Convergent and Divergent Ligand Specificity among PDZ Domains of the LAP and Zonula Occludens (ZO) Families*

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We present a detailed comparative analysis of the PDZ domains of the human LAP proteins Erbin, Densin-180, and Scribble and the MAGUK ZO-1. Phage-displayed peptide libraries and *in vitro* affinity assays were used to define ligand binding profiles for each domain. The analysis reveals the importance of interactions with all four C-terminal residues of the ligand, which constitute a core recognition motif, and also the role of interactions with more upstream ligand residues that support and modulate the core binding interaction. In particular, the results highlight the importance of site^{-1}, which interacts with the penultimate residue of ligand C termini. Site^{-1} was found to be monospecific in the Erbin PDZ domain (accepts tryptophan only), bispecific in the first PDZ domain of ZO-1 (accepts tryptophan or tyrosine), and promiscuous in the Scribble PDZ domains. Furthermore, it appears that the level of promiscuity within site^{-1} greatly influences the range of potential biological partners and functions that can be associated with each protein. These findings show that subtle changes in binding specificity can significantly alter the range of biological partners for PDZ domains, and the insights enhance our understanding of this diverse family of peptide-binding modules.

Many specialized cellular functions associated with multicellular organisms are dependent upon specialized membrane domains (1). Particularly well studied examples of membrane specialization are found in the cells of the epithelia and endothelia, which exhibit apicobasal polarity arising from the differential sorting of plasma membrane proteins to apical and basolateral compartments and from polarization of the cytoskeleton (2, 3). Apicobasal polarity is maintained by junctional complexes that are responsible for lateral adhesion between cells and act as barriers to diffusion between different compartments (4, 5). The molecular interactions governing cellular polarity have attracted increasing attention, as it has become apparent that loss of polarity is linked to uncontrolled cell proliferation, a hallmark of tumorigenesis (6–8).

In vertebrates, the junctional complexes include tight junctions (4), which are located in the most apical region of the lateral membrane, and adherens junctions (5), which are located immediately basal to tight junctions. Arthropods contain septate junctions rather than tight junctions, and these are located basally rather than apically to the adherens junctions (Fig. 1A). Adherens junctions maintain cell-cell adhesion, whereas tight/septate junctions maintain apico-basal polarity and regulate diffusion of solutes through the paracellular pathway. To a large extent, these distinct functions are a consequence of the protein composition of each type of junction. Tight junctions contain three types of integral membrane proteins: claudins, occluding, and junctional adhesion molecule. Adherens junctions contain integral membrane proteins of the cadherins family, which in turn associate with the cytoplasmic proteins α-, β- and γ-catenin. Inside the cell, diverse cytoplasmic proteins associate with either the claudin-occludin complex in tight junctions or the cadherin-catenin complex in adherens junctions. Many of these proteins belong to families that contain PDZ (PSD-95/Discs-large/ZO-1)[2] domains (4, 5); in the present study, we have focused on two of these families.

The LAP (leucine-rich repeats and PDZ domains) family members contain 16 leucine-rich repeats, two LAP-specific domains, and one or more PDZ domains (3, 9) (Fig. 1B). In mammals, the family includes Erbin and its close homologue Densin-180, which each contain one PDZ domain, and Scribble (Scrib), which contains four PDZ domains (Fig. 1B). Although expression of Densin-180 is brain-specific (10, 11), Erbin and Scrib are expressed in both neurons and epithelia (12, 13). In polarized epithelial cells, both proteins are localized on the lateral membrane and at adherens junctions (13–15). Genetic studies have shown that Scrib is a neoplastic tumor suppressor in *Drosophila*, as genetic mutations lead to loss of apicobasal polarity and concomitant overproliferation (16, 17). In addition, studies in *Caenorhabditis elegans* have shown that LET-413, a LAP family member, is critical for normal assembly of adherens junctions (18). Thus, members of the LAP family are involved in the organization of junctional complexes and in the establishment and maintenance of cell polarity.

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[2] The abbreviations used are: PDZ, PSD-95/Discs-large/ZO-1; CAT, catenin; BSA, bovine serum albumin; Densin-PDZ, the PDZ domain of human Densin-180; DLG, Discs-large; ELISA, enzyme-linked immunosorbent assay; Erbin-PDZ, the PDZ domain of human Erbin; GST, glutathione *S*-transferase; GUK, guanylate kinase; GUKH, guanylate kinase holder; LAP, leucine-rich repeats and PDZ; MAGUK, membrane-associated guanylate kinase; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PSD-95, postsynaptic density-95; Scrib-PDZ*n*, the *n*th PDZ domain of human Scrib; TOPK, T-LAK cell-originated protein kinase; ZO, zonula occludens; ZO1-PDZ*n*, the *n*th PDZ domain of human ZO-1.
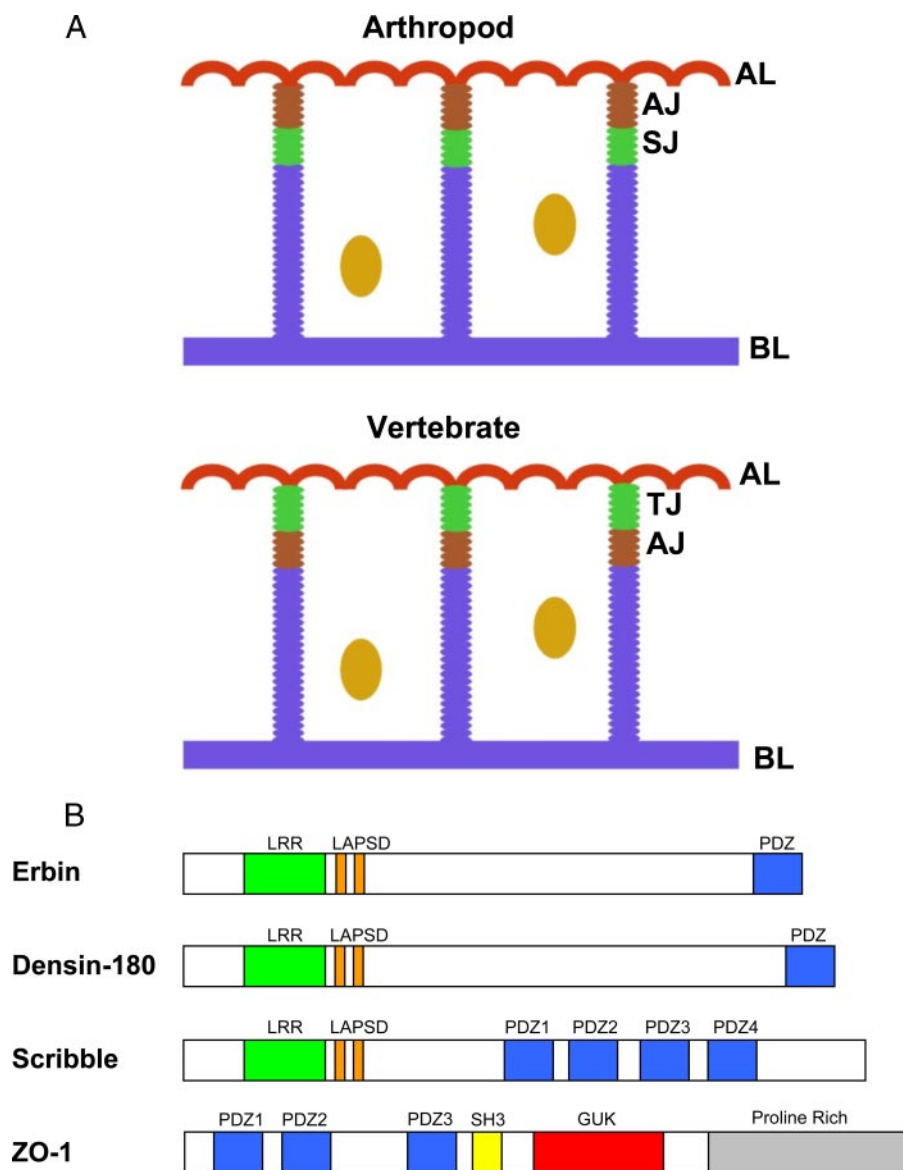
PDZ Domain Specificity

FIGURE 1. **PDZ domain-containing proteins associated with junctional complexes.** *A*, domains and junctions of epithelial cells in arthropods and vertebrates. Adherens junctions (*AJ*) and tight junctions (*TJ*) or septate junctions (*SJ*) in arthropods, separate the apical layer (*AL*) from the basolateral layer (*BL*). *B*, domain organization of the human LAP family members and ZO-1. In addition to PDZ domains, the LAP family members also contain leucine-rich repeats (*LRR*) and LAP-specific domains (*LAPSD*). ZO-1 belongs to the MAGUK family, which is characterized by the presence of PDZ domains, a Src homology-3 (*SH3*) domain, a GUK domain, and a proline-rich region.

