antisense primer 5'-cgc gga tcc tta ttc act atg gca cct gcc agt-3'. The PCR product was digested with EcoRI and BamHI and cloned into the GAL4 BD vector, pBridge. PDZK1 PDZ3 cDNA was amplified with sense primer 5'-ccg gaa ttc ttg aaa ctg ctg ccc cac cag ccc cgg-3' and antisense primer 5'-cgc gga tcc tta ccg agc cag gct ata gat gct ctc tgc-3'. The PCR product was digested with EcoRI and BamHI and cloned into the GAL4 BD vector, pBridge. PDZK1 PDZ4 cDNA

Id	Aliases	Alone	amino acid transport (61638)	anion transport (33200)	calcium ion transport (12243)	carbohydrate transport (59068)	synaptic transmission (47308)
949	SCARB1 CLA1 SRB1 CLA-1 SR-BI CD36L1 scavenger receptor class B member 1	1319	25	4	2	41	1
6550	SLC9A3 NHE3 solute carrier family 9 (sodium/hydrogen exchanger) isoform 3	592	37	58	5	21	0
1244	ABCC2 DJS MRP2 cMRP ABC30 CMOAT ATP -binding cassette sub-family C (CFTR/MRP) member 2	1211	95	368	0	111	0

Table 2. Function patterns correlation between two ligands calculation. The number of queries that returned at least one result was considered as a positive result. The positive result correlation between SCARB1 (Gene ID: 949) and SLC9A3 (Gene ID: 6550) was 4, the correlation between SCARB1 (Gene ID: 949) and ABCC2 (Gene ID: 1244) was 3, and the correlation between SLC9A3 (Gene ID: 6550) and ABCC2 (Gene ID: 1244) was 3.

was amplified with sense primer 5'-ccg gaa ttc gcg gag ggt cac aag ccc aag-3' and antisense primer 5'-cgc gga tcc tta ctt age ttg gaa gta gct gta ggc-3'. The PCR product was digested with EcoRI and BamHI and cloned into the GAL4 BD vector, pBridge. PDZK1 full length cDNA was amplified with sense primer 5'-cgc gga tcc aaa tgg cgt cca cct tca acc cc-3' and antisense primer 5'-cgc gga tcc tca cat ctc cgt gtc ttc aga -3'. The PCR product was digested with BamHI and cloned into the GAL4 BD vector, pBridge. All bait plasmids had a stop codon following the PDZ domain. All the constructed bait plasmids were confirmed by DNA sequencing.

Construction and characterization of the Y2H random peptide library and Y2H screening of this library

A high diversity random peptide library was constructed using an improved methodology based on our previously described method [21]. The library was constructed based on the following principles. Genomic DNA digested with a frequent-cutting restriction enzyme recognizing four nucleotides will theoretically consist of small DNA pieces with average length of 256 nucleotides, and on average around 10^7 fragments can be generated from a genome of 3×10^9 bases. A peptide library translated from these fragments will have sufficient diversity for some protein interaction screening experiments, 1.4×10^7 clones were obtained for the random peptide library. The GAL4 BD-PDZ fusion bait plasmid was transformed into the yeast strain CG1945 using the lithium acetate protocol as previously described [22].

Validation screening of the PDZ ligand library

Validation screening is a one-to-one Y2H testing of all the PDZ ligands in PDZ ligand library. The PDZ ligand library was the collection of all the non-redundant positive clones isolated from random peptide library screening by other PDZ

domains previously [22]. The one-to-one Y2H method was used for validation screening of the current PDZ ligand library consisting of 242 non-redundant PDZ ligand clones. There were 124 (51.24%), 71 (29.34%), 17 (7.02%), 2 (0.80%), and 28 (11.57%) for class I, class II, class III, class IV and other ligands, respectively.

