An Exquisitely Specific PDZ/Target Recognition Revealed by the Structure of INAD PDZ3 in Complex with TRP Channel Tail

Graphical Abstract

Highlights

- Specific binding between INAD PDZ3 and TRP channel discovered
- A 15-residue tail of TRP is required for specific INAD PDZ3 binding
- Structure of the INAD PDZ3/TRP tail complex reveals a new PDZ-binding mode
- PDZ/target interactions can be exquisitely specific

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In Brief

PDZ domains bind to the C-terminal tail of target proteins with modest affinities and specificities. Ye et al. report an exquisitely specific PDZ/target interaction between scaffold protein INAD and the TRP channel in Drosophila photoreceptors, which is likely to have broad implications in other PDZ-mediated target interactions.

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An Exquisitely Specific PDZ/Target Recognition Revealed by the Structure of INAD PDZ3 in Complex with TRP Channel Tail

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SUMMARY

The vast majority of PDZ domains are known to bind to a few C-terminal tail residues of target proteins with modest binding affinities and specificities. Such promiscuous PDZ/target interactions are not compatible with highly specific physiological functions of PDZ domain proteins and their targets. Here, we report an unexpected PDZ/target binding occurring between the scaffold protein inactivation no afterpotential D (INAD) and transient receptor potential (TRP) channel in Drosophila photoreceptors. The C-terminal 15 residues of TRP are required for the specific interaction with INAD PDZ3. The INAD PDZ3/TRP peptide complex structure reveals that only the extreme C-terminal Leu of TRP binds to the canonical αβ/ββ groove of INAD PDZ3. The rest of the TRP peptide, by forming a β hairpin structure, binds to a surface away from the αβ/ββ groove of PDZ3 and contributes to the majority of the binding energy. Thus, the INAD PDZ3/TRP channel interaction is exquisitely specific and represents a new mode of PDZ/target recognitions.

INTRODUCTION

Modular protein-protein interactions play central roles in organizing and coordinating complex eukaryotic signal transductions (Pawson and Nash, 2003). PDZ domains are one of the protein-interacting modules occurring very abundantly in the proteomes of metazoans (Feng and Zhang, 2009; Luck et al., 2012; Ye and Zhang, 2013). PDZ domain proteins play vital roles in diverse biological processes, including organizing signal transduction complexes, clustering membrane receptors/ion channels, and trafficking of vesicles and/or proteins. A canonical PDZ domain contains ~90 amino acids and folds into a partially open β barrel composed of six β strands with each end of the barrel capped with an α helix. Typically, a PDZ domain recognizes a short stretch of peptide fragment (mostly four to six residues) situated at the very carboxyl tail of a target protein via a common binding surface known as the αβ/ββ groove (Doyle et al., 1996; Harris and Lim, 2001; Zhang and Wang, 2003). Some PDZ domains also recognize internal peptide sequences from target proteins via the same αβ/ββ groove (Hillier et al., 1999; Penkert et al., 2004). Based on the distinct C-terminal peptide-binding preferences, PDZ domains have been classified into discrete categories: class I PDZ domains recognize the consensus sequence Ser/Thr-X-Ψ-COOH, where X is any amino acid and Ψ represents a hydrophobic residue; class II domains prefer Ψ-X-Ψ-COOH; and class III domains prefer Asp/Glu-X-Ψ-COOH (Songyang et al., 1997). However, accumulating evidence indicates that the classifications that focused on the last four amino acids of binding targets are too restrictive to explain the functional and structural characteristics of PDZ domains accurately. Large-scale, systematic PDZ/target interaction screening studies (Stiffler et al., 2007; Tonikian et al., 2008), as well as many individual studies (reviewed in Feng and Zhang, 2009; Ye and Zhang, 2013), have shown that PDZ domains both within the same class and sometimes even from different classes share overlapping bindings to short carboxyl peptides. Such promiscuous PDZ/target interactions often are at odds with specific cellular functions known for many PDZ domain proteins as well as their binding targets. In addition, the majority of the reported PDZ/target peptide interactions display rather weak binding affinities (with Kd in the range of a few μM to tens of μM). In some intensively studied PDZ domain proteins such as DLGs and PICK1, a single PDZ domain can bind to several dozens of different targets. Obviously, this is not compatible with specific functional roles of many PDZ scaffold/target protein complexes revealed by in vivo studies. For example, numerous PDZ domain proteins and PDZ-binding motif-containing receptors/ion channels/cell adhesion molecules are known to co-exist in various specific cellular compartments such as postsynaptic densities in neurons and inter-cellular junctions in polarized epithelia (Sheng and Hoogenraad, 2007; Subbaiah et al., 2011). How such promiscuous PDZ/target interactions can support highly specific cellular functions of many known PDZ-binding targets such as glutamate...
receptors and many ligand-gated receptors in postsynaptic densities is conceptually hard to rationalize. It is possible that our current understanding of PDZ/target interactions is partial, and specific and high-affinity PDZ/target interactions with different binding modes have escaped our detection.

