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Supramodular Nature of GRIP1 Revealed by the Structure of Its PDZ12 Tandem in Complex with the Carboxyl Tail of Fras1

Jiafu Long[1,2]†, Zhiyi Wei[1]†, Wei Feng[1], Cong Yu[1], Yan-xiang Zhao[3]* and Mingjie Zhang[1]*

[1]*Department of Biochemistry, Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong*

[2]*Center for Chemical Genomics and Drug Discovery, College of Life Science, Nankai University, 94 Weijin Road, Tianjin 300071, P.R. China*

[3]*Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong*

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The scaffold protein GRIP1 (glutamate receptor interacting protein 1) binds to and regulates both the trafficking and membrane organization of a large number of transmembrane proteins. Mutation of *GRIP1* in mice displays essentially the same phenotype of the mutations of *Fras1* or *Frem2*, which are the animal models of the human genetic disorder Fraser syndrome. However, the molecular basis governing the interaction between GRIP1 and Fras1/Frem2 is unknown. Here, we show that interaction between Fras1 and GRIP1 requires the first two PDZ domains (PDZ1 and PDZ2) to be connected in tandem, as the folding of PDZ1 strictly depends on the covalent attachment of PDZ2. The crystal structure of GRIP1 PDZ12 in complex with the Fras1 C-terminal peptide reveals that the PDZ12 tandem forms a supramodule in which only the peptide-binding groove of PDZ1 is bound with the Fras1 peptide. The GRIP1 PDZ12/Fras1 peptide complex not only provides a mechanistic explanation of the link between GRIP1 and the Fraser syndrome but may also serve as a foundation for searching for potential mutations in GRIP1 that could lead to the Fraser syndrome.

Keywords: GRIP1; PDZ tandem; protein trafficking; Fraser syndrome; Fras1

Introduction

The GRIP (glutamate receptor interacting protein) family proteins, GRIP1 and GRIP2/ABP, contain six to seven PDZ domains and were originally identified by their interaction with GluR2/3 subunits of AMPA receptors.[1–3] Since their discovery, numerous proteins have been identified to interact with the GRIP family proteins, and the majority of the interactions between GRIP and their targets are mediated by the PDZ domains of GRIP. Some of the GRIP-binding proteins include EphB receptor kinases, ephrin-B ligands,[4–6] the adaptor protein liprin-α,[7] neuronal RasGEF GRASP-1,[8] the type 5 metalloproteinase,[9] and the cellular matrix proteins Fras1 and Frem2.[10,11] Accumulating data indicate that the GRIP family proteins are ideally suited as adaptors modulating the delivery and organization of transmembrane proteins at the surfaces of membranes.[7,10,12–14] Interruption of GluR2–GRIP interaction led to the inhibition of AMPA receptor accumulation at synapses.[1,15] The surface expression of EphB2 receptor tyrosine kinase was greatly reduced when endogenous GRIP1 was knocked down by small inhibitory RNA.[14] Consistent with the protein trafficking function of the GRIP family proteins, the microtubule motor protein KIF5 (also

*Corresponding authors. E-mail addresses: Yanxiang.Zhao@inet.polyu.edu.hk; mzhang@ust.hk.

† J. L. and Z. W. contributed equally to this work.

Abbreviations used: GRIP, glutamate receptor interacting protein; CD, circular dichroism; HSQC, heteronuclear single quantum coherence; PDB, Protein Data Bank; FITC, fluorescein isothiocyanate.

known as kinesin 1) was shown to bind directly to a region of GRIP1 between PDZ6 and PDZ7.[12]