Candidate ligand prediction and confirmation using a yeast two-hybrid system

For each PDZ, consensus-binding sequences were deduced from sequence alignment of positive clones and comparative analysis of positive sequences. Tailfit software was used to search the IPI database to retrieve the potential ligand proteins whose carboxyl termini matched the consensus-binding sequences [23]. All ligands were analyzed by GOfact [24] (<http://www.hupo.org.cn/GOfact>) for Gene Ontology (GO) [25] annotation; GOfact supplies fundamental information (processes, functions and components) for these ligands. Because PDZK1 PDZ domains commonly mediate the formation of protein complexes at the membrane, the ligands found at the membrane were chosen for one-to-one Y2H confirmation. The GAL4 AD plasmids expressing the carboxyl-terminal 13 amino acid residues of the candidate ligands of PDZK1-PDZ1 were first constructed based on our previously described method [22]. Each constructed plasmid was co-transformed with PDZK1-PDZ1 bait plasmid into yeast strain CG1945. The same procedure was used for the candidate ligands of PDZK1-PDZ2, PDZK1-PDZ3 and PDZK1-PDZ4.

Ligand association analysis by the relationship of their biological processes through literature mining

GOfact is an integrated strategy for functional analysis in large scale proteomic research by gene ontology [25]. We used Gofact [24] (<http://www.hupo.org.cn/GOfact>) to search the

Process A:	Process B:	A∩B	Association rate of A to B $\frac{A \cap B}{A}$	Association rate of B to A $\frac{A \cap B}{B}$
1420	106046	77	$\frac{77}{1420}$ (0.054)	$\frac{77}{106046}$ (0.0007)

Table 3. Association rate calculation. The number of publications for A (phosphate metabolism) is 1420 for B (excretion) is 106046, number of literature co-occurrences of the two processes is 77. As shown in the table above, the association rate of A to B is 0.054.

ligands for their GO annotation. MILANO (Microarray Literature-based Annotation) was used as the literature mining tool; it is a custom annotation of microarray results using automatic literature searches [26].

Ligand association by similar patterns of functions: The list of Gene IDs of ligands were pasted into the Primary Search Term field of the MILANO program, and the list of all the biological processes of these ligands with quotation marks were used to cross-reference search terms in the Secondary Search Term field. The number of publications reflects the relationship between the genes and biological processes. We focused on Pubmed search results, which could serve as an annotation table.

As shown in Table 2, the program's output is a table containing the number of publications for each gene-biological process combination. The number of queries that returned at least one result was considered as a positive result. The positive result correlation between SCARB1 (Gene ID: 949) and SLC9A3 (Gene ID: 6550) was 4, the correlation between SCARB1 (Gene ID: 949) and ABCC2 (Gene ID: 1244) was 3, and the correlation between SLC9A3 (Gene ID: 6550) and ABCC2 (Gene ID: 1244) was 3, so SCARB1 and SLC9A3 were considered more likely to be functionally associated than the other two pairs.

We analyzed the correlation of all the identified ligands, and the ligands with higher correlation were considered more likely to be functionally associated.

Ligand association by closely related functions: In the MILANO search, we pasted the list of all the biological processes of the identified ligands with quotation marks into both the Primary Search Term and Secondary Search Term fields to search Pubmed. The association rate was used to evaluate the relationship between these biological processes. The association rate represents the correlation of each pair of biological processes (Table 3). The higher the association rate of the biological processes, the more likely it is that the pair of ligands is associated.

The number of publications for process A is 1420, and for process B is 106046, the number of literature co-occurrences of the two processes is 77. As shown in the Table 3, the association rate of A to B is 0.054, and the association rate of B to A is 0.0007.

The association rates for each pair of biological processes were arranged from high to low. If two ligands were in biological processes with a high association rate, we considered these two ligands to have a higher probability of being functionally associated.