ZO-1 is localized exclusively at tight junctions in polarized epithelia, but in non-epithelial cells that lack tight junctions, the protein is associated with adherens junctions (22–24). ZO-1 also associates with adherens junction markers in nonpolarized epithelial cells, as for example, ZO-1 and E-cadherin colocalize at initial sites of cell-cell contact during the establishment of apicobasal polarity (25–28). Distinct junctional complexes form as polarization proceeds, and ZO-1 and E-cadherin segregate into the tight or adherens junctions, respectively (27). In contrast with ZO-1, the LAP family members are excluded from the tight junctions of polarized epithelia and, instead, are confined to the adherens junctions and the basolateral layer (13).

The LAP and ZO families present an interesting example of proteins that exhibit both convergence and divergence in terms of structural organization and cellular function. The proteins all utilize PDZ domains to mediate interactions with intracellular binding partners, and we thought it would be enlightening to examine in detail the similarities and differences between these domains.

PDZ domains are peptide-binding modules, and as many as 440 PDZ domains in 259 different proteins have been predicted within the human genome (29). They are usually embedded in large scaffolding proteins along with additional PDZ domains and/or other peptide-binding modules, and thus, PDZ domains function to assemble their ligands into large complexes with other scaffold-associated molecules (30–32). As the biological functions of PDZ domains are intimately associated with ligand specificity, there is great interest in understanding the molecular basis for PDZ domain function.

Structural studies have revealed that PDZ domains share a common fold and most recognize protein C termini in a sequence-specific manner (33–40). Peptides bind in a groove located between a β-strand and an α-helix, and in most cases, the C-terminal carboxylate is critical for high affinity binding, as it is hydrogen-bonded to backbone amides in a conserved "carboxylate-binding" loop. In addition, the last four ligand residues form hydrogen bonds with the PDZ domain β-strand in an anti-parallel β-sheet conformation. These backbone-medi-

The membrane-associated guanylate kinase-like homologues (MAGUKs) represent another family that is associated with junctional complexes; these proteins contain PDZ domains, a Src homology-3 (SH3) domain, and an inactive guanylate kinase-like (GUK) domain that is involved in protein-protein interactions (1, 4, 19). The MAGUK zonula occludens-1 (ZO-1) and its close homologues (ZO-2 and ZO-3) each contain three PDZ domains (Fig. 1*B*) and are involved in assembling protein complexes at tight junctions (4, 19). ZO-1 has also been implicated as a tumor suppressor, because down-regulation of its expression is coupled to breast cancer progression (20). However, it is likely that ZO-1 acts as a tumor suppressor only in certain cancers, as its function depends on many different factors (4), and it is overexpressed in some cancers (21).

ated interactions are common to most PDZ domain-ligand complexes, and thus, it is the nature of the interactions with the ligand side chains that determines the specificity and biological function of each unique PDZ domain.

Essentially all PDZ domains prefer ligands containing a hydrophobic residue at the C terminus (position^0),[3] and early studies have suggested that specificity is determined mainly by interactions with the side chain at position^{-2} (35, 39, 41). These findings led to a classification scheme based on the specificity of site^{-2}, and two major classes of PDZ domain specificities were proposed (class I (X[T/S]$X\phi_{COOH}$) and class II ($X\phi X\phi_{COOH}$), where X is any amino acid and ϕ is a hydrophobic amino acid). However, recent studies have revealed a more complex picture of selectivity that depends critically on all four C-terminal ligand side chains and is also influenced by residues further upstream (33, 34, 36–38, 40, 42–44). For example, our studies of the Erbin PDZ domain (Erbin-PDZ) have demonstrated that it is a class I domain, but high affinity ligand recognition also depends on a Trp at position^{-1}, a Glu/Asp at position^{-3}, and an aromatic residue at position^{-4} (38). Erbin-PDZ was originally identified as a binding partner for ErbB2 by yeast two-hybrid methods (45), but it was subsequently shown that the PDZ domain binds with much higher affinity to three p120-like catenins (δ-CAT, ARVCF (armadillo protein deleted in velo-cardiofacial syndrome), and p0071) (46, 47), which terminate in an identical tetrapeptide sequence (DSWV$_{COOH}$) that closely matches the optimal binding motif for Erbin-PDZ ([E/D][T/S]WV$_{COOH}$). In contrast, the C terminus of ErbB2 (DVPV$_{COOH}$) differs significantly at position^{-1}.

This example and others have made it clear that detailed analysis and comparison of many proteins will be required to ascertain and explain the full range of ligand specificities supported by the PDZ domain fold. A high resolution understanding of the molecular basis for PDZ domain function should in turn provide considerable insights into the mechanisms whereby ligand specificity and promiscuity dictate biological function.

Herein, we report an in-depth, comparative analysis of the binding specificities of the PDZ domains of the human LAP family and ZO-1. We have used phage-displayed peptide libraries and *in vitro* affinity assays to map the binding specificity of each domain in fine detail. The specificity profiles were used to rationalize known natural interactions and, also, to predict novel, putative interactions that may explain the physiological functions of each protein. In an accompanying article (48), we used structural analysis to delineate the molecular basis for similarities and differences in ligand recognition. Taken together, these studies provide a comprehensive view of how PDZ domains use a common fold to mediate a diverse array of biological functions.

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[3] The C terminus of a PDZ domain ligand is designated as position^0, and the remaining positions are numbered with negative integers for which the absolute value decreases toward the C terminus. The corresponding binding sites on the PDZ domain are numbered in a corresponding manner as site^0, site^{-1}, etc.

EXPERIMENTAL PROCEDURES

Materials—Enzymes and M13-KO7 helper phage were from New England Biolabs. Maxisorp immunoplates plates were from Nalge NUNC International (Naperville, IL). *Escherichia coli* XL1-blue was from Stratagene. Bovine serum albumin (BSA) and Tween 20 were from Sigma. Anti-glutathione S-transferase (GST) antibody was from Zymed Laboratories. Plasmid pGEX, horseradish peroxidase/anti-GST antibody conjugate, and glutathione-Sepharose 4B were from Amersham Biosciences. 3,3′, 5,5′-Tetramethyl-benzidine/H$_2$O$_2$ peroxidase substrate was from Kirkegaard and Perry Laboratories, Inc. NeutrAvidin was from Pierce Biotechnology, Inc. Anti-mouse IgG antibody conjugate was from Jackson ImmunoResearch Laboratories. The Quick-coupled T7 T$_N$T *in vitro* translation system was from Promega.

Synthetic Peptides—Peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) protocols, cleaved off the resin with 2.5% triisopropylsilane and 2.5% H$_2$O in trifluoroacetic acid, and purified by reverse-phase high performance liquid chromatography. The purity and mass of each peptide were verified by liquid chromatography/mass spectrometry.

Library Construction and Sorting—A peptide library was fused to the C terminus of a mutant M13 major coat protein designed for high valency display (49) as described (46, 50). The library consisted of random decapeptides encoded by 10 consecutive degenerate codons (NNK, where N = A/C/G/T and K = G/T) and contained 2×10^{10} unique members.

Phage pools from the library were cycled through rounds of binding selection with a GST·PDZ fusion protein coated on 96-well Maxisorp immunoplates as the capture target. Phage were propagated in *E. coli* XL1-blue with M13-KO7 helper phage and 10 mM isopropyl 1-thio-β-D-galactopyranoside. After three or four rounds of binding selection, individual phage clones were analyzed in a high-throughput phage ELISA (51), and positive clones were subjected to DNA sequence analysis.

Protein Purification—GST·PDZ fusion proteins were produced and purified using the pGEX *E. coli* expression system as recommended by the manufacturer. For each PDZ domain, protein fragments spanning the following amino acids of the full-length protein were produced: Erbin-PDZ (1217–1371), Densin-PDZ (1424–1537), Scrib-PDZ1 (718–829), Scrib-PDZ2 (857–983), Scrib-PDZ3 (982–1100), Scrib-PDZ4 (1099–1219), ZO1-PDZ1 (414–506), ZO1-PDZ2 (584–660), ZO1-PDZ3 (819–901).

Affinity Assays—The binding affinities of peptides for Erbin-PDZ were determined as IC$_{50}$ values using the AlphaScreen^{TM}, a bead-based chemiluminescence assay. The IC$_{50}$ was defined as the concentration of peptide that blocked 50% of the chemiluminescence arising from the interaction of anti-GST acceptor beads coated with GST·Erbin-PDZ and streptavidin donor beads coated with biotinylated peptide (biotin-TGWETWV$_{COOH}$). Assays were performed at room temperature in white opaque 384-well plates (25 μl/well) under subdued lighting to reduce nonspecific chemiluminescence. The assay buffer consisted of PBS, 0.5% Tween 20, 0.1% bovine γ-globulin, 1 ppm proclin. The reaction mixture

contained fixed concentrations of anti-GST acceptor beads (16 μg/ml), biotin-TGWETWV$_{COOH}$ (36 nM), and GST·Erbin-PDZ (3 nM). Serial dilutions of peptide were added followed by addition of streptavidin donor beads (20 μg/ml). The mixture was incubated at room temperature for 1 h and read on an AlphaQuest plate reader set at 1 s/well.