Recent genetic studies in mice have provided solid evidence supporting GRIP1 as a scaffold protein for the trafficking of transmembrane proteins. *GRIP1*$^{-/-}$ mice display severe defects in multiple organs including skin (blistering, cryptophthalmos, and syndactyly), kidney (renal agenesis), and brain (hemorrhagic blisters), and these phenotypes are markedly comparable to the human Fraser syndrome and the animal models of this human genetic disorder.[10,16–19] Mutations of two extracellular matrix proteins, Fras1 and Frem2, resulted in Fraser syndrome in humans and the blebbed (*bl*)[17,18] and the myelencephalic blebs (*my*)[11,20,21] phenotypes in mice, respectively. Both Fras1 and Frem2 contain a PDZ-domain-binding motif at their extreme carboxyl tail, and the PDZ-binding motifs of Fras1 and Frem2 have been shown to directly interact with the PDZ1–3 cassette of GRIP1.[10] In *GRIP1*$^{-/-}$ mice, trafficking of Fras1 to the basement membranes of epidermis is disrupted, and epidermal–dermal junctions are broken.[10,16] In another mouse model of Fraser syndrome called the eye-bleb (*eb*), *GRIP1* is disrupted by the deletion of exons 10 and 11, and the mutant *GRIP1* lacks the C-terminal 4 PDZ domain.[10] Despite the direct functional link between GRIP1 and the Fraser syndrome proteins Fras1 and Frem2, the molecular basis of the interaction between GRIP1 and Fras1/Frem2 is still unknown.

In this work, we show that the PDZ12 tandem of the GRIP1 PDZ1–3 cassette forms an obligatory folding unit. The folding of PDZ1 strictly depends on the covalent attachment of PDZ2. The crystal structure of GRIP1 PDZ12 in complex with the Fras1 C-terminal peptide, together with detailed biochemical studies, reveals that the PDZ12 tandem forms a supramodule in which the peptide-binding groove of PDZ1 is accessible and occupied by Fras1. In contrast, the target-binding groove of PDZ2 is completely occluded by the back side of PDZ1. Two salt bridges play critical roles in "stitching" the two PDZ domains together. Single point mutations of any of these salt-bridge-forming residues in the PDZ1 and PDZ2 interface disrupted GRIP1's binding to Fras1. We speculate that such mutations of GRIP1 could account for certain populations of the Fraser syndrome patients who bear no mutations in either *Fras1* or *Frem2*.

Results

The PDZ12 tandem of GRIP forms an obligatory structural unit

The seven PDZ domains of GRIP1 are organized into three cassettes: PDZ1–3, PDZ4–6, and PDZ7. Such domain organization of GRIP is highly conserved throughout the evolution, suggesting that each cassette plays specific roles. A number of binding partners for the PDZ4–6 cassette and for PDZ7 have been identified, and the biochemical basis and the cellular roles of many of these interactions have been suggested.[1,2,4–7,14,22–24] In contrast, the only known binding partners of the PDZ1–3 cassette are Fras1 and Frem2,[10] two closely related transmembrane cell matrix proteins. The biochemical and structural basis of the PDZ1–3 cassette and Fras1 (or Frem2) interaction is not known. Additionally, it is puzzling that no specific PDZ domain in the PDZ1–3 cassette has been assigned to interact with Fras1 or Frem2.

As the first step in elucidating the structure and function of the PDZ1–3 cassette of the GRIP family scaffold proteins, we evaluated the folding properties of each isolated PDZ domain using NMR and circular dichroism (CD) spectroscopic techniques (Fig. 1). The narrow backbone amide chemical shift distribution pattern of the ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectrum of PDZ1 indicated that the isolated PDZ1 is unfolded (Fig. 1a), and this conclusion is supported by the random coil-like CD spectrum of PDZ1 (Fig. 1f). In contrast, the sharp and well-dispersed backbone amide resonances of PDZ2 (Fig. 1b) and PDZ3 (Fig. 1c) indicated that both PDZ2 and PDZ3 in their isolated forms are well folded. We discovered with surprise that the entire ^{1}H–^{15}N HSQC spectrum of the PDZ12 (Fig. 1d, blue peaks) is well dispersed and that the peak number of the spectrum matches well with two folded PDZ domains. The CD spectrum of PDZ12 also indicated that the PDZ tandem is well folded and lacks a significant level of random coil structure. Additionally, there are gross chemical shift differences between the HSQC spectra of the isolated PDZ2 and PDZ2 in the PDZ12 tandem (red *versus* blue peaks in Fig. 1d), indicating that the conformation of PDZ2 in the PDZ12 tandem differs significantly from the conformation of the isolated PDZ2. The NMR and CD data (Fig. 1d and f) strongly indicate that the PDZ12 tandem forms an integral structural unit. In this unit, PDZ2 induces *de novo* folding of PDZ1, and in return, PDZ1 also significantly alters the conformation of PDZ2. Finally, we compared the HSQC spectrum of PDZ123 with those of the PDZ12 tandem and the isolated PDZ3 (Fig. 1e). Since the quality of the PDZ123 spectrum is not sufficiently high, we were not able to detect all the peaks from the three domains. Nevertheless, the overlap of the peaks (i.e., PDZ12 + PDZ3 matches with PDZ123) in the well-resolved regions (see the highlighted regions in Fig. 1e) indicated that minimal interaction could be observed between PDZ12 and PDZ3. Taken together, our spectroscopic studies revealed that the PDZ1–3 cassette of GRIP1 is composed of two structural units: the obligatory PDZ12 tandem and the well-folded, structurally independent PDZ3 domain.

