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CHAPTER FIVE

Emerging Themes in PDZ Domain Signaling: Structure, Function, and Inhibition

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Abstract

Post-synaptic density-95, disks-large and zonula occludens-1 (PDZ) domains are small globular protein—protein interaction domains widely conserved from yeast to humans. They are composed of ~90 amino acids and form a classical two α-helical/six β-strand structure. The prototypical ligand is the C-terminus of partner proteins; however, they also bind internal peptide sequences. Recent findings indicate that PDZ domains also bind phosphatidylinositides and cholesterol. Through their ligand interactions, PDZ domain proteins are critical for cellular trafficking and the surface retention of various ion channels. In addition, PDZ proteins are essential for neuronal signaling, memory, and learning. PDZ proteins also contribute to cytoskeletal dynamics by mediating interactions critical for maintaining cell—cell junctions, cell polarity, and cell migration. Given their important biological roles, it is not surprising that their dysfunction can lead to multiple disease states. As such, PDZ domain—containing proteins have emerged as potential targets for the development of small molecular inhibitors as therapeutic agents. Recent data suggest that the critical binding function of PDZ domains in cell signaling is more than just glue, and their binding function can be regulated by phosphorylation or allosterically by other binding partners. These studies also provide a wealth of structural and biophysical data that are beginning to reveal the physical features that endow this small modular domain with a central role in cell signaling.

1. INTRODUCTION

The post-synaptic density-95, disks-large and zonula occludens-1 (PDZ) domain was identified over 20 years ago (Cho et al., 1992; Itoh et al., 1993; Woods and Bryant, 1991). It is highly represented in eukaryotes and prokaryotes (Ponting, 1997) and in the human genome over 500 proteins contain PDZ domains (SMART database) (Schultz et al., 1998). The typical PDZ domain contains approximately 90 amino acids and a tertiary fold that includes six beta strands (β1—β6) and two alpha helices

(α1 and α2). PDZ domains themselves have no enzymatic activity but generally recognize the 5–10 C-terminal residues of binding partners acting as scaffolds to mediate the assembly of protein complexes. Through these protein complexes, PDZ proteins regulate a vast number of biological processes including protein trafficking, signal transduction, cell–cell junctions, and cell polarity and adhesion. Because of their critical biological significance and association with disease, PDZ proteins, and consequently PDZ domains, have become attractive therapeutic targets. The structural and dynamic properties of PDZ domains have been studied, and in many cases found to aid in fine-tuning their scaffolding and signaling functions. This chapter highlights emerging concepts in PDZ domain structure, specificity, and cellular function and its multifaceted role in human disease.

2. CELLULAR FUNCTION OF PDZ DOMAIN–CONTAINING PROTEINS AND THEIR LINK TO DISEASE

PDZ proteins are broadly classified into subfamilies based on their overall domain architecture (Harris and Lim, 2001). Fig. 1 shows the four subfamilies of PDZ proteins and highlights many of the proteins discussed in this review. The first subfamily comprises membrane-associated guanylate kinase (MAGUK) proteins that contain multiple PDZ domains and a signature PDZ-SH3-GK structural "supramodule." A second subfamily is composed of essentially only PDZ domains, functioning exclusively as scaffolding proteins. PDZ proteins may also contain domains with enzymatic functions (e.g., phosphatase, guanine nucleotide exchange, etc.) and these proteins comprise a third subfamily of PDZ proteins. Finally, a growing list of PDZ proteins form a separate PDZ subfamily characterized by the presence of diverse protein–protein interaction domains that continue to show novel structural and regulatory properties.

PDZ proteins have many diverse functions but are widely known for their role in regulating neuronal synaptic signaling and cell–cell junctions in most cell types. This function is largely due to the direct binding of PDZ domains with target proteins possessing unique C-terminal PDZ-binding motifs (PBMs) (see Section 3). These interactions help coordinate the trafficking of transmembrane proteins (usually ion transporters) from the Golgi to the plasma membrane and participate in their stabilization at the membrane. The regulation of ion-channel trafficking can ultimately have a significant effect on synaptic signaling. PDZ domains can also act

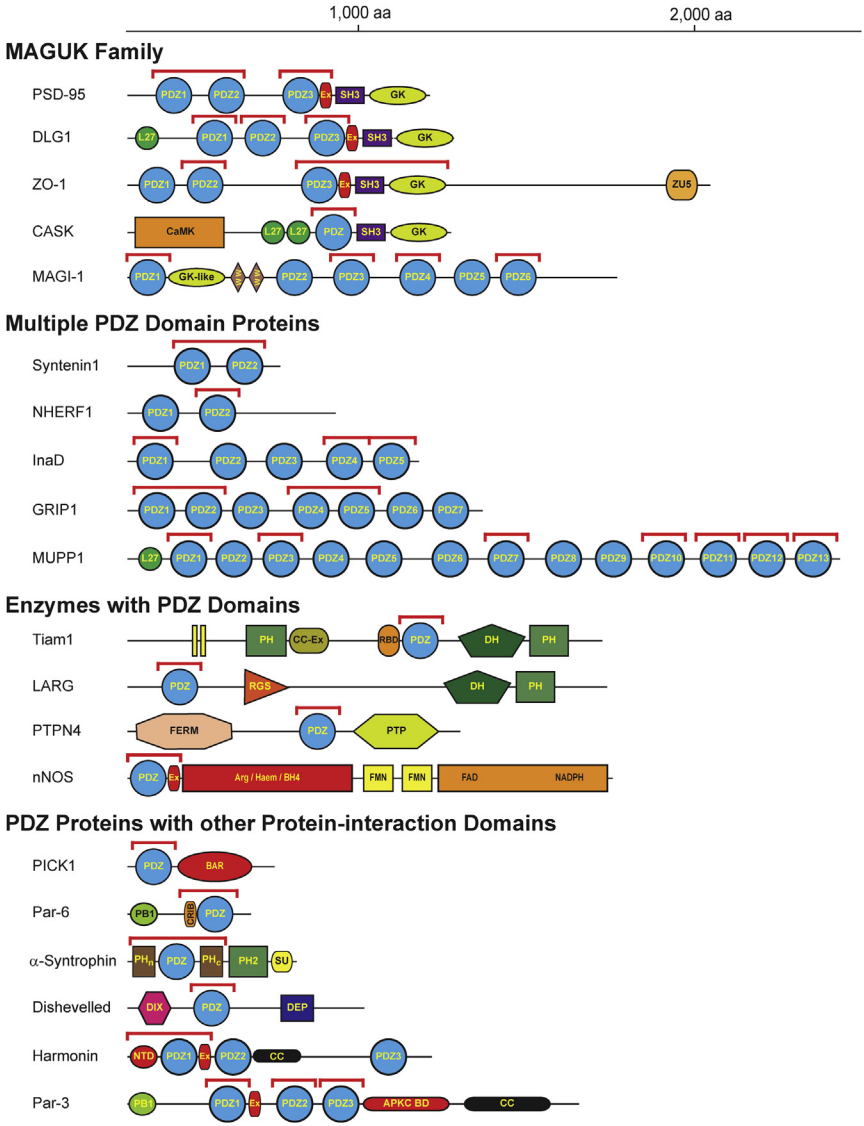

Figure 1 Distinct classes of PDZ-containing proteins. Proteins are scaled to the length of their primary sequence. *Red brackets* indicate that the structure of that region has been determined.

as scaffolds to recruit structural and regulatory components of cell–cell junctional complexes to the cytoplasmic membrane. In this section, we will discuss the specific roles of representative PDZ domain proteins involved in important physiological processes associated with disease.

2.1 Coordination of Pre- and Postsynaptic Signaling in Neuronal Cells

Communication between pre- and postsynaptic neurons (i.e., synaptic transmission) is an essential part of neuronal cell signaling. Critical for this function are the many excitatory receptor proteins whose function is, in part, regulated by PDZ-containing proteins. In excitatory glutamatergic synapses, the neurotransmitter glutamate is released from presynapses and sensed by two ionotropic receptors—α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors—both of which are able to transport Na^+, K^+, and Ca^{2+} (Madden, 2002). Metabotropic glutamate receptors (mGluR) can control ion channels indirectly through G-proteins (Niswender and Conn, 2010). After ion channel opening, neuronal signals are further decoded and relayed to the postsynaptic density (PSD) region of an adjacent cell. This signal relay is mediated by a set of PDZ-containing proteins including PICK-1 (protein interacting with C kinase 1), GRIP (glutamate receptor interacting protein), CASK (calcium/calmodulin-dependent serine protein kinase), Shank-3, and most importantly, PSD-95, a major constituent of the PSD (Fig. 2A).

2.1.1 AMPA Receptor

The AMPA receptor (AMPAR) is a heterotetramer membrane-bound protein comprised of a glutamate receptor 2 (GluR2) dimer and two subunits of GluR1, GluR3, or GluR4. AMPAR is involved in long-term depression (LTD) and long-term potentiation (LTP) that are critical for memory and learning in mammals (Huganir and Nicoll, 2013). Studies showed that overexpression of PSD-95, PSD-93, and SAP-102 led to the enhancement in both the frequency and amplitude of excitatory postsynaptic current along with an increase in the number of AMPARs in the PSD region (El-Husseini et al., 2000), whereas RNA knockdown of PSD-95 or PSD-93 proteins led to 50% reduction of AMPAR molecules at synapses (Schluter et al., 2006). These data indicate that trafficking of AMPAR is regulated by PSD-95 family proteins. A later study confirmed that this regulation is mediated through endocytosis of AMPAR (Bhattacharyya et al., 2009). However, PSD-95 and SAP-102 knockdown were only able to reduce the AMPAR-mediated neuronal signal transmission in immature synapses, implying developmentally controlled regulation of AMPAR trafficking (Sans et al., 2000). Among PSD-95 family members, only Dlg1 (also known as SAP-97) interacted directly with AMPAR (Leonard et al., 1998). Dlg1 can also bind the kinesin motor and myosin-V to deliver AMPAR-containing vesicles along actin and microtubules to dendrites. In contrast, PSD-95 mediates AMPAR trafficking through both a C-terminal PDZ interaction and via an auxiliary

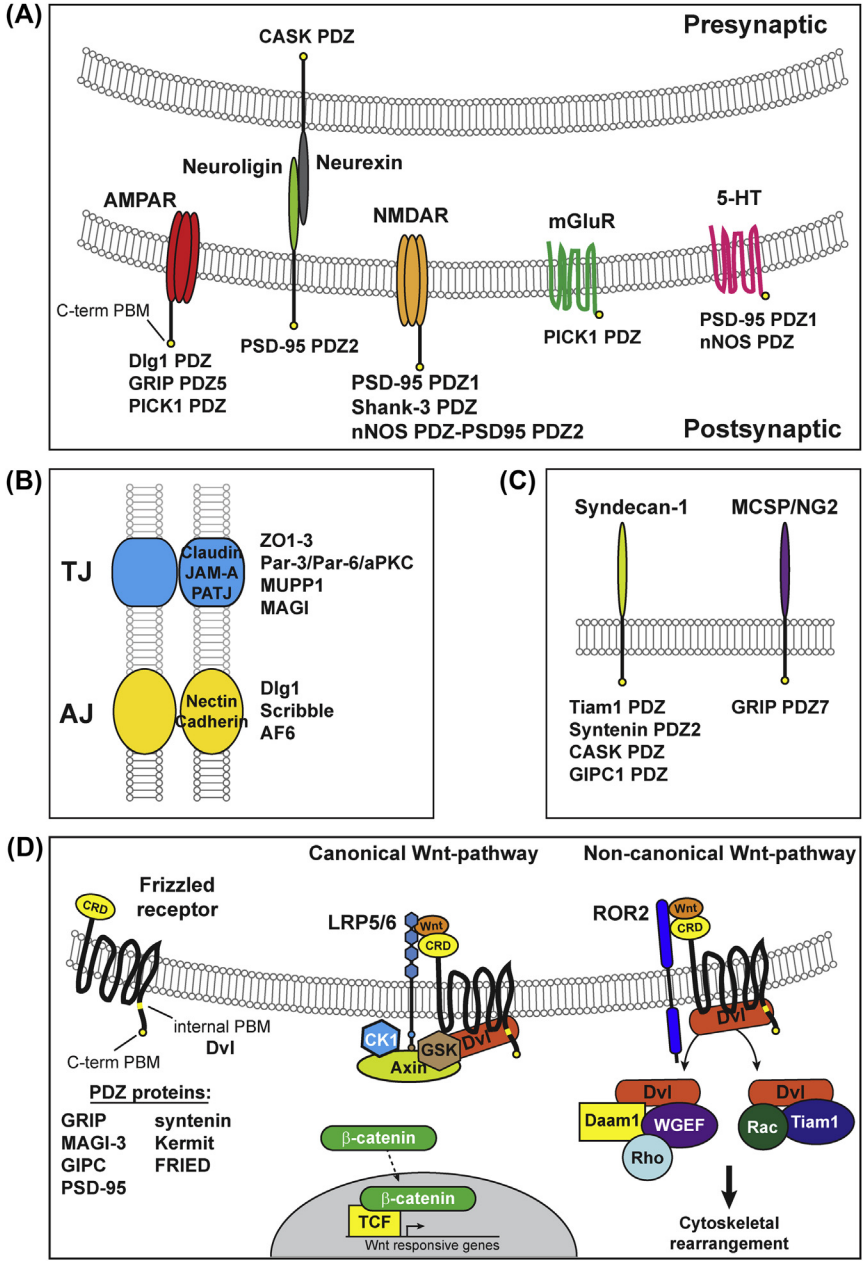

Figure 2 PDZ domain proteins are involved in a variety of signal transduction pathways. (A) PDZ domains interact with neuronal receptor proteins. A neuronal synapse is depicted showing PDZ proteins and several receptors they bind. (B) Adhesion proteins and associated PDZ domain proteins found at tight junctions (TJs) and adherens junctions (AJs). (C) Proteoglycan adhesion proteins that target of PDZ domain proteins. (D) A simplified representation of the Wnt pathway, both the canonical and noncanonical branches of the Wnt pathway are shown. PDZ proteins that interact with the

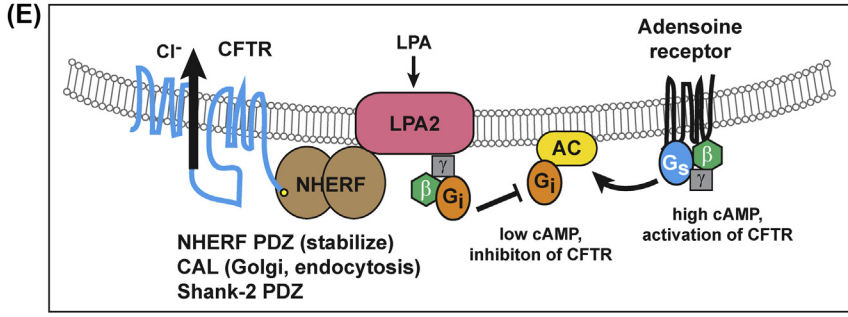

Figure 2 (***continued***).

scaffold protein (i.e., stargazin), which bridges the interaction between AMPARs and PSD-95 (Chen et al., 2000).

Other PDZ proteins, such as GRIP and PICK-1 are known to directly bind to AMPARs through the C-termini of the GluR2 subunits. Like Dgl1, GRIP was able to directly interact with a kinesin motor protein and facilitate the targeting of AMPAR to synapses (Fig. 2A) (Schnapp, 2003). Interestingly, phosphorylation on GluR2 PBM differently regulates these interactions—phosphorylation disrupts the with GluR2/GRIP interaction without interfering with the GluR2/PICK-1 interaction. Together, these studies show that PDZ domain—mediated interactions regulate AMPAR trafficking to the synapse, which is critical for synaptic plasticity.

2.1.2 NMDA Receptor

NMDA receptors (NMDAR) are heterotetramer glutamate receptors, consisting of two GluN1 subunits and a combination of GluN2 and/or GluN3 subunits. Like APMAR, NMDAR is involved in regulating LTP and synaptic plasticity. NMDARs were found to interact with SAP-102, PSD-93, and PSD-95 in brain and hippocampus extracts (Fig. 2A) (Lau et al., 1996; Sans et al., 2003). These interactions are mediated through the PDZ domains and the C-termini of NMDAR subunits, such as GluN2A and GluN2B, and may be enhanced by additional interactions, such as binding to the putative SH3-binding motif found in GluN2A subunit (Cousins et al., 2009). PSD-95 proteins also regulate cellular trafficking, membrane

Frizzled receptor are listed. (E) Regulation of CFTR by PDZ-domain proteins. CFTR is associated with NHERF2 and the LPA2 GPCR in a complex. On lysophosphatidic acid (LPA) activation of LPA2, adenylate cyclase (AC) is inhibited through G_i resulting in low cAMP levels and inhibition of CFTR. Activation of AC occurs through G_s (e.g., adenosine receptor), which increases cAMP levels and CFTR activity. *Adapted from Li, C., Naren, A.P., 2010. CFTR chloride channel in the apical compartments: spatiotemporal coupling to its interacting partners. Integr. Biol. (Camb.) 2, 161—177.*

targeting, and retention of NMDARs. NMDARs are assembled at the endoplasmic reticulum and trafficking is dependent on its interaction with the PSD-95 family proteins SAP-102 and Dlg1 (Sans et al., 2003). In addition, CASK, another PDZ domain–containing protein, was found to interact with the GluN2B and GluN2A subunits to mediate NMDAR trafficking through Golgi networks and Golgi outposts (Jeyifous et al., 2009). Like the AMPAR, the PDZ-mediated trafficking processes for NMDAR occurred predominantly during development rather than in mature neurons (El-Husseini et al., 2000; Elias et al., 2008).

In addition to regulating the cellular trafficking of NMDAR, PSD-95 is also involved in the assembly of protein interaction networks in the PSD region. The PSD-95 PDZ1 domain recognizes the C-termini of the GluN2A and GluN2B subunits of NMDAR, whereas PDZ2 domain binds to the neuronal nitric oxide synthases (nNOS) (Fig. 2A). The formation of NMDAR/PSD-95/nNOS complex plays significant roles in learning, memory, and social behavior. On the other hand, disruption of this complex couples glutamate signaling to excessive production of NO, which ultimately leads to excitotoxicity (Sattler et al., 1999). Excitotoxicity has been found in many disorders including ischemic stroke, neuropathic pain, and depression (Doucet et al., 2012; Sattler et al., 1999). The guanylate kinase (GK) domain of PSD-95 interacts with the GK-associated protein (GKAP), which together bind the Shank-3 PDZ domain to form a ternary complex. Shank-3 is another important scaffold protein that anchors NMDAR to the actin cytoskeleton via PDZ domain–mediated interactions (Naisbitt et al., 1999). Moreover, disruption of these interactions has been seen in synaptic dysfunction and autism spectrum disorders (Jiang and Ehlers, 2013).

Thus, several PDZ-containing proteins are critical for physiological synaptic function and involved in pathological synaptic disorders. Effective small molecule inhibitors that target unique PDZ domains might be useful for the treatment of excitotoxicity, chronic pain, and Alzheimer's disease rather than targeting ion channels or receptors that usually lead to side effects (D'Mello et al., 2011; Gardoni, 2008; Ittner et al., 2010). More importantly, these inhibitors could also be used as molecular probes to investigate the role of PDZ domains in regulating synaptic dynamics at the PSD and other signaling pathways.

2.1.3 G-Protein Coupled Receptors

mGluR is a glutamate receptor and a member of the G-protein coupled receptors (GPCR) superfamily that regulates ion channels indirectly through

downstream signal transduction mediated by G-proteins. Most mGluRs contain PBMs that are recognized by PDZ domains. For instance, mGluR7 interacts with the PICK-1 PDZ domain to regulate receptor cell surface expression and Ca^{2+} channel voltage gating (Fig. 2A) (Perroy et al., 2002). Moreover, mutating C-terminal residues of mGluR7 disrupted receptor trafficking by impeding normal excitatory signaling resulting in epilepsy-like symptoms in rats and mice (Bertaso et al., 2008).

The serotonin (5-HT) GPCR receptor also contains a PBM and interactions between these proteins are important for synaptic signaling. In particular, the 5-HT receptor couples to nNOS signaling and NO production in neuronal cells through a PDZ/receptor interaction (Fig. 2A) (Manivet et al., 2000). PDZ/receptor interactions are also involved in agonist-induced desensitization, which provides yet another way of fine-tuning GPCR signaling (Romero et al., 2011). For example, the 5-HT receptor/PSD-95 PDZ1 domain interactions mediated neuropathic pain in a rat model of diabetic neuropathy (Pichon et al., 2010). Thus, disruption of this PDZ domain—mediated interaction could have antihyperalgesic effects for acute and chronic pain.

2.2 Establishment and Maintenance of Cell—Cell Junctions and Cell—Matrix Adhesions

2.2.1 Adherens and Tight Junction Proteins

Adherens junctions (AJs) and tight junctions (TJs) are important for the integrity of tissues formed by epithelial cells. AJs provide the physical attachment for cell—cell and cell—extracellular matrix (ECM) interactions. In contrast, TJs function by forming a semipermeable barrier that regulates the flow of solutes into the paracellular space (the space between cells). Importantly, both AJs and TJs contribute to apical—basal polarity of cells, which is critical for the physiological function of tissues. Disruption of this polarity leads to the epithelial-to-mesenchymal transition (EMT), a hallmark of tumor cells (Thiery, 2002).

PDZ proteins are important for both AJ and TJ function (Fig. 2B). One of the best-known examples is the ZO family of proteins (ZO-1 through 3) (Fanning et al., 2012). ZO family proteins interact with claudins, a transmembrane protein family found in TJs. Specifically, claudins 1—8 are known to directly bind to the first PDZ domain of the three ZO isoforms using relatively conserved C-termini sequences (-K/S/T-D/N/E/Q-YV_{coo-}) (Itoh et al., 1999). This interaction is important for the assembly of TJ strands, a set of continuous intramembranous strands observed using electron microscopy. Epithelial cells lacking ZO-1 and ZO-2 fail to

maintain the TJ permeability barrier, which can be rescued by reexpression of ZO-1 or ZO-2 (Umeda et al., 2006). The second PDZ domain of ZO proteins is unique because it mediates homo- and heterodimerization through PDZ−PDZ domain interactions with other ZO isoforms that are required for the recruitment of claudins to TJs (Umeda et al., 2006; Utepbergenov et al., 2006). The third PDZ domain in ZO-1 interacts directly with the Ig-like transmembrane protein JAM-1 (junctional adhesion molecule 1), which also interacts with a PDZ domain in the Par complex.