INAD (inactivation no afterpotential D) is one of the best characterized PDZ scaffold proteins, and it has served as a model protein to understand how scaffold proteins can effectively and specifically organize and regulate biological signaling events. INAD contains five PDZ domains (Figure 1A), each of which is responsible for binding to a specific signaling protein required for the Drosophila photo-transduction cascade, thus forming a highly efficient and specific multi-protein signaling complex (also known as signalsome) (Hardie and Raghu, 2001; Montell, 1999; Tsunoda et al., 1997). Genetic and cell biology evidence has shown that INAD PDZ2 is required for binding to eye-PKC, PDZ3 for interaction with the transient receptor potential (TRP) channel, and PDZ5 for engaging PLC\(b^n\)/NORPA (Adamski et al., 1998; Chevesich et al., 1997; Huber, 2001; Huber et al., 1996; Kimple et al., 2001; Li and Montell, 2000; Mishra et al., 2007; Shieh and Zhu, 1996; Tsunoda et al., 1997; Tsunoda and Zuker, 1999; van Huizen et al., 1998; Wang and Montell, 2007). In the microvilli of fly photoreceptors, INAD forms a near stoichiometric complex with TRP, presumably via highly specific PDZ domain-mediated interactions (Huber et al., 1996). This interaction works as a molecular anchor in mediating normal localization of photo-transduction signalsome in rhabdomere (Tsunoda et al., 2001). However, the current understanding of PDZ/target recognitions cannot fully explain the highly specific INAD-organized photo-transduction complex in fly eyes.
Here, we show that INAD PDZ3 binds to the TRP channel tail with a mode entirely different from current known PDZ/target interactions. A 15-residue C-terminal tail of TRP forms a β hairpin structure and binds to a surface of INAD PDZ3 outside the canonical target-binding αβ/ββ groove. Amino acid residues from both strands of the TRP β hairpin are engaged in binding, and thus the INAD PDZ3/TRP interaction is highly specific and with high affinity. The INAD PDZ3/TRP interaction revealed here represents a new paradigm for PDZ/target recognitions capable of supporting specific cellular functions of PDZ domain binding targets.

RESULTS

INAD PDZ3 Functions as an Independent Structural Unit to Interact with TRPCT

We showed previously that the PDZ45 tandem of INAD binds to the C-terminal tail of TRP containing nine or 15 residues with a very weak affinity ($K_d$ $\sim$140 μM). The interaction between INAD and the TRP tail peptides was enhanced by more than 1,000-fold by using the PDZ345 tandem and by extending the TRP C-terminal tail from the last nine amino acids to the last 15 amino acids (TRPCT) (Liu et al., 2011). Since the isolated PDZ3 could not be purified at the time of the study, the PDZ345 tandem was used instead to characterize the interaction between INAD and TRP. By combining our biochemical data with earlier reported cell biology findings, we proposed that TRP uses two discrete elements, its carboxyl tail and an internal sequence (the $\beta^{1264}$STV$^{1266}$ motif), to bind to the PDZ45 tandem and PDZ3 of INAD, respectively (Li and Montell, 2000; Liu et al., 2011; Peng et al., 2008; Shieh and Zhu, 1996) (see Figure 1B1 for the proposed interaction model). However, direct biochemical and structural data have been lacking to support such an INAD PDZ345/TRPCT interaction model.

To further characterize the INAD PDZ345/TRPCT interaction model, we performed a series of nuclear magnetic resonance (NMR) and biochemical experiments. First, we tested whether PDZ3 indeed directly interacts with PDZ45 forming an integral (NMR) and biochemical experiments. First, we tested whether PDZ345 indeed directly interacts with PDZ45 forming an integral INAD PDZ3 functions as an independent structural unit of INAD and is solely responsible for binding to TRPCT.