The interaction between GRIP1 and Fras1 is mediated by the PDZ12 tandem

We next characterized the interaction between Fras1 and the GRIP1 PDZ1–3 cassette in detail. We

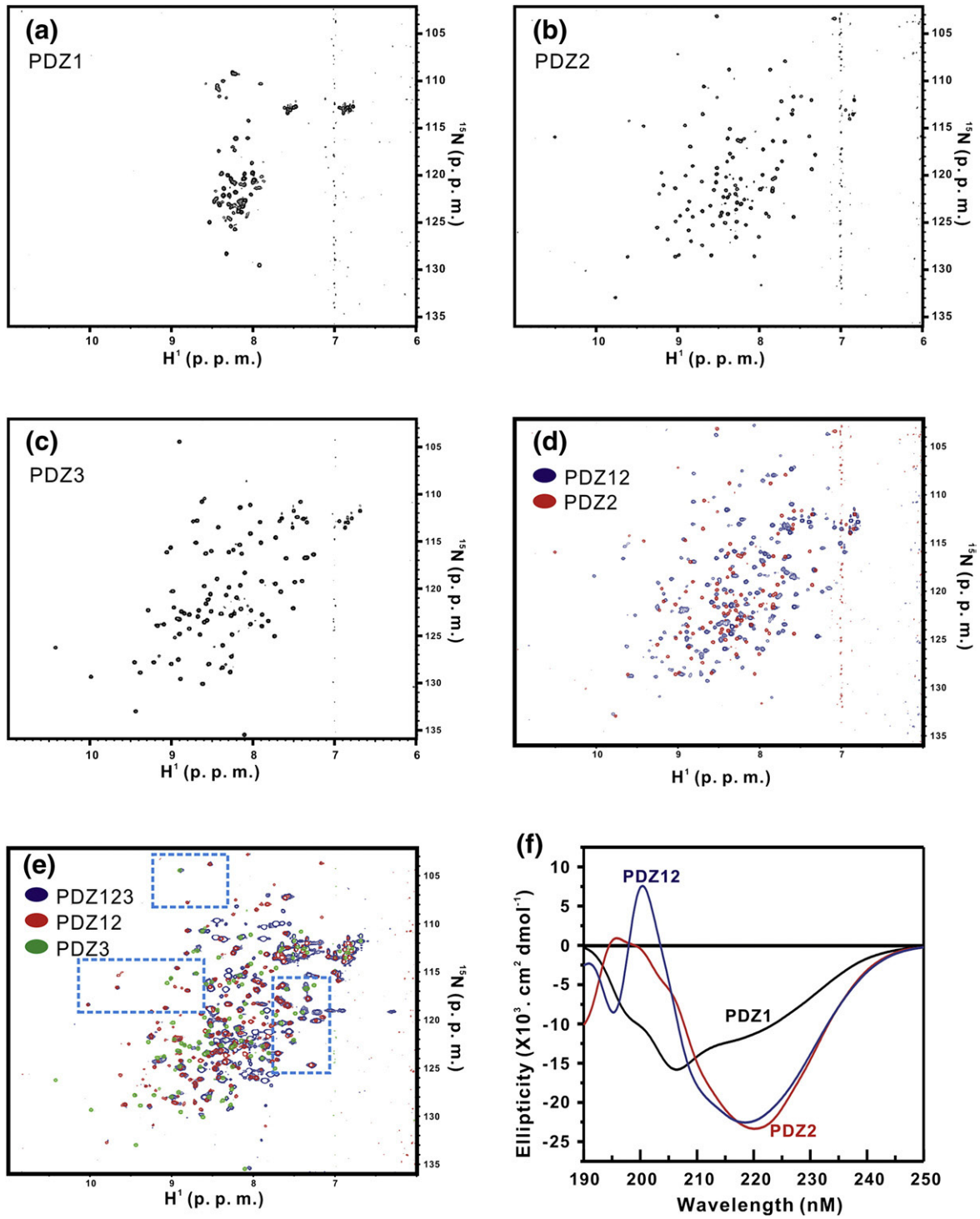

Fig. 1. Folding properties of the PDZ1–3 cassette of GRIP1. The figure shows the ^{1}H,^{15}N HSQC spectra of the isolated PDZ1 (a), PDZ2 (b), and PDZ3 (c). The figure also compares the HSQC spectrum of the PDZ12 tandem with that of the isolated PDZ2 (d) and the entire PDZ1–3 cassette (e). The CD spectra shown in (f) are used to characterize the overall folding of PDZ1, PDZ2, and PDZ12.

used an N-terminal fluorescence-labeled peptide encompassing the last 10 amino acid residues of Fras1 (referred to as the Fras1 peptide) to measure the binding affinities of Fras1 to the three isolated PDZ domains of the PDZ1–3 cassette and their combinations (Fig. 2). As expected, the isolated PDZ1 showed no detectable binding to the Fras1, as the domain is not folded. The isolated PDZ2 and PDZ3 or PDZ23 in tandem, although properly folded, showed very weak binding (K_d in hundreds of micromolars) to the Fras1 peptide, and such weak binding is likely to be physiologically irrelevant. Interestingly, the PDZ12 tandem showed robust binding to the Fras1 peptide with a K_d of ~9.4 μM,

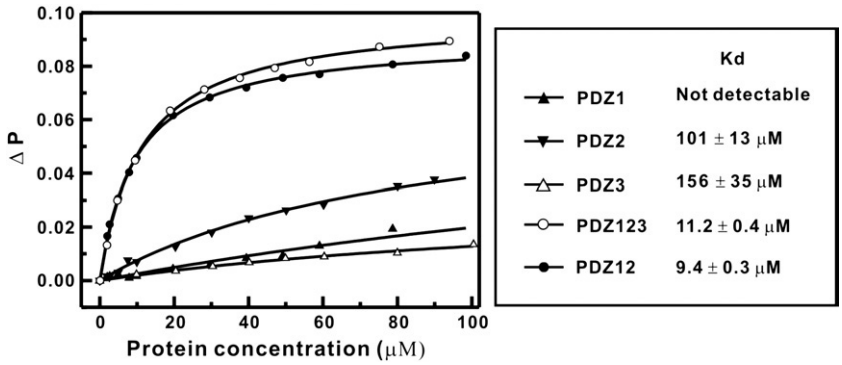