	GENE ID	Name	Carboxyl terminus	
PDZ1	10158	MAP17	STPM	[49]
	6550	SLC9A3	STHM	[50]
	116085	SLC22A12	STQF	[48]
	1244	MRP2	STKF	[49]
	55867	SLC22A11	STSL	[51]
	1182	CLC-3B	STTL	[52]
	54868	TMEM104	ETKL	✓
	8884	SLC5A6	ETSL	✓
	66035	SLC2A11	STEL	✓
	80704	SLC19A3	STKL	✓
PDZ2	6583	SLC22A4	ITAF	[50]
	6584	SLC22A5	STAF	[53]
	116085	SLC22A12	STQF	[48]
	114571	SLC22A9	VTQF	✓
	6568	SLC17A1	HTRL	[50]
PDZ3	6569	SLC34A1	ATRL	[12,50,55]
	6565	SLC15A2	KTKL	[29]
	28230	SLC21A13	KTKL	[50]
	65010	SLC26A6	VTRL	[50]
	1080	CFTR	DTRL	[11]
	6755	SSTR5	TSKL	[28]
	10786	SLC17A3	LTRL	✓
	55824	PAG1	ITRL	✓
	7224	TRPC5	TTRL	✓
	80704	SLC19A3	STKL	✓
	9229	DLGAP1	QTRL	✓
	22839	DLGAP4	QTRL	✓
	25789	C19orf4	LTKL	✓
	160728	SLC5A8	GTRL	✓
	5420 ^a	PODXL	DTHL	✓
PDZ4	6539 ^a	SLC6A12	ETHL	✓
	6753 ^a	SSTR3	ISYL	✓
	165829 ^a	GPR156	PTLV	✓
	8884 ^a	SLC5A6	ETSL	✓
	3357 ^a	HTR2B	VSYV	✓
	116085	SLC22A12	STQF	[48,50]
	6584	SLC22A5	STAF	[53]
	10158	MAP17	STPM	[49]
	10080	CFTR	DTRL	[11]
	11216	D-AKAP2	STKL	[50]
	55867	SLC22A11	STSL	[51]
	66035	SLC2A11	STEL	✓
	7224	TRPC5	TTRL	✓
	80704	SLC19A3	STKL	✓
	7223	TRPC4	TTRL	✓

Table 4. C-termini recognized by each PDZ domains in PDZK1. ^aThe ligand proteins identified directly from the validation screening of the PDZ ligand library.

Fig. 1. Strategy for systematic analysis of a simple adaptor protein PDZK1. The binding properties of four PDZ domains in PDZK1 were characterized by yeast two-hybrid screening of a random peptide library and high-throughput validation screening of a specialized PDZ ligand candidate library. The C termini represent native proteins in the library and enable the direct discovery of novel PDZ ligand proteins from a single round of validation screening. The candidate ligand proteins were predicted by protein database searches using consensus-binding sequences, followed by manually filtering with biological information such as subcellular localization. The C-terminal 13 amino acid residues of the predicted candidate ligand proteins were cloned and co-transformed into CG1945 yeast cells with four PDZK1 PDZ bait plasmids. Following the systematic and comprehensive identification of PDZK1 ligands, MILANO was used as a literature mining tool to analyze ligand associations by their similar patterns of functions and their closely related functions. The interactions of the top 10 functionally associated ligand pairs were studied in a yeast three-hybrid system.

Interaction of functionally associated ligand pairs in a yeast three-hybrid system

The pBridge vector contains two distinct multiple cloning sites to allow for the expression of the DNA-BD fusion as well as a third protein. In our pBridge three-hybrid system, the PDZ domain was fused to the Gal 4 BD domain, and one ligand was fused to the Gal 4 AD domain, the third protein in BD vector can influence the interaction. The coding sequence for the carboxyl-terminal 13 amino acid residues of one ligand from the functionally associated ligand pairs was cloned into MCSII which is located downstream of an HA epitope and a second NLS. The resulting fusion protein in conditionally expressed from the MET25 promoter in response to methionine levels in the medium. The plasmid with both PDZK1 and one ligand was co-transformed with another functionally associated ligand in an AD (pGADT7) plasmid; the transformants were selected on plates with SD/-His/-Leu/-Trp/-Met medium. Transformed yeasts were plated on SD/-Trp-Leu for a transformation efficiency control, and LacZ assays were repeated to verify results if the growth and LacZ assays were both negative.

Results

Our strategy involved the following seven steps, as illustrated in Figure 1. The binding properties of four PDZ domains in PDZK1 were characterized by yeast two-hybrid screening of a random peptide library and high-

throughput validation screening of a specialized PDZ ligand candidate library. The C termini represent native proteins in the library and enable the direct discovery of novel PDZ ligand proteins from a single round of validation screening. The candidate ligand proteins were predicted by protein database searches using consensus-binding sequences, followed by manually filtering with biological information such as subcellular localization. The C-terminal 13 amino acid residues of the predicted candidate ligand proteins were cloned and co-transformed into CG1945 yeast cells with four PDZK1 PDZ bait plasmids. Following the systematic and comprehensive identification of PDZK1 ligands, MILANO was used as a literature mining tool to analyze ligand associations by their similar patterns of functions and their closely related functions. The interactions of the top 10 functionally associated ligand pairs were studied in a yeast three-hybrid system.