The Partition-defective (Par) polarity complex is another PDZ-containing complex critical for cell junction integrity. The core complex consists of Par-3, Par-6, and atypical protein kinase C (aPKC), and the Cdc42 GTPase is critical for its activation. Both Par-3 and Par-6 contain PDZ domains (see Fig. 1). Par-6 might be considered the hub of complex assembly because it interacts with the other components—it binds aPKC via its N-terminal PB1 domain, Cdc42 through the Cdc42/Rac interactive-binding (CRIB) motif and Par-3 likely through interactions between the Par-6 PDZ and Par-3 PDZ1 domains. Activation of the complex occurs through the Par-6 CRIB/Cdc42•GTP interaction, connecting the Cdc42 activity with aPKC signaling. Importantly, the Cdc42 interaction allosterically regulates subsequent Par-6 PDZ domain ligand binding events (Peterson et al., 2004). The Par-3 PDZ3 domain interacts with the C-terminal PBM of JAM-1 to recruit Par-3 and the Par-6−aPKC subcomplex to the regions where ZO protein and claudins localize and control TJ formation (Itoh et al., 2001). Unlike the Par-3 PDZ1 domain, PDZ2 was shown to interact with phosphatidylinositide phosphates (PIPs). This interaction works in a cooperative manner with the Par-3 PDZ3/PTEN interaction to integrate PIP signaling with the establishment of cell polarity (Feng et al., 2008; Wu et al., 2007a).

2.2.2 Proteoglycan Receptors

Proteoglycan transmembrane adhesion receptors also bind PDZ-containing proteins. This receptor family consists of three major classes, heparan sulfate proteoglycans (HSPG), melanoma chondroitin sulfate proteoglycan/neuron/glia-type 2 antigen (MCSP/NG2) and part-time proteoglycans (neuropilin-1 and CD44), which under some circumstances act as glycoproteins (Couchman, 2010). Proteoglycans usually sense extracellular molecules, transducing extracellular cues to the signaling apparatus located at the cytoplasmic C-termini that ultimately regulates cell adhesion, migration, and invasion. Dysregulation of this signaling leads to the disruption of various processes (e.g., cell polarity, cell proliferation, immune function, and angiogenesis) typically associated with different vascular diseases and cancers.

The syndecan family of proteoglycans, a prominent member of HSPG class, contains four isoforms expressed in different tissues and cells (Lambaerts et al., 2009). Syndecans work coordinately with integrin and growth factor coreceptors. Their extracellular polysaccharide chains bind collagen, chemokines, and cytokines to coordinately promote cell—matrix adhesion. Their short C-terminal cytoplasmic PBMs interact with several cytoplasmic PDZ-containing proteins, including syntenin, GIPC, CASK, and Tiam1 (Fig. 2C) (Cohen et al., 1998; Gao et al., 2000; Grootjans et al., 2000; Shepherd et al., 2010). These proteins are thought to cluster the syndecan receptors and relay signaling cues to downstream effectors (Tkachenko et al., 2006; Woods et al., 2000). The syntenin PDZ2 domain was found to regulate the endocytic pathway of syndecan (Zimmermann et al., 2005). Syntenin can also link syndecans to other cell surface receptors and kinases, such as E-cadherin and ephrin-B through its tandem PDZ domains, establishing signaling crosstalk (Lin et al., 1999; Zimmermann et al., 2001). In addition to syndecans, other proteoglycans interact with PDZ-domain proteins. For example, MCSP/NG2 clustering was mediated by GRIP-1 PDZ7 domain (Fig. 2C) (Karram et al., 2005).

Proteoglycan signaling through Rho GTPases controls the dynamics of the actin cytoskeleton and contributes to the establishment of cell adhesion. Rho GTPases are regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GAPs enhance GTP hydrolysis, and thus shut down the GTPase signaling, whereas GEFs catalyze the exchange of GDP to GTP to propagate the signaling event (Hodge and Ridley, 2016). Rho GEFs can harbor PDZ domains or PBMs and couple proteoglycan receptors to Rho GTPase signaling. For example, syndecan1 is linked to Rac1 signaling through the interaction between the syndecan1 PBM and the PDZ domain of Tiam1, a Rac1-specific GEF. Both cell migration and cell adhesion were impaired on disrupting the PDZ/syndecan-1 interaction (Shepherd et al., 2010). Moreover, bioinformatics studies indicate that ~40% of Rho GEFs contain a PBM at their C-terminus (Garcia-Mata and Burridge, 2007). Because the number of GEF proteins is 3-fold greater than the number of Rho GTPases, incorporation of PDZ domains and/or PBMs into GEFs can provide signaling specificity.

2.2.3 PDZ Proteins as Targets for Pathogenic Viruses

It is now apparent that PDZ domain proteins are important cellular targets of viruses (Table 1). Adenovirus (Ad), a dsDNA virus responsible for severe respiratory and gastrointestinal infections, was the first virus identified to

Table 1 PDZ Proteins Targeted by Viruses

Virus	Viral Proteins and C-Terminal Sequences	PDZ Target	Consequence
Adenovirus	E4-ORF1: $ATLV_{coo-}$	Dlg1 PDZ1 and PDZ2 domains MAGI-1 PDZ1 domain ZO-2 PDZ1 domain MUPP-1 PDZ7 domain PDZ10 domain	(1) Disruption of tight junction (TJ) formation and loss of apical–basal polarity (2) Constitutive activation PI3K signaling pathway to block cell death
Human Papillomavirus (HPV)	Oncoprotein E6: $ETQL_{coo-}$ (HPV-16) $ETQV_{coo-}$ (HPV-18)	Dlg1 PDZ1 and PDZ2 domains Scribble PDZ domains MAGI-1/3 PDZ1 domain PATJ PDZ4 MUPP-1 PDZ domains	(1) Ubiquitin-mediated degradation of target protein (2) Disruption of TJ integrity (3) Increased morphological transformation in human keratinocytes
Human T-cell leukemia virus (HTLV)	Tax protein: $ETEV_{coo-}$	Dlg1 PDZ domains Scribble PDZ2 and PDZ4 domains	(1) Suppression of cell cycle arrest (2) Enhancement of the transforming activity of infected cells
	Envelope glycoproteins (Env): $ESSL_{coo-}$	Dlg1 PDZ domains	Facilitate viral cell–cell transmission and viral spread.
SARS virus	E envelop protein: $DLLV_{coo-}$	PALS PDZ domain	Mis-targeting/retention of PALS1 at Golgi and delaying TJ formation
Influenza A virus	Nonstructural protein 1 (NS1): $ESEV_{coo-}$	Dlg1 PDZ1 and PDZ2 domains Scribble PDZ1 and PDZ2 domains	(1) Disruption of TJ formation during viral infection (2) Reduction of cell apoptosis
Rabies Virus	E envelop protein: $ETRL_{coo-}$	PTPN4 PDZ domain	(1) Attenuation of rabies virulence (2) Trigger apoptosis of infected cells
Tick-borne flaviviruses	NS5 protein: $MYYS_{coo-}$	Scribble PDZ4 domain	Inhibition interferon-stimulated JAK-STAT signaling and innate immunity

have proteins that interact with a cellular PDZ protein (Davison et al., 2003). Several Ad virus subgroups (A, B, and D) induced tumors in rodent cells and within subgroup D, only serotype Ad9 could induce tumors in rats (Hierholzer, 1992). Ad-infected rat cells displayed distinct mammary tumors, including fibroadenoma, phyllodes tumor, and solid sarcoma (Javier et al., 1991). Further analysis showed that the gene inducing these tumors was found in the E4 region of the viral genome, a complex transcriptional unit with six open reading frames (ORF). The E4-ORF1 encodes a protein containing a C-terminal PBM ($ATLV_{coo-}$), which was the first identified PBM in a viral protein and capable of interacting with both TJs (e.g., ZO-2, PATJ and MUPP-1) and AJs proteins (e.g., Dlg1) (Javier and Rice, 2011). Accumulating evidence indicates that loss of cell polarity and cell–cell junctions (i.e., tight and adherens junctions) are important events required for tumorigenesis (Soler et al., 1999). Dlg1 relies on the interaction between its PDZ2 domain and the PBM of adenomatous polyposis coli (APC) for proper establishment of anterior–posterior cell polarity. Disruption of this interaction by the viral E4-ORF1 PBM resulted in increased cellular invasion (Lee et al., 1997). Additionally, the E4-protein/Dlg1 interaction was recruited to cytosolic membranes resulting in activated Ras/PI3K signaling to support cell proliferation and survival (Frese et al., 2003). The E4-ORF1 also interacted with ZO-2, PATJ, and MUPP-1 through PDZ domain interactions sequestering these junctional proteins in the cytosol to prevent the formation of normal cell–cell junctions (Latorre et al., 2005). Thus, the Ad9 E4-ORF1 is capable of interacting with various adhesion proteins to disrupt tight junctions and cell polarity; it also interacts with Dlg1 to translocate it to the cell membrane to activate the oncogenic PI3K-signaling pathway.

Human papillomaviruses (HPVs) contain over 100 serotypes and are the major cause of genital warts and tumors (zur Hausen, 2002). HPVs are further categorized into high-risk (e.g., HPV-16 and -18) and low-risk based on their capacity for promoting the EMT and cause cervical cancers (Watson et al., 2003). In HPV-derived cancer cells, the E6 and E7 oncoproteins are highly expressed. These proteins contributed to the tumorigenesis cooperatively, yet by distinct mechanisms—overexpression of E7 caused proliferation of cells, whereas E6 mainly led to metastasis (Song et al., 2000). The C-termini of the E6 oncoproteins in high-risk HPV contain PBMs ($ETQV/L_{coo-}$) (Table 1), which are absent in low-risk E6 oncoproteins (Lee et al., 1997). Dlg1 and Scribble were the first two PDZ-containing proteins identified as E6-binding partners (Kiyono et al., 1997; Nakagawa and Huibregtse, 2000).

Other proteins, such as Na^+/H^+ exchanger regulatory factor-1 (NHERF-1), MAGI-1, PSD-95, ZO-2, and β-2 syntrophin, were later found to interact with E6 (Pim et al., 2012; Thomas et al., 2016). Again, most of these proteins are involved in establishing cell polarity and maintaining cell–cell junctions. The HPV E6/PDZ domain interactions occurred at different stages in cell metastasis and were regulated by phosphorylation (Pim et al., 2012). In particular, the HPV E6/Dlg1 PDZ1 and PDZ2 interactions regulated tumorigenesis by disrupting cell polarity. The HPV E6/Sribble interaction stimulated the Ras/MAPK signaling to regulate tumorigenesis (Dow et al., 2008). Furthermore, HPV oncoproteins caused degradation of their targets through the ubiquitin–proteasome pathway (Gardiol et al., 1999; Huh et al., 2007). Specifically, E6 was able to recruit the ubiquitin protein ligase E3A to ubiquitinylate p53, a well-known tumor suppressor, leading to its degradation (Huibregtse and Scheffner, 1991). Indeed, most of the E6-targeting proteins were degraded by an E6-mediated protease (Javier and Rice, 2011). Therefore, PDZ protein interactions with the C-terminus of E6 not only disrupt normal protein–protein interactions but also promote degradation of the target protein, ultimately leading to the loss of the proper cell–cell junctions and the development of tumors.

The human T-cell leukemia virus (HTLV) is a causative agent of adult T-cell leukemia. It is known to contain two subtypes, HTLV-1 and HTLV-2. HTLV-1 leads to cellular transformation by activating cAMP and NFκB signaling, whereas HTLV-2 has not been related to any lymphoma malignances (Matsuoka and Jeang, 2007). Analogous to the HPV E6 oncoprotein, the oncoprotein Tax from HTLV-1 has a PBM at its C-terminus ($ETEV_{coo-}$), whereas HTLV-2 Tax does not contain this motif (Hirata et al., 2004). In vitro kinase studies showed that the casein kinase 2 (CK2) phosphorylated three Ser/Thr residues near or at the PBM of the Tax protein. A phosphomimetic mutation prevented the binding between Tax protein and Dlg1, implying a phosphorylation-based regulation (Bidoia et al., 2010). Tax was found to interact with the PDZ domain–containing proteins Scribble and Dlg1 to interfere with cell polarity and immune function, which helped maintain persistent HTLV-1 infections facilitating the development of T-cell leukemia and lymphoma (Arpin-Andre and Mesnard, 2007; Suzuki et al., 1999). In addition to Tax, the HTLV-1 envelop protein (Env) also harbors a PBM ($ESSL_{coo-}$) that was shown to bind all three PDZ domains in Dlg1 (Blot et al., 2004). Moreover, the glucose transporter 1 (GLUT1) also forms clusters at the "virological synapse" and acts as a cellular receptor for HTLV-1. GLUT1 functions together with Env to

form cell–cell contacts and facilitate cell transmission of the virus. Its clustering is recruited and mediated by the interaction between its PBM and Dlg1 PDZ domains (Yoshida et al., 2008).

Other disease-causing viruses, such as rabies, severe acute respiratory syndrome (SARS), influenza, tick-borne encephalitis, and Dengue all possess PBMs in either nonstructural or envelop proteins (Golebiewski et al., 2011; Prehaud et al., 2010; Teoh et al., 2010; Werme et al., 2008). For example, the envelope glycoprotein (G) in the rabies virus contains a PBM that is critical for virulence. In one virulent strain this PBM is $QTRL_{coo-}$, which interacts with the PDZ domains of the microtubule associated serine-threonine kinases 1 and 2 (MAST1 and MAST2) to promote virus infection. Interestingly, a nonvirulent attenuated strain was found to have a single mutation in its G envelope protein PBM ($\mathbf{Q}\rightarrow\mathbf{E}TRL_{coo-}$). Yeast two-hybrid (Y2H) analyses determined that the mutant PBM was capable of binding several new proteins through PDZ domain interactions, including -protein tyrosine phosphatase non-receptor type 4 (PTPN4). Surprisingly, the G envelope-PBM/PTPN4-PDZ interaction was sufficient to trigger cell apoptosis leading to viral attenuation rather than virulence. Therefore, a single Q to E mutation in the viral PBM was capable of regulating the switch from survival to apoptosis in the infected cells (Prehaud et al., 2010). In the SARS coronavirus, the E envelop protein contains a PBM that interacts with the PDZ domain of PALS1, a member of MAGUK protein family and serves as a TJ regulator (Teoh et al., 2010). In SARS-infected cells, PALS1 was mislocated to the Golgi leading to the disruption of TJs and apical–basal polarity, implying a role for the PBM in pathogenesis of SARS-derived lung syndrome (Teoh et al., 2010). Finally, the NS1 protein from the influenza A virus has a PBM at its C-terminus. Both the avian and human viral NS1 proteins had PBMs, but their sequences were slightly different. The avian NS1 PBM sequence is $ESEV_{coo-}$ and caused a more severe infection than the human version ($RSKV_{coo-}$) (Soubies et al., 2010). Subsequently, it was shown that the avian PBM disrupted TJs by binding to Scribble and Dlg1, implying a pathological role in respiratory infection (Golebiewski et al., 2011).

The widespread conservation of PBMs in virus families indicates that this is a common feature and future studies are expected to discover additional examples. The PBMs of most nononcogenic viruses (e.g., rabies, SARS, and influenza) interfere with the establishment of cell polarity and cell junction maintenance and may contribute to viral transmission. This also leads to virulence by impacting the inflammatory responses during infection

(Jackson et al., 2008). For viruses with oncogenic potential (e.g., Ad, HPV, and HTLV), loss of cell polarity and disruption of the normal cell—cell junctions are the initial steps for metastasis. PBMs harbored by oncogenic viruses typically bind to and inactivate proteins that play crucial roles in establishing and maintaining cellular junction events by either altering subcellular localization or mediating their degradation. PBM/PDZ interactions are also involved in inhibiting the tumor suppressors (e.g., APC) and activating oncoproteins (e.g., PI3K) through association. Indeed, many PBM/PDZ interactions found in oncogenic viruses ultimately result in deregulation of the downstream APC/PI3K signaling, a key pathway for cell proliferation and survival (James and Roberts, 2016). In addition, these interactions can inhibit apoptosis and promote proliferation resulting in tumorigenesis along with viral replication. Thus, effective disruption of interactions between cellular PDZ domains and viral PBMs using small molecule inhibitors may be a feasible therapeutic strategy against viral infection and viral-mediated tumorigenesis (see Section 4).

2.3 Other PDZ Domain Proteins in Cell Signaling Pathways

2.3.1 Wnt Signaling

The Wnt signaling pathway is critical for embryonic development, cell proliferation, and cell migration. Importantly, this pathway is regulated by PDZ domain interactions. The Wnt protein is a secreted growth factor conserved from *Drosophila* to *Homo sapiens* (Clevers, 2006). The cell surface receptor for Wnt is Frizzled, a seven transmembrane helix protein with an additional extracellular ligand-binding domain and a cytoplasmic domain. The cytoplasmic domain has two independent PBMs, an internal membrane proximal PBM recognized by the Dishevelled (Dvl) PDZ domain and a membrane distal C-terminal PBM recognized by several PDZ domains (Fig. 2D). The Dvl PDZ domain is able to recognize all Frizzled isoforms, which are expressed under tight spatiotemporal regulation (Malbon and Wang, 2005). However, other PDZ domains have unique specificities for targeting the membrane distal PBM. For example, PSD-95 PDZ2 recognizes Frizzled-1, 2, 4, and 7, whereas Kermit (*Xenopus laevis* ortholog of GIPC) specifically binds to Frizzled-3 and 7 (Hering and Sheng, 2002). Moreover, Frizzled receptors are coupled to distinct downstream signaling molecules, such as syndecan, PKC, APC, and Rho GTPases, via different PDZ domain interactions to regulate a wide spectrum of cellular functions (Wawrzak et al., 2009). Crosstalk between Wnt signaling and these

pathways is important for synapse formation, early heart morphogenesis, and neural crest induction in *Drosophila* and *Xenopus* (Ataman et al., 2006; Hamblet et al., 2002; Wallingford and Harland, 2002). Therefore, distinct PDZ domain proteins interact with different Frizzled receptors contributing to the specificity and complexity of Wnt signaling.

Wnt/Frizzled/Dvl signaling is divided into canonical and noncanonical pathways based on the destiny of β-catenin (Fig. 2D). In the absence of Wnt, the canonical Wnt-signaling pathway results in β-catenin degradation and downregulation of planar cell polarity (Clevers, 2006). On Wnt binding to Frizzled and the LRP5/6 coreceptor, Dvl and is recruited to the membrane resulting in the block of β-catenin degradation. The β-catenin transcriptional coactivator is now available to enter the nucleus and promote TCF-dependent transcription to regulate cell movement and establish cell polarity. Dvl can also interact with different effectors, such as Dapper, β-arrestin, CK1 and 2, through its PDZ domain to reinforce β-catenin degradation and planar cell polarity signaling. The noncanonical Wnt pathway leads to planar cell polarity, in part, through the recruitment of the ROR2 tyrosine kinase coreceptor to the Frizzled receptor and the activation of Rho GTPases (Fig. 2D). Cytoplasmic Dvl forms a complex with the adapter protein Daam1 and the WGEF, a RhoA GEF (Habas et al., 2001; Liu et al., 2008b; Tanegashima et al., 2008). In addition, Dvl can form a complex with Tiam1, a Rac1 GEF (Cajanek et al., 2013; Saxena et al., 2015). These complexes rearrange the actin cytoskeleton to reinforce cell polarity. Because of the central role of Dvl and its PDZ domain in regulating the two Wnt pathways, Dvl is thought to function as a hub where distinct interactions dictate downstream binding partners and signaling events (Gao and Chen, 2010). Interestingly, a recent study found that Dvl is autoinhibited through the interaction of the PDZ domain and its own PBM. Conformational changes between the autoinhibited and open states can distinctly regulate canonical and noncanonical pathways (Qi et al., 2017). The Dvl PDZ domain also plays crucial roles in Wnt-driven tumorigenesis. For instance, abnormal activation of Wnt signaling along with upregulation of Dvl drives overexpression of cMyc and Cyclin D1 genes associated with colon cancer, melanomas, and leukemia (Clevers, 2006; Morin et al., 1997). Consequently, understanding the mechanism that fine-tunes PDZ-domain recognition in Wnt signaling would be extremely useful for dissecting Wnt biology. Additionally, the Dvl PDZ domain is an attractive cellular target for development of potent inhibitors for cancer therapies.

2.3.2 CFTR Signaling

Cystic fibrosis transmembrane conductance regulator (CFTR) is an anion (Cl^-) channel regulated by local cAMP concentration, protein kinase A (PKA) activity, and protein–protein interactions (Anderson et al., 1991). It functions to control the ion/fluid balance required for the clearance of mucus. PDZ domain–containing proteins are key regulators of CFTR signaling (Fig. 2E). A key PDZ domain–containing protein regulator is the NHERF (Raghuram et al., 2001). NHERF-1, 2, 3, and 4 have been shown to interact with the C-terminal PBM of CFTR to anchor this channel to the apical membrane in epithelial cells. Other PDZ proteins such as CAL (CFTR-associated ligand) and Shank-2 also interact with CFTR. Unlike the aforementioned PDZ proteins, CAL interacts with CFTR to localize and retain it to the Golgi and mediate the endocytosis process (Li and Naren, 2010). Thus, PDZ domain-mediated interactions with CFTR play critical roles in membrane recruitment and internalization of this ion channel to further modulate ion conductivity and mucus thickness.