INAD PDZ3 Binds to TRP Channel with High Affinity

The average backbone amide peak intensity of TRPCT in the PDZ45/INAD PDZ345/TRPCT complex is essentially the same as that of PDZ3 (3.68 $\times$ 10$^6$ ± 0.37 $\times$ 10$^6$ versus 3.39 $\times$ 10$^6$ ± 0.34 $\times$ 10$^6$), and larger than that of PDZ45 (1.74 $\times$ 10$^6$ ± 0.17 $\times$ 10$^6$), indicating that PDZ3 rotates faster than PDZ45 does in the PDZ345/TRPCT complex. This result further confirms that INAD PDZ3 has no structural coupling with PDZ45. The amino acids (12 residues) located in the linker region between PDZ3 and PDZ45 in the PDZ345/TRPCT complex are likely to be unstructured, as their backbone amides undergo rapid exchange with solvent and are not observable at the buffer (pH 7.8). The above NMR-based evidence conclusively demonstrates that PDZ3 functions as an independent structural unit of INAD and is solely responsible for binding to TRPCT.
increased dissociation of GB1-TRPCT from the PDZ345/TRPCT complex (Figure 2D). Interestingly, addition of excess amounts of a nine-residue TRP tail peptide resulted in minimal GB1-TRPCT dissociation from PDZ345 (Figure 2D1), indicating that the N-terminal six residues of TRPCT are critical for its binding to INAD PDZ3. Finally, the $K_d$ value of the PDZ3/TRPCT complex measured by analytical ultracentrifugation is 0.13 $\mu$M (Figures 2B and S2), which is essentially the same as that of the PDZ345/TRPCT complex measured by the same method (Liu et al., 2011).

The Overall Structure of the PDZ3/TRPCT Complex

The highly homogeneous HSQC spectrum of the PDZ3/TRPCT complex (Figure 1D1) indicates that the complex adopts a stable, mono-dispersed conformation favorable for crystallization. Indeed, crystals of the PDZ3/TRPCT complex were readily obtained and diffracted to resolutions up to 1.75 Å. The structure of the PDZ3/TRPCT complex was determined by the molecular replacement method using the PDZ domain of TIP-1 (PDB: 3DIW) as the searching model (Table S1).

The structure of the PDZ3/TRPCT complex reveals a totally unexpected target-binding mode for any PDZ domains known to date (Figure 3A). Except for the N-terminal first residue, the conformation of the rest 14 residues of TRPCT in the complex is well defined. The N-terminal ten residues of TRPCT (i.e., from Ile(-4) to Gly(-13)) form a $\beta$ hairpin and extensively interact with a surface formed by $\beta_B$, $\beta_C$, and $\alpha_A$ of PDZ3 via both hydrophobic and charge-charge interactions (Figure 3A3). The last four residues of TRPCT, instead of forming a $\beta$ strand paring in antiparallel with $\beta_B$ of PDZ, insert vertically into the carboxyl residue-binding pocket at the end of the $\alpha_B$/$\beta_B$ groove. The carboxyl group and the side chain of Ile0 bind to the carboxyl-residue-binding pocket of INAD PDZ3 following the canonical PDZ/carboxyl peptide recognition mode (Figure 3A3). Therefore, INAD PDZ3 uses two discrete surfaces, one involves part of the canonical target-binding $\alpha_B$/$\beta_B$ groove and the other a much larger one outside the $\alpha_B$/$\beta_B$ groove, to bind to a 15-residue carboxyl peptide from TRP.

A set of NMR experiments were used to validate that the interaction seen in the crystal structure of the PDZ3/TRPCT complex also occurs in solution. A number of characteristic nuclear Overhauser effects (NOEs) [e.g., the inter-$\beta$ strand NOEs originating between Ile-4 and Arg-14 and between Arg-6 and Ser-11; Figure S3] clearly support the formation of the antiparallel $\beta$ hairpin...
structure of TRPCT in the complex in solution. Fully consistent with the structure of the PDZ3/TRPCT complex presented here, removal of the most upstream two residues Gly(-13) and Arg(-14) led to a large decrease of the binding between INAD PDZ3 and TRPCT (Peng et al., 2008), presumably because of weakening or even disruption of the β hairpin structure of TRPCT (Figure 3A).

In addition, INAD PDZ3 contains an N-terminal extension folding into a stable α helix (αN) which extensively interacts with a hydrophobic surface formed by residues from βD, βF, and αB of PDZ3 (Figures 3B and S4). The entire αN is likely required for the stable folding of PDZ3 as removal of part of or the entire αN led to formation of inclusion bodies when we tried to express truncated PDZ3 (data not shown). Therefore, INAD PDZ3 represents another example of PDZ domains with an extension sequence modulating the core PDZ domain structure and function (Wang et al., 2010).