Fig. 2. The Fras1 peptide specifically binds to the PDZ12 tandem of GRIP1. Fras1 peptide binding properties of the isolated PDZ1, PDZ2, and PDZ3 as well as the PDZ12 and PDZ123 repeats of GRIP1 studied by fluorescence-anisotropy-based titration experiments. The *y*-axis (ΔP) denotes the fluorescence polarization changes of the FITC-labeled Fras1 peptide induced by binding to various PDZ domains.

indicating that the integral PDZ12 structural unit is required for GRIP1 to bind to Fras1. Consistent with the NMR-based observation that minimal interaction could be observed between PDZ12 and PDZ3 (Fig. 1e), the PDZ1–3 cassette has essentially the same binding affinity to Fras1 as the PDZ12 tandem does. However, from the data shown in Figs. 1 and 2, we cannot conclude whether the Fras1 peptide binds to PDZ1 or PDZ2 or both in the PDZ12 tandem, as either of the two PDZ domains can influence the conformation of the other. To resolve this issue and to uncover the mechanistic basis of the PDZ12 tandem-mediated GRIP1/Fras1 interaction, we decided to determine the complex structure of the GRIP1 PDZ12 tandem in complex with the Fras1 peptide. We first attempted to solve the PDZ12/Fras1 complex structure by NMR spectroscopy. However, the broad line widths of the complex spectra proved that NMR-based structural determination of the complex was challenging. Therefore, we resorted to X-ray crystallography for the structure determination of the PDZ12/Fras1 complex (Table 1).