2.4 Diseases Related to PDZ Proteins and Their Interaction Partners

A substantial literature shows that PDZ/PBM interactions are involved in many significant cellular processes, thus it is not surprising that dysfunction of these interactions contributes to a variety of human malignancies (Table 2). Three general diseases linked to PDZ domain proteins are neurological disorders, cancer, and cystic fibrosis (CF), which will be discussed here.

Neurological disorders are linked with several PDZ domain–containing proteins. Loss of active Dlg1 was associated with altered glutamate signaling in schizophrenic patients (Toyooka et al., 2002). PSD-95 has been shown to interact with three major glutamate receptors such as AMPAR, NMDAR, and 5-HT receptor and the downstream effector nNOS. Other PDZ proteins, such as PICK-1 and GRIP1, interact with C-terminal PBM of AMPAR to relay signaling cascades. These and other protein–protein interactions govern the organization and plasticity of synapses and contribute to various neurologic disorders, including pain, depression, and stroke. PSD-95 interacts with a network of proteins to establish the PSD region. The interaction with neuroligin (NLGN), a postsynaptic transmembrane critical for neuronal function, occurs through its C-terminal PBM. PSD-95 interacts with GKAP through its GK domain, whereas the C-terminus of GKAP associates with the Shank-3 PDZ domain. In the presynaptic cleft, the ligand

Table 2 Function, Disease, and Inhibition of PDZ-Mediated Interactions

PDZ	Function	Disease	Inhibitor
PSD95 PDZ1/NMDAR PSD95 PDZ2/nNOS	Assembly of the NMDAR/PSD95/nNOS complex and regulation central excitatory synapses	Stroke, cerebral ischemic damage	Tat-NR2B9c Tat-NPEG4(IETDV)2 ZL006 (Fig. 7A.1) IC 87201(Fig. 7A.2)
PSD95 PDZ1/Serotonin receptor (5-HT_{2A})	Regulation of GPCR signaling and neuronal excitation	Neuropathic pain	Indole derivative, compound 8b (Fig. 7A.3)
PICK-1 PDZ/ErbB2 PICK-1 PDZ/AMPA GluR2 PICK-1 PDZ/dopamine transporter (DAT)	Regulation of the surface expression of receptors and transporters	Cancer, neuropathic pain, depression, and schizophrenia	FSC231 (Fig. 7A.4) CSC-03 (Fig. 7A.5)
Shank-3 PDZ/GKAP	Anchoring postsynaptic membrane proteins and regulation of synaptic activity	Autism and mental retardation	Tetrahydroquinoline carboxylate compound 36 (Fig. 7A.6)
CASK PDZ/NRXN PSD-95 PDZ3/NLGN Syntrophin PDZ/NLGN3	Regulation of NRXN/NLGN mediated trans-synaptic complex formation	Autism spectrum disorders (ASD)	
Dvl PDZ domain/Frizzed-7	Activation of Wnt signaling	Colon and skin cancer, and leukemia	3289−8625 (Fig. 7A.7) FJ9 (Fig. 7A.8) J01-017a (Fig. 7A.9) Sulindac (Fig. 7A.10)
Syntenin1 PDZ1/c-Src tyrosine kinase	mediate posttranslational signaling process and promote tumor invasion	Glioblastoma multiforme cancer and melanoma	113B7 (PDZ1i) (Fig. 7A.11)

(*Continued*)

Table 2 Function, Disease, and Inhibition of PDZ-Mediated Interactions—cont'd

PDZ	Function	Disease	Inhibitor
AF-6 PDZ/Bcr LARG PDZ/CD44 Tiam1 PDZ/sydecan1	Regulation of Rho GTPase activity and cytoskeleton dynamics, cell adhesion and migration	Acute lymphoblastic leukemia, breast cancer, and prostate cancer	2-thioxo-4-thiazolidinone derivative (Fig. 7A.12)
Par-6 PDZ/Par-3 Par-3 PDZ/PTEN Dlg1 PDZ1/β-catenin	Assembly and maintenance of tight junction and cell proliferation	Epithelial-to-mesenchymal transition in tumor progression	
NHEFR2 PDZ domain/LPA (lysophosphatidic acids)	Regulation of CFTR Cl^-channel	Cystic fibrosis	CO-068 (Fig. 7A.13)

of NLGN is neurexin (NRXN), which is recognized by the CASK PDZ domain. CASK then binds to Mint1 protein, which mediates presynaptic signaling by its PDZ domain. The interactions between NRXN, NLGN, and other PDZ domain proteins are important for synaptic signal transduction and required for synaptic efficacy and plasticity. Dysfunction in this network leads to cognitive diseases, such as autism spectrum disorder and schizophrenia (Sudhof, 2008).

PDZ domain–mediated interactions play significant roles in tumorigenesis. The upregulation of the Wnt-signaling pathway leads to the transcription of cMyc and Cyclin D1 and is associated with tumor formation and cancer progression (Weeraratna et al., 2002; Yuzugullu et al., 2009). Indeed, the PDZ domain–containing protein Dvl is highly overexpressed in breast and lung cancer, whereas deletion of its PDZ domain leads to downregulation of c-myc expression and suppression of tumorigenesis in malignant mesothelioma (Nagahata et al., 2003; Uematsu et al., 2003b). These data suggest that PDZ domain–mediated interactions in Wnt signaling can drive tumorigenesis. PDZ domains in cell–cell junctional proteins are regulators of EMT, an important process where cell–cell adhesion is lost and invasion is promoted. Specifically, PDZ-mediated interactions in the Par complex are critical for apical–basal polarity and dysregulated in many tumors. In addition, the Par complex is a downstream effector of TGF signaling, which can promote cell transformation (Aranda et al., 2008). Additionally, both PDZ domains and PBMs are found in Rho family GEF proteins, and dysfunction of these interactions results in the inappropriate activation of GTPase signaling and actin remodeling, a potential link to cancer metastasis. Overexpression of PDZ domain–containing GEF LARG was found in patients having high propensity to develop acute myeloid leukemia (Rujkijyanont et al., 2007). Overexpression of the Tiam1 GEF has been shown in many cancers such as breast, colon, prostate, and melanoma and contributes to the invasion and metastasis (Adams et al., 2010; Choi et al., 2010; Engers et al., 2006; Xu et al., 2010). Cell adhesion receptors, CADM1 and syndecan1, can directly bind to the PDZ domain of Tiam1 leading to activation of the Rac1 GTPase and rearrangement of the actin cytoskeleton (Masuda et al., 2010; Shepherd et al., 2010). Reciprocally, disruption of Tiam1 PDZ domain–mediated interactions affects Rac1 activation and cell migration (Shepherd et al., 2010). Moreover, some tumor suppressors, such as APC and the PTPN phosphatase, harbor PBMs at their C-termini. Disruption of PDZ/PBM interactions in these proteins results in cell

proliferation and is found in cancers (Miyoshi et al., 1992; Pedemonte et al., 1998). Importantly, these interactions are frequently the cellular targets for oncogenic viruses (Javier, 2008). Viruses also target host-cellular junctional PDZ proteins such as Dlg1, MAGI, and ZO-2. Perturbation of the cognate PDZ interactions coupled with the hijacking tumor suppressor proteins allows oncogenic viral proteins to promote cell migration and transformation, facilitating the initiation and development of mammary tumors, lymphoma malignances, and cervical cancers. Viruses without oncogenic potential also interact with cell junctional proteins to increase their replication, disseminate in the host, and transmit to new host cells.

A number of proteins play crucial roles in the trafficking and function of CFTR, the molecular cause of CF. CFTR is a Cl^- channel activated by local cAMP and functions to regulate the ion/fluid balance for the clearance of mucus. Dysregulation of CFTR signaling impairs transportation of ions across the epithelial membrane and causes the accumulation of thick, viscous mucus in the lungs that blocks the airway (Riordan, 2008). This also leads to various CF-related disorders in the pancreas, kidneys, and intestine. CFTR ΔF508 (a deletion of residue F508) is the most common mutation in CFTR and results in inefficient folding of the protein, decreased channel activity, and rapid degradation (Guggino and Stanton, 2006). The rapid degradation of ΔF508 CFTR is mediated by the interaction of the CFTR PBM with the CAL PDZ protein (Cheng et al., 2002; Lukacs et al., 1993; Swiatecka-Urban et al., 2005). The CAL PDZ domain functions as an important regulator of CFTR endocytic trafficking and lysosomal degradation, reducing the CFTR levels at the plasma membrane (Cheng et al., 2004). In contrast, the NHERF-1 and -2 PDZ domains interact with and stabilize CFTR activity at the membrane (Raghuram et al., 2001). However, the NHERF-2 PDZ also binds to the lysophosphatidic acid 2 (LPA2) receptor at the apical membrane (Lin and Lai, 2008), and this binding can activate G_i GTPase signaling to reduce the local cAMP concentration and PKA activity (Fig. 2E) (Li et al., 2005a). This, in turn, causes the loss of CFTR activity resulting in respiratory and gastrointestinal defects.

Together, PDZ/PBM interaction networks regulate synaptic transmission and plasticity and the establishment and maintenance of cell adhesion and junction. Therefore, dysregulation of these interactions contributes to the development of neurological disease, cancers, and viral infection. Additionally, a variety of PDZ-mediated protein complexes fine-tune the

activity of CFTR, which directly affects CF. Targeting these PDZ/PBM interactions with small-molecule inhibitors is an emerging area that shows promise for dissecting the biology and development of novel therapeutic agents to treat disease (see section 5).

3. CANONICAL PDZ DOMAIN–BINDING MOTIFS AND NOVEL LIGANDS

PDZ domains consist of ~90 amino acids folded into six β strands and two α helices arranged in a β1–β2–β3–α1–β4–β5–α2–β6 pattern with loops of variable length connecting the elements of secondary structure (Fig. 3A). The N- and C-termini of PDZ domain are relatively close in space, supporting its role as small globular protein–protein interaction module. The C-terminal peptide ligand interacts with the PDZ domain through a binding groove formed by the β2 stand and α2 helix and makes additional interactions with residues located along the β1–β2 loop, known as the carboxylate-binding loop. All PDZ domains share this conserved β-barrel fold, yet have variations on the canonical structure. For example, the Erbin PDZ domain contains only one α helix, lacking the short α helix between the β3 and β4 strands (Birrane et al., 2003), whereas the Tiam1 PDZ domain has only five β strands (Liu et al., 2013). However, the absence of these structural elements does not affect the global structure of the PDZ domain. Other structural elements, in particular the β2–β3 loop region, can vary in length. Importantly, loop length has been correlated with dynamic motions and contributes to ligand specificity (Shepherd et al., 2010; Tyler et al., 2010).

3.1 C-Terminal PDZ-Binding Motifs

The canonical binding motif of a PDZ domain is the C-terminus of cognate interaction proteins. For convenience and by convention, the extreme C-terminal residue of the PDZ domain ligand is defined as position 0 (or P_0) and the remaining residues are numbered in reverse order (P_{-1}, P_{-2}, P_{-3}, etc.) from the C- to N-terminus. Similarly, individual binding pockets or sites in the PDZ domain are described by S_X, where X indicates the ligand residue proximal to the pocket. Using this notation, the side chain of the P_0 residue in the ligand is described as interacting with residues in the S_0 pocket of the PDZ domain (Fig. 3B).

Initial studies suggested that the PBMs are short, linear peptide regions (typically four amino acids) derived from C-termini of natural proteins.

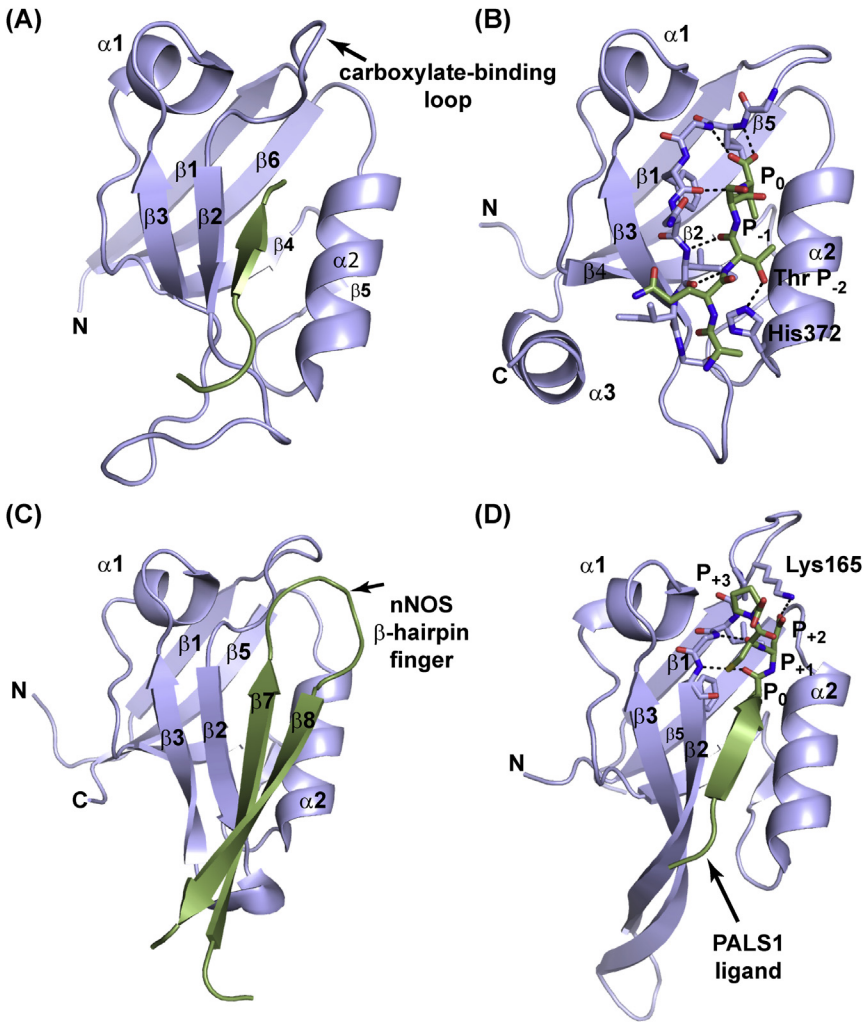

Figure 3 Structures of PDZ domains and their interaction with peptide ligands. (A—B) PDZ domains interacting with C-terminal peptide ligands. The ligand is colored green. Panel A shows the complex between hDlg1 PDZ1/APC peptide ligand (PDB code: 3RL7). Panel B shows the PSD-95 PDZ3/CRIPT peptide ligand complex (PDB code: 1BE9). (C—D) Structures of PDZ domains that interact with internal peptide ligands. The ligand is colored in green. (C) The α1-Syntrophin PDZ/nNOS complex (PDB code: 1QAV). (D) The Par-6 PDZ/PALS1 ligand (PDB code: 1X8S).

One of the most significant studies used a synthetic peptide library approach to screen for potential ligands for individual PDZ domains (Songyang et al., 1997). Immobilized PDZ domains were used to screen an oriented peptide library and the retained peptides were chemically sequenced as a pool. These

data were then used to determine the consensus binding motif for several PDZ domains, giving rise to original classification scheme. The two major classes described were: class I (X—S/T-X-$\mathbf{\Phi}_{coo-}$) and class II (X-$\mathbf{\Phi}$-X-$\mathbf{\Phi}_{coo-}$), where $\mathbf{\Phi}$ represents a hydrophobic residue and X can be any residue (Fig. 4A), which suggested a strong selectivity at P_0 and P_{-2}. PDZ domains also display unique amino acid sequence signatures that are consistent with the ligand classification scheme. For example, PDZ domains that bind class I ligands have either a conserved His or Arg residue in the α2 helix near the S_{-2} pocket that forms a hydrogen bond with a Ser/Thr hydroxyl of the ligand at position P_{-2} (Fig. 3B). In contrast, PDZ domains that do not have a basic residue at P_{-2} (e.g., Val/Leu/Asp) select for

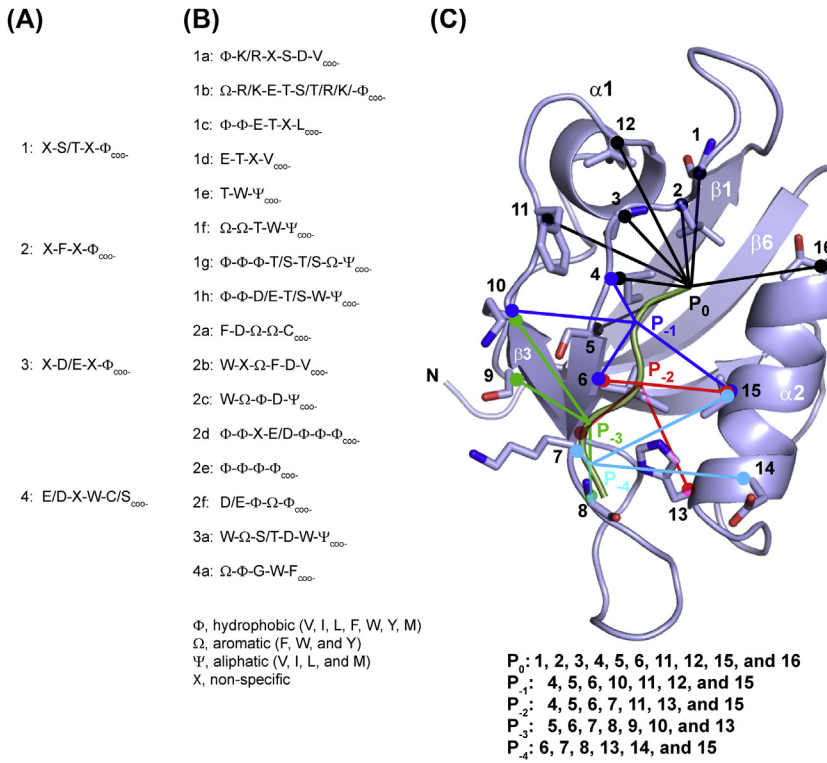

Figure 4 Classification of PDZ/ligand interactions. (A) The original classification based on the identity of last four residues of the ligand, and particularly the P_{-2} residue. (B) Detailed classification based on a large-scale phage display study using peptide residues from P_0 to P_{-6} (Tonikian et al., 2008). (C) The α1-syntrophin PDZ showing predicted residues involved in PBM-binding specificity. The 16 residues that interact with the P_0 to P_{-4} residues of the peptide ligand are labeled (PDB code: 2PDZ).

hydrophobic residues (e.g., Phe or Tyr). Subsequent studies with the nNOS and Mint1 PDZ domains identified new PBMs that did not fit either class I and II consensus sequences. Accordingly, class III (X-D/E-X-Φ_{coo-}) and IV (D-X-W-C_{coo-}) were proposed (Fig. 4A) (Bezprozvanny and Maximov, 2001; Maximov et al., 1999; Songyang et al., 1997; Stricker et al., 1997).

Modifications of the original peptide library screening technique were carried out to increase the size of the library and efficiency of screening. These methods include SPOT synthesis and one-bead-one-compound strategy. SPOT synthesis allows parallel synthesis and screening of peptides on individual "spots" on a sheet of cellulose membrane (Frank, 2002). Further modification of SPOT synthesis allowed for a free C-terminus that made it useful for profiling PDZ-mediated interactions (Boisguerin et al., 2004). These studies identified many new PBMs for known PDZ domains, including the Erbin PDZ domain (Boisguerin et al., 2007; Wiedemann et al., 2004). The one-bead-one-compound strategy utilizes a specialized resin coated with a peptide ending with $X_{-4}X_{-3}X_{-2}X_{-1}X_{0,COOH}$. X_{-4}—X_0 represents any amino acid and thus constitutes a library with theoretical diversity of 20^5. This approach was first used to screen binding partners of NHERF-1 PDZ domain and recently used to find potential ligands for both the Tiam1 and Tiam2 PDZ domain (Joo and Pei, 2008; Shepherd et al., 2011). By constructing a position-weighted matrix, analysis of the ligand amino acid sequence data allowed for classification into four distinct subgroups for both Tiam1 and Tiam2 PDZ domains. Thus, even within a PDZ protein several distinct motifs may exist. Validation of individual peptides led to the conclusion that the Tiam1 and Tiam2 PDZ domain specificities were overlapping, but distinct (Shepherd et al., 2011). Phage display methods have also been used to determine the specificity of PDZ domains. Tonikian et al. (2008) discovered over 3000 peptide ligands that bind 82 human and *Caenorhabditis elegans* PDZ domains. Analysis of the identified peptide sequences indicated that at least the last seven C-terminal residues of ligands were involved in PDZ domain interactions. Using positions P_0 through P_{-6}, PDZ ligands could be categorized into distinct classes (Fig. 4B). This classification included the original four classes, but further expanded into a total of 16 classes. Importantly, these classes were conserved in both the *C. elegans* and human PDZ domains, implying that specificity is not significantly more complex with increasing genome size (Tonikian et al., 2008). The combination of phage display and next-generation sequencing produces a large database of individual variants that can be analyzed using bioinformatic methods. This methodology has been used to successfully

discover new binding partners of PDZ domains, including those from viral proteins (Ivarsson et al., 2014; McLaughlin and Sidhu, 2013). Some binding pairs displayed high specificity but weak affinity, which might be further optimized through rapid evolution (Ernst et al., 2009). Hence, phage display has been powerful in providing a broader classification, defining novel ligand-binding specificity and identifying new binding partners of PDZ domains. However, the prediction of potential PDZ/PBM interaction based on phage display data should be examined carefully as it may be biased toward hydrophobic interactions, whereas natural PBMs frequently contain hydrophilic peptides as well (Luck and Trave, 2011).