Systematic identification of PDZK1 ligands

The potential ligand proteins of the four PDZK1 PDZ domains in the human proteome were retrieved by searching the IPI database with the consensus-binding sequences using Tailfit software [27]. The GO annotation of these ligands was searched using GOfact (<http://www.hupo.org.cn/GOfact>) which provided the

PDZ1		PDZ2		PDZ3		PDZ4	
3	IHETKL*	4	AKVTKF*	49	FRQTRL*	13	ILTTAL*
17	IFSTYL*	6	VKITRF*	20	KKSTRL*	22	IASTKL*
35	LLSTWL*	9	ARVTPF*	11	TDITRL*	36	FSTRL*
40	IRETLL*	10	VKITTF*	6	HSGTRL*	39	FSTAM*
41	LRSTAL*	14	HIITKF*	14	EKETRL*	42	FSTPL*
67	IESTFL*	16	HIITKF*	43	QKITRL*	46	ILSTSL*
6	IVSTSL*	17	HIITKF*	13	MESTRL*	68	FQSTPL*
27	ICTTLL*	21	HIITKF*	9	QNITKL*	74	EESTKL*
76	LNSTYL*	2	HIITKF*	45	TKWTRL*	102	FSTPL*
81	FSTRL*	3	CHITRF*	36	VRVSR*	104	FSTAM*
29	LKSTFM*	18	LKITKF*	29	AEVTRL*	108	EISTHL*
56	CNETRL*	19	YKSGPF*	17	RGGTRL*	126	LSTSL*
	SE-T-x-LM ^a		VIS-T-x-F ^a	41	YKMTRL*	69	LSTSM*
8	IFNTYF*	3.43	QKITRL*	54	MKSTRL*	1.3	IHETKL*
10	LKVACF*	1.3	IHETKL*		ST-KR-L ^a	76	FSTKF*
18	IHSEHF*	20	LSYSVH*	34	DSGTWL*		ST-T-x-FLM ^a
36	SHSTYF*			16	HGGTWL*		
	TAE-YCH-F ^a			50	FKCTYL*		
31	VEQTNL*			T50	GRQTFL*		
46	MEQTYI*			D87	GEITYL*		
	E-Q-T-x-IL ^a				T-WFY-L ^a		
37	VFSSAL*			2.6	VKITRF*		
66	KWCSHL*				T-KRH-F ^a		
	SC-S-x-L ^a						
82	EKEHYL*						
3.45	TKWTRL*						
3.16	HGGTWL*						
Z3.27	LESIFL*						

Table 5. Positive binding sequences for the PDZK1 domains.
^a:Consensus sequence, x, any amino acid. They were obtained from random peptide library screening except the number in bold were obtained from validation screening.

fundamental information (processes, functions and components) of these ligands. Because PDZ domains commonly mediate the formation of protein complexes at the membrane, the ligands which localize at the membrane were chosen for one-to-one Y2H confirmation. 22 candidate ligand proteins which had been previously been shown to interact with the four PDZK1 PDZ domains by other independent experiments (Table 1) were also included in the functional association study. The one-to-one Y2H method was employed to confirm the candidate interactions. The candidate ligand proteins chosen for Y2H confirmation were selected manually using biological information such as subcellular localization (membrane). The C-terminal 13 amino acid residues of the candidate ligand proteins were cloned and co-transformed into CG1945 yeast cells with four PDZK1 PDZ bait plasmids. As shown by a “v” in Table 4, several novel ligands have been confirmed by the Y2H system.