Overall structure of GRIP1 PDZ12 in complex with the Fras1 peptide

The crystal structure of GRIP1 PDZ12 (residues 48–243) in complex with the Fras1 peptide was solved by the molecular replacement method. In the final refined model at 2.3 Å resolution, each asymmetric unit contains two molecules of GRIP1 PDZ12. The two PDZ12 molecules in each asymmetric unit are almost identical (overall RMSD of 0.67 Å), and small differences are located in the loop regions of the two domains. In the crystal, each PDZ12 tandem binds to one molecule of the Fras1 peptide, and the binding is mediated via the target recognition groove of PDZ1 (Fig. 3a).

Consistent with the NMR-based study in Fig. 1, the crystal structure of the GRIP1 PDZ12/Fras1 peptide complex showed that the PDZ12 tandem consists of two well-folded PDZ domains (PDZ1 and PDZ2) connected by a nine-residue loop (referred to as the $Loop_{1-2}$) (Fig. 3a). The two PDZ domains pack with each other side by side in a front-to-back manner, and the well-defined $Loop_{1-2}$ packs with one end the PDZ2 β-barrel. The entire PDZ12 tandem forms a compact and structurally integrated supramodule (Fig. 3a and b). The overall structures of the two PDZ domains in PDZ12 are very similar (Fig. 3c and d, an RMSD value of 1.23 Å when compared by the 87 aligned residues between the two domains). In the PDZ12 supramodule, the target-binding groove of PDZ2 is occluded by the βA-, αA-, and the αA/βD-loop of PDZ1 and, therefore, is completely inaccessible to its potential targets. In contrast, the target-

Table 1. Statistics of X-ray crystallographic data collection and model refinement

Data collection	
Space group	$P2_12_12_1$
Unit cell parameters (Å)	
a	59.10
b	75.92
c	126.38
Resolution range (Å)	30–2.3 (2.42–2.3)
No. of total reflections	211,810 (20,674)
No. of unique reflections	25,986 (3700)
I/σ	30.2 (4.4)
Completeness (%)	99.9 (99.8)
R_{merge} (%)[a]	6.0 (35.5)
Structure refinement	
Resolution (Å)	30–2.3 (2.36–2.30)
R_{cryst}/R_{free} (%)[b]	19.9 (23.5)/24.7 (24.8)
r.s.m.d. bonds (Å)/angles (°)	0.008/1.09
No. of reflections	
Working set	24,632
Test set	1344
No. of atoms	
Protein atoms	2952
Peptide atoms	56
Water molecules	212
Average *B*-factor (Å^2)	
Main chain	48.1/43.3[c]
Side chain	50.1/45.4[c]
Peptide	48.3
Water	49.6
Ramachandran plot (%)	
Most favored regions	96.6
Additionally allowed	3.4
Generously allowed	0.0

Numbers in parentheses represent the value for the highest-resolution shell.

[a] $R_{merge} = \sum|I_i - I_m| / \sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry related reflections.

[b] $R_{cryst} = \sum||F_{obs}| - |F_{calc}|| / \sum|F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure factors, respectively. $R_{free} = \sum_T||F_{obs}| - |F_{calc}|| / \sum_T|F_{obs}|$, where T is a test data set of about 5% of the total reflections randomly chosen and set aside prior to refinement.

[c] Values for the two monomers (A/B) in one asymmetric unit.