Detailed Interactions between PDZ3 and TRPCT

Detailed analysis of the PDZ3/TRPCT interaction provides insights into the exquisite target-binding selectivity of INAD PDZ3. The C-terminal Leu0 of TRPCT is the only residue of TRP occupying the canonical PDZ-binding groove, and should be sufficient for excluding other proteins from binding. Trp-1 serves as a key residue to mediate the complex formation by extending its aromatic ring into the core of the binding interface between TRP and PDZ3 (Figures 4A and 4B1). The side chains of Trp-1 form hydrophobic contacts with Met378 from βB of PDZ3 and Ile-4 from the peptide itself on one side and a cation-π interaction with Arg397 from PDZ3 on the other side. Phe374 from PDZ3 further stabilizes the Trp-1/Arg397 interaction by forming another cation-π interaction with Arg397 (Figure 4B1). Finally, the side-chain amide of Trp-1 forms a hydrogen bond with the backbone of Gly-13 (Figure 4B1). Deletion of the last four amino acids of TRP (\(^{3}\text{SGWL}\)) completely disrupted the interaction between INAD and TRP, demonstrating that other residues play important roles in the binding are Val-9 and Ser-11, which are located in the turn of the TRPCT β hairpin and interact with Val409 on the αA/βD loop of PDZ3 (Figure 4B2). Ser-11 also interacts with Asp395 through hydrogen bonds and the side chain of Asp395 forms hydrogen bonds with the main chain of the TRPCT hairpin loop (Figure 4B2). It was reported that substitutions of Ser-11 and Val-9 with Ala or Asp significantly weakened the binding between TRP and INAD (Li and Montell, 2000; Peng et al., 2008; Shieh and Zhu, 1996). Since the \(^{11}_{\text{STV}}\text{βB}-\text{motif}\) within PDZ3 resembles the classical type I PBM, it was hypothesized that this TRP internal peptide fragment binds to the αB/β groove of PDZ3 (Peng et al., 2008; Shieh and Zhu, 1996). It is now clear that, although important for the PDZ3 binding, the \(^{11}_{\text{STV}}\text{βB}-\text{motif}\) in PDZCT neither binds to the canonical αB/β groove nor forms a β strand structure in the complex. Therefore, caution should be exercised in inferring so-called internal PDZ-binding motifs based on mutation-based experiments. Finally, Lys-12 forms a salt bridge with Glu398 from the βC/βA loop of PDZ3 (Figure 4B2). Consistent with the above structural analysis, substitutions of Asp395, Glu398, and Val409 in PDZ3 with Ala, individually, decreased the binding between the full-length INAD and TRPCT (Figure 4C). Amino acid sequence analysis has revealed that the residues forming the binding interface between INAD PDZ3 and TRPCT are highly conserved among the different insect species, indicating that the interaction mode observed between INAD PDZ3 and TRPCT in Drosophila melanogaster is also adopted by other insects with compound eyes (Figures 4D and 4E).

The InaD\(^{215}\) allele has been reported to be defective in fly visual signaling, and the mutant flies contain a M442K point mutation in PDZ3 (Shieh and Zhu, 1996; Tsunoda et al., 1997; Tsunoda and Zuker, 1999). The PDZ3/TRPCT structure provides a mechanistic explanation to the mutation-induced functional defect of the InaD\(^{215}\) allele. Met442 is located in the second last residue of βB, and forms a part of the folding core of PDZ3 (Figure S5). The M442K mutation is likely to destabilize or even
disrupt the overall folding of PDZ3 and thus impair its binding to TRP. Indeed the M442K mutant of the full-length INAD has diminished binding to TRPCT in an in vitro binding assay (Figure 4C).

DISCUSSION

Our results provide an answer to the conundrum of target-binding specificities by canonical PDZ domains. In the previously known canonical PDZ/target interactions, a short carboxyl ligand peptide binds to the $\alpha B/\beta B$ groove of a PDZ domain usually by augmenting its $\beta B$ strand (Figure 5A). Because of the limited length of the $\alpha B/\beta B$ groove, only a short C-terminal ligand peptide (four to five residues and certainly less than ten) is sufficient to bind to a corresponding PDZ domain. In addition, most of the reported PDZ/ligand interactions have modest binding affinities (typically with dissociation constants in the range of a few to dozens of $\mu$M). Accordingly, the majority of PDZ domains display rather promiscuous bindings to overlapping target proteins (i.e., a given PDZ domain can often bind to several or even dozens of different PBM-containing targets, and a given PBM can bind to many different PDZ domains). Such promiscuous PDZ/target interactions are not compatible with highly defined functions known for many PDZ targets, including many ion channels and receptors.