The binding properties of the four PDZ domains in PDZK1 were characterized experimentally in the study (Table 5). The binding properties of PDZ1, PDZ3 and PDZ4 are similar. These PDZ domains showed predominant preference for hydrophobic amino acids at the extreme carboxyl terminus (P0), and the results indicated a strong consensus located at the C-terminal

No	Name	Sequence	Binding domain
2.19	Binding sequence	-YKSGPF	PDZ2
6753	SSTR3	-MRISYL	PDZ3
165829	GPR156	-LKPTLV	PDZ3
5420	PODXL	-EEDTHL	PDZ3
25789	C19orf4	-LDLTKL	PDZ3
160728	SLC5A8	-SNGTRL	PDZ3
3357	HTR2B	-ERVSYV	PDZ3

Table 6. The target-binding preference of full length PDZK1 differs from that of the separate PDZ domains.

Target protein	Correlation	Ligand 1	Ligand 2	Reference
BAT3	40	EDN1	PTH	[31]
	32	END1	PRNP	
	32	PRNP	PTH	[32]
	30	CDK4	PTN	
	29	CDK4	END1	
	28	IGFBP5	PTH	
	27	CDK4	PRNP	[34-36]
	27	PELP1	PTH	
	26	CDK4	PELP1	[33]
	26	EDN1	IGFBP5	
SAT	37	EPHB6	TP53	[37]
	36	HTATIP	TP53	
	30	EPHB6	HTATIP	[38]
	25	TP53	SEPHS1	
	24	PTN	TP53	[39]
	23	HTATIP	SEPHS1	
	22	EPHB6	PTN	
	22	EPHPB6	SEPHS1	
	21	APLP1	TP53	
	20	APLP1	EPHB6	

Table 7. Ligand association by similar patterns of functions.

four amino acid residues. PDZ2 showed unique properties. It showed a predominant preference for aromatic amino acids at the extreme carboxyl terminus (P0).

In a previous study, only one G-protein-associated protein was found to interact with PDZK1 [28]. In our study, other G-protein-associated proteins such as SSTR3, GPR156 and HTR2B were identified as PDZK1 potential ligands. It seems that PDZK1 can interact with many G-protein-associated proteins, and these G-protein-associated proteins may functionally regulate transporters via their roles in the G-protein signaling pathway. Some new biological processes from PDZK1 ligands identified in our study were also found, such as “cell-cell signaling” (DLGAP1&DLGAP4) and “immune response” (PAG1).

Systematic ligand identification results (Table 4) indicated that one ligand can bind to two or more PDZ domains in PDZK1. For example, CFTR can bind PDZ1, PDZ3 and PDZ4 [11]. It has been reported that CFTR channel open probability is increased by the formation of

Table 8. A: BAT3 ligand association by closely related functions. *: associate rate of B to A. B: SAT ligand association by closely related functions. *: associate rate of B to A.

Association rate*	Process A	Ligands in process A	Process B	Ligands in process B	Reference	
A						
0.957265	G1/S transition of mitotic cell cycle regulation of progression through cell cycle	CDK4	cell cycle	CDK4	[33]	
0.840362		CDK4	Cell cycle	CDK4		
0.8	dorsal/ventral pattern formation	PCTK1 END1	transcription	IKBKAP SETDB1 PELP1		
0.767726	spliceosome assembly signal transduction	SMN1	RNA splicing	SMN1		
0.722222		IGFBP5 EDN1	G-protein signaling, phospholipase D activating pathway	END1		
0.714286	protein amino acid phosphorylation	CDK4 IKBKAP PCTK1	transcription	IKBKAP SETDB1 PELP1		
0.672332	protein import into mitochondrion transcription	TOMM20	protein targeting to mitochondrion	TOMM20		
0.581114		IKBKAP SETDB1 PELP1	chromatin modification	SETDB1		
0.576923	signal transduction	EDN1 IGFBP5	epidermal growth factor receptor signaling pathway	TDGF1		
0.571429	signal transduction	EDN1 IGFBP5	protein amino acid phosphorylation	IGFBP5 IKBKAP PCTK1		
B						
0.975	apoptosis	TP53 FLJ13391 APLP1	caspase activation via cytochrome c	TP53	[39]	
0.927602	signal transduction	TLE1	transmembrane receptor protein tyrosine phosphatase signaling pathway	PTN		
0.918596	apoptosis	APLP1 TP53 FLJ13391	caspase activation	HSPE1	[39]	
0.870934	cell cycle	TP53 RIF1	negative regulation of progression through cell cycle	TP53		
0.84056	cell cycle	TP53 RIF1	regulation of progression through cell cycle	PTN		
0.8	apoptosis	TP53 FLJ13391	regulation of mitochondrial membrane permeability			
0.75	signal transduction	APLP1 TLE1	androgen receptor signaling pathway	TP53 HTATIP		
0.714286	transcription	CHD3 TP53 SETDB1H TATIP MYST2	protein amino acid phosphorylation	EPHB6		
0.714286	transport	ABCB8	protein amino acid phosphorylation	EPHB6		
0.6	transcription	TP53 SETDB1 MYST2 HTATIP CHD3	negative regulation of helicase activity			[56]
				TP53		[37]
				TP53		[40]