Protein microarray technology has been developed and applied to identify and quantify PDZ/ligand interactions. This technology uses recombinant PDZ domain proteins spotted onto an aldehyde-presenting glass plate in a 96-well format followed by the addition of fluorescently labeled synthetic peptides as a platform to investigate PDZ/ligand specificity and affinity. The initial study used 22 PDZ domains and 20 PBMs to benchmark the method, resulting in the characterization of 520 PDZ/ligand combinations (Stiffler et al., 2006). A more comprehensive study used 157 mouse PDZ domains and 217 peptides derived from the mouse proteome (Stiffler et al., 2007). This large-scale study found that the P_0 residue of the PBM was less important in defining specificity and affinity than previously thought, and that residues P_{-1} through P_{-4} all contributed significantly to PDZ binding. Further analyses of this dataset suggested that PDZ domains do not fall into unique specificity classes; instead, selectivity is a property distributed throughout the PBM and PDZ domain. Therefore, it was concluded that the ligand-binding specificity of PDZ domains from the mouse proteome was intrinsically optimized, even though it could be further regulated by expression level and cellular localization. In a subsequent study, the PDZ array dataset was used to train a predictive model established using an interaction matrix. This matrix constituted 38 possible interactions, formed between 16 residues on the binding surface of the α1-syntrophin PDZ domain and 5 residues from its cognate C-terminal ligand (Fig. 4C). Based on these interactions, a Bayesian estimation protocol was developed to predict the binding capacity of PDZ domains using only their primary sequence as input. The model had reasonable predictive power and accuracy in identifying new interactions in other organisms and changes in affinity after PDZ mutations (Chen et al., 2008b). This study is noteworthy because the prediction was based exclusively on the primary sequence of the PDZ domain. These interactions came from 16 different positions in the PDZ domain but only 5 in the peptide, suggesting that

several residues in PDZ domain cooperate to recognize a single position in the peptide, reinforcing the idea that residues from P_0 to P_{-4} cooperatively contribute to ligand-binding process.

The techniques discussed so far have been mostly in vitro and used to predict potential PDZ/PBM interactions that might occur in cells; however, they neglect the influence of other cellular factors and domains in full-length proteins on specificity. To assess the PDZ/ligand interactions in vivo, a quantitative fluorescence resonance energy transfer (FRET)—based method was developed (You et al., 2006). This method takes advantage of CyPet and YPet, an optimized pair of cyan and yellow fluorescent protein, to detect a FRET signal with expanded dynamic range in a high-throughput and real-time fashion in *Escherichia coli* cells using fluorescence-activated cell sorting (Nguyen and Daugherty, 2005). The method was applied to the PSD-95 PDZ2 domain to screen for ligands from a random peptide library. A new set of ligands were identified, which were missed in a previous Y2H screening experiment (Bassand et al., 1999). In addition, direct binding and an apparent affinity (K_d) could be determined by this method. This method, in principle, could provide a direct measure of PDZ/ligand-binding affinity in a cellular environment. With the advancement of technology, it could be widely used in yeast and mammalian cells to identify novel in vivo PBMs in a quantitative and real-time manner. The main limitation of FRET-based systems has been the relatively low S/N ratio during image detection. This limitation can be overcome by luminescence resonance energy transfer (LRET)—based methods. Rajapakse et al. (2010) have developed a novel LRET method to characterize PDZ/ligand interactions in mammalian cells (Rajapakse et al., 2010). Briefly, the proteins to be examined are fused to *E. coli* dihydrofolate reductase (eDHFR) labeled with a luminescent terbium complex (TMP-Lumi4) or GFP. On binding, this pair readily produces an LRET signal. The longer lifetime and larger Stokes shift of this LRET signal enables time-resolved detection of well-separated emission signals. In this first application, the ZO-1 PDZ1 domain was fused to eDHFR, whereas the C-terminal PBM of claudin1 was incorporated into GFP. A positive interaction between PDZ1/claudin1 was detected with more than six fold signal over negative control. Thus, LRET techniques may prove to be a powerful *in vivo*-imaging method to investigate PDZ domain—based interactions in mammalian cells. Further modification of this technique incorporating an efficient probe delivery and 96 or higher well plate format could enable the screening of potential PDZ ligands or small-molecule inhibitors in a high-throughput fashion.

In summary, PBMs of C-terminal peptides are the predominant ligands of PDZ domains. After many PBMs were identified using efficient screening methods, the original PDZ/PBM classification appeared to be oversimplified. The classification based on the chemical properties of P_{-2} alone is not sufficient to explain and predict the binding specificity. With the advancement of a wide range of large-scale techniques, the classification of these PDZ domains has been challenged and evolved. Moreover, it is now becoming clear that regions outside the C-terminal four residues can also be important in defining affinity and specificity (Bezprozvanny and Maximov, 2001; Birrane et al., 2003; Luck et al., 2012; Tyler et al., 2010; Vaccaro and Dente, 2002). In particular, there is evidence that residues outside the canonical binding groove also contribute to binding specificity. Although much has been learned about the rules for binding specificity, predicting ligand partners from sequence alone is still a formidable challenge. Because the sheer number of PDZ domains is vast, it is likely new insights and unexpected modes of binding remain to be discovered. Novel large-scale high-throughput screens (in vitro and in vivo) combined with quantitative analyses of binding will likely contribute significantly to future studies (Reich et al., 2014).

3.2 Internal Binding Motifs

Although the canonical PBM is the C-terminus of partner proteins, PDZ domains can also bind to their partners using noncanonical internal motifs that do not involve the C-terminus. The first identified internal PDZ peptide motif was found in nNOS and mediated the interaction with the α1-syntrophin PDZ domain (Hillier et al., 1999). This interaction helps subcellular localization of the complex in skeletal muscle cells, where NO is produced on muscle contraction and leads to dilation of arteries (Brenman et al., 1995). Syntrophin and nNOS form a PDZ−PDZ heterodimer mediated by a β-hairpin finger motif found at the C-terminus of the nNOS PDZ domain (Fig. 3C). The β-hairpin finger docks into the canonical syntrophin PDZ domain ligand groove using the β-turn located between the two β-strands of the β-hairpin finger. Importantly, the penultimate β-strand packs into the binding groove rather than the classical free C-terminus. This internal peptide-binding mechanism did not impact the ligand-binding pocket of the nNOS PDZ domain, which could still bind canonical C-terminal peptide ligands. Thus, the heterodimer configuration provides syntrophin with the capacity to bind new proteins via the nNOS PDZ domain.

The Par-6 PDZ domain has also been found to bind a noncanonical internal ligand. The Par-6 PDZ domain recognizes an internal peptide of

PALS1 through a different mechanism compared to the α1-synthrophin PDZ (Hurd et al., 2003; Penkert et al., 2004). Instead of a β-hairpin finger motif, the Par-6 PDZ domain uses an extended eight amino acid linear motif. The crystal structure of Par-6 PDZ in complex with an internal PALS1 peptide showed that the P_0 Val (using the same convention as C-terminal ligands) and three additional residues C-terminal of this Val (referred as P_{+1}, P_{+2}, and P_{+3}) also contributed to the interaction (Fig. 3D). Within this extension, the side chain of P_{+1} Asp formed a hydrogen bond with the side chain of Lys165 found in the PDZ domain carboxyl binding loop, mimicking the role of the C-terminal carboxyl group. Mutating P_{+1} Asp to Ala or Asn abolished binding to the PDZ domain, suggesting an important role for the proper conformation of the carboxylate-binding loop. The Par-6 PDZ domain also binds to canonical ligands such as the C-terminus of Crb3. Moreover, the binding between the Cdc42 and the Par-6 CRIB motif enhances the Crb3 ligand affinity to the PDZ domain allosterically by regulating the conformation of the carboxyl-binding loop. Interestingly, Cdc42 binding to the CRIB domain did not enhance PALS1 affinity suggesting that the same loop was already in the appropriate conformation to bind the internal ligand (Penkert et al., 2004).

In addition to syntrophin and Par-6 PDZ domains, other PDZ domains bind internal targets. For example, the Dvl PDZ binds an internal ligand in the Frizzled receptor whereas the β2-syntrophin PDZ domain binds an internal sequence in the Tiam1 GEF (Mack et al., 2012; Wong et al., 2003). Despite a lack of structural details for these interactions, they play critical roles in regulating cell—cell junction, muscle contraction, and cell proliferation. More recently, a study using all the PDZ domains from *C. elegans* found that internal sequences mediated noncanonical binding on a genome-wide scale, much more frequently than previously appreciated—~51% of the PDZ domains did not associate with a classical C-terminal ligand (Lenfant et al., 2010). Similarly, a recent study used a Y2H and a random internal sequence library to determine that 14 out of 24 tested PDZ domains are capable of binding internal peptide sequences (Mu et al., 2014). These studies underscore the prevalence, and likely the significance of internal PDZ-binding motifs has been underestimated. The capacity for binding internal ligands in addition to C-terminal ligands enable PDZ domains to sense distinct binding signals and connect signaling pathways in an unprecedented manner, complicating PDZ signaling. Finally, the biophysical rationale for internal ligand specificity has yet to be determined, and future studies should help elucidate the rules for specificity.

3.3 Intra- and Interprotein PDZ–PDZ Domain Interactions

PDZ domain–containing proteins can dimerize and even oligomerize. This oligomerization can be mediated by PDZ domains, as first seen in the INAD protein (Xu et al., 1998). PDZ-mediated self-association was found later in EBP50 (Fouassier et al., 2000) and also with NHERF-1/2 homo- and heterodimer (Lau and Hall, 2001). Structural studies showed the interaction surface of PDZ6 homodimer from GRIP-1 (Fig. 5A) (Im et al., 2003b). This surface contained antiparallel packing of the β1 strand with a buried surface area over 600 $Å^2$. Moreover, this β1 strand–mediated dimerization did not disturb the ligand-binding groove. Therefore, this PDZ domain dimer can be formed in both apo and ligand-bound states without affecting PBM binding. Further analysis of this GRIP-1 PDZ6 dimer interface showed Tyr671 was an important residue as the Y671D mutation disrupted the

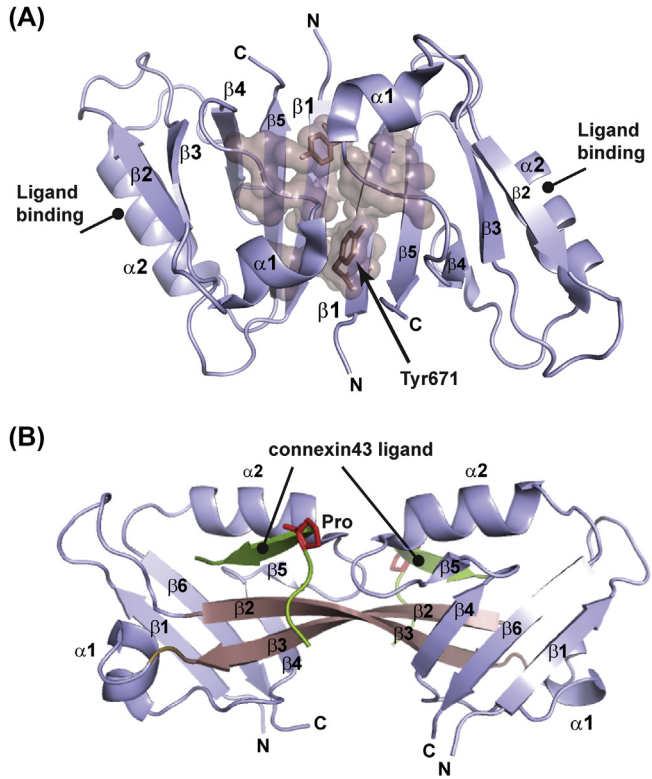

Figure 5 PDZ–PDZ domain interactions. (A) The GRIP-1 PDZ6 dimer (PDB code: 1N7E) and (B) ZO-1 PDZ2 dimer (PDB code: 3CYY) are shown. The interaction surface for the GRIP-1 dimer shown in *brown*.

hydrophobic interaction leading to monomeric species in solution. A similar dimer packing was found in the Shank-1 PDZ domain dimer, although the orientation of β1 strand was altered (Im et al., 2003a). This new β1 strand orientation caused a change in the binding pocket, which might facilitate recognition of the ligand for the dimer more efficiently at sites near the cytoplasmic membrane. Both structures suggest a new dimeric interaction of proteins mediated by the PDZ domain. These interactions in both proteins are crucial for forming homodimers or heterodimers, likely facilitating the assembly of multiprotein complexes including rhodopsin, protein kinase C, and calmodulin (Xu et al., 1998).

The ZO-1 PDZ2 domain offers another variation on PDZ dimerization. ZO-1 was found to form a stabilized dimer both in vitro and in vivo, and this dimerization required the second PDZ domain (Utepbergenov et al., 2006). A subsequent structural study highlighted the unique β-strand—swapped dimer (Fig. 5B) (Chen et al., 2008a; Fanning et al., 2007). The β2 strand from one monomer was inserted into the adjacent PDZ domain packing next to the β3 strand. A large number of interdomain interactions, including hydrogen bond and hydrophobic contacts were identified that stabilized the dimer. These interactions were more extensive than those found with GRIP-1 or Shank-1 PDZ dimers. This unique topology made the binding pocket available for two ligands simultaneously. Interestingly, the proline residue at P_{-5} in the connexin43 ligand turned the N-terminal residues by almost 90° to avoid a steric clash with the second ligand. The N-terminal residues were accommodated by a charge—charge interaction network found in the enlarged binding pocket around the extended β2-β3 strand. The dimer was necessary for ligand binding as a mutational insertion in the β2/β3 loop prevented dimer formation and impeded binding with the connexin43 C-terminus. Sequence alignment showed that the ZO-2 and ZO-3 PDZ2 domains are highly conserved with ZO-1, suggesting a similar dimerization mechanism. Indeed, ZO-2 PDZ2 homodimers and ZO-2/ZO-1 heterodimers were formed in vitro and in vivo. Also, the NMR structure of the dimerized ZO-2 PDZ2 showed a similar domain-swapped topology, supporting a general role of dimerization throughout the ZO family (Wu et al., 2007b). In summary, the swapped dimer in PDZ2 domain coupled with other structural features enhanced its ligand (such as in connexin43)-binding specificity and affinity, contributing to the effective association of ZO-1 to the connexin membrane channel. Furthermore, this specific dimer packing could promote the self-association and clustering of this scaffold protein at the cytoplasmic membrane, which

might trigger the oligomerization of other cell junction proteins at the membrane through other PDZ domain (such as PDZ1 or PDZ3)—mediated interaction.

Although these individual PDZ studies describe several cases of PDZ—PDZ interactions, their generality was still a question. A recent large-scale study has provided some insight for the overall frequency of PDZ—PDZ dimerization. Using protein microarray technology, Chang et al. utilized 157 mouse PDZ domains to asses 12,403 potential dimerization events (Chang et al., 2011). Primary hits were further validated using a solution-based fluorescence polarization (FP) assay with 37 PDZ dimer interactions between 46 PDZ domains confirmed. This indicated that ~30% of mammalian PDZ domains are involved in dimer interactions. Among these interactions, the tighter ones (with $K_d < 5$ μM) were confirmed in vivo in the context of full-length proteins. Bioinformatics studies showed this dimerization functioned significantly in assembling and strengthening the multiple protein complexes. The mechanism by which these newly identified PDZ domains dimerize is not known and additional structural work waits completion. Further investigation should also focus on the specific cellular function of the identified PDZ pairs.

3.4 Phosphatidylinositol and Cholesterol Binding

It is well known that cell signal transduction pathways can be regulated by a variety of protein—protein and protein—lipid interactions. PIPs are a major class of signaling phospholipids found in mammalian cell membranes. PIPs can recognize and recruit proteins to cellular membranes through diverse protein—PIP-binding domains (e.g., PH, FYVE, ANTH, BATS, FERM, PX and PTB) (Balla, 2005; Kutateladze, 2006, 2010; Ravichandran et al., 1997). More recently, it has become evident that PDZ domains can also bind PIPs.

The PDZ1 and PDZ2 domains of syntenin1 were the first PDZ domains reported to interact with PIPs. Syntenin1 was found to localize to cellular membranes in a process that depended on phospholipase C activation and the formation of $PI(4,5)P_2$. Moreover, this localization was lost after the breakdown of $PI(4,5)P_2$ (Zimmermann et al., 2001). The $PI(4,5)P_2$-binding sites on the two syntenin1 PDZ domains were localized to basic residues in the carboxylate-binding loop and α2 helix through mutational analysis (Zimmermann et al., 2002). Recently, the structure of syntenin1 PDZ2 domain bound to both $PI(4,5)P_2$ lipid and a C-terminal peptide from Frizzled-7 was solved (Fig. 6A). Although only the inositol ring and phosphates

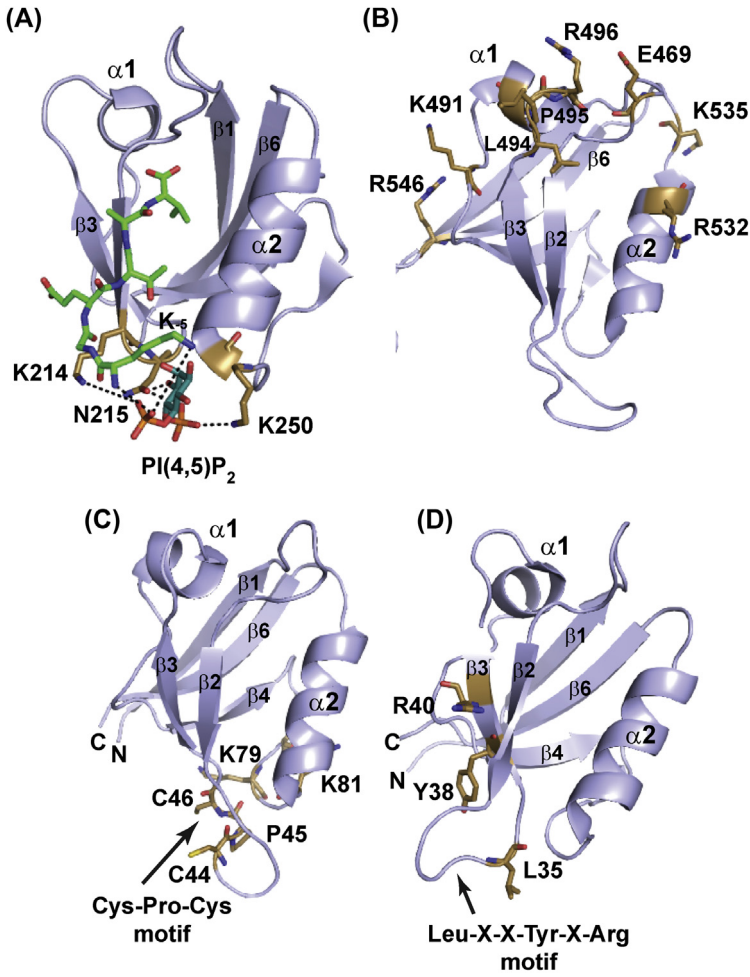

Figure 6 PDZ domains that interact with phosphatidylinositol and cholesterol. (A) The syntenin-1 PDZ2/Frzziled-7/PI(4,5)P_2 complex (PDB code: 4Z33). (B) Residues in Par-3 PDZ2 having chemical shift perturbations on titration with PI(3,4,5)P_3 (PDB code: 2OGP). (C) Residues in PICK-1 PDZ/PI(3,4,5)P_3 (PDB code: 2PKU). (D) NHERF-1 PDZ/cholesterol ligand (PDB code: 1G9O). Residues involved in lipid binding are labeled and shown in *gold*.

4 and 5 were unambiguously modeled, it precisely showed the lipid-binding site. The PIP_2 head group was bound into a pocket, formed mostly by positively charged residues in the β2−β3 loop (K214) and α2 helix (K250). Interestingly, the P_{-5} Lys residue in the Frizzled-7 ligand also interacted with phosphate 5 of the inositol ring. This unprecedented interaction, along

with SPR studies, indicated cooperative binding between phospholipid and peptide ligand in the syntenin1 PDZ2 domain (Egea-Jimenez et al., 2016).

The Par-3 PDZ2 domain binds to PI(3,4,5)P_3, and this interaction was found to be important for the regulation of cell polarity via the Par polarity complex. Using an NMR-based titration assay, PI(3,4,5)P_3 was found to bind the Par-3 PDZ2 domain near the α2−β6 and β1−β2 loops (Fig. 6B). This surface contains several charged residues that were shown to be important for PIP binding, indicating the significance of electrostatics in phospholipid recognition (Wu et al., 2007a). A similar patch of positively charged residues was shown to be important for binding PIPs in the PICK-1 PDZ domain (Fig. 6C). Moreover, a conserved peptide motif (Cys−Pro−Cys) near the β2−β3 loop was shown to facilitate the phospholipid association to the PICK-1 PDZ domain via penetration into the membrane. By utilizing this interaction, the PICK-1 PDZ domain controlled synaptic trafficking of AMPARs in neurons (Pan et al., 2007).

The different phospholipid-binding surfaces described above indicate several possible PDZ−PIP-binding modes and beckon the question of whether classical PDZ domain ligands and phospholipids generally bind simultaneously or are mutually exclusive. In the case of the Par-3 PDZ2 domain, binding data suggested peptide and phospholipid binding were mutually exclusive (Wu et al., 2007a). Similarly, competitive binding was found for the ZO-1 (and ZO-2) PDZ2 domains as binding of PI(4,5)P_2 prevented the interaction with peptide ligands (Meerschaert et al., 2009). In contrast, the two conserved phospholipid-binding regions in the PICK-1 PDZ domain did not overlap with the peptide ligand-binding site, suggesting that both ligands bind simultaneously and contribute to membrane recruitment (Pan et al., 2007). The CASK PDZ domain also showed synergy between PIP and ligand binding. Binding of PIP(4,5)P_2 was enhanced 4-fold in the presence of the peptide ligand (Ivarsson et al., 2013). Together, these studies indicate that the particular structural context of the interaction will dictate whether C-terminal peptide ligands and PIPs bind simultaneously or are mutually exclusive. In the case of simultaneous binding there are hints that the interaction may be cooperative. Future efforts will be necessary to fully understand the biophysical nature and physiological significance of this cooperativity.