The binding affinities of peptides for the Scrib PDZ domains and ZO1-PDZ1 were determined as IC$_{50}$ values using a competition ELISA as described (44). The IC$_{50}$ value was defined as the concentration of peptide that blocked 50% of PDZ domain binding to immobilized peptide. Assay plates were prepared by immobilizing an N-terminally biotinylated peptide (RRWFETDL$_{COOH}$, HRVRETWV$_{COOH}$, RSWFETDL$_{COOH}$, or WRWTTWL$_{COOH}$ for Scrib-PDZ1, -PDZ2, -PDZ3 or ZO1-PDZ1, respectively) on Maxisorp immunoplates coated with neutravidin and blocked with BSA. A fixed concentration of GST·PDZ fusion protein (0.7, 6.6, 0.7, or 3.6 nM for Scrib-PDZ1, -PDZ2, -PDZ3, or ZO1-PDZ1, respectively) in PBS, 0.5% BSA, 0.1% Tween 20 (PBT buffer) was preincubated for 3 h with serial dilutions of peptide and then transferred to the assay plates. After a 15-min incubation, the plates were washed with PBS, 0.05% Tween 20, incubated with a mixture of anti-GST antibody (0.5 μg/ml) and horseradish peroxidase/rabbit anti-mouse IgG antibody conjugate (1:2000 dilution) in PBT buffer, washed again, and detected with 3,3′,5,5′-tetramethyl-benzidine/H$_2$O$_2$ peroxidase substrate. For IC$_{50}$ values less than or greater than 100 μM, the standard deviations were less than 20 or 40%, respectively.

Pull-down Assays—GST or GST·PDZ fusion protein was bound to glutathione-Sepharose 4B beads following standard protocols, and bound proteins were quantified by SDS-PAGE using known amounts of BSA as standards. Wild-type ARVCF, or a mutant ARVCF in which the last three amino acids (SWV$_{COOH}$) were replaced by alanines (AAA$_{COOH}$), was produced by *in vitro* transcription/translation using the T$_N$T^{TM} Quick-coupled T7 transcription/translation system (Promega) according to the manufacturer's instructions. Beads carrying protein (2–10 μg) were incubated with 5–10 μl of *in vitro* translated wild-type or mutant ARVCF for 2 h at 4 °C in binding buffer (25 mM Tris, 50 mM NaCl, 20 mM MgCl$_2$, 0.1% Tween 20, 1 mM dithiothreitol, pH 7.5). The beads were washed with binding buffer containing 150 mM NaCl, and bound proteins were analyzed by SDS-PAGE, autoradiography, and densitometry.

Peptides corresponding to the 11 C-terminal amino acids of wild-type or a mutant (T233R) Claudin 16 were coupled to beads, and 25 μl of beads (2–10 μg of coupled peptide) were incubated for 2 h at 4 °C with 1–2 mg of GST·PDZ fusion protein in binding buffer (25 mM Tris, 50 mM NaCl, 20 mM MgCl$_2$, 0.1 mM EDTA, 0.1% Tween 20, pH 7.5). The beads were washed five times with binding buffer containing 150 mM NaCl, and bound proteins were fractionated by SDS-PAGE on 10% gels and blotted onto polyvinylidene difluoride membranes. Membranes were stained with Ponceau S for 5 min and then washed with water.

RESULTS AND DISCUSSION

Binding Specificity Profiles for the PDZ Domains of the LAP Proteins—As we and others have shown, combinatorial peptide libraries can be used to survey the ligand preferences of PDZ domains (38, 41, 44, 46, 52, 53). These combinatorial methods are particularly effective as guides for the design of synthetic peptide ligands for use in *in vitro* binding assays, which quantitatively assess the contributions of individual ligand side chains to binding affinity and specificity (38).

We used phage-displayed peptide libraries to explore the binding specificity profiles of the PDZ domains of the human LAP family members Erbin, Densin-180, and Scrib. Each PDZ domain was screened separately against a library of random decapeptides displayed in a high valency format by fusion to the C terminus of the M13 major coat protein (49). The library was constructed with degenerate codons that encode for all 20 natural amino acids and, also, for a stop codon that allows for the display of shorter peptides. We were successful in obtaining specific binding clones for each domain except the fourth PDZ domain of Scrib (Scrib-PDZ4). As the four PDZ domains of Scrib are highly homologous, it is not clear why the selection failed for Scrib-PDZ4, but we speculate that Scrib-PDZ4 may be less stable than the other domains. We sequenced binding clones from each of the successful experiments and obtained many unique sequences that could be aligned and analyzed for homology (Fig. 2). The sequence data were also used to guide the design of a panel of synthetic peptides for affinity assays to compare and contrast the binding specificities of the different domains in detail (Table 1 and Fig. 3). For each domain, the combined results of these analyses were used to derive a specificity profile in the form of a preferred binding motif (Fig. 4).

As observed in previous studies (38, 46), Erbin-PDZ prefers ligands typical of a class I PDZ domain (Thr/Ser at position^{-2}) and exhibits significant preferences at each of the last six positions of the ligand (Fig. 4). As expected, the very similar PDZ domain of the Erbin homologue Densin-180 (Densin-PDZ) exhibits a binding motif that is essentially identical to that of Erbin-PDZ. The related but more divergent Scrib PDZ domains exhibit binding motifs that are similar to those for Erbin-PDZ and Densin-PDZ but also exhibit noteworthy differences.

In comparing the binding motifs for the LAP PDZ domains, it is apparent that they all prefer hydrophobic residues at positions upstream of position^{-3}, but the preference is for general hydrophobic character rather than for any particular residue. Structural analyses of Erbin-PDZ and other PDZ domains have shown that these upstream ligand residues can interact with PDZ domains through contacts with the loop that connects β-strands β2 and β3 (38, 42, 48). This may be a general strategy that PDZ domains use to modulate and fine-tune the affinities of ligand interactions that are predominantly mediated by interactions with the four C-terminal residues (38, 42).

Considering the four C-terminal positions, it appears that all of the LAP PDZ domains exhibit conserved, stringent preferences for ligand side chains at position^{-3} (Glu/Asp) and at position^{-2} (Thr/Ser). However, there are clear differences in the specificities observed at the two C-terminal positions, and these differences have significant impact on the overall ligand preferences of the individual domains. Ligands for the PDZ domains of Erbin and Densin-180 exhibit high conservation of a C-terminal Trp-Val$_{COOH}$ sequence, and affinity assays for Erbin-PDZ show a stringent requirement for Val^0, as even replacement with highly homologous residues (Leu or Ile) com-

A Erbin-PDZ **B** Densin-PDZ **C** Scrib-PDZ1 **D** Scrib-PDZ2 **E** Scrib-PDZ3 **F** ZO1-PDZ1 **G** ZO1-PDZ3
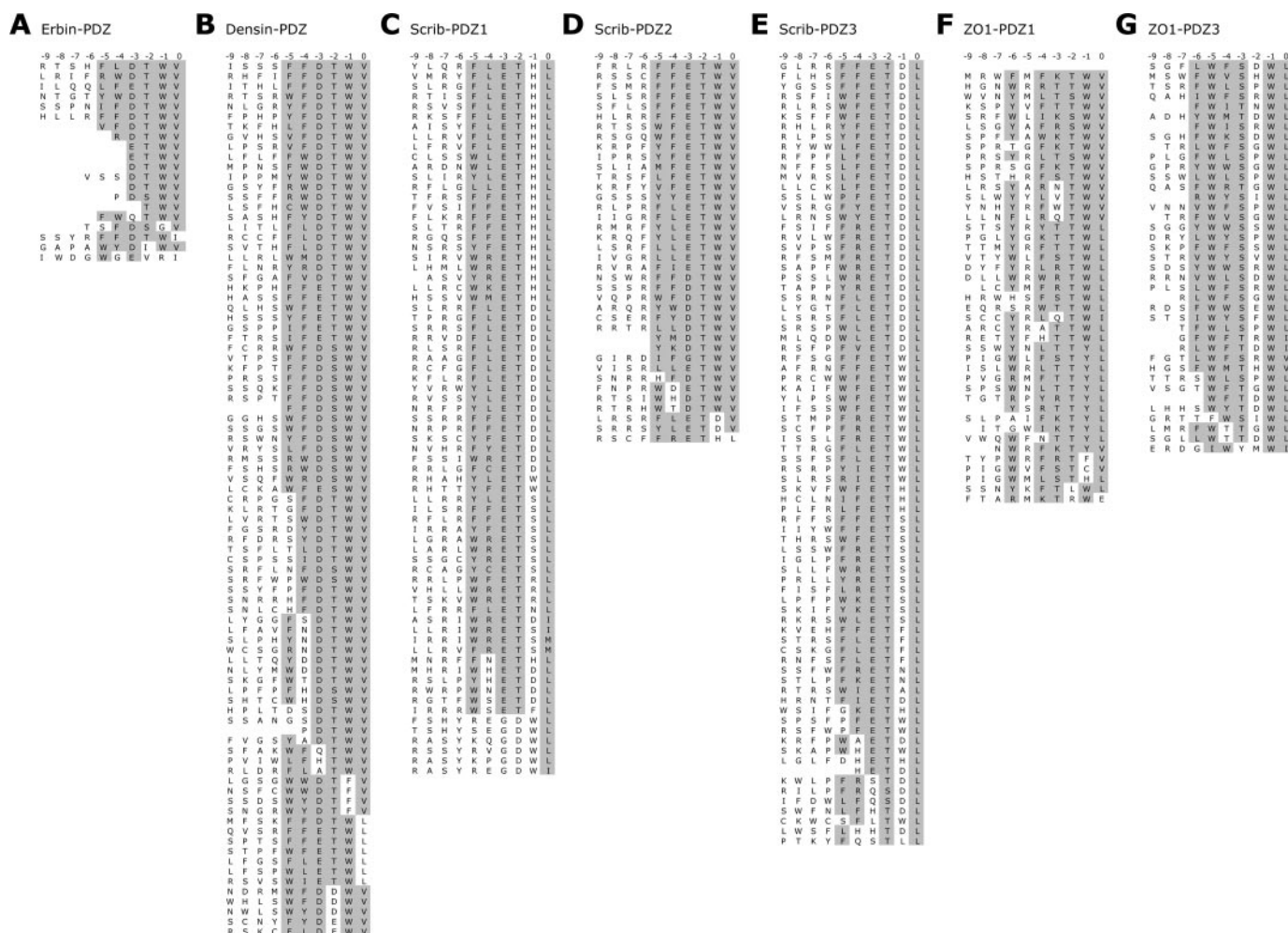
FIGURE 2. **Peptide ligands for the LAP and ZO-1 PDZ domains.** Sequences are shown for peptides selected from a completely random phage-displayed library screened against Erbin-PDZ (*A*), Densin-PDZ (*B*), Scrib-PDZ1 (*C*), Scrib-PDZ2 (*D*), Scrib-PDZ3 (*E*), ZO1-PDZ1 (*F*), and ZO1-PDZ3 (*G*). *Gray shading* indicates sequences that match the optimal binding motifs defined in the legend for Fig. 4.