The INAD PDZ3/TRPCT complex structure presented in this work reveals an entirely unexpected target recognition mode for a PDZ domain (Figure 5B). Instead of binding only to a short carboxyl peptide, INAD PDZ3 recognizes a 15-residue tail peptide from TRP. Besides the very C-terminal residue which binds to PDZ3 with the same mechanism as the canonical PBMs, the rest of the upstream 14 residues form a $\beta$ hairpin structure and bind to a site outside the $\alpha B/\beta B$ groove of the PDZ domain.
to the carboxyl-binding pocket of PDZ3, the upstream residues C-terminal tail of TRP and PDZ3 of INAD. In addition to binding binding between INAD and TRP is mediated by a 15-residue structural analysis demonstrated that the strong and specific interaction between INAD PDZ3 and TRP C-terminal tail. Biochemical and functions of both PDZ domain scaffold proteins and their bind- ing targets will be valuable for understanding cellular interactions may follow the mechanism analogous to that observed in the INAD PDZ3/TRP complex in order to fulfill their specific cellular functions.

The requirement of at least 15 residues of TRPCT for its specific and high-affinity binding to INAD PDZ3 revealed in this study also indicates that caution is needed when interpreting widely accepted screening methods for PDZ/peptide ligand interactions. In the past, library-based methods, either using synthetic peptide libraries or phage displayed libraries, have been successfully used to identify numerous target peptides both for individual PDZ domains and for proteome-wide collections of PDZ domains, as well as for elucidating underlying mechanisms governing target-binding specificities of PDZ domains (Fuh et al., 2000; Songyang et al., 1997; Stiffler et al., 2007; te Velthuis et al., 2011; Tonikian et al., 2008). However, because of the practical limitations imposed by the size of ligand libraries, the lengths of peptide ligands screened are no more than ten residues. Therefore, interactions of PDZ domains with target peptides longer than those that can be covered by library-based methods have escaped detection. Since such elongated target peptides are likely to bind to their cognate PDZ domains with higher specificity and affinity, identification of such interactions will be valuable for understanding cellular functions of both PDZ domain scaffold proteins and their binding targets.

In summary, we discovered a novel PDZ interaction mode between INAD PDZ3 and TRP C-terminal tail. Biochemical and structural analysis demonstrated that the strong and specific binding between INAD and TRP is mediated by a 15-residue C-terminal tail of TRP and PDZ3 of INAD. In addition to binding to the carboxyl-binding pocket of PDZ3, the upstream residues of TRPCT form a β hairpin and bind to a surface outside the canonical target-binding αB/βB groove. The INAD PDZ3/TRP-binding mode represents a clever trick that Mother Nature de-
building and adjustment were completed using COOT (Emsley et al., 2010). All structure figures were prepared by PyMOL (http://pymol.sourceforge.net).

Analytical Ultracentrifugation
Sedimentation equilibrium experiments were performed using a Beckman proteomelab XL-I ultracentrifuge equipped with Beckman 50TI rotor and six sector cells at three different concentrations (absorption at 280 nm of 0.3, 0.6, and 0.9, respectively). Different concentrations of the INAD PDZ3/TRPCT complexes were centrifuged at 17,000 rpm. Samples were equilibrated for 72 hr at 17,000 rpm, and scans were taken every 12 hr and the data were buffer corrected. Data were analyzed using Sedfit and Sedphat programmes (http://www.analyticalultracentrifugation.com/default.htm). Data fitting was performed using a monomer-dimer association model and with simulated annealing algorithms (Lebowitz et al., 2002).

ACCESSION NUMBERS
The atomic coordinates of the INAD PDZ3/TRPCT structure have been deposited in the PDB under the accession code PDB: 5F67.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2015.12.013.

AUTHOR CONTRIBUTIONS
F.Y., W.L., and M.Z. designed experiment and analyzed data. F.Y., W.L., and Y.S. performed experiments. F.Y., W.L., and M.Z. wrote the manuscript. M.Z. coordinated the research.

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Recognition of unique carboxyl-terminal motifs by distinct PDZ domains.