Given that PDZ domains are highly prevalent, several large-scale studies have probed the extent to which PDZ domains interact with phospholipids. An early study by Wu et al. (2007a) surveyed 74 human PDZ domains for their ability to bind PIPs in vitro and found that ∼25% of PDZ domains

were capable of these interactions. Using a cell-based assay, Ivarsson et al., (2011, 2013) identified one Drosophila PDZ domain and 53 out of 256 (~22%) human PDZ domains as capable of interacting with PIPs. These observations were followed with biochemical validation and mutagenesis, reiterating the point that PDZ/PIP interactions are diverse and generally do not share a common motif. A more recent study combined surface plasmon resonance (SPR) and bioinformatics to predict the lipid-binding capacity of PDZ domains at a genomic level (Chen et al., 2012). The authors showed that nearly 40% of the 70 mouse, rat, and human PDZ domains bound to PIP molecules presented in anionic lipid vesicles. These PDZ domains were then used to develop an algorithm that included amino acid sequence and physiochemical information to predict PDZ/PIP interactions of 2000 PDZ domains found in 20 species. Validation of the PDZ/PIP interactions indicated that ~90% of the predictions were correct, suggesting that nearly 30% of all PDZ domains bind PIPs. Finally, the authors developed a general classification scheme based on the proximity of the predicted lipid-binding site to the canonical C-terminal peptide–binding groove. Similar to other studies, it was found that PIP binding could influence peptide binding depending on the extent of overlap between the two binding sites.

Cholesterol, another major lipid component of the cell plasma membrane, was also found to bind PDZ domains. A recent study used an SPR assay to screen PDZ domains that bind cholesterol (Sheng et al., 2012). Of the 30 PDZ domains investigated, 12 were found to bind cholesterol with an affinity of μM or better. Using NHERF-1 PDZ1 domain as a model system, a cholesterol-binding motif was identified near the β2–β3 loop and β3 strand region of the PDZ surface, away from the canonical peptide-binding site. This motif contains a consensus recognition sequence, Leu–X–X–Tyr–X–Arg, where X represents any amino acid (Fig. 6D). This consensus-binding motif might be useful for identifying additional cholesterol-binding PDZ domains. Functionally, the cholesterol-binding capacity of the NHERF-1 PDZ domain affected the Cl^- channel activity of CFTR, a known binding partner. In particular, mutations that disrupted cholesterol binding also interfered with the membrane association of NHERF-1 and colocalization with CFTR, leading to a pronounced reduction in CFTR-mediated current. More recently, Dvl2 was found to bind cholesterol, and this interaction was essential for activation of the canonical Wnt-signaling pathway (Sheng et al., 2014).

In summary, progress has been made in the field of the PDZ/lipid interaction. Several large-scale studies indicate phospholipid binding as a

relatively common feature of PDZ domains. Functional analyses showed that phospholipid binding can have a major role on regulating canonical PDZ-domain interactions, and thus the activity of PDZ proteins. Furthermore, biochemical and structural studies revealed diverse phospholipid-binding sites on PDZ surfaces suggesting structural diversity in PDZ/PIP interactions (Gallardo et al., 2010). Despite the first PDZ/PIP interaction being identified more than a decade ago, these interactions and their functional significance are still poorly understood. Given that only one PDZ/PIP co-complex structure has been determined to date, how phospholipid binding affects ligand binding is still not clear. Future investigations should focus on the detailed structural characterization of PDZ/phospholipid interactions and the functional interplay between peptide and lipid binding. Likewise, a few initial studies indicate that cholesterol binding to PDZ domains can be important, but the extent to which they occur, their precise binding site, and their physiological role(s) will require additional studies.

4. INHIBITION OF PDZ DOMAIN/LIGAND INTERACTIONS

There have been many efforts placed on developing inhibitors targeting protein–protein interactions involved in disease-related cellular signaling. We have already described many PDZ domain interactions that serve significant biologically roles in cellular signaling and whose dysfunction leads to various malignancies including neuronal toxicity, neuropathic pain, cancers, and CF (see Section 2). Moreover, biophysical and biochemical properties of PDZ domain–mediated protein–protein interactions have been very well characterized. Although PDZ domains share similar modes of ligand binding, their unique subcellular compartmentalization and tightly regulated complex assembly potentially make them selective for targeting, avoiding severe side effects of other common large protein families (e.g., GPCR and kinases). Hence, PDZ domains have emerged as important "druggable" targets (Blazer and Neubig, 2009; Dev, 2004; Gardiol, 2012; Grillo-Bosch et al., 2013; Manzo-Merino et al., 2013; Romero et al., 2011). A further incentive for targeting PDZ-mediated interactions is that small molecule inhibitors might be developed that could overcome the permeability, bioavailability, and high cost associated with monoclonal antibodies. Moreover, nonclinical grade inhibitors can be employed as powerful probes to dissect the specificity of PDZ-dependent signaling cascades (Zhang et al., 2011b). Below, we describe

several PDZ-domain inhibitors identified as potential lead compounds for the treatment of a variety of conditions, including neuropathic pain, cancer, and CF.

4.1 PDZ Domain Inhibitors in Neuronal Disorders

As described in previous sections, many synaptic junctional complexes are assembled through PDZ-mediated interactions and thus critical for synaptic formation and plasticity (Fig. 2A). One of most important PDZ-containing proteins is PSD-95. The first two PDZ domains from PSD-95 (PDZ1 and PDZ2) bind to NMDAR and nNOS to form an NMDAR/PSD-95/nNOS ternary complex (Christopherson et al., 1999; Sattler et al., 1999). Thus, PSD-95 PDZ1 and PDZ2 couple NMDAR signaling to nNOS and NO production. Importantly, overproduction of NO leads to neurotoxicity and ischemic brain damage (Huang et al., 1994), results in the elevation of sensitivity associated with the central nervous system (central sensitivity), and promotes acute thermal hyperalgesia, a well-known inflammatory response that leads to pain sensitivity from thermal stimuli (Ji and Strichartz, 2004). Decreasing PSD-95 expression by an antisense oligonucleotide was able to suppress neurotoxicity without affecting NMDAR activity (Sattler et al., 1999) making PSD-95 an attractive target for inhibitor for development to treat stroke and systemic neuropathic pain. Initial efforts by the Tymianski group used a natural C-terminal PDZ ligand as antagonist to disrupt PSD-95/NMDAR signaling in vivo (Aarts et al., 2002). A nonapeptide derived from the C-terminus of the NR2B subunit of the NMDAR ($KLSSIESDV_{coo-}$, NR2B9c) was fused to the cell-membrane transduction domain of the human immunodeficiency virus type 1 (HIV-1) Tat protein to form a Tat-peptide fusion (Tat-NR2B9c). Adding Tat-NR2B9c into cultured cortical neurons suppressed NO production by disrupting the PSD-95/NMDAR/nNOS ternary complex formation without affecting NMDAR activity. Furthermore, intraperitoneal injection of Tat-NR2B9c into mice showed pronounced effects in reducing ischemic neuronal damage after stroke onset. Although there are many other PDZ domain–containing proteins that participate in neuronal signaling cascades, Tat-NR2B9c perturbed the NMDAR/PSD-95 interaction with strikingly high specificity (Cui et al., 2007). Therefore, this inhibitory peptide was effective in reducing stroke damage in mice without deleterious side effects that generally occur after blocking NMDAR. Indeed, this inhibitor peptide successfully provided neuroprotection in higher-order gyrencephalic primates on stroke, suggesting that it might be a useful therapeutic in humans

(Cook et al., 2012a,b). The Tat-NR2B9c peptide is being developed as a drug (NA-1) by NoNO Inc. (Toronto, Canada) to treat acute ischemic stroke, subarachnoid hemorrhage, and procedurally induced strokes. Importantly, it has already advanced through Phase 1 and Phase 2 clinical trials in the United States and Canada (http://nonoinc.ca/). The Phase 2 study clearly showed that NA-1 could reduce ischemic brain damage in patients (Hill et al., 2012). A recent study found that NA-1 was also capable of reducing neonatal stoke damage and an effective neuroprotection regent in neonatal hypoxic-ischemic brain injury (Xu et al., 2016).

The Strømgaard group has taken a similar approach, but with optimization of the Tat-NR2B9c peptide to avoid poor pharmacokinetics and stability issues. Initial truncation and mutational studies showed that the C-terminal five residues of the NR2B subunit ($IESDV_{coo-}$) is the minimal peptide required to maintain high affinity (Bach et al., 2008). As PSD-95 PDZ1 and PDZ2 are both capable of binding the $IESDV_{coo-}$ peptide, a dimeric Tat-fused peptide inhibitor linked by four polyethylene glycol (PEG4) subunits was developed to simultaneously target both PDZ domains (Bach et al., 2012). The combination of the two PDZ ligands (i.e., multivalency) had a synergetic effect on affinity, creating a new ligand with ~1000-fold greater affinity (K_d ~5 nM) than the Tat-NR2B9c ligand. Substitution of the central oxygen in the PEG4 linker with nitrogen (*N*PEG) [Tat-*N*PEG4$(IETDV)_2$ or Tat-*N*-dimer] showed an almost five fold enhancement in stability ($T_{1/2}$ ~4900 m) compared with the monomeric Tat-NR2B9c ($T_{1/2}$ ~1100 m) in human blood plasma. Moreover, modifying this peptide with a Tat peptide synthesized from D-amino acids (ReTat-*N*-dimeric) showed no sign of degradation in plasma. As proof of principle, the Tat-*N*-dimer inhibitor was capable of reducing ischemic brain damage by 40% in a mouse stroke model. Currently, this is the most efficient PSD-95 antagonist and a promising therapeutic agent against stroke (Avilex Pharma has been formed to develop these compounds, http://www.avilexpharma.com/). Because other types of acute brain injury, such as traumatic brain injury, share similar pathophysiological mechanisms as stroke, the efficacy of the dimer inhibitor was tested in animals with traumatic brain injury. However, no neuroprotective effect by this inhibitor was found in two recent studies using different animal models of traumatic brain injury (Sommer et al., 2017a; Sommer et al., 2017b,c).

Peptidomimetics, chemically modified natural peptides, are alternative peptide-like inhibitors designed to be more stable in vivo and have an

increased affinity for PDZ domains. Modification of Tat-NR2B9c by truncation and methylation of the glutamate in the peptide ($E_{Me}TAV_{coo-}$) resulted in a chemically smaller inhibitor with essentially the same affinity as the parent $IESDV_{coo-}$ peptide. Additional optimization identified that an N-terminal chemical substitution of the peptide (N-cyclohexylethyl-$ETAV_{coo-}$) had a ~20-fold increase in affinity compared with the WT peptide (Bach et al., 2008). In many cases, an additional advantage of peptidomimetics is enhanced stability against proteases, often achieved by peptide cyclization. The first example of this strategy for a PDZ domain was applied to the interaction between the PSD-95 PDZ3 domain and the CRIPT peptide. A cyclic peptide was synthesized by linking the P_{-1} and P_{-3} amino acids in the CRIPT peptide with β-alanine, which enhanced the affinity up to 10-fold. In addition, peptide cyclization promoted rapid onset and duration of inhibition in cellular assays due to an increased stability against proteases (Piserchio et al., 2004). Several constrained cyclic peptides were designed to reduce the entropic changes on binding compared with linear peptides. In general, these cyclic peptides were capable of binding to the PSD-95 PDZ1 domain with high affinity (Udugamasooriya and Spaller, 2008). Incorporation of a $(Arg)_7$ N-terminal extension to this peptidomimetic resulted in cell-permeable compounds. The cyclic peptide CN2097 (R7-CC-YK[KTE(β-Ala)]V) showed decreased hyperexcitability of neurons and alleviated thermal hyperalgesia in rats (LeBlanc et al., 2010).

The molecular weight and polarity features of peptides are potential drawbacks as inhibitors for use in vivo. In particular, short half-lives in the blood stream, excretion by human body, and poor transportation through cell membranes and the blood—brain barrier limit their applicability. Peptidomimetics might overcome some of these liabilities, but they are still relatively large. In contrast, small molecule inhibitors in principle do not have these limitations and have their own benefits, in particular displaying higher stability in cells and blood plasma (Wang et al., 2008). To date, many small molecule inhibitors have been developed to target neuronal PDZ domains (see Fig. 7 and Table 2). Two indole-based compounds, ZL006 and IC87201, were identified that target the PSD-95 PDZ2/nNOS interaction using in silico docking and high-throughput methods, respectively. Both compounds readily crossed the blood—brain barrier and inhibited the PSD-95 PDZ2/nNOS interaction effectively, with IC_{50}s of 82 nM and 31 μM, respectively. ZL006 showed neuroprotective effects against cerebral ischemia in rats (Zhou et al., 2010), whereas IC87201 suppressed mechanical allodynia (i.e., neuropathic pain caused

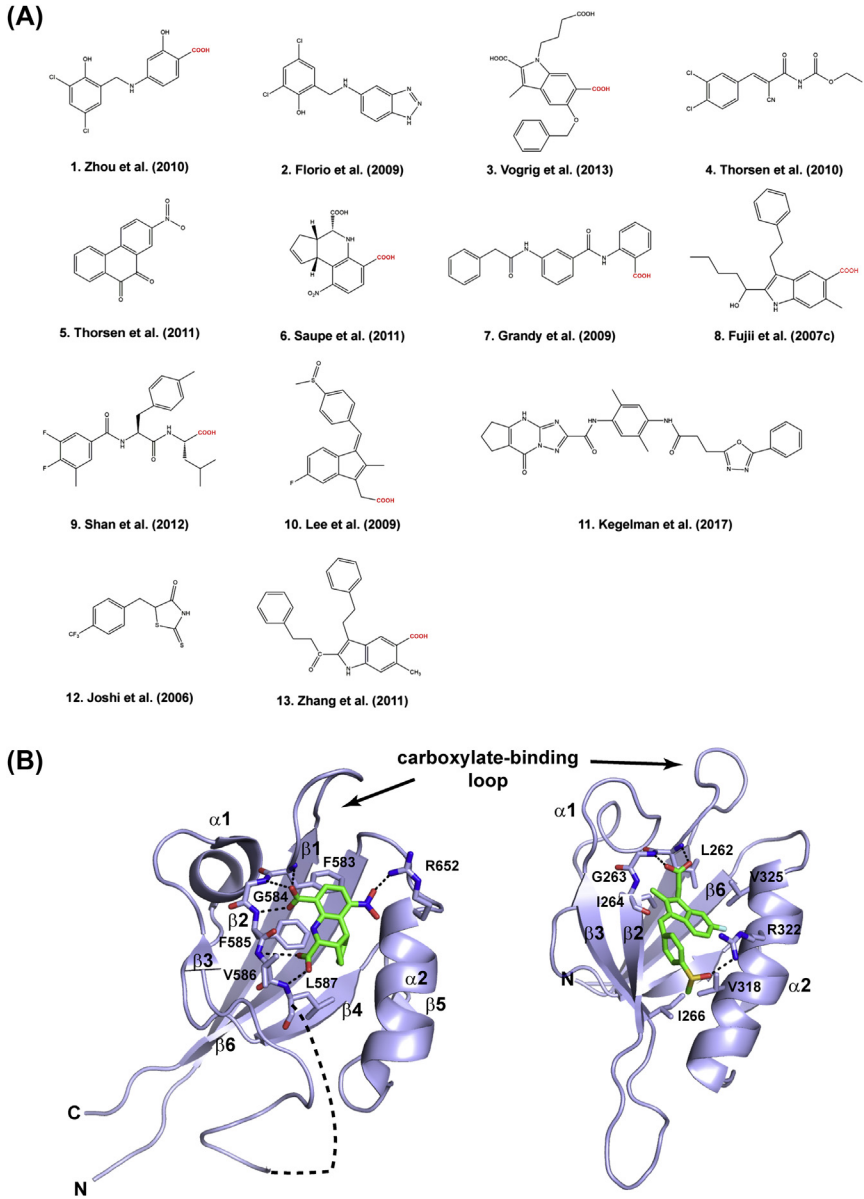

Figure 7 PDZ domain inhibitors. (A) The chemical structures of small-molecule inhibitors that target PDZ domains. Carboxylate groups are highlighted in red. (B) The structure of PDZ domain/inhibitor complexes. Left panel, crystal structure of tetrahydroquinoline carboxylate compound (A.6) bound to the Shank-3 PDZ domain (PDB code: 3O5N). Right panel, solution NMR structure of sulindac (A.10) bound to Dvl PDZ domain (PDB code: 2KAW). Residues involved in hydrogen bonding with each compound are labeled.

by innocuous stimuli) induced by chronic constriction of the sciatic nerve (Fig. 7A.1 and A.2) (Florio et al., 2009). Neither of the compounds interfered with normal NMDAR or nNOS function and showed no major side effects. However, a recent study employed a battery of biochemical methods, yet failed to validate the direct interaction between either of these compounds and the PDZ domains in PSD-95 or nNOS (Bach et al., 2015). Thus, further mechanistic studies will be required to understand the precise molecular mechanism of action of ZL006 and IC87201. Several other indole-based compounds were identified and optimized by in silico docking and solution NMR methods to target the PSD-95 PDZ1/5-HT_{2A} serotonin receptor interaction (Boucherle et al., 2011). One particular analogue (8b) was effective at suppressing the mechanical hyperalgesia in a rat model of neuropathic pain (Fig. 7A.3) (Boucherle et al., 2011; Vogrig et al., 2013).

Other PDZ-containing proteins associated with synapses are potential targets for inhibition. For example, the PICK-1 and Shank-3 have roles in chronic pain, autism spectrum disorders, and schizophrenia (see in Section 2). Among these, the PICK-1 PDZ domain has been shown to be involved in cocaine-induced plasticity, neuronal toxicity after cerebral trauma, and neuropathic pain (Bell et al., 2009; Bellone and Luscher, 2006; Garry et al., 2003). Consequently, the PICK-1 PDZ domain has been targeted for the development of small molecule inhibitors. A recent study developed a FP-based assay using a dye-labeled C-terminal peptide from the dopamine transporter (DAT) to identify inhibitors that disrupt PICK-1 PDZ interactions (Thorsen et al., 2010, 2011). The assay was optimized on a 96-well microtiter plate format and used to screen a 43,380 compound library from Neurosearch A/S (Denmark). Six chemically distinct compounds with affinities ranging from $\sim$5 to 200 μM were characterized, and the compounds FSC231 and CSC-03 were chosen for further study (Fig. 7A.4 and A.5). Competition-binding experiments determined that FSC231 had a $K_i \sim 10$ μM, similar to that found for the DAT peptide. Moreover, this compound was selective and did not disrupt three other PDZ/ligand interactions. Bioactivity studies showed that FSC231 was capable of passing the cell membrane and blocking PICK-1 PDZ *in* COS7 cells. Importantly, FSC231 blocked both LTD and LTP in hippocampal CA1 neurons, consistent with the inhibition of PICK-1 function in synaptic plasticity (Thorsen et al., 2010). These results suggest that FSC231 is a promising lead compound for the development of small-molecule therapeutics to treat PICK-1—associated conditions such as neuropathic pain, excitotoxicity, and cocaine addiction.

Inhibitors targeting Shank-3 PDZ domain interactions were also identified by high throughput screening (HTS) methods. Using an FP-based 384-well format, two compounds containing a tetrahydroquinoline chemical scaffold (K_is ~10 μM) were identified from the ChemBioNet library (Saupe et al., 2011). Structure activity relationship (SAR) studies identified two additional potent compounds. In addition, the X-ray crystal structure of Shank-3 PDZ complexed with compound 36 (a quinoline derivative) was determined (Saupe et al., 2011) (Fig. 7A.6 and B). The crystal structure provided valuable insight into PDZ/inhibitor interactions; in particular, it showed that the carboxylate at position R_6 is critical for binding. Unfortunately, no cell-based or in vivo studies were performed, and thus the biological efficacy of these compounds remains unknown.

4.2 PDZ Domain Inhibitors in Cancer

A variety of PDZ proteins and their interactions are crucial for the regulation of cell polarity, development, and proliferation. Dysfunction in the control of these processes, either by loss or inappropriate activation of PDZ proteins, has been found in several human malignancies, including cancer. In some specific cases, PDZ domain interactions are potential targets for the development of inhibitors and offer therapeutic value for the treatment of cancer (Subbaiah et al., 2011). The best-studied example is the Dvl protein, which contains a single PDZ domain. The Dvl PDZ domain interacts with the Frizzled receptor and is involved in Wnt signaling that activates transcription of many oncogenic genes, such as Myc and Cyclin D1 (MacDonald et al., 2009). Moreover, inappropriate activation of Wnt signaling by upregulation of Dvl has been implicated in tumorigenesis of breast, colon, prostate, leukemia, and lung cancer, whereas downregulation of Dvl inhibited tumor growth (Bui et al., 1997; Mizutani et al., 2005; Uematsu et al., 2003a, 2003b). Thus, inhibiting Dvl and its PDZ domain—mediated interaction could have significant therapeutic value for the treatment of cancer.

Using a structure-based in silico screening approach, Zheng and colleagues identified several small molecules from the National Cancer Institute 3D structure database as potential inhibitors (Shan et al., 2005). NMR-based experiments showed that NSC668036 bound to the Dvl PDZ domain, whereas competition-binding experiments estimated the affinity for this PDZ domain to be ~237 μM. The NSC668036 compound was bound to the Dvl PDZ domain specifically, as no binding was observed with either PSD-95 PDZ1 or GRIP-1 PDZ7 domains. Importantly, in vivo signaling assays in *Xenopus* embryos showed that NSC668036 blocked canonical

Wnt signaling at the Dvl level. Subsequent screens using a refined docking algorithm and multiple libraries (NCI, Chemical Diversity and Sigma–Aldrich) identified additional compounds with higher affinity (Grandy et al., 2009). In particular, the compound 3289–8625 was found to bind the canonical Dvl PDZ domain–binding pocket with a dissociation constant of ~11 μM (Fig. 7A.7). This compound was biologically active as it inhibited Wnt signaling in *Xenopus* embryos and suppressed the proliferation of prostate cancer–derived PC-3 cells. Independently, Fujii et al. (2007c) identified the compound FJ9 using an AlphaScreen assay developed for the Dvl PDZ/Frz receptor interactions. This compound contained an indole-2-carbinol scaffold (Fig. 7A.8) and bound the Dvl PDZ domain directly. FJ9 was able to suppress canonical Wnt signaling and cell growth in both melanoma and non–small cell lung cancer cell lines. Finally, FJ9 had a marked inhibition of tumor growth in a mouse xenograft model. Together, the development and use of Dvl PDZ inhibitors directly show that inhibition of Dvl PDZ interactions is linked to tumor growth in distinct model biological systems, providing several lead compounds for further development as cancer therapeutics.