TABLE 1

IC$_{50}$ values for synthetic peptides binding PDZ domains of the LAP family

The IC$_{50}$ value is the mean concentration of peptide that blocked 50% of PDZ domain binding to an immobilized high affinity peptide ligand. The N termini of the peptides were acetylated. Deviations from the sequence of peptide L1 are in bold text.

| Peptide | Sequence | | | | | | | | IC$_{50}$ | | | |
	-7	-6	-5	-4	-3	-2	-1	0	Erbin	Scrib-PDZ1	Scrib-PDZ2	Scrib-PDZ3
											μM	
L1	R	S	W	F	E	T	W	V	0.01	2.2	4.7	3.9
L2	R	S	W	F	E	T	W	**L**	0.6	4.3	100	1.9
L3	R	S	W	F	E	T	W	**I**	0.3	3.7	40	3.8
L4	R	S	W	F	E	T	**D**	V	2.1	4.6	22	8.4
L5	R	S	W	F	E	T	**E**	V	2.6	4.2	47	18
L6	R	S	W	F	E	**S**	W	V	0.01	7.1	15	8.8
L7	R	S	W	F	**D**	T	W	V	0.01	27	6.5	70
L8	R	S	W	F	E	T	**D**	**L**	21	3.6	50	1.6
L9	R	S	W	F	E	T	**E**	**L**	16	1	52	2.6
L10		**R**	**F**	**W**	E	T	W	V	0.1	1.9	2.7	3.2
L11			**W**	E	T	W	V		0.1	25	4.2	3.6
L12		**R**	**F**	**W**	E	T	**D**	V	6.1	3.9	6.4	5.9
L13			**W**	E	T	**D**	V		4.1	48	18	5.3

promises binding ~50-fold (Table 1, compare peptide L1 to L2 and L3). Furthermore, detailed analysis of specificity for position^{-1} shows that substitution of Trp^{-1} by any other amino acid is highly detrimental (Fig. 3).

In contrast, the first and third PDZ domains of Scrib are more promiscuous in their specificity for the two C-terminal ligand residues (Fig. 2). Both domains accommodate the three aliphatic side chains (Val, Leu, Ile) equally well at site^0 (peptides L1, L2, and L3), and substitutions for Trp at position^{-1} have only modest effects (<5-fold, compare peptide L1 with L4 and L5). Indeed, a detailed analysis of site^{-1} specificity of Scrib-PDZ1 shows that substitution of Trp^{-1} by other residues
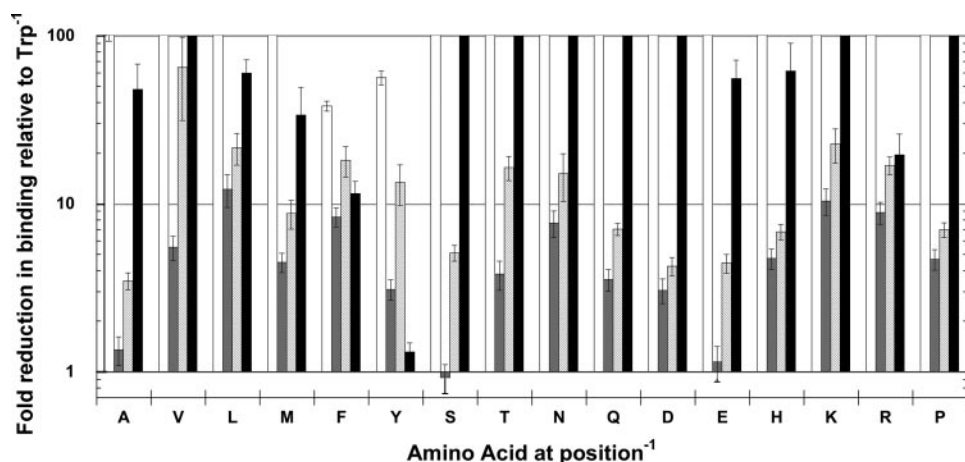
FIGURE 3. **Binding specificity at site^{-1}.** The effects of different amino acids substitutions for Trp at ligand position^{-1} (*x* axis) were assessed as -fold reduction in binding (*y* axis) by making substitutions in the context of a heptapeptide (RFWETWV$_{COOH}$). The affinities of peptide ligands were estimated as IC$_{50}$ values by competition ELISA. Greater than 100-fold reductions in binding were beyond the sensitivity range of the assays. The following PDZ domains were analyzed: Erbin-PDZ (*white bars*), Scrib-PDZ1 (*gray bars*), Scrib-PDZ2 (*cross-hatched bars*), and ZO1-PDZ1 (*black bars*).

FIGURE 4. **Binding specificity profiles for the PDZ domains of the LAP proteins and ZO-1.** For each domain, the specificity at each site is shown, as deduced from the phage data and affinity assays. ϕ = aromatic/aliphatic (Phe, Tyr, Trp, Leu, Ile, Val, Lys, Arg). Lys and Arg were included with the aromatic/aliphatic amino acids because of the aliphatic portions of their side chains.

C-terminal Val^0 over those containing Leu^0 or Ile^0 ($\sim$10–20-fold; Table 1). At position^{-1}, Scrib-PDZ2 also prefers ligands containing a Trp^{-1}, but this preference is rather modest in comparison with that of Erbin-PDZ (Fig. 3).

In summary, the binding specificities of the PDZ domains of Erbin and Densin-180 are essentially identical, and these domains exhibit strong preferences for particular side chains at all four C-terminal positions of the ligand. The binding specificity of Scrib-PDZ2 is similar to that of Erbin-PDZ, except that site^{-1} is considerably more promiscuous and exhibits only a modest preference for Trp^{-1}. Finally, the binding specificities of Scrib-PDZ1 and Scrib-PDZ3 have diverged even further from the optimal binding motif for Erbin-PDZ, and this has been accomplished by a further loosening of the binding preference at site^{-1} combined with a more promiscuous site^0.

It is worth noting that the Scrib PDZ domains are more promiscuous than Erbin-PDZ, partly because Erbin-PDZ is highly specific for Trp^{-1}, whereas the Scrib PDZ domains are not capable of recognizing any side chain at position^{-1} particularly well. Thus, the favorable interaction between Erbin-PDZ and ligands with a Trp^{-1} side chain can be considered to be an additional interaction, which the Scrib PDZ domains are not capable of making. As a result, Erbin-PDZ can recognize optimal ligands with affinities in the low nanomolar range, whereas even the optimal ligands for the Scrib PDZ domains only bind with affinities in the low micromolar range (Table 1). In other words, the Scrib PDZ domains are promiscuous because, unlike Erbin-PDZ, they are unable to bind any natural peptides with affinities in the nanomolar range but, instead, bind to a broad range of peptides with affinities in the micromolar range. Nonetheless, ligands that prefer the Scrib PDZ domains over Erbin-PDZ can be derived from a high affinity Erbin-PDZ ligand by introducing changes that are tolerated by the former but not the latter (Table 1, compare peptide L1 with L8 and L9). In addition, it may also be possible to modulate overall and relative affinities by exploiting differences in the specificities of PDZ domains for ligand side chains upstream of position^{-3} (Table 1, compare peptide L10 with L11 and L12 to L13).

Binding Specificity Profiles for the PDZ Domains of ZO-1— We also used phage-displayed peptide libraries to analyze the binding specificities of the three PDZ domains of human ZO-1. We obtained positive results for the first and third domains (ZO1-PDZ1 and ZO1-PDZ3) but not for the second domain (ZO1-PDZ2).