CFTR dimers through an interaction with a common PDZ adaptor protein like PDZK1. The bivalent binding could bring two CFTR molecules into proximity, reminiscent of

the bivalent binding ability of PDZ3-4 or PDZ3-3. At high concentrations, PDZK1 binding was mediated primarily via the PDZ3 domain, which had the highest affinity. As

Table 9A

Correlation	Ligand 1	Ligand 2	Reference
17	CFTR	NHE3	[41]
15	BCR	CFTR	
15	BCR	NHE3	
14	BCR	NHERF1	[42]
14	CFTR	NHERF1	
14	MRP2	NHE3	
14	NHE3	NHERF1	[44]
13	BCR	NHERF2	
13	CFTR	NHERF2	[43]
13	CFTR	DRA	[45]

Table 9. A: PDZK1 ligand association by similar patterns of functions. B: PDZK1 ligand association by closely related functions. *: associate rate of B to A.

Table 9B

Association rate*	Process A	Ligands in process A	Process B	Ligands in process B
0.926778	regulation of Rho protein signal transduction	BCR	signal transduction	D-AKAP2 SSTR5 HTR2B GPR156 SSTR3
0.714286	protein amino acid phosphorylation	BCR SLK	transport	CFEX SLC22A9 SLC17A3 URAT1 SLC19A3 OAT4 DRA MRP2
0.571429	protein amino acid phosphorylation	BCR SLK	signal transduction	D-AKAP2 SSTR5 HTR2B GPR156 SSTR3
0.553191	intracellular signaling cascade	BCR NHERF2	signal transduction	D-AKAP2 SSTR5 HTR2B GPR156 SSTR3
0.502513	G-protein signaling, coupled to cyclic nucleotide second messenger	SSTR3 SSTR5	signal transduction	D-AKAP2 SSTR5 HTR2B GPR156 SSTR3
0.498316	cell-cell signaling	DLGAP4 DLGAP1	signal transduction	D-AKAP2 SSTR5 HTR2B GPR156 SSTR3
0.342105	regulation of pH	NHE3 CLCN3	transport	CFEX SLC22A9 SLC17A3 URAT1 SLC19A3 OAT4 DRA MRP2
0.34072	fluid secretion	OCTN1 NPT2	transport	CFEX SLC22A9 SLC17A3 URAT1 SLC19A3 OAT4 DRA MRP2
0.337931	urate transport	URAT1	excretion	DRA
0.336788	carnitine transport	OCTN2	organic cation transport & cation transport	OCTN1 TRPC4 TRPC5

a result, the bivalent binding was reduced and the channel switched back to a less active state [11]. We believe that different numbers or different species of ligands bind to different sites to facilitate the formation of different complexes, leading to different functions in different conditions.

Most of PDZK1’s ligands are transporters. PDZ domain-containing proteins (PDZK1, PDZK2, NHERF1 and NHERF2) that directly interact with the C-terminus

of transporters have been reported [29]. Some of these PDZ proteins have been suggested to regulate transporters via at least two pathways, i.e. stabilization at the cell-surface and direct modulation of transporter function. Therefore, it is possible that membrane transport of therapeutic agents is not only governed by drug transporters themselves, but also indirectly by PDZ proteins [30].

Each ligand plasmid identified by a separate PDZ

Table 10. Interactions that were influenced and the influencers. The rows in bold were predicted by word/phrase without quotation marks in Pubmed search only, the others were predicted by word/phrase without and with quotation marks in Pubmed search.