Subsequent studies used more sophisticated computational tools to guide the design and synthesis of new potent inhibitors for the hit-to-lead stage of drug development. In particular, the use of a computational pharmacophore model for the NSC668036 inhibitor and additional in silico searching identified 15 related compounds with ~6–28-fold tighter affinity for the Dvl PDZ domain (Shan and Zheng, 2009). Additional virtual screening with a higher diversity of substituents yielded nine more related compounds (Shan et al., 2012). These compounds were then used to develop three-dimensional quantitative structure-activity relationship (3D-QSAR) profile. Guided by the 3D-QSAR profile and using "compound 16" as a reference, several novel compounds were designed, synthesized, and tested for binding to the Dvl PDZ domain. The compound J01-017a (Fig. 7A.9) emerged from the pool of optimized compounds as having the highest affinity (K_d ~1.5 μM) of any compound for the Dvl PDZ domain to date. This compound is currently being tested for biological activity. Concurrently, the known anti-inflammatory drug Sulindac was identified to interact with Dvl PDZ domain and block canonical Wnt signaling in *Xenopus* embryos (Fig. 7A.10) (Lee et al., 2009). The solution NMR structure of the complex showed that the carboxylic acid group in Sulindac forms hydrogen bonds with the PDZ carboxylic-binding loop (Fig. 7B). In addition, the oxygen atom in the (methylsulfinyl)benzene ring of Sulindac

interacted with a unique Arg residue (Arg322) side chain in α2 helix of Dvl PDZ. The structure explained the observed specificity of Sulindac as it did not bind three other PDZ domains. Thus, Sulindac is a good example of a "repurposed" drug with novel therapeutic value.

The PDZ-containing proteins GIPC-1, PTPN-4, syntenin, AF-6, and MAGI-1 are involved in tumorigenesis and potential targets for inhibitor development. GIPC-1 contains a single PDZ domain that interacts with the C-termini of transmembrane receptors (e.g., insulin-like growth factor receptor 1 or IGF-1R) and cytosolic proteins (e.g., the regulator of G-protein signaling 19 or RGS19). It is upregulated in breast, ovarian, and pancreatic cancers and promotes tumor proliferation and invasion (Muders et al., 2006, 2009; Rudchenko et al., 2008; Wang et al., 2010b). This is due in part to the role of GIPC-1 in endosomal trafficking and stabilization of IGF-1R at the cell surface. Knockdown of GIPC-1 in cancer cells inhibits proliferation and promotes apoptosis through the function of the PDZ domain, suggesting that inhibition of this domain may be a novel target for therapeutic development (Chittenden et al., 2010; Muders et al., 2006). To this end, Mukhopadhyay and colleagues used an N-terminally myristoylated octapeptide derived from the C-terminus of RGS19 (N-myristoyl-PSQSSSEA$_{coo-}$ or CR1023) to disrupt the GIPC-1 PDZ domain interactions in cells to inhibit proliferation, survival (Muders et al., 2009), and tumor growth in vivo (Wang et al., 2010b). Modification of CR1023 at P_{-1} and P_{-3} with the bromobenzoyl group into the amine side-chains of Lys yielded the peptidomimetic inhibitor CR1166 [N-myristoyl-PSQSK(N-4-bromobenzoyl)SK(N-4-bromobenzoyl)A$_{coo-}$], which had the greatest biological activity cells and in vivo, significantly reducing both breast and pancreatic tumor growth in mice (Patra et al., 2012).

The human protein tyrosine phosphatase non-receptor type 4 (PTPN4) promotes tumorigenesis by protecting cancer cells against death. PTPN4 contains a single PDZ domain and its interaction with the C-terminus of the envelope glycoprotein (G) of the rabies virus is a critical determinant of virulence. Importantly, the wild-type envelope glycoprotein (G) PBM does not bind the PTPN4 PDZ domain, allowing both cell survival and virulence. In contrast, a single mutation in the envelope protein G (G**E**TRL$_{coo-}$ to G**Q**TRL$_{coo-}$) permits the interaction of the PTPN4 PDZ domain and leads to cellular apoptosis and attenuation of virulence (Prehaud et al., 2010). Based on these observations, Wolff and colleagues devised a therapeutic strategy to induce apoptosis in malignant brain tumors, such as glioblastoma, using peptides that target the PTPN4 PDZ domain

(Babault et al., 2011). Guided by the crystal structures of two PTPN4 PDZ domain complexes (with Cyto13-att: GETRL$_{coo-}$ and GluN2A16: ISEDV-$_{coo-}$), a series of peptides with a broad range of affinity were designed by incorporating positively and negatively charged residues at P_{-4} and P_{-1}, respectively. This optimized peptide was fused to a HIV-1 Tat peptide to aid in cell permeability (Tat-***R***ET***E***V$_{coo-}$) and showed ~150-fold enhancement in affinity in vitro. Moreover, it induced apoptosis in ~70% of cells of a highly invasive glioblastoma cell line, which exceeded the effectiveness of Shepherdin, an established compound that induces apoptosis (Plescia et al., 2005). Therefore, this newly engineered peptide might be an efficient antiproliferative agent for malignant glioblastoma.

The protein syntenin1 contains two PDZ domains that have recently been found to play central roles in tumor invasion and angiogenesis. Different binding partners as well as downstream regulators of syntenin1 PDZ domains, including Src kinase, MAPK, and focal adhesion kinase, have been identified that promote the progression and invasion of a variety of cancers, including breast cancer, melanoma, and glioblastoma multiforme (GBM) (Kegelman et al., 2015). Radiotherapy is a common treatment for GBM. However, cells surviving radiotherapy had increased invasiveness mediated by syntenin1 PDZ domain interactions. Thus, inhibiting syntenin1 PDZ domain interactions might complement radiotherapy. Combining fragment-based screening by NMR with in silico docking identified a small molecule (113B7) that specifically interacted mostly with the PDZ1 domain and part of the PDZ1–PDZ2 interface of syntenin1 (Fig. 7A.11). Moreover, it did not bind to the isolated syntenin1 PDZ2 domain. This example suggests that tandem PDZ domains might have additional specificity compared with individual domains. Cellular studies found that 113B7 inhibited cell invasion mediated by syntenin1, similar to the effect of syntenin1 knockdown. In a GBM animal model, 113B7 treated mice had increased survival. The combination of 113B7 with radiotherapy produced significant effects in decreasing GBM invasion and enhanced survival. Together, these data suggest that an effective PDZ inhibitor can complement traditional radiotherapy to combat aggressive cancers, such as GBM (Kegelman et al., 2017).

Acute lymphoid leukemia fusion partner on chromosome 6 (AF-6, afadin, or MLLT4) was originally identified as a fusion partner of the mixed lineage leukemia (MLL) gene involved in lymphoblastic and myeloid acute leukemia. AF-6 contains two Ras-associated domains and a single PDZ domain (Mandai et al., 2013). The PDZ domain binds the C-terminus of several target proteins, including transmembrane adhesion receptors

(e.g., NRXN and nectin) and kinases (e.g., Eph receptors and Bcr). The interaction with Bcr is important for regulating Ras signaling, cell−cell junctions, and oncogenic potential (Liedtke et al., 2010; Radziwill et al., 2003; Zhang et al., 2005). Thus, the AF-6 PDZ domain may be a useful target for inhibitor development. The initial efforts for identifying small molecule inhibitors targeting the AF-6 PDZ domain used an NMR spectroscopy approach for identifying lead compounds and medicinal chemistry for lead optimization. A small library of 5000 compounds was screened using ^{15}N-labeled PDZ domain and 2D ^{1}H−^{15}N-heteronuclear single quantum correlation experiments to identify weakly binding compounds. This screen led to three classes of compounds, including one containing a thiazolidinone scaffold with a $K_d \sim 460$ μM. A secondary screen of analogues verified the importance of several functional groups. Additional SAR studies identified that a trifluoromethyl phenyl ring (4-$CF_3C_6H_4$) appended to the thiazolidinone scaffold (compound 5f) improved the affinity for the AF-6 PDZ to $K_d \sim 100$ μM (Fig. 7A.12). The solution structure of the complex indicated that the trifluoromethyl phenyl ring bound deeply into the hydrophobic peptide-binding groove (Joshi et al., 2006). Unfortunately, this compound did not have high binding specificity, as it also bound to the α1-syntrophin PDZ domain. More recently, these same authors reported a study aimed at developing compounds based on the 5f scaffold with higher affinity and specificity (Vargas et al., 2014). Modification at position R_6 improved the affinity and specificity of the parent compound. Compound 10, containing a morpholine-phenylcarbonylmethyl moiety, had the highest affinity ($K_d \sim 4.9$ μM) for the AF-6 PDZ domain and did not bind to the Shank-3 or PSD-95 PDZ1 and PDZ3 domains, but showed weak binding to the Dvl1, PSD-95 PDZ2, and α1-syntrophin PDZ domains. NMR chemical shift perturbation experiments and molecular modeling indicated that compound 10 bound in the same peptide groove as the parent compound, but extended into a new subpocket (Ala80 pocket). The series of compounds were capable of disrupting the AF-6/Bcr interaction in cells, suppressing RTK/Ras signaling and maintaining the cells in the nonproliferative state. Further development of these compounds may have potential value in the treatment of leukemia.

Membrane-associated guanylate kinase with an inverted repeat family proteins (MAGI 1−3) are atypical members of the MAGUK superfamily located at tight junctions and regulators of cellular trafficking (Laura et al., 2002). MAGI proteins have important functions in regulating cell−cell contacts and stabilizing target proteins (e.g., AMPARs) at the membrane.

Importantly, MAGI proteins have been implicated in cancer through their interactions with the PTEN tumor suppressor and HPV-16 and -18 E6 viral proteins (Feng et al., 2014). In particular, the interaction between PDZ2 of MAGI-2 and -3 with PTEN suppresses AKT kinase signaling and apoptosis (Tolkacheva et al., 2001; Wu et al., 2000). In contrast, the first PDZ domain of all MAGIs was found to be a cellular target of HPV-16 and -18 E6 PBMs, and the interaction resulted in MAGI-1 degradation, subsequent loss of cell—cell junction integrity, and the progression of human cervical carcinomas (Kranjec and Banks, 2011; Thomas et al., 2001). Thus, several groups have begun campaigns to identify small-molecule probes to further understand the role of MAGI proteins in PTEN signaling and inhibit their interaction with HPV proteins.

Inhibition of MAGI-3 PDZ2 was first attempted using an in silico docking approach. A homology model of the MAGI-3 PDZ2 with a C-terminal PTEN peptide was constructed and a variety of heterocyclic core structures were docked into the peptide-binding site. This docking approach found that an indole-3-carbinol chemical scaffold could preserve the required geometry and distance to support an interaction with the PDZ domain, whereas still accessing a critical histidine residue located in the α1 helix to produce a covalent bond (Fujii et al., 2007a). This compound was active in cells showing $\sim$3-fold decrease in AKT signaling (Fujii et al., 2003). Moving the 3-hydroxymethyl to another position produced an indole-2-carbinol scaffold, which acted as a reversible inhibitor for MAGI-3 PDZ domain. This compound is similar to the FJ9 scaffold mentioned above (see Fig. 7A.8) (Fujii et al., 2007b). An SPR-based HTS was recently developed to discover potential inhibitors targeting the MAGI-1 PDZ1/HPV-16 E6 interaction (Choulier et al., 2013). In this assay, a C-terminal HPV-16 E6 peptide was covalently attached to the sensor chip and the PDZ domain with and without compound flowed over the chip. Screening 1120 compounds Prestwick Chemical Library (France) yielded four inhibitors with IC_{50}s estimated to be between 10 and 50 μM. Interestingly, one compound (methacycline hydrochloride) was found to bind the peptide rather than the PDZ domain, whereas the other three compounds bound to the MAGI-1 PDZ1 domain. No cell-based assays were performed, and hence the potential therapeutic value of these compounds is unknown.

4.3 PDZ Domain Inhibitors in Cystic Fibrosis

As mentioned in Section 2, CF is caused by a variety of CFTR mutations. The most common disease-causing mutation, ΔF508 CFTR (a deletion of

residue F508), results in inefficient folding of the protein, rapid degradation, and decreased channel activity (Guggino and Stanton, 2006). The decreased CFTR function impairs transportation of ions across the epithelial membrane and causes the accumulation of thick viscous mucus in the lungs that blocks the airway (Riordan, 2008). Several inhibitors have been developed to target CFTR for the treatment of CF. These compounds are either "correctors" (Pedemonte et al., 2005) or "potentiators" (Van Goor et al., 2006) capable of correcting the folding of ΔF508 CFTR or potentiating ion flow through the channel, respectively. However, these modulators are still not capable of rescuing ΔF508 CFTR activity to wild-type levels. This, in part, is due to the rapid degradation of ΔF508 CFTR mediated by the interaction of the CFTR PBM with the CAL PDZ protein (Cheng et al., 2002; Lukacs et al., 1993; Swiatecka-Urban et al., 2005). Inhibition of CAL (and CAL PDZ interactions) by RNA interference or mutation of the CAL PDZ domain resulted in an increase in ΔF508 CFTR cell-surface expression, suggesting that CAL PDZ/CFTR PBM inhibition is a viable strategy to treat CF, particularly in the presence of other therapeutic corrector and potentiator compounds (Cushing et al., 2008; Wolde et al., 2007).

To identify and engineer potent peptide inhibitors of CAL PDZ domain interactions, Boisguerin and colleagues screened a library of C-terminal peptides for high-affinity ligands (Vouilleme et al., 2010). After several rounds of optimization, a decameric peptide inhibitor, iCAL36$_{10}$ (ANSRWPTSII$_{coo-}$) was obtained with a K_i of 17 μM for the CAL PDZ and little affinity ($K_i > 1$ mM) for the NHERF-1 and -2 PDZ domains (two PDZ proteins known to interact with the CFTR PBM). Further N-terminally modification of iCAL36$_{10}$ with fluorescein (*F**-iCAL36$_{10}$) improved the affinity ($K_i \sim 1.3$ μM) for the CAL PDZ domain and rendered the peptide cell permeable. In cells, the *F**-iCAL36$_{10}$ peptide blocked CAL PDZ-domain interactions, increased the half-life of ΔF508 CFTR at the cell surface, and enhanced current efflux (Cushing et al., 2010). A series of peptide inhibitors were designed using the structure-based computational design algorithm (known as *K**) (Roberts et al., 2012) and the solution structure of the CAL PDZ/CFTR PBM (Piserchio et al., 2005). Among the top scoring designs, the hexapeptide kCAL01 (WQVTRV$_{coo-}$) was found to have highest affinity ($K_i \sim 2.3$ μM), comparable to the iCAL36$_{10}$ peptide. This newly designed peptide had similar efficiency as *F**-iCAL36$_{10}$ in restoring ΔF508 CFTR cell-membrane expression and Cl^- efflux in CF patient–derived bronchial cells. The

recent crystal structure of the CAL PDZ/iCAL36$_{10}$ complex coupled with mutagenesis was able to rationalize the iCAL36$_{10}$ design (Amacher et al., 2013, 2014a).

The use of inorganic metals and chemical modifications of iCAL36$_{10}$ peptide derivatives was explored to enhance the affinity to the CAL PDZ domain. Incorporating rhodium (II) tetracarboxylate [$Rh_2(OAc)_4$] into the Glu at P_{-6} of the peptide yielded the E_{Rh}WPTSII$_{coo-}$ metallopeptide. This metallopeptide complex was bound to the CAL PDZ domain with an affinity of 0.56 μM, a 75-fold increase in binding compared to the unmodified peptide. This increase was accomplished through a direct interaction with His301 of CAL PDZ and the metal, as the H301A mutation decreased the affinity significantly compared to CAL PDZ WT (Kundu et al., 2012). In a separate study, the the role of individual residues in the iCAL36$_{10}$ peptide for PDZ binding was probed by incorporating a substituted Nζ-lysine (e.g., acetylation or halogenated benzoic acid) as chemical modifications (Amacher et al., 2014b). However, these modifications generally had small effects on peptide affinity. Together, these studies have explored a large chemical space and provides an excellent template for the development of future peptidomimetic inhibitors.

NHERF-1 and -2 are PDZ-containing proteins that interact with and stabilize CFTR activity at the membrane (Raghuram et al., 2001). The NHERF-2 PDZ1 domain also binds to the C-terminus of LPA2. This interaction initiates the G_i protein—signaling pathway, which inhibits adenylate cyclase effectively reducing the local cAMP concentration to ultimately downregulate CFTR activity. Thus, the NHERF-1/2 PDZ domain is another potential target for inhibition to rescue the CFTR function (Fig. 2E). A structure-based in silico design was first performed using the indole-3-carbinol inhibitor, identified for MAGI-3 PDZ2 domain (Fujii et al., 2007b). Unique chemical moieties were added or replaced at specific positions of this scaffold to generate diverse analogues targeting the NHERF-1 PDZ domain. In particular, replacing the methyl group at P_{-3} pocket with carboxyl acid improved the potency possibly by mimicking the favorable salt bridge between P_{-3} Asp and PDZ Arg40. Similarly, the indole-2-carbinol—based inhibitor prevented the covalent bonding with the PDZ domain to work as a reversible inhibitor (see Fig. 7A.8) (Fujii et al., 2007a). Another inhibitor, CO-068, targeting the NHERF-2 PDZ domain was successfully

discovered using AlphaScreen-based HTS (Zhang et al., 2011b) (Fig. 7A.13). This compound displayed an inhibitory effect against NHERF-2 PDZ/LPA2 peptide with IC_{50} ~60 μM. In contrast, the IC_{50} of CO-068 for both the NHERF-1 PDZ/CFTR and NHERF-2 PDZ/CFTR interactions was greater than 120 μM, suggesting high specificity for the target complex. CO-068 disrupted the NHERF-2 PDZ/LPA2 interaction in cells without affecting the NHERF-2 PDZ/CFTR interaction, and the treated cells had increased cAMP levels and CFTR channel function. This same study used a pig tracheal submucosal gland secretion model to show that compound CO-068 potentiates CFTR-dependent fluid secretion. These data suggest that CO-068 could restore the decreased CFTR activity and ultimately the impaired airway clearance in CF patients. More potent compounds could be further developed using the current chemical scaffold for use as a therapeutic reagent for CF.

As described above, a number of PDZ domains have been successfully targeted by small molecules or peptides for therapeutic benefit. These successes provide the impetus for continued development of novel inhibitors. However, challenges remain. First, the robustness of inhibition of relatively weak PDZ domain interactions for therapeutic benefit remains an open question for many of the systems studied. However, the inhibitors that simultaneously targeted the PDZ1 and PDZ2 domains of PSD-95 suggest that increasing affinity using multi-valency can result in suitably robust compounds (Bach et al., 2012). Second, the specificity of targeting PDZ domain interactions still has not been fully validated. Given that there are hundreds of PDZ domains in the human genome, the possibility of nonspecific targeting could be a real issue that requires additional studies to resolve. However, targeting lager fragments containing multiple PDZ domains, rather than an isolated PDZ domain, might provide the required specificity. Syntenin is a good example of this, where the 113B7 inhibitor targeted PDZ1 and the linker between PDZ1 and PDZ2, but not PDZ2 (Kegelman et al., 2017). This example suggests that the interfaces between PDZ domains and their adjacent domains might provide greater specificity than the isolated PDZ domain. Thus, PDZ domains in the context of larger fragments likely contain unique environments that might be exploited to obtain greater inhibitor specificity. Thus, the verdict is still out on how effective targeting PDZ domain interactions will be for treating disease, but it is clear that the current developed probes have great utility for providing insight into the biology of a number of signal transduction systems and disease states.

5. REGULATION OF PDZ DOMAIN/LIGAND INTERACTIONS

5.1 Phosphorylation

Phosphorylation is a common mechanism used for the regulation of cellular signaling. There is now mounting literature about regulation of PDZ domain signaling by phosphorylation. Overall, phosphorylation was found in both PBMs and PDZ domains, and it can be positive or negative.

5.1.1 Phosphorylation of the PDZ-Binding Motif

Residues in the PBM often contain Ser, Thr, or Tyr amino acids that can be phosphorylated. Generally, phosphorylation disrupts PDZ/ligand interactions. For instance, a study showed that phosphorylation of serotonin 5-HT_{2C} receptor at either the P_{-1} or P_{-2} Ser disrupted the interaction with the MUPP-1 PDZ10 (Parker et al., 2003). This result was quantified using isothermal titration calorimetry (ITC) and showed ~37- or 26-fold decrease in affinity on phosphorylation of P_{-1} or P_{-2} Ser, respectively (Sharma et al., 2007). The phosphorylation of the 5-HT_{2C} receptor at the P_{-1} Ser is a key mechanism for receptor resensitization (Backstrom et al., 2000). Phosphorylation can also regulate internalization of receptors in synaptic transmission. For example, phosphorylation of the P_{-2} Ser in the PBM of the NR2b subunit of the NMDA receptor regulated the interaction between NR2b and PSD-95 or SAP-102. CaMKII and the CK2 complex drove NR2b phosphorylation and selectively removed this subunit from the cell surface and decreased surface expression of the receptor (Chung et al., 2004; Sanz-Clemente et al., 2013). Phosphorylation also controlled endocytic sorting of the β2-adrenergic receptor by modulating the PDZ domain interaction (Cao et al., 1999). Phosphorylation at P_{-3} Ser of the AMPAR GluR2 PBM prevented the interaction with GRIP-1 PDZ5, but not the PICK-1 PDZ domain (Chung et al., 2000). Phosphorylation also regulated the AMPAR internalization, prevented its surface accumulation, depressed its signal transmission and affected LTD (Seidenman et al., 2003).