The alignment of ligand sequences for ZO1-PDZ1 reveals a binding motif that resembles those of the LAP PDZ domains but also exhibits significant differences (Fig. 2*F*). ZO1-PDZ1 exhibits class I binding specificity, as Thr/Ser occur with high frequency at position^{-2}, and as in the case of Scrib-PDZ1 and

reduces binding by $\sim$10-fold at most, and some substitutions have no effect (Fig. 3). Interestingly, Scrib-PDZ2 exhibits a binding motif that is intermediate between the highly specific motifs for the PDZ domains of Erbin and Densin-180 and the more promiscuous motifs for Scrib-PDZ1 and Scrib-PDZ3 (Fig. 4). Like Erbin-PDZ, Scrib-PDZ2 prefers ligands containing a

-PDZ3, all three aliphatic side chains (Leu, Ile, Val) occur at position[0]. However, unlike the Scrib PDZ domains, ZO1-PDZ1 exhibits a clear preference for particular sequences at position[−1], as binding sequences contain either Trp[−1] (similar to Erbin-PDZ) or Tyr[−1] (unique to ZO1-PDZ1). In contrast with the LAP PDZ domains, Glu/Asp residues are conspicuously absent from position[−3] in the ZO1-PDZ1 ligands, and instead, this position is occupied mostly by Thr/Ser or Arg/Lys. Beyond position[−3], there are strong preferences for hydrophobic residues at position[−4] and aromatic residues at position[−6] but no clear preference at the intervening position[−5]. Thus, the binding motif for ZO1-PDZ1 exhibits sequence preferences at six of the last seven ligand positions (Fig. 4).

The binding motif for ZO1-PDZ3 is also similar to those for the other PDZ domains, but there are peculiarities unique to ZO1-PDZ3. Considering the last two ligand positions, ZO1-PDZ3 resembles Erbin-PDZ in that it clearly prefers ligands containing Trp[−1], but it resembles Scrib-PDZ1, Scrib-PDZ3, and ZO1-PDZ1 in that it accommodates all three aliphatic side chains at position[0] (Fig. 2G). Most interestingly, ZO1-PDZ3 differs from most PDZ domains characterized to date in that it exhibits no preference at position[−2]; the ligand alignment reveals highly diverse sequences at this position with no clear bias. At position[−3], ZO1-PDZ3 prefers ligands containing Ser/Thr[−3], and this preference is different from that of the LAP PDZ domains but somewhat similar to that of ZO1-PDZ1 (Fig. 4). Considering the positions preceding position[−3], there is a preference for hydrophobic character at the −4 and −6 positions and position[−5] is dominated by Trp.

To better understand the details of ligand specificity for ZO1-PDZ1, we conducted affinity assays with a panel of synthetic peptides based on an optimal ligand derived from the phage display data. As in the case of Erbin-PDZ, the optimal ligand binds to ZO1-PDZ1 extremely tightly, exhibiting an IC$_{50}$ value in the low nanomolar range (Table 2, peptide Z1). However, unlike Erbin-PDZ, substitution of the C-terminal Val by Leu has only a modest detrimental effect on binding (~3-fold, compare peptides Z1 and Z2 or Z3 and Z4), as expected from the phage display data. Also in agreement with the phage display data, substitution of Trp[−1] by Tyr[−1] did not affect binding significantly (compare peptides Z1 and Z3 or Z2 and Z4). However, a detailed analysis of specificity at site[−1] reveals that ZO1-PDZ1 is not promiscuous like the Scrib PDZ domains but, rather, can be best characterized as bispecific; it binds with high affinity to ligands with either a Trp[−1] or Tyr[−1], but affinity is reduced severely by substitution with other amino acids (Fig. 3). Even the highly conservative substitution of Phe[−1] for Tyr[−1] attenuates binding by ~10-fold, and in terms of most other substitutions, ZO1-PDZ1 can be considered to be as specific as Erbin-PDZ.

We also explored the effects of substitutions at other positions within the optimal ligand. Substitution of Thr[−2] by Ser (Table 2, peptides Z1 and Z5) or of Thr[−3] by Ser or Lys (compare peptide Z1 with Z6 and Z7) caused only modest 3–5-fold reductions in affinity, which is consistent with the phage-derived binding motif (Fig. 4). At the three positions preceding position[−3], various aromatic/aliphatic residues were well tolerated (Table 2, compare peptide Z1 with Z8–Z12). To better

TABLE 2

IC$_{50}$ values for synthetic peptides binding to ZO1-PDZ1

The IC$_{50}$ value is the mean concentration of peptide that blocked 50% of PDZ domain binding to an immobilized high affinity peptide ligand. The N termini of the peptides were acetylated. Deviations from the sequence of peptide Z1 or E1 are in bold text.

Peptide	Sequence							IC$_{50}$
	−6	−5	−4	−3	−2	−1	0	
								μM
Z1	W	R	R	T	T	W	V	0.0049
Z2	W	R	R	T	T	W	**L**	0.02
Z3	W	R	R	T	T	**Y**	V	0.0071
Z4	W	R	R	T	T	**Y**	**L**	0.013
Z5	W	R	R	T	**S**	W	V	0.021
Z6	W	R	R	**S**	T	W	V	0.028
Z7	W	R	R	**K**	T	W	V	0.014
Z8	W	R	**F**	T	T	W	V	0.0048
Z9	W	R	**L**	T	T	W	V	0.0027
Z10	W	**M**	R	T	T	W	V	0.0062
Z11	W	**V**	R	T	T	W	V	0.011
Z12	**Y**	R	R	T	T	W	V	0.014
Z13		R	R	T	T	W	V	0.12
Z14			R	T	T	W	V	0.17
Z15				T	T	W	V	1.2
Z16					T	W	V	3.5
Z17						W	V	>400
E1			W	E	T	W	V	0.75
E2			**F**	E	T	W	V	1.3
E3			**Y**	E	T	W	V	0.92
E4			**A**	E	T	W	V	7.4
E5			W	**A**	T	W	V	0.8
E6			W	**D**	T	W	V	16
E7			W	E	**V**	W	V	3.3
E8			W	E	**A**	W	V	15
E9			W	E	T	W	**L**	4.2
E10			W	E	T	W	**I**	6.2
E11			W	E	T	W	**A**	>400
E12			W	E	T	W	**V**[a]	>400

[a] The C terminus was amidated.

appreciate the contributions of individual residues to binding affinity, we also subjected the optimal peptide to a sequential truncation scan starting from the N terminus (Table 2, compare peptide Z1 with Z13–Z17). Deletion of Trp[−6] reduced binding by almost 25-fold (Z13), but further deletion of Arg[−5] did not have an appreciable effect (Z14). Deletion of Arg[−4] reduced binding further by ~7-fold (Z15), but deletion of Thr[−3] only caused a further 3-fold reduction in affinity (Z16). The C-terminal tripeptide bound to ZO1-PDZ1 with an affinity in the low micromolar range, but the affinity was ~700-fold weaker than that of the optimal heptapeptide (Table 2, compare peptide Z1 with Z16), and further truncation to the C-terminal dipeptide (Z17) essentially abolished binding. Overall, these results are in good agreement with the binding motif predicted by phage display, as they demonstrate that an optimal C-terminal tetrapeptide constitutes a core recognition motif that binds to ZO1-PDZ1 with an affinity in the low micromolar range, and affinity can be improved to the low nanomolar range by additional favorable interactions with hydrophobic side chains at the −4 and −6 positions.

We also analyzed the binding affinity of ZO1-PDZ1 for a panel of peptides based on a previously characterized optimal ligand for Erbin-PDZ (Table 2, peptide E1), and this analysis provided additional information about ligand recognition by ZO1-PDZ1. The analysis confirmed the importance of a hydrophobic side chain at position[−4], as substitution by Ala reduced binding by ~10-fold (compare peptide E4 with E1, E2, and E3). At position[−3], substitution of the Glu side chain by Ala (pep-

PDZ Domain Specificity

TABLE 3

IC$_{50}$ for putative natural ligands binding to LAP PDZ domains

The IC$_{50}$ value is the mean concentration of peptide that blocked 50% of PDZ domain binding to an immobilized high affinity peptide ligand. Values for IC$_{50} \leq 100$ μM are in bold text. The N termini of the peptides were acetylated.

Natural ligand	Sequence								IC$_{50}$			
	−7	−6	−5	−4	−3	−2	−1	0	Erbin PDZ	Scrib PDZ1	Scrib PDZ2	Scrib PDZ3
									μM			
δ-CAT	P	A	S	P	D	S	W	V	**6.6**	>400	**82**	>400
ARVCF	P	Q	P	V	D	S	W	V	**3**	>400	**43**	>400
HPV-E6	R	T	R	R	E	T	Q	L	>400	**7.1**	>400	**5.3**
GUKH	L	P	S	F	E	T	A	L		**1.7**	200	**3.4**
TOPK	V	E	A	L	E	T	D	V	**21**	**33**	**26**	**30**
RhoGEF-16	R	L	R	V	E	T	D	V	**70**	**40**	**39**	**25**
Kv1.4	A	K	A	V	E	T	D	V	**50**	**40**	**31**	**32**
Kv1.5	D	T	S	R	E	T	D	L	>400	**31**	**100**	**5.4**
β-CAT	L	A	W	F	D	T	D	L		**71**	>400	**29**
α-ACTN-1/4[a]	A	L	Y	G	E	S	D	L		200	>400	**22**
ZO-2	S	R	Y	R	D	T	E	L	>400	**50**	300	**100**
NR2B[b]	L	S	S	I	E	S	D	V	**37**	**60**	**100**	200
NR2D[b]	F	S	S	L	E	S	E	V	**54**	>400	200	**100**

[a] The C termini of α-actinin-1 and -4 are identical.