Presence	Binding domain	Effect	Interaction	Binding domain	Method for prediction
D-AKAP2	PDZ4	Interruption	DLGAP1	PDZ3	Related functions
HTR2B	PDZ3	Interruption	DLGAP1	PDZ3	Related functions
D-AKAP2	PDZ4	Interruption	BCR	PDZ1	Related functions
CFTR	PDZ1,3,4	Interruption	PAG1	PDZ3	Patterns of functions
PAG1	PDZ3	Interruption	CFTR	PDZ1,3,4	Patterns of functions
SSTR5	PDZ3	Rescue	HTR2B	PDZ3	Related functions

domain was co-transformed with full length PDZK1 bait plasmid into yeast strain CG1945. We found that some ligands identified by PDZ domains did not interact with the full length PDZK1 in the Y2H system. Three-dimensional structures of the full length protein may influence these ligands' binding properties. Our current study demonstrates that the ligands identified by the separate PDZ domains were different from the full length protein. This underscores the notion that the results identified by PDZ domains should be carefully considered.

As shown in Table 6, the binding sequences of the PDZ domains did not bind to full length PDZK1.

Assessing the validity of this method for predicting functional associations of the ligands

SAT, BAT3 and ZNF145 were chosen from a high-throughput protein-protein interaction study [13] whose ligand numbers ranged from 31 to 40. The average number of articles for the ligands of SAT, BAT3 and ZNF145 in Pubmed were over 500. These were chosen to assess the validity of the literature mining method, since this literature mining method is probably only suitable for the ligands of interesting proteins which have been studied extensively.

Based on ligand association by similar patterns of functions, the top 10 correlations of BAT3 and SAT were analyzed; some predicted ligands associations were supported by other independent experiments (Table 7). So far, there has been no experimental support for ZNF145. It has been shown that END1 regulates PTH expression of human parathyroid cells [31] and can induce an increase in CDK4 kinase activity [32], END1 also proved inhibitory to the IGFBP5 endopeptidase inducing property of FSH [33]. PTH has been shown to stimulate the production of insulin-like growth factors (IGFs) and insulin-like growth factor-binding proteins (IGFBPs) [34-36]. It has been shown that depletion of HTATIP inhibits the ability of TP53 to activate BAX and PUMA transcription [37]. Chung HJ et al. demonstrated that SEPHS1 was able to affect cell viability upon ionizing radiation via modulation of TP53 activity [38]. APLP1 was confirmed to be a novel transcriptional target of TP53

by in vivo and in vitro characterization [39].

Based on ligands association by closely related functions, the top 10 association rates of BAT3 and SAT were analyzed (Table 8); some predicted ligands associations were supported by other independent experiments. So far there has been no experimental support for ZNF145 yet. TP53-CHD3 interactions via SUMO-1 were detected by Adrian Minty et al [40]. The three pairs of ligands (IGFBP5&END1, TP53&HTATIP and TP53&APLP1) were described.

Some predicted ligand associations were supported by experiments from other laboratories. But the association of other ligands, which were not predicted by our method, could not be ruled out. With the accumulation of relevant information, the strategy can be repeated again to find new functional associations of the ligands.

Prediction of functional associations of PDZK1 ligands by similar patterns of functions and closely related functions

45 interactions were identified, including 23 novel interactions and 22 known interactions, among four PDZ domains and 35 ligand proteins. The PDZK1 ligands previously reported and those identified in our study were analyzed together by this literature mining method.

We used MILANO as a literature mining tool to analyze PDZK1 ligand association by similar patterns of functions and closely related functions. The top 10 ligand association correlations were analyzed by similar patterns of functions (Table 9A) or closely related functions (Table 9B). So far, there has been no experimental support for PDZK1 by the second method, but for four pairs of ligands, the interaction of one ligand with PDZK1 was found to be influenced by the presence of its counterpart in yeast three hybrid study (Table 10).

As shown in Table 9A, CFTR has been demonstrated to regulate the activity and expression of the Na⁺/H⁺ exchanger, NHE3 [41]. Bossard, F. et al. revealed that NHERF1 specifically determined the apical plasma membrane expression of DeltaF508-CFTR [42]. Favia, M. et al. demonstrated that the deletion of any of the NHERF2 binding domains inhibited both PKA-

dependent apical CFTR expression and its activation [43]. NHERF1 and NHERF2 bind directly to the exchanger, NHE3 [44]. Ko, S.B. et al. demonstrated that CFTR markedly activates Cl^- and $\text{OH}^-/\text{HCO}_3^-$ transport by a member of the SLC26 family, DRA [45].