Phosphorylation has not only been seen in the PBMs of cell surface receptors but also in the PBMs of different virus proteins. HTLV-1 Tax-1 was phosphorylated by CK2 at P_{-2} Thr in the PBM. Moreover, this phosphorylation disrupted the interaction between Tax-1 PBM and hDlg PDZ, though the biological consequence of this interaction was not determined (Bidoia et al., 2010). High-risk HPV E6 proteins were previously shown to interact with Dlg PDZ domain through their PBMs. This interaction

normally induces degradation of Dlg. However, phosphorylation of the P_{-2} Thr in PBM disrupted this interaction and Dlg was no longer targeted for degradation (Kuhne et al., 2000).

A larger scale study using synthetic phosphorylated peptides library found that phosphorylation also disrupted ligand interactions with AF-6, Erbin, and α-1-syntrophin PDZ domains, with the greatest effect at the P_{-1} and P_{-2} positions (Boisguerin et al., 2007). More recently, a study determined that 31/146 proteins that have a class I PBM and a potential phosphorylation site at P_{-1} position could be phosphorylated, reinforcing the idea that this position is important for PDZ/PBM regulation (Pangon et al., 2012).

Phosphorylation does not always disrupt PDZ/PBM interactions, and several cases show it can bolster PDZ/PBM binding. Phosphorylation of the P_{-2} Thr in the PTEN PBM disrupted the interactions with Dlg1 PDZ2 and MAST PDZ, but promoted the association with two other proteins yet to be identified (Adey et al., 2000). PKC-mediated phosphorylation of the MRP2 (an ABC protein family member) PBM at P_{-3} Ser enhanced the binding to its cognate PDZ domains (Hegedus et al., 2003). Likewise, P_{-3} Ser or Thr phosphorylation of C-termini of FATZ Z-discs protein family members (FATZ1: EpTEEL, FATZ2: EpSEDL, and FATZ3: EpSEEL) resulted in tighter binding to the ZASP/cypher PDZ domain compared the unphosphorylated form (von Nandelstadh et al., 2009). Overall, phosphorylation modulates the PDZ/ligand interactome in both positive and negative manners.

Despite all the biochemical and biological analyses on the phosphorylation of PDZ/PBM interactions, structural information remains scarce. Phosphorylation of P_{-7} Tyr (pTyr) in ErbB2 decreased the Erbin PDZ/ErbB2-binding affinity almost three fold. The crystal structure of the Erbin PDZ/pErbB2 (containing pTyr) complex was solved. Interestingly, the structure failed to resolve the interactions as the electron density of residues beyond P_{-4} was not interpretable, implying that the weaker binding is connected to the conformational flexibility in this region (Birrane et al., 2003). Another study found that pTyr had little effect on the ligand binding in the Tiam1 PDZ/syndecan-1 (SDC1) interaction. The phosphorylation of P_{-1} tyrosine (TKQEEFpYA) of SDC1 modestly increased the binding affinity from 27 to 19 μM (Shepherd et al., 2010). The structure of Tiam1 PDZ complexed with SDC1 and phosphorylated SDC1 (pSDC1) revealed the molecular rationale for this result. Namely, phosphorylation of the Tyr side chain induced a conformational switch, flipping the Tyr side chain toward the K879 in S_{-1} pocket and facilitating an electrostatic interaction

with a phosphate in pTyr (Fig. 8A). Interestingly, this phosphorylation also regulated the protein dynamics by decoupling the allosteric β3-α1 site from ligand-binding site (see details in Section 5.2) (Liu et al., 2013).

As PBM phosphorylation can both enhance and suppress the binding with different PDZ domains, it could be a means for selecting particular PDZ/PBM interactions. Regulation of PDZ/ligand-binding events via phosphorylation and selection of specific PDZ interaction partners is best exemplified in the SDC1 and AMPAR systems (Fig. 8A). Internalization of SDC1 was shown to be dependent on the syntenin-1 PDZ2/SDC1 interaction in a phosphorylation-dependent manner, whereby phosphorylation of SDC1 at Tyr P_{-1} prevented SDC1 internalization (Sulka et al., 2009).

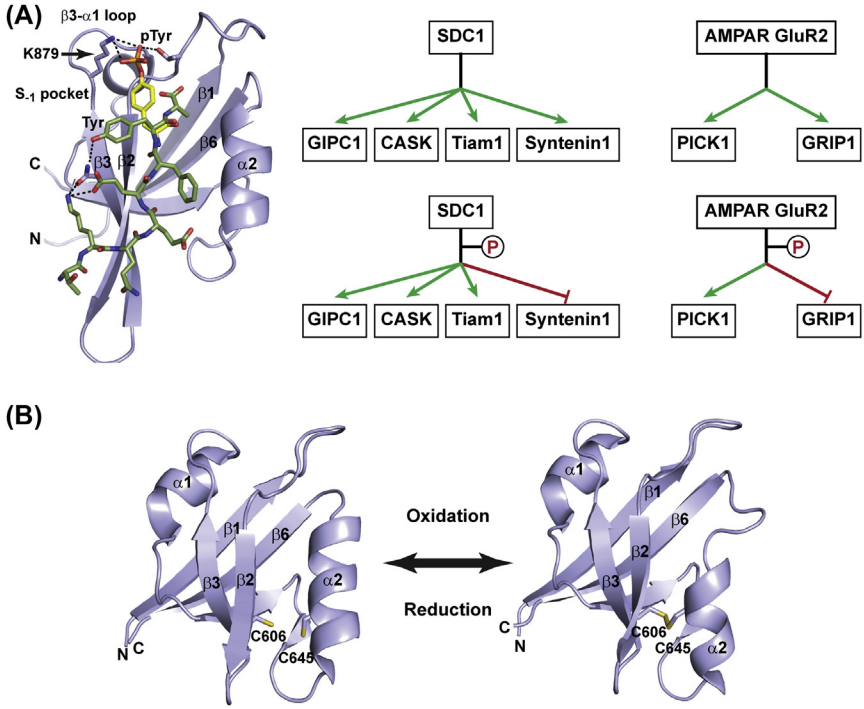

Figure 8 Regulation of PDZ signaling by phosphorylation and redox environment. (A) Left panel: The structures of Tiam1 PDZ domain bound to SDC1 and pSDC1. The conformational change in Tyr P_{-1} (yellow sticks) after phosphorylation is highlighted (PDB codes: 4GVC and 4GVD). Right panel: Phosphorylation regulates SDC1 and AMPAR signaling by modulating PDZ-ligand interactions. (B) Regulation of PDZ-ligand binding by an intramolecular disulfide bond. Disulfide bond formation in the INAD PDZ5 domain remodels the ligand pocket to regulate ligand binding (PDB code: 2QKT and 2QKU).

A structural model of the syntenin1 PDZ2/pSDC1 structure suggested that two negatively charged residues (D204 and D224) in the S_{-1} pocket were likely electrostatically unfavorable, preventing the interaction with pTyr. Interestingly, these residues (or other acidic residues) were not found in the S_{-1} pocket of either GIPC or CASK PDZ domain. Therefore, it is reasonable to predict that these PDZ domains might accommodate both the pSDC1 and SDC1 ligands. Thus, phosphorylation of P_{-1} Tyr might serve as a switch between activation of SDC1/Tiam1/Rac1 signaling and the SDC1 recycling pathway (Liu et al., 2013; Roper et al., 2012). A phosphorylation-based switch was also found in AMPAR signaling. The C-terminus of GluR2 subunit of AMPAR is the ligand for the PICK-1 PDZ and GRIP-1 PDZ5 domains. As mentioned above, phosphorylation at the P_{-3} Ser residue selectively blocked the interaction with GRIP-1 but did not affect the interaction with the PICK-1 PDZ domain. Additionally, P_{-7} Tyr phosphorylation of the GluR2 subunit also regulated the PDZ binding. Likewise, this phosphorylation did not affect binding to the PICK-1 PDZ domain, whereas the GRIP-1 PDZ5 domain interaction was severely disrupted. Importantly, each phosphorylation event was able to selectively activate the PICK1-mediated cell surface receptor internalization pathway, which would induce internalization and loss of the surface pool of GluR2 (Chung et al., 2003; Hayashi and Huganir, 2004). It would be interesting to know the combined effect of P_{-3} Ser and P_{-7} Tyr phosphorylation, which has not been investigated.

The extent to which differential regulation of PDZ/ligand interactions and pathways occurs is not clear because of the few cases reported in the literature. New high-throughput methods are needed to discover more phosphorylated ligands, and the context with which they occur. Furthermore, biochemical and structural investigations of these phosphorylation events will be necessary to understand the molecular origins of this regulation. These studies will be required for exploiting these interactions to enable rationale control PDZ domain interactions via protein engineering or small molecules.

5.1.2 Phosphorylation of PDZ Domains

Phosphorylation of PDZ domains that affect PBM interactions was first seen in SAP-97 and PSD-95. CaMKII-mediated phosphorylation of Ser-73 in PSD-95 PDZ1 domain disrupted its interaction with the NR2A subunit from NMDAR (Mauceri et al., 2007). Similarly, Ser phosphorylation was identified in NHERF-1 PDZ1 domain and shown to reduce the binding

to both the β2-adrenergic receptor and CFTR (Voltz et al., 2007). A recent study reported a comprehensive analysis of phosphorylation of four different sites on the three PDZ domains of PSD-95 (Pedersen et al., 2017). These phosphorylation sites were identified in vivo, although the kinases responsible for modifying these sites have not been identified. The phosphorylation sites were produced by the "expressed protein ligation" method targeting a Ser residue (Ser-73) located in the β1−β2 loop, three Tyr residues in the β5 strand, the β5−α2 loop, and the additional α3 helix. The phosphorylated PDZ domains maintained their structure but were generally thermodynamic destabilized. Phosphorylation of these sites suppressed PBM binding, but to differing extents (up to 1.2−11-fold). Therefore, PDZ phosphorylation seems to regulate ligand binding by fine-tuning the interaction rather than acting as a binary on/off switch. Interestingly, a 4.5-fold enhancement of affinity was found with the stargazin ligand after phosphorylation of Tyr397 (pTyr397) in the atypical α3 helix of the PDZ3 domain, a region distal from the ligand-binding pocket. This was proposed to be mediated by salt bridges formed among two Arg residues (Arg 318 and 319) in stargazin and pTyr397 in PDZ3. However, pTyr397 also reduced the affinity between PDZ3 domain and other ligands, including the CRIPT ligand. The molecular mechanism of this result was reported in an NMR study and involved the rapid association and dissociation of the α3 helix to the core of PDZ3, resulting in a lowered affinity for CRIPT ligand (Zhang et al., 2011a). Moreover, pTyr397 controls the interdomain packing between PDZ3 and the adjacent SH3 domain, an unprecedented role for phosphorylation in a PDZ-containing protein. Phosphorylation of a flanking region near a PDZ domain to regulate ligand binding was also found in the AF-6 PDZ protein. Here, phosphorylation of a threonine residue located 100 amino acids upstream of the PDZ domain impaired ligand binding (Radziwill et al., 2003). The structural mechanism for this phosphorylation-based allosteric regulation is not clear, but likely occurs through protein dynamics and conformational change.

The interplay between PDZ domain and PBM phosphorylation and phospholipid binding has also been seen. Phosphorylation of a C-terminal region of the syntenin1 PDZ domains was critical for high-affinity binding with phospholipids (Wawrzyniak et al., 2012). The C-termini of many cell surface receptors contain PBMs, therefore phospholipid and PBM binding may be coincidental. Moreover, a recent study showed that in vitro phosphorylation of PICK-1 PDZ domain was enhanced ~10-fold after adding lipids into the reaction. This implied that the recruitment of PDZ domain to

the membrane was an important step for following kinase-mediated reaction (Ammendrup-Johnsen et al., 2012).

In summary, phosphorylation of PDZ domains can modulate their interaction with ligands both positively and negatively. This typically depends on the identity of the residues surrounding the phosphorylation site, which often contain charged side chains. In contrast to the PBM phosphorylation, the study of PDZ phosphorylation sites and consequences is underdeveloped, possibly owing to the challenges of obtaining phosphorylated PDZ domains. Phosphomimetic mutations, such as Asp and Glu residues, could be used, but they often do not represent authentic phosphorylation effectively, especially in the case of pTyr (Pedersen et al., 2017). Therefore, in vitro phosphorylation by recombinant kinases (Zhang et al., 2011a) or the expressed protein ligation–based semisynthetic strategy (Pedersen et al., 2017) shows great promise for biochemical and structural characterization of these interactions. Although our knowledge of how phosphorylation regulates ligand binding is limited, design of a phosphoswitchable PDZ domain has been achieved, suggesting the possibility that novel PDZ domains with switchable ligand binding can be designed for novel purposes (Smith et al., 2013).

In addition to phosphorylation, reduction–oxidation (redox)-based modification is another mechanism that can regulate PDZ-domain interactions. A conformational change in the PDZ5 domain of INAD, a multi-PDZ domain scaffolding protein critical for vision, was found to be dependent on the formation of intramolecular disulfide bond (Mishra et al., 2007). Formation of this disulfide bond bridged helix α2 and strand β3, distorting the normal ligand-binding groove typically seen in PDZ domains. Under reduced conditions, the disulfide bond was broken allowing helix α2 to relax back to the typical "open" position to accommodate a ligand (Fig. 8B). Importantly, this oxidation/reduction cycle of disulfide and the accompanying distortion/relaxation of the ligand-binding pocket was light-dependent in vivo. Moreover, a C645S mutant in the PDZ domain that prevented disulfide formation showed defects in visual response and loss in refractory period, consistent with a dynamic conformational change mediating a regulatory switch in the INAD visual signaling pathway. Interestingly, a separate study found that the isolated PDZ5 was stable in the oxidized state, whereas it remained in the reduced state when linked to PDZ4. The presence of PDZ4 changed the local environment, raising the redox potential of the disulfide in the PDZ5 domain. Therefore, PDZ5 ligand binding was regulated by the redox environment and further

allosterically controlled by the PDZ4 domain (Liu et al., 2011). Whether redox regulation of PDZ domain interactions is widespread is unknown and will require future studies.

5.2 PDZ Domain Dynamics and Allostery

The three-dimensional structures of PDZ domains at atomic resolution have been exceptionally useful for characterizing the details of protein–ligand interactions. However, it is becoming increasingly clear that proteins accomplish their biological functions (e.g., signal transduction, enzymatic catalysis, ligand binding, etc.) through multiple conformational states and the interconversion between them. Thus, protein dynamics is an important aspect in structure–function relationships (Henzler-Wildman and Kern, 2007; Tzeng and Kalodimos, 2011; Wand, 2013). Dynamics is also an important mechanism for allosterically regulating protein function. Protein dynamics on fast (ps-ns) and slow (μs-ms) timescales can be studied by solution NMR methods. Here, we first discuss fast timescale motions in the PDZ domains, which provide insight into changes in dynamics at the ligand-binding site and regions "distal" from it associated with allostery (see below). Later, we describe slower timescale motions associated with larger scale conformational changes in PDZ domains, which may also contribute to allosteric control of binding events.

5.2.1 Intrinsic Dynamics and Allosteric Behavior

NMR spectroscopy and computational simulations are widely utilized to monitor the dynamics of individual atoms in proteins. Sophisticated NMR analysis of fast timescale (pico-to nanosecond, ps-ns) motions results in two parameters: S^2 (order parameter) that describes the amplitude and τ_e that describes the timescale of bond vector dynamics, respectively. The $^{15}N–H$ bond vector is utilized to describe the backbone dynamics, whereas the methyl symmetry axis (collinear with $C–CH_3$ bond vector) describes methyl-containing residues (Igumenova et al., 2006). In addition to fast timescale motions, slower dynamical motions can also be studied by NMR. These dynamics, on the micro- to millisecond (μs-ms) timescale, are generally studied by Carr–Purcell–Meiboom–Gill relaxation dispersion methods (Baldwin and Kay, 2009; Loria et al., 2008; Palmer, 2014).

The degree of backbone rigidity on the fast timescale has been studied in many PDZ domains and is typically correlated with stable elements of secondary structure. In apo PDZ domains, the loops connecting the secondary structures and at N-, C-termini are generally highly dynamic and

flexible, and usually contain glycine residues that serve as hinges (Walma et al., 2002). Several PDZ domains show missing electron density in the β1−β2 loop in the absence of ligand (Fuentes et al., 2004; Law et al., 2009; Shepherd et al., 2010). Moreover, structural alignment of various PDZ domains showed that β1−β2 and β2−β3 loops had higher root mean square deviation than other secondary structures. Nevertheless, these regions become rigidified after ligand binding as shown by NMR studies of various PDZ domains (Charbonnier et al., 2011; Fuentes et al., 2004; Liu et al., 2008a, 2013; Zhang et al., 2010). In a few cases, the NMR resonance signals of the β2−β3 loop were extremely broadened in the NMR spectra (e.g., Leukemia-associated RhoGEF [LARG] [Liu et al., 2008a] and Tiam2 PDZ domains [unpublished data]) due to extensive dynamic motions.

Fast-timescale dynamics of side chains, namely those with methyl groups, can be monitored using methyl groups ($C-CH_3$ vector) as the appropriate spin−relaxation vector. Moreover, their motions do not appear to correlate with secondary structure, and they are generally more sensitive to perturbations (e.g., ligand binding and mutations) because they exhibit larger fluctuations than those seen along the backbone (Sapienza and Lee, 2010). PDZ domains are excellent model systems for methyl-based experiments as they are relatively small (∼90 amino acids) and typically contain 50−60 methyl-bearing residues evenly distributed throughout the protein. The side-chain dynamics of the second PDZ domain of the phosphatase 1e (PTP1e PDZ2) alone and complexed with a peptide derived from the RA-GEF2 protein showed changes in dynamics on ligand binding in the binding cleft, the β1−β2 and β2−β3 loops and other regions not directly involved in ligand binding (Fuentes et al., 2004). In particular, methyl groups up to ∼11 Å from the ligand-binding pocket were found to have perturbed dynamics on ligand binding and these regions were termed "distal surface 1" and "distal surface 2." Distal surface 1 is located adjacent to the peptide-binding site near the N-terminus of β6 and β4−β5, whereas distal surface 2 is located near helix α1 (Fig. 9A). A similar dynamic response was found with a distinct binding peptide, confirming the robustness of the dynamic transmission induced by ligand binding (Zhang et al., 2010). A subsequent study compared the methyl side-chain dynamics of apo Erbin PDZ, PDZ3 from PSD-95, and PDZ2 from PTP1e and showed their dynamics to be highly similar (correlation coefficient of 0.65) (Law et al., 2009). Given that PDZ domains contain ∼30% sequence identity, these results suggest that protein dynamics is a functionally conserved property of the PDZ-domain family.

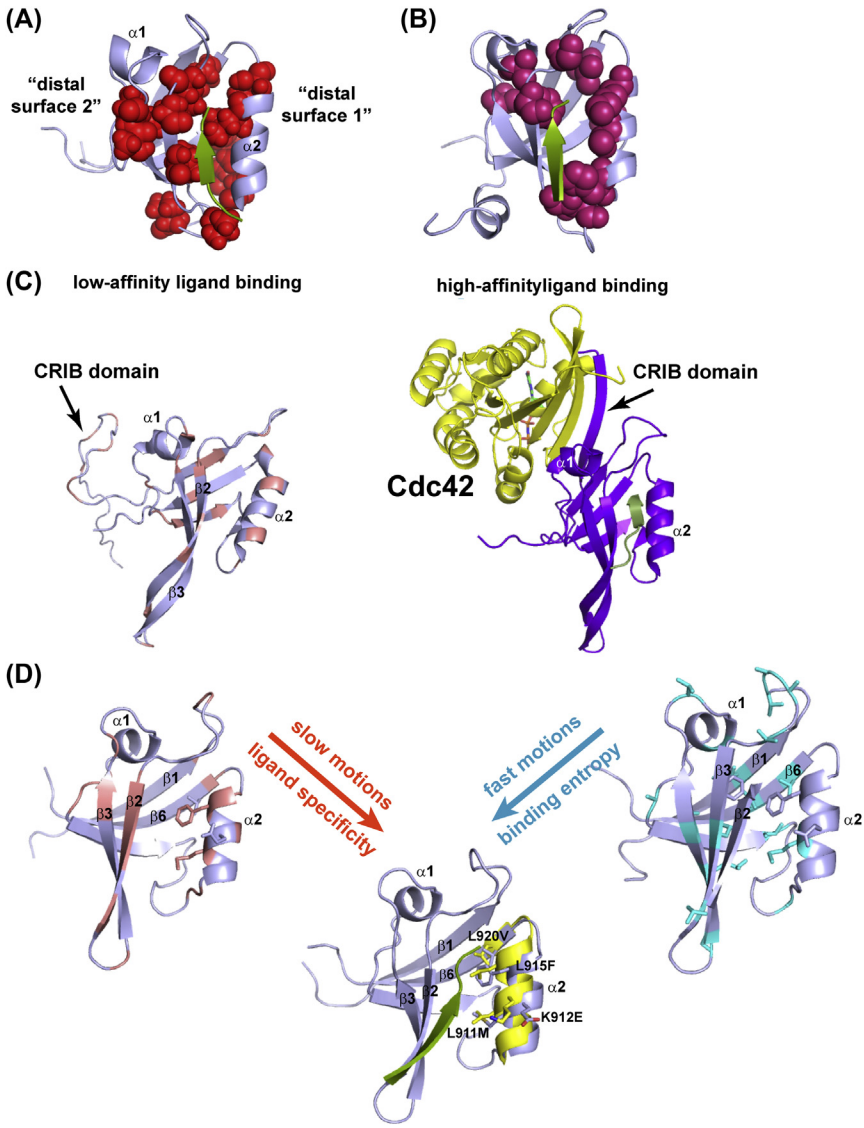

Figure 9 Allosteric- and dynamics-based regulation of PDZ domains. (A) Changes in methyl dynamics of the PTP1E PDZ2 domain (PDB code: 1D5G) on ligand binding determined by NMR. The two surfaces allosterically connected to the binding site are labeled. (B) Long-range statistical coupling network identified in PSD-95 PDZ3 (PDB code: 1BE9). (C) Left panel: Residues in the Par6 PDZ domain (PDB code: 1RY4) that show slow dynamic motions. Right panel: Cdc42 binding to the CRIB motif (PDB codes: 1NF3). This interaction allosterically enhances PDZ-ligand binding by triggering a conformational change in ligand-binding groove and α1 helix of Par6 PDZ domain. (D) Coincidence of fast and slow dynamic motions in the Tiam1 QM PDZ domain that regulates both ligand-binding affinity (through entropy) and specificity, respectively (PDB codes: 3KZD and 4NXR).