[b] The last six or seven residues of NR2A or NR2C are identical to those of NR2B or NR2D, respectively.

tides E1 and E5) did not affect binding, but substitution by Asp reduced binding by ~20-fold (compare peptide E6 with E1 and E5), suggesting that certain side chains may be excluded because of unfavorable interactions. Interestingly, Val was reasonably well tolerated at position^{-2} (peptide E7), indicating that ZO1-PDZ1 is not restricted exclusively to type I ligands; but nonetheless, the presence of an optimal Thr^{-2} side chain contributes favorably to binding, as substitution by Ala reduced binding by 20-fold (peptide E8). At position^{0}, the data confirm that a Val side chain is optimal, but Leu and Ile are also well tolerated (compare peptide E1 with E9 and E10). Truncation of the C-terminal side chain by substitution with Ala essentially abolished binding (peptide E11) as did blocking of the C terminus by amidation (peptide E12).

Affinity Assays for Known and Putative Natural Ligands of the LAP PDZ Domains—Erbin-PDZ has been shown to bind to the C termini of the p120-like catenins ARVCF and δ-CAT both *in vitro* and *in vivo* (46, 47). Several natural ligands for the PDZ domains of Scrib have been identified by conventional biochemical methods; these include a human papillomavirus oncoprotein, HPV-E6 (54), a synaptic protein in *Drosophila* (guanylate kinase holder, GUKH) (55), and a mammalian guanine nucleotide exchange factor (βPIX) (56). We synthesized peptides corresponding to the C termini of these proteins and estimated affinities for the PDZ domains of Scrib and Erbin (Table 3). Scrib-PDZ2, but not Scrib-PDZ1 or -PDZ3, exhibited appreciable affinity for the C termini of ARVCF and δ-CAT, suggesting that Scrib-PDZ2 may have a function similar to that of Erbin-PDZ. This result was in agreement with the results of our phage display experiments, which demonstrated that the binding specificity of Scrib-PDZ2 resembles that of Erbin-PDZ, whereas the binding specificities of the other Scrib PDZ domains are more divergent. Conversely, the C termini of HPV-E6 and GUKH bind with high affinity to Scrib-PDZ1 and -PDZ3 but do not exhibit appreciable affinity for Scrib-PDZ2 or Erbin-PDZ. Again, these findings are in good agreement with the binding specificities defined by phage display, as the C-terminal Leu^{0} found in the HPV-E6 and GUKH sequences is well tolerated by Scrib-PDZ1 and -PDZ3 but not by Scrib-PDZ2 (Table 1, compare peptides L1 and L2). In the case of GUKH,

although we confirmed an interaction with Scrib, our findings contrast with previous domain-mapping studies that lead to the conclusion that GUKH interacts mainly with *Drosophila* Scrib-PDZ2 (55). The discrepancy may be because the previous study used qualitative yeast two-hybrid methods with *Drosophila* Scrib, whereas we are studying the human homologue. In any case, the results of our quantitative *in vitro* assays are internally consistent (Tables 1 and 3). We did not measure affinities for the βPIX C terminus, but the sequence AWDETNL$_{COOH}$ is a good match with the binding motifs for Scrib-PDZ1 and Scrib-PDZ3 (Fig. 4), and we predict high affinity interactions with these domains.

In addition to measuring affinities for known ligands of the LAP PDZ domains, we also used the phage-derived binding motifs to search for additional, putative natural ligands. We searched the human proteome data base for C termini matching a binding motif representing a composite of the binding motifs for the LAP PDZ domains ([D/E][T/S][D/E/W][L/V/I]$_{COOH}$). We note that this search was not exhaustive, because we did not account fully for the promiscuous nature of site^{-1} in the Scrib PDZ domains and we did not consider ligand residues upstream of the last four. Nonetheless, the search allowed us to identify interesting putative ligands, which we analyzed by *in vitro* affinity assays.

Each putative ligand exhibited appreciable affinity for at least one of the LAP PDZ domains, and most exhibited affinity for multiple domains (Table 3). Overall, the binding profiles agreed with the phage-derived specificity profiles, as Erbin-PDZ and Scrib-PDZ2 recognized a similar set of ligands, whereas the sets of ligands recognized by Scrib-PDZ1 and -PDZ3 were similar to each other. Scrib-PDZ2 is restricted to ligands that terminate with a Val^{0}, whereas Scrib-PDZ1 and -PDZ3 do not discriminate between ligands that terminate in either a Val^{0} or a larger Leu^{0}. Thus, most of the putative ligands for Scrib-PDZ2 represent a subset of the putative ligands for the other Scrib PDZ domains, which also recognize an additional group of ligands that are excluded by the restrictive site^{0} of Scrib-PDZ2. It is notable that, of all the natural C termini we tested, only those of δ-CAT and ARVCF exhibit appreciable affinity for Scrib-PDZ2 but not for Scrib-PDZ1 or -PDZ3. This may be because these

two ligands contain an Asp^{-3} but most of the other ligands contain a Glu^{-3}, and although Scrib-PDZ2 does not discriminate between these two side chains, Scrib-PDZ1 and -PDZ3 exhibit a marked preference for Glu^{-3} over Asp^{-3} (>10-fold; Table 1, peptides L1 and L7). Alternatively, or in addition, it is possible that residues upstream of position^{-3} in δ-CAT and ARVCF make favorable binding contacts with Scrib-PDZ2 but not with Scrib-PDZ1 or -PDZ3.

Some putative ligands are intracellular proteins that mediate biological functions that can be linked logically to the known physiological functions of Scrib. β-CAT has been shown to interact with several other PDZ domain-containing proteins (57–61), and it is a component of the cadherin-catenin complex, which also includes the p120-like catenins δ-CAT and ARVCF (5, 46). Because Erbin associates with this complex through δ-CAT/ARVCF (46), it is possible that Scrib associates with β-CAT through interactions with Scrib-PDZ1 and/or Scrib-PDZ3 and with δ-CAT/ARVCF through interactions with Scrib-PDZ2. Multiple binding interactions between the Scrib PDZ domains and the cadherin-catenin complex may compensate for the fact that the affinities of δ-CAT and ARVCF for Scrib-PDZ2 are somewhat weaker than those for Erbin-PDZ. It has been shown recently that E-cadherin is required for the localization of human Scrib at cell-cell junctions where it colocalizes with β-CAT (13). These findings are consistent with our results, which suggest that interactions between the Scrib PDZ domains and β-CAT and/or δ-CAT/ARVCF indirectly link Scrib to E-cadherin. ZO-1 and ZO-2 have also been shown to interact with ARVCF (Ref. 24 and see below), and thus, the affinity of Scrib-PDZ1 and -PDZ3 for the C terminus of ZO-2 may be biologically significant, as at least under some conditions, ZO-2 and Scrib may be co-localized by interactions with the cadherin-catenin complex. Indeed, a recent report has shown that ZO-2 and Scrib interact at the cell-cell junctions of unpolarized cells prior to the segregation of ZO-2 to the tight junctions (62).

Putative interactions between Scrib-PDZ3 and the two non-muscle isoforms of the actin-binding protein α-actinin (α-ACTN-1 and -4) may provide a link between Scrib and the cytoskeleton; such a link seems reasonable in light of observations that Scrib plays a role in maintaining cell shape and polarity (3, 16, 17). Interestingly, α-ACTN-1 and -4 link the PDZ-containing protein MAGI-1 to the cytoskeleton (63), and other PDZ-containing proteins also associate directly (ZO-1) (23) or indirectly (human DLG (Discs-large) and CASK) (64, 65) with the actin cytoskeleton. Thus, the association of PDZ-containing proteins with the cytoskeleton appears to be a common mechanism whereby scaffolding proteins build and maintain signaling complexes and cell polarity.

The C terminus of the T-LAK cell-originated protein kinase (TOPK) exhibits good affinity for the PDZ domains of Scrib and Erbin (Table 3). Interestingly, this mitotic kinase was first identified as a PDZ-binding kinase by virtue of the interaction of its C terminus with the second PDZ domain of human DLG, and it was hypothesized that its intracellular localization and function may be mediated by interactions with PDZ domains (66). More recently, it has been shown that TOPK is phosphorylated by cdk1/cyclin B, and the activated form binds to microtubules

and is localized to spindle fibers during mitosis (67). Taken together, these results and our findings suggest that TOPK may interact with many different PDZ domain-containing proteins and, in this way, may be localized near substrates, which may include the PDZ domain-containing proteins and/or their binding partners.