Predicting functional associations of the ligands used terms without quotation marks in Pubmed search

We also searched words/phrases without quotation marks in Pubmed (supplemental data). Without quotation marks, two new predicted functionally associated ligand pairs were found. The associations were supported by other independent experiments, for APLP1&HSPE1 [13] and NHE3&NHERF2 [44], while two pairs, END1&IGFBP5 [33] and CHD3&TP53 [40], which had supporting evidences too, were lost in the search.

The interactions of functionally associated ligand pairs

The interactions of the top 10 functionally associated ligand pairs were tested in a three-hybrid system to see if one ligand's interaction with PDZK1 can be influenced by the presence of another ligand. These interactions (Table 10) may be the mechanism for the functional regulation of the protein complex in a biological process. It provides potential targets for intervention.

The data from HPRD (<http://www.hprd.org>) and EXPASY (<http://www.expasy.org>) demonstrated that some functionally associated ligand pairs from Table 10 share common expression sites and subcellular locations, such as: DLGAP1&D-AKAP2; DLGAP1&HTR2B; CFTR&PAG1; HTR2B&SSTR5. There is no evidence that BCR&D-AKAP2 share common expression sites and subcellular locations.

As shown in Table 10, interaction of six ligands with PDZK1 can be influenced by another ligand's presence. Possible mechanisms of influence are i): these ligand pairs compete for the same PDZ domain (for example, both DLGAP1 and HTR2B can bind to PDZ3) and ii): allosteric effects from one ligand inserted into one PDZ domain binding pocket causes structural changes in another PDZ peptide binding groove, thus regulating the binding (for example, the binding of DLGAP1 to PDZ3 might inhibit the binding of D-AKAP2 to PDZ4). In this relatively high-throughput Y3H study, the results could only provide clues for future verification in cells.

Interestingly, HTR2B identified by separate PDZ domains does not interact with full length PDZK1, but the interaction can be rescued by the ligand SSTR5. Some

ligands that bind to full length proteins need the presence of another ligand.

It is not common that one ligand's interaction with PDZK1 was influenced by the presence of another. 54 pairs of other predicted ligands were tested in yeast three-hybrid system, only in six pairs list in Table 10, interactions were influenced.

Currently, only the interactions between two ligands can be studied due to the limitations of the yeast three-hybrid system. In this relatively high throughput study, the carboxyl-terminal sequences were used instead of the full length ligands for studying the interactions of functionally associated ligand pairs. The reason that some functionally associated ligand pairs predicted by the literature mining method may not have been shown to interact may be in part because carboxyl-terminal amino acids take up less space than the full length proteins.

Discussion

This data mining method requires extensive research for each potential ligand because it is based on ligand association by closely related known functions. The top 10 association rates were analyzed for PDZK1. So far, there has been no experimental support for the predictions for PDZK1, even though the method seemed to work well in predictions for SAT and BAT3. We believe that the research of PDZK1 functions may not yet be extensive enough for this method, or the predictions have not yet been verified by experiments.

We searched words/phrases with or without quotation marks in our literature mining, the results were similar (supplemental data). With quotation marks, the results are more precise. Without quotation marks, the results may contain more indirect clues which can help to relate functions of the two ligands.

To define closely related functions, we used all the biological process of these identified proteins in a MILANO search. If the biological process could be more specific to the protein, the results would be more reliable. Currently, the biological processes from the GO annotation are not specific enough. The reason that we could still use these GO annotations to define closely related functions for protein associations was because of the strict preconditions that these proteins all bind to same protein, PDZK1. Without evidence from experimental interactions, the association analysis would be full of false positives.

This strategy can be easily extended to a variety of peptide-binding domain families that act as adaptor

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Abbreviations

AD (activation domain); BD (binding domain); HPRD (human protein reference database); PCR (polymerase chain reaction); SPOT synthesis (synthesis of peptides on cellulose membranes); Y2H (yeast two-hybrid); Y3H (yeast three-hybrid); ORF (open reading frame).

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