The long-range changes in dynamics have been validated in other PDZ domains using different methods, including molecular dynamics (MD) simulations (Gerek and Ozkan, 2011) and binding kinetics studies (Gianni et al., 2006). Importantly, the residues in PDZ domains experimentally found to be dynamically perturbed are consistent with a network of energetically connected residues ("sparse energetic network") originally identified in the third PDZ domain of PSD-95 (Lockless and Ranganathan, 1999) (Fig. 9B). These coevolved residues are distributed sparsely on the primary sequence of the PDZ domain, but form a physically continuous network in the tertiary structure to link the active site with allosteric sites (Halabi et al., 2009). The sparse network idea has progressed into the "protein sectors" concept, which defines these coevolved energetically or dynamically coupled residues as the basic architectural unit of three-dimensional structure. A high-throughput study found that mutations at sector positions had pronounced effects, such as switching the ligand-binding specificity, compared to those in nonsector positions (McLaughlin et al., 2012). Moreover, some mutations did not switch specificity directly, but worked coordinately with other mutations in an epistatic manner. These so-called "class-bridging" mutations also resided in sector regions to maintain the original specificity and they worked allosterically to regulate the dynamics and function of the binding site (Raman et al., 2016).

Although the general dynamic response pattern in PDZ domains is conserved, specific perturbations can produce subtle variations in the pattern for an individual PDZ domain. One simple way to change the dynamic distribution (i.e., dynamic response) is by mutagenesis of residues at or near the ligand-binding site. Using a series of PDZ mutants in PTP1e PDZ2, a thorough side-chain dynamics study found that residue I20 served as a hub of this dynamic network, linking the ligand-binding site to the long-range distal sites (Fuentes et al., 2006). The importance of this residue was also captured in a MD study of the PSD-95 PDZ3 domain (Ota and Agard, 2005). Interestingly, a recent study showed that a single phosphorylation event of the ligand was also capable of propagating dramatic changes in dynamics, indicating that natural perturbations can also modulate dynamics and potentially ligand affinity (Liu et al., 2013).

Protein dynamics is important for enzymatic catalysis and ligand binding (Eisenmesser et al., 2005; Tzeng and Kalodimos, 2012). In addition, dynamics in PDZ domains can affect ligand binding by modulating both affinity and specificity. A recent MD study on PSD-95 PDZ3 CRIPT complex showed that two charged residues in the β2−β3 loop interact

with P_{-4} and P_{-7} residues in the ligand to form transient salt bridge interactions, providing an explanation for their role in supporting ligand-binding affinity. Interestingly, these interactions were not readily obvious in previous crystallography studies (Mostarda et al., 2012). Further, a "specific-Gaussian network model" analysis of various PDZ domains compared the dynamics pattern in class I and class II PDZ domains (Gerek et al., 2009). Both classes of ligands changed the dynamics in the common β1−β2 loop. However, class I ligand binding also disturbed the motions in β2−β3 loop, whereas class II affected motions in helix α2. These studies suggest that the binding selectivity in PDZ domains can be modulated distinctly by the dynamics of specific residues. Indeed, the study by McLaughlin et al. (2012) showed that mutating two residues in the sector positions in PSD-95 PDZ3 could result in class switching by changing the binding preference from class I to class II ligand. Importantly, one of these residues was located in the β2−β3 loop and not directly involved in ligand binding, reinforcing the idea that dynamics in this loop contributes to ligand specificity.

A significant role for fast-timescale dynamics in regulating PDZ-domain ligand binding was found in PSD-95. The structure of PSD-95 PDZ3 showed that an extended C-terminal helix, namely the α3 helix, packs against the core PDZ domain. Deletion of this helix ($PDZ3^{\Delta\alpha3}$) did not affect the overall structure of the PDZ domain, but surprisingly weakened the binding affinity for the CRIPT peptide ~25-fold. ITC experiments suggested that the binding difference was driven by conformational entropy. The α3 helix does not directly participate in CRIPT ligand binding, and desolvation effects in binding were found negligible. Furthermore, NMR experiments showed that the side chains of the PDZ domain became more flexible on the ps—ns timescale after removing α3 helix, whereas in the presence of ligand there was little difference in the dynamics of PDZ3 with and without α3 helix. Thus, the fast-timescale dynamics contributed to the entropic penalty, reducing ligand affinity after removal of the α3 helix (Petit et al., 2009). This result was significant as only a few cases of pure dynamics-based regulation of ligand binding had been previously reported (Frederick et al., 2007; Popovych et al., 2006; Tzeng and Kalodimos, 2009). More importantly, this regulation was accomplished through an allosteric region, distal from the canonical PDZ-binding pocket. A recent follow-up study found that native state protein volume fluctuations is associated with the allostery in PSD-95 PDZ3. The PSD-95 $PDZ3^{\Delta\alpha3}$ had a significantly expanded volume compared to PDZ3 with the α3 helix.

The unique packing of the PDZ3$^{\Delta\alpha3}$ domain was correlated to and works in concert with the increased internal motions to control the functional allostery, i.e., ligand binding (Law et al., 2017).

A second example of allostery-based regulation of binding is found in the Par-6 PDZ domain. Par-6 is an important scaffolding protein, part of Par-6/Par-3/aPKC cell polarity complex (Lin et al., 2000). The protein contains an N-terminal PDZ domain followed by a CRIB motif. The PDZ domain recognizes a model class I peptide (VKESLV$_{COOH}$) with an affinity of ~80 μM. The adjacent CRIB motif binds to the Cdc42 GTPase and this interaction bolstered PDZ ligand binding ~13-fold. This GTPase/Par-6 interaction is of crucial importance for tight junctions in epithelial cells. A structural study found large structural deviations in both the carboxyl-binding loop and α2 helix of the Par-6 PDZ with and without Cdc42 binding and canonical PDZ domains. In presence of Cdc42, the Par-6 PDZ domain resembled a canonical PDZ domain with a suitable conformation for tight ligand binding (Fig. 9C). Moreover, the shift between these distinct conformations in the carboxyl-binding loop suggested inherent dynamics (Peterson et al., 2004). In the absence of Cdc42, the CRIB motif is disordered and flexible, gaining structure on Cdc42 binding. A disulfide stabilized CRIB—PDZ mutant showed that high-affinity ligand binding was driven by a dipeptide conformational switch in the carboxyl-binding loop. This conformational change, driven by Cdc42/CRIB binding and stabilization of CRIB motif packing against α1 helix of the Par-6 PDZ, relied on the dynamics of carboxyl-binding loop (Whitney et al., 2011). Detailed examination of μs—ms timescale dynamics revealed conformational exchange throughout the entire PDZ domain, including the carboxyl-binding loop and involved partial unfolding of the PDZ domain (Fig. 9C). This interdomain-based regulation of the Par-6 PDZ domain implied that intrinsic protein dynamics plays a role in allosteric regulation of ligand binding. Interestingly, the region involved in this regulation spanned the α1 helix to the carboxyl-binding loop and α2 helix, consistent with the dynamics and energetic pathway proposed by NMR- and *in silico*-based studies (Fuentes et al., 2004; Lockless and Ranganathan, 1999). Moreover, the Crumbs ligand binding to Par-6 was also regulated by the Cdc42/CRIB-mediated allostery, suggesting a common mechanism for C-terminal ligands in Par-6 PDZ (Whitney et al., 2016). In contrast, Cdc42 did not regulate binding to the Pals1 protein that binds the Par-6 PDZ domain through an internal sequence (Peterson et al., 2004). The thermodynamic and structural rationale for this is unknown and will require additional work.

Distinct timescales of dynamics control PDZ/ligand binding by different mechanisms, as shown by the PSD-95 and Par-6 PDZ examples. A recent study found both fast and slow dynamics coexisted in the same protein—a quadruple mutant (QM) of Tiam1 PDZ domain—and act distinctly to regulate ligand binding (Fig. 9D) (Liu et al., 2016). The Tiam1 QM PDZ domain was engineered with four mutations in the ligand-binding pocket designed to switch its specificity to that of its homolog, Tiam2 PDZ domain (Shepherd et al., 2011). Binding experiments showed that the QM had a broadened specificity, reminiscent of the Tiam2 PDZ domain. Analysis of backbone and side chain fast-timescale dynamics and ITC data showed that conformational entropy contributed to ligand-binding affinity, similar to PSD-95 PDZ3. In contrast, slow dynamics was found throughout QM but not in WT, suggesting that slower conformational switching between multiple conformations was related to specificity by enabling the selection of a wider repertoire of ligands. Although not identical, the QM PDZ domain shares similar features found in the apo PDZ domains of AF-6 (Niu et al., 2007) and the Par-6 (Whitney et al., 2013), in particular, conformational exchange in the ligand-binding site near the β2−β3 loop and α2 helix.

The propagation of dynamics is one way for a PDZ domain to transmit allosteric signals. This mechanism of signal transmission involves no significant conformational change relying only on the entropic contribution from fast-timescale dynamics (Wand, 2013; Wand et al., 2013), as shown in Tiam1 QM PDZ and PSD-95 PDZ3. In contrast, slow-timescale dynamics in the Tiam1 QM, AF-6, and Par-6 PDZ domains suggest an ensemble of slowly interconverting states. In this scenario, the upstream-signaling input (i.e., ligand binding) to this domain would likely stabilize a unique conformer, such as Cdc42 rendering the binding loop in Par-6 PDZ suitable for effective ligand binding, to further propagate the signal relay. Conversely, the native state might have a unique conformation, which on ligand binding could be redistributed or shifted into multiple conformations that are necessary for signaling (Swain and Gierasch, 2006). Moreover, selection of a unique conformation by evolutionary pressure could provide novel PDZ specificity. Given the wide distribution of PDZ domain proteins in the human genome, each unique signal that a PDZ domain senses, and ultimately transmits, could represent a different dynamic state (Smock and Gierasch, 2009). Although enticing, this idea requires further experimental testing in multiple PDZ-domain systems.

5.2.2 PDZ Domain Allostery in Larger Fragments

Allostery commonly refers to regulation of function by a distally located second site. In PDZ domains, allostery is defined as regulation of ligand binding from sites distal to the well-defined ligand-binding pocket, such as the α1 helix in Par-6 PDZ discussed above. Allostery can also originate from noncanonical extensions of the classical PDZ structure, such as the α3 helical extension in the PSD-95 PDZ3 domain. Here, we will discuss PDZ domain allostery derived from structural extensions, such as larger fragments or domains. This allostery was unraveled by a set of biophysical chemistry studies, rather than merely by NMR-based analyses. Indeed, a recent review predicted that one-third of PDZ domains contain functionally important extensions at either the N- and C-terminus of a PDZ domain or both (Wang et al., 2010a). The predicted extensions were generally short, suggesting relatively tight packing against the PDZ domain core. Initial structural studies showed that a number of PDZ domains contained regions of secondary structure beyond their canonical fold at their N- or C-termini (Bhattacharya et al., 2010; Doyle et al., 1996). These extended regions were required for correct folding and ligand binding. In particular, the MAGI PDZ1 had a tendency to aggregate during purification without a 25-residue long C-terminal extension, even though it was unstructured in both apo and ligand-bound states. Moreover, this C-terminal extension affected ligand binding. Surface plasmon resonance (SPR) studies showed that mutations in three residues located in this extension decreased ligand affinity up to five fold. Intermolecular nuclear Overhauser effect indicated a direct interaction between residues of this region and the peptide ligand. Thus, the extended C-terminal region in the MAGI PDZ1 provided an additional binding site for its ligand (Charbonnier et al., 2011).

Longer PDZ domain extensions can also modulate function. In the NHERF-1 PDZ2, a 30-amino acid C-terminal extension formed a helix-turn-helix motif, which stabilized the core PDZ domain and increased its affinity ~10-fold to a C-terminal ligand from CFTR (Bhattacharya et al., 2010). Intriguingly, three point mutations at NHERF-1 identified from patients with chronic kidney disease were located at this motif (Karim et al., 2008). Biochemical studies showed that these mutations reduced the stability of the PDZ2 domain in cells, leading to the incorrect assembly of phosphate transporters at the cell membrane. Following this motif was an additional C-terminal ~100 amino acid extension, known as an ezrin binding (EB) domain that recognizes the FERM domain in ezrin. The EB domain negatively regulated PDZ/ligand binding and when bound to the

FERM domain increased PDZ/ligand affinity ~20-fold (Li et al., 2005b). NMR combined with SAXS studies showed that an extended C-terminal loop in the EB domain interacted with the PDZ-binding groove. However, in the presence of the FERM domain a conformational change relieved this inhibition (Bhattacharya et al., 2010; Cheng et al., 2009). Taken together, PDZ/ligand binding can be bolstered or suppressed by distinct N- and C-terminal extensions to allosterically regulate the stability of a PDZ domain and its accessibility to ligand.

PDZ domains can be regulated by both N- and C-terminal extension regions simultaneously, as seen in the harmonin PDZ domain. Harmonin is a scaffolding protein that plays an important role in the development of cilia in photoreceptors and hair cells. Harmonin interacts with Sans, another scaffolding protein in these cells (Reiners and Wolfrum, 2006). Disruption of these protein−protein interactions results in Usher syndrome, a common form of hereditary vision and hearing loss (Kremer et al., 2006). A construct from the protein harmonin containing an N-terminal region, PDZ1 domain, and C-terminal 25-residue extension formed a stable supramodule (NPDZ1). X-ray crystallography studies clearly showed that the C-terminal extension in NPDZ1 packed against the linker region between the N-terminal domain and the PDZ1 domain through hydrophobic and electrostatic interactions. This supramodule was different from that typically formed by tandem PDZ domains. Biochemical studies showed that NPDZ1 interacted separately with the SAM domain or C-terminal PBM region of Sans, binding each with μM affinity. However, the tandem SAM-PBM region bound NPDZ1 with 1:1 stoichiometry and nM affinity (Yan et al., 2010). The structure of the harmonin/Sans complex showed that the C-terminal tail of Sans inserted into the classical binding pocket of PDZ1, whereas helix α1 of the SAM domain packed into a new pocket formed by the PDZ α2−β5 region and the C-terminal extension. Thus, the PDZ domain and its extensions formed a supramodule to create a novel ligand-biding site. This binding site integrates a novel subsite and the canonical PDZ-binding site to provide a stable harmonin/Sans complex, necessary for the function of cilia in visual and hearing cells.

As many PDZ proteins contain more than one PDZ domain, the influence of one PDZ domain on another is critical for understanding the overall function. This is particularly relevant as most biochemical and structural studies examine only one PDZ domain. However, more recent studies indicate that allosteric regulation can occur between adjacent PDZ domains.

Studies of the PTP-BL PDZ2 showed promiscuity in ligand binding as it recognizes class I, II, and III ligands (van den Berk et al., 2007). However, in the context of the double PDZ1—PDZ2 construct (PDZ12), the PDZ2 domain preferred class I ligands. NMR studies indicated that a physical interaction between PDZ1 and PDZ2 occurred through a site located at the α1/β1 region of PDZ2, opposite to the ligand-binding groove. Structural models suggested that the PDZ1—PDZ2 interaction might change the configuration of Ile48 in PDZ2, next to the α1 helix to form a hydrophobic interaction with P_{-1} Leu in class III ligands. This allosteric regulation was reminiscent of the Par-6 PDZ domain as both allosteric sites contained α1 helix.

PDZ domains can also allosterically control the binding affinity of an adjacent PDZ domain. The best example of this is found in GRIP-1. The interaction between GluR2 and GRIP-1 was found to be mediated by the PDZ4 and PDZ5 domains in tandem. Interestingly, the binding stoichiometry was shown to be 1:1, but neither PDZ4 nor PDZ5 alone was able to bind to GluR2 (Zhang et al., 2001). Further study verified that PDZ5 was the bona fide GluR2-interacting PDZ domain as it had a well-defined PDZ-binding groove, whereas the binding grove of PDZ4 was incapable of ligand binding (Feng et al., 2003). Biochemical data showed that PDZ5 was stabilized by the N-terminal PDZ4 and the intra-domain linker region. Similarly, the GRIP-1 PDZ1 alone was not stable and it did not to bind to its ligand unless PDZ2 was present. Structural studies showed that the two PDZ domains packed against each other to form a compact intertwined domain with a large interface (~580 $Å^2$). Unlike PDZ4, PDZ2 contained a well-defined binding groove. However, it was still unable to bind ligand as the "front-to-back" packing mode in PDZ12 tandem blocked its binding site. Thus, PDZ1 was the only active-binding subunit and it was regulated allosterically by PDZ2 (Long et al., 2008).

Regulation of PDZ/ligand binding by an adjacent PDZ domain is also found in syntenin1 and PSD-95. Chemical denaturation of the syntenin1 constructs showed that the unfolding energy of PDZ12 was in between that of PDZ1 and PDZ2, suggesting that the tandem PDZ12 construct was associated into a folded and cooperative supramodule (Kang et al., 2003b). Unlike the tandem PDZ45 domains of GRIP-1, both PDZ1 and PDZ2 of syntenin1 had intact binding grooves. However, their binding specificity was not identical—PDZ1 bound class I and III ligands, whereas PDZ2 bound class I and II ligands. This degenerate binding preference,

coupled with a unique orientation between the tandem PDZ domains in which PDZ2 is orientated approximately 90 degrees from PDZ1, was proposed to provide two scaffolding modes observed in the crystal structures of PDZ12 (Grembecka et al., 2006; Kang et al., 2003a,b). In the first scaffolding mode, the two PDZ domains recognize multimeric membrane receptors with class I C-terminal tails (e.g., IL5Rα). In the second scaffolding mode, signals relayed from a class III receptor (e.g., syndecan4) to a class II downstream signaling molecule (e.g., merlin) can be transduced effectively by a single protein.

PSD-95 is a prototypical member of the MAUGK family that contains a C-terminal SH3 domain, a GK domain, and three PDZ domains. The first two PDZ domains are connected by a short, five amino acid linker suggesting that it functions as a supramodule (Long et al., 2003). Both PDZ domains were capable of binding to peptide ligands derived from the Kv1.4 potassium channel. Moreover, Kv1.4 binding synergy was observed with the PDZ12 domains compared to the individual PDZ domains. This simultaneous and cooperative binding induced clustering of the potassium channel. The NMR structure of tandem PDZ12 domain/ligand complex showed the two binding grooves were aligned in almost parallel orientation. However, the structural mechanism of ligand recognition was controversial, as subsequent crystal studies using a divalent peptide showed antiparallel binding for the two domains (Sainlos et al., 2011). A recent study reported an antiparallel orientation in the apo state based on the distance restraints calculated from single molecule FRET experiments (McCann et al., 2011). Interestingly, the orientation of PDZ12 alone was same as that in the full length PSD-95, but distinct from the orientation observed in the crystal structure of the ligand-bound state. The discrepancy in peptide orientations captured by different techniques suggests that PDZ12 may sample multiple conformations once bound to ligands. Consistent with this, NMR-relaxation experiments and MD simulations showed that the interdomain linker becomes flexible on ligand binding. The loss of the limited interdomian mobility created more conformational entropy to facilitate ligand binding (Wang et al., 2009). A SAXS study of PSD-95 PDZ12 showed that the interdomain distance became larger after dimer ligand binding, further supporting the flexible behavior of this supramodule in the bound state (Bach et al., 2012). Overall, these distinct conformations suggest more than one scaffolding mode for PSD-95, similar to that seen in syntenin1. Dimeric or multimeric receptors can be recognized by PDZ repeats in a parallel orientation, whereas monomeric receptors can be targeted by one

of the PDZ domains in antiparallel orientation, with other downstream-signaling molecules relayed by another PDZ (McCann et al., 2011). A follow-up smFRET study found that PSD-95 formed complexes with different receptors and downstream effectors in a transient and short-lived manner. Thus, the binding kinetics in PDZ-mediated interactions may play more critical roles than affinities in forming complexes (McCann et al., 2011). Integration of multiple biophysical techniques, in particular single molecule methods, should enable new insights into the dynamics and allosteric interplay between adjacent PDZs in scaffolding distinct receptors and downstream effectors.

6. CONCLUSIONS

Although PDZ domains were identified over 20 years ago, they continue to provide new surprises with respect to their function and regulation. Their primary function is as a protein–ligand interaction domain in cell signaling and they participate in many biological processes, including ion channel trafficking, neuronal development, and cell–cell junctions. They bind a diverse set of ligands—C-termini and internal sequences of proteins, phosphatidylinositol, and cholesterol—and binding can be regulated by phosphorylation and allosterically by protein interactions. Despite knowing much about the structure and specificity of individual PDZ domains, it is now clear that in the context of full-length proteins and larger order complexes PDZ domains possess more complex functions and regulatory mechanisms. Thus, PDZ domains are not just "beads-on-a-string," but adaptable protein–ligand interaction modules with the capacity to integrate their structure and function with other protein domains/regions. Because of their biological importance, individual PDZ domains and their interactions have been targeted for the development of inhibitors for a number of human diseases. To this end, several therapeutics have been developed that show promise. Given that there are over 500 PDZ domains in the human genome and only a small fraction have been well characterized, it is very likely that the future holds many new surprises.

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