Some of the putative ligands for Scrib, Erbin, and (likely) Densin-180 are integral membrane proteins of a type that are well known binding partners for various PDZ domains but have not been reported to interact with the LAP family. These include Shaker-type potassium channels (Kv1.4 and Kv1.5) and all four NR2 subunits of *N*-methyl-D-aspartate (NMDA) glutamate receptors (NR2A-D). Whether these interactions occur *in vivo* will depend on whether the putative ligands are expressed and localized in the same tissues and subcellular compartments as the LAP proteins. In *Drosophila* neurons, Scrib forms a tripartite complex with GUKH (see above) and DLG (55), whereas in mammalian cells, both Erbin and Densin-180 associate with the DLG orthologue, PSD-95 (postsynaptic density-95) (12, 68). The PDZ domains of *Drosophila* DLG and mammalian PSD-95 bind to and cluster Shaker-type potassium channels (69–72), and the PSD-95 PDZ domains also bind to NMDA receptors (73, 74). Thus, it seems reasonable that, like their interaction partner DLG/PSD-95 (31, 75, 76), the LAP family members may be involved in assembling ion channels and receptors into signal transduction complexes in neurons. Mutations in the gene that encodes for *Drosophila* Scrib cause abnormalities in synapse structure and function, and it has been hypothesized that elimination or mislocalization of calcium buffering or sensing molecules may play a role in these defects (77). In light of these observations, it is significant that Scrib may be involved in clustering NMDA receptors, which are channels for calcium influx (31).

The biological functions of RhoGEF-16 (Rho guanine exchange factor-16) are not yet known, but an interaction between it and Scrib may be physiologically relevant, because Scrib binds to another guanine exchange factor, βPIX (Ref. 56 and see above). Scrib may also be involved in vesicle trafficking (16, 17) and in the regulation of synaptic vesicle dynamics (77), and βPIX binds to Rac1 and Cdc42, two Rho GTPases that are involved in reorganizing the cystoskelton and possibly in promoting exocytosis (56). Furthermore, mutations in Scrib and βPIX impair calcium-dependent exocytosis in response to membrane depolarization by high potassium (56). In this regard, it is notable that the Scrib PDZ domains bind to βPIX and may also bind to Shaker-type potassium channels and NMDA receptor-calcium channels; thus, Scrib could link Rac1/Cdc42-mediated exocytosis to potassium-mediated calcium influx.

Interactions of ZO-1 with Natural Ligands—The ZO-1 PDZ domains have been shown to interact with several natural proteins, including members of the large families of claudins (78, 79) and connexins (80–83), junctional adhesion molecule (84, 85), ARVCF (24), and also the ZO-1 homologues ZO-2 and ZO-3 (86). We did not obtain peptide-phage data for ZO1-PDZ2, but this domain has been shown to interact with the C terminus of connexin-43 (DLEI$_{COOH}$) (81, 82) and with the second PDZ domains of ZO-2 and ZO-3 through a noncanonical

binding mode that does not involve recognition of a free C terminus (23, 27, 86). Connexin-45 has also been shown to interact with the PDZ-containing region of ZO-1 (80, 83), but it has not been clarified as to which PDZ domains are involved. The C terminus of connexin-45 ($SVWI_{COOH}$) is a good match for the phage-derived binding motifs for both ZO1-PDZ1 and -PDZ3 (Fig. 4); thus, we would predict that the connexin-45/ZO-1 interaction is mediated by ZO1-PDZ1 and/or -PDZ3. In osteoblasts, ZO-1 may serve as a scaffold to assemble gap junction channels composed of connexin-43 and connexin-45 (80). In this regard, the distinct specificities of the ZO-1 PDZ domains provide a simple mechanism for channel assembly; PDZ2 and PDZ1/PDZ3 are likely responsible for recruiting connexin-43 and connexin-45, respectively.

ZO1-PDZ1 is responsible for interactions with members of the claudin family (78, 79), which also agrees with our data, as most claudins terminate in a dipeptide (YV_{COOH}) that matches the phage-derived binding motif (Fig. 4). The claudins, family of integral membrane proteins of which there are at least 19 members in humans, have been shown to constitute the backbone of tight junction strands (79, 87). Qualitative *in vitro* binding assays have been used to show a direct interaction between ZO1-PDZ1 and the C-terminal sequences of claudin-1–8, an interaction that requires the C-terminal YV_{COOH} sequence (79). We used competition ELISAs with synthetic peptides to quantitatively assess the affinities of six claudin C termini for ZO1-PDZ1, and we found that the IC_{50} values were in the low micromolar range (Table 4). Interestingly, the binding affinity of claudin-1 for ZO1-PDZ1 has been estimated previously to be in the low nanomolar range, but the assay relied on capturing the PDZ domain with a GST·claudin-1 fusion protein immobilized on glutathione beads (79). It has become apparent that assays of this type, which use ligands immobilized on solid surfaces, can significantly overestimate affinities due to avidity effects induced by polyvalent binding (32, 46, 88). In contrast, we used a solution-phase competition assay, which gives a more accurate estimate of monomeric binding affinities. It is interesting to compare the results of the two assays, as they provide complementary information. Although solution-phase measurements provide accurate estimates of intrinsic affinities, the solid-phase capture assay may mimic the *in vivo* situation in which claudins embedded within tight junctions are likely to present clusters of C termini for polyvalent interactions with PDZ domains. Thus, polyvalent avidity effects can amplify the low micromolar intrinsic affinities by three orders of magnitude and generate extremely tight effective affinities in the low nanomolar range.

An interesting aspect of the alignment in Table 4 is that most of the claudin C termini are atypical of type I PDZ ligands, as they do not contain the defining Thr/Ser^{-2}. In fact, position^{-2} is remarkably diverse among the sequences, and based on the commonly accepted ligand preferences of class I PDZ domains (41), one would not necessarily predict that the claudins would bind to ZO1-PDZ1 with high affinity. However, our phage-derived binding motif reveals that ZO1-PDZ1 recognizes ligands not only through interactions with the last three residues but also can derive significant binding energy from interactions with upstream residues. Indeed, when the entire phage-derived

TABLE 4

IC_{50} values for claudin C termini binding to ZO1-PDZ1

The IC_{50} value is the mean concentration of peptide that blocked 50% of PDZ binding to an immobilized high affinity peptide ligand. The N termini of the peptides were acetylated. Sequences that match the optimal binding motif for ZO1-PDZ1 (see Fig. 4) are in bold text.

Claudin	Sequence							IC_{50}
	−6	**−5**	**−4**	**−3**	**−2**	**−1**	**0**	
								μM
1	S	S	G	**K**	D	**Y**	**V**	
2	**Y**	S	L	**T**	G	**Y**	**V**	13
3	**Y**	D	**R**	**K**	D	**Y**	**V**	
4	A	A	A	**S**	N	**Y**	**V**	
5	**Y**	D	**K**	**K**	N	**Y**	**V**	
6	**Y**	P	T	**K**	N	**Y**	**V**	
7	N	S	S	**K**	E	**Y**	**V**	
8	**Y**	S	**R**	**S**	Q	**Y**	**V**	4.6
9	**L**	D	**K**	**R**	D	**Y**	**V**	
10	**F**	D	**K**	N	A	**Y**	**V**	42
11	H	A	**K**	**S**	A	H	**V**	
12	**P**	V	**V**	**S**	H	T	T	
14	**Y**	R	**L**	N	D	**Y**	**V**	29
15	**Y**	G	**R**	N	A	**Y**	**V**	
16	**Y**	A	**V**	D	**T**	R	**V**	
17	**K**	T	S	**T**	**S**	**Y**	**V**	2
18	P	S	**K**	H	D	**Y**	**V**	
19	A	A	A	**R**	E	**Y**	**V**	
20	H	N	**L**	**K**	D	**Y**	**V**	61

binding motif is considered, each claudin exhibits homology at multiple positions (Table 4), and thus it appears that ZO1-PDZ1 recognizes most claudins by making favorable interactions with the C-terminal dipeptide and with residues at the −3, −4, and/or −6 positions. One exception to this rule appears to be claudin-16, which has been shown to interact with ZO-1 (78) but terminates in a sequence (TRV_{COOH}) that contains a mismatch with the optimal ZO1-PDZ1 binding motif at position^{-1}. However, Arg is only moderately disfavored in comparison with Tyr at position^{-1} of ZO1-PDZ1 ligands (Fig. 3), and substitution of Ala for Arg^{-1} in the claudin-16 C terminus does not compromise binding to ZO-1 (78). Furthermore, claudin-16 is the only claudin that contains an optimal Thr at position^{-2}, which likely compensates for the mismatch at position^{-1} (Table 4). Thus, we predicted that the interaction between claudin-16 and ZO-1 is mediated by ZO1-PDZ1; we confirmed this prediction with *in vitro* binding assays, which show that the C terminus of claudin-16 binds to ZO1-PDZ1 but not to ZO1-PDZ2 or -PDZ3 (Fig. 5*A*).

We speculate that the C-terminal sequences of claudins may have evolved under selective pressure to maintain affinity for ZO1-PDZ1, while avoiding interactions with most other class I PDZ domains. This may be especially crucial for clustered membrane proteins, because polyvalent avidity effects can significantly enhance the apparent affinities of relatively weak interactions (see above). In such a scenario, it would make sense to avoid the presentation of canonical type I C termini that would be recognized by many PDZ domains and, instead, to rely on interactions that are more specific to ZO1-PDZ1 (*i.e.* Tyr at position^{-1} and residues upstream of position^{-2}). For example, although ZO1-PDZ1 and Erbin-PDZ exhibit similarities in their optimal binding motifs (Fig. 4), the Tyr^{-1} found in most claudins is disfavored for binding to Erbin-PDZ and the Asp/Glu^{-3} favored by Erbin-PDZ is not found in claudins; thus, these differences could serve to discriminate against Erbin-PDZ. Although ZO1-PDZ1 prefers Thr/Ser at position^{-2}, other
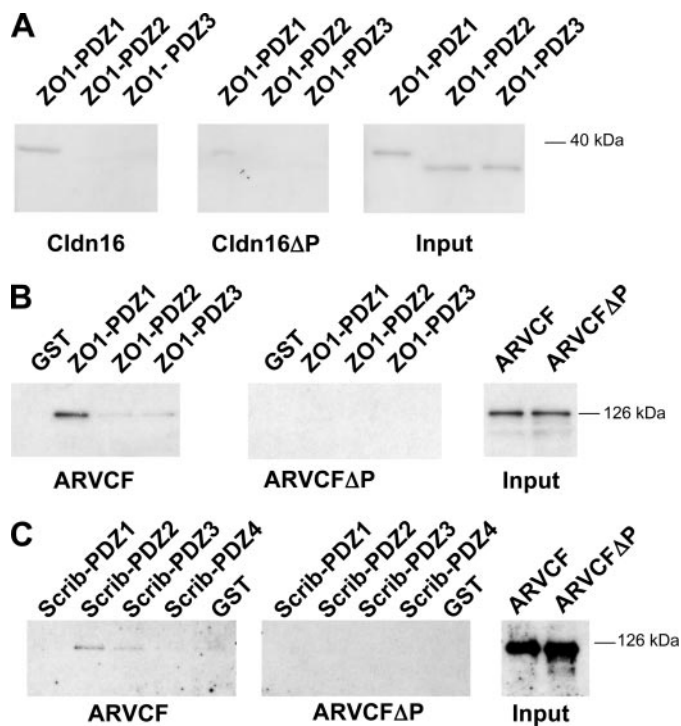
FIGURE 5. **Binding of Claudin-16 and ARVCF to PDZ domains.** *A*, claudin-16 binds to ZO1-PDZ1. Peptides corresponding to the C terminus of claudin-16 (*Cldn16*) or a claudin-16 mutant with the PDZ-binding motif inactivated (*Cldn16ΔP*) were coupled to beads and incubated with GST·PDZ fusion proteins as labeled. PDZ domains bound to the beads were fractionated by SDS-PAGE, transferred to membranes, and stained with Ponceau S. An aliquot of each GST·PDZ fusion protein (*Input*) was analyzed to ensure that similar amounts were added to the binding reactions. *B* and *C*, ARVCF binds to ZO1-PDZ1 and Scrib-PDZ2. GST or GST·PDZ fusion proteins were coupled to glutathione beads and incubated with *in vitro* translated, radioactively labeled ARVCF or an ARVCF mutant with the PDZ-binding motif inactivated (*ARVCFΔP*). Protein bound to the beads was analyzed by SDS-PAGE and autoradiography. An aliquot of the *in vitro* translated material (*Input*) was analyzed to confirm that similar amounts had been added to the binding reactions.

residues do appear among the phage-derived ligands (Fig. 5*A*), and thus a canonical type I binding motif is not an absolute requirement for high affinity ligands of ZO1-PDZ1. Claudin-16 is an exception to this generalization, as it is the most distantly related member of the claudin family (78), and its C terminus does conform to the canonical type I consensus. Consequently, in addition to the common interaction with ZO1-PDZ1, claudin-16 may interact with other class I PDZ domains that would not bind to most of the other claudins, which may in turn lead to divergence of biological function.

Convergence and Divergence in the Binding Specificities of the LAP and ZO PDZ Domains—ZO-1 is localized at tight junctions in fully polarized epithelial/endothelial cells, but it is localized at adherens junctions in cells that lack tight junctions (22, 23). Even in epithelial cells, ZO-1 colocalizes and associates with adherens junction markers in the initial stages during the establishment of apicobasal polarity (24, 27). ZO-1 binds directly to both types of multipass membrane proteins found in tight junctions; it binds to claudins through its first PDZ domain (79) (see above) and to occludin through its GUK domain (89, 90). The interaction with claudins results in the recruitment of ZO-1 to tight junctions (79). On the other hand, experiments with occludin-deficient embryonic stem cells have

shown that tight junctions form in the absence of occludin and that ZO-1 is still localized at the tight junctions (91). Furthermore, experiments with connexin-occludin chimeras suggest that the interaction between ZO-1 and occludin is responsible for the localization of occludin to tight junctions (92). In contrast, localization of ZO-1 to adherens junctions is dependent upon binding of its GUK domain to α-CAT, which binds to β-CAT associated with E-cadherin (23, 89). Recently, it has been shown that the PDZ domain-containing region of ZO-1 binds to ARVCF, which results in the recruitment of ARVCF to adherens junctions (24). The C terminus of ARVCF matches the optimal binding motif for ZO1-PDZ1 at four positions (QP*VDSWV*$_{\text{COOH}}$), and thus we predicted that the interaction is mediated by ZO1-PDZ1. We confirmed the prediction with *in vitro* binding assays, which showed that ARVCF binds to ZO1-PDZ1 but not to ZO1-PDZ2 or -PDZ3 (Fig. 5*B*).

It has been shown previously that Erbin-PDZ binds to the C terminus of ARVCF, and this interaction recruits Erbin to the cadherin-catenin complex (46). We predicted that Scrib-PDZ2 should also bind to the C terminus of ARVCF, and we confirmed the prediction with *in vitro* binding assays that showed an interaction between ARVCF and Scrib-PDZ2 but not the other Scrib PDZ domains (Fig. 5*C*). Although the interaction between Scrib-PDZ2 and ARVCF is not as strong as that between ARVCF and Erbin-PDZ (Table 3) or ZO1-PDZ1 (Fig. 5), it is possible that an association between Scrib and the cadherin-catenin complex could be strengthened by additional interactions mediated by the other PDZ domains. For example, Scrib-PDZ1 and -PDZ3 may interact with β-CAT (Table 3), and thus Scrib could simultaneously bind to ARVCF and β-CAT, which in turn independently associate with cadherins (see above).

Thus, ZO-1 and Erbin (and likely Scrib) can bind to ARVCF and become associated with the cadherin-catenin complex at adherens junctions. In each case, recognition is mediated by a PDZ domain that recognizes the C terminus of ARVCF and makes use of favorable binding contacts with the Trp side chain at position^{-1}. However, ZO-1 differs from Erbin in that ZO1-PDZ1 can also bind with high affinity to ligands that contain Tyr^{-1}, whereas Erbin-PDZ greatly prefers Trp^{-1} and Glu/Asp^{-3} (Figs. 2 and 3). These differences have important biological consequences, as ZO1-PDZ1 can interact strongly with tight junction claudins that contain Tyr^{-1} and lack Glu/Asp^{-3} (Table 4). As a result, in polarized epithelial cells, ZO-1 is found exclusively at tight junctions, whereas Erbin is excluded from tight junctions and remains associated with the cadherin-catenin complex in the basolateral layer (46). Claudins are integral membrane proteins, and as described above, the polyvalent presentation of membrane-bound claudin C termini may greatly amplify the strength of the interaction with ZO1-PDZ1. In contrast, ARVCF is a soluble protein, and the interactions of individual ARVCF molecules are likely to be relatively independent of each other. As a result, although both ARVCF and claudins interact strongly with ZO1-PDZ1, the polyvalent nature of the claudin interaction may allow it to out-compete the ARVCF interaction, which could explain why ZO-1 associates exclusively with claudins once tight junctions have formed.

PDZ Domain Specificity

Conclusions—Herein we have presented a detailed comparative analysis of the PDZ domains of the LAP family and the MAGUK ZO-1. In an accompanying article (48), we used structural analysis to examine the molecular basis underlying our findings of both similarities and differences in the ligand specificities of these PDZ domains. Our results emphasize the importance of PDZ domain interactions with all four C-terminal residues of the ligand, which constitute a core recognition motif. Ligand recognition is further modulated by interactions with residues upstream of the core motif, which act as an auxiliary recognition motif that can enhance both the affinity and specificity of the interaction. The results highlight the key contributions of site^{-1}. The relevance of site^{-1} to PDZ domain biology is underscored by the comparison of the PDZ domain of Erbin with those of Scrib and ZO-1, as it appears that the level of promiscuity within site^{-1} profoundly impacts the range of biological partners, and thus of biological functions, that can be associated with each protein. Overall, the results enhance our understanding of PDZ domain function and provide valuable guidelines for further studies of this remarkably diverse family.

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