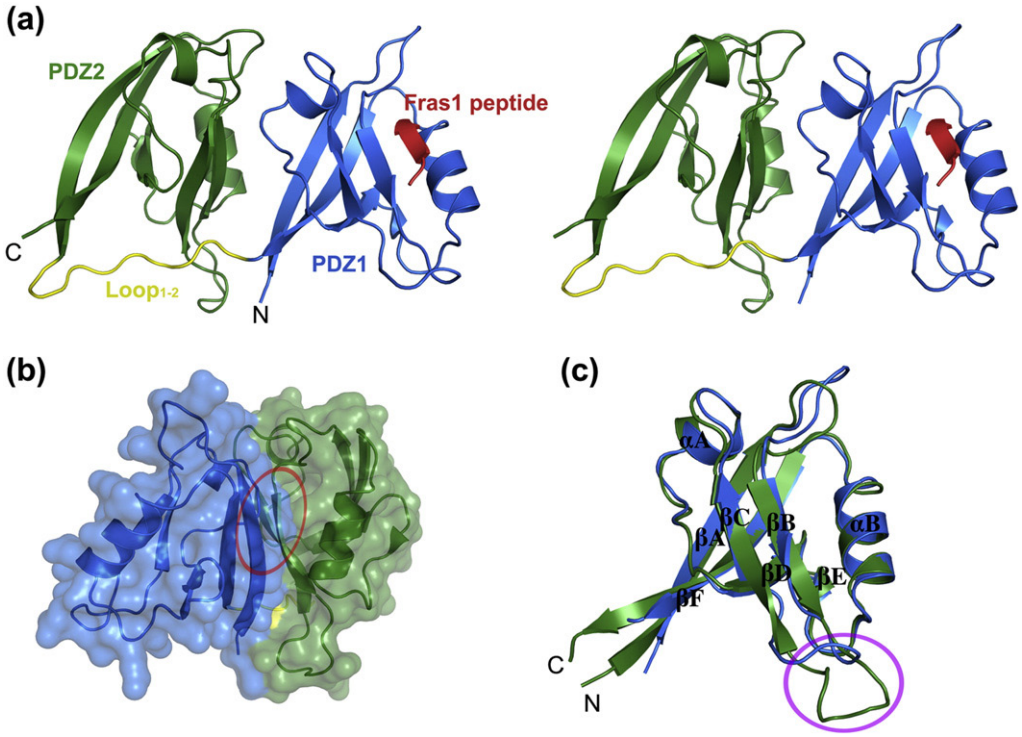

Fig. 3. The overall structure of the GRIP1 PDZ12 tandem in complex with the Fras1 peptide. (a) Ribbon diagram representation showing the stereoview of the backbone structure of the GRIP1 PDZ12 tandem in complex with the Fras1 peptide. PDZ1 (residues 48–135), PDZ2 (residues 145–240), and $Loop_{1-2}$ (residues 136–144) are colored blue, green, and yellow, respectively. (b) A semitransparent surface representation of the PDZ12 tandem showing that the two PDZ domains interact with each other in a front-to-back fashion to form a structurally intact supramodule. The figure also illustrates that the hypothetical target-binding groove of PDZ2 (highlighted with a red oval) is occupied by residues from the βA-, αA-, and the αA/βD-loop regions of PDZ1 and, therefore, inaccessible to peptide ligands. (c) Overlay plot of the backbone structures of PDZ1 and PDZ2 showing the similarity of their overall conformation. The different conformation of the βB/βC-loop between the two PDZ domains is highlighted by a purple oval.

binding groove of PDZ1 in the PDZ12 tandem is fully exposed and occupied by the Fras1 peptide (Fig. 3a). The X-ray structure of the GRIP1 PDZ12/Fras1 complex, together with the NMR data in Fig. 1, demonstrates that GRIP1 PDZ12 functions as a structurally intact supramodule. The intimate interaction between PDZ1 and PDZ2 is essential for the proper folding and, therefore, target (Fras1 in this case) binding of PDZ1.

The interface of PDZ1 and PDZ2

The intimate interaction between PDZ1 and PDZ2 buries a total of ~580 $Å^2$ surface area in the interface of the two PDZ domains (excluding $Loop_{1-2}$). The PDZ1 and PDZ2 interface is primarily stabilized by buried charge–charge interactions. Only a few weak hydrogen bonds and sparse hydrophobic interactions were found in the PDZ12 interface (Fig. 4b). Specifically, two salt bridges formed by one Asp–Arg pair (Asp93–Arg186) at the top and one Asp–Arg pair (Asp99–Arg167) at the bottom of the PDZ12 interface "glue" the two PDZ domains together (Fig. 4a). We further note that the guanidium group of Arg167 from PDZ2 is situated right above the aromatic ring plane of Tyr134 located at the end of PDZ1 βF. This cation–π interaction between Arg167 and Tyr134 not only directly contributes to the binding between PDZ1 and PDZ2 but should also further stabilize the salt bridge formed by the Asp99–Arg167 pair. We speculate that the salt bridge formed by the Asp99–Arg167 pair likely plays a more significant role in the interdomain stability of PDZ12. Consistent with their critical roles in the interdomain stability inferred from the structural analysis, these five residues located in the interface (i.e., Asp93–Arg186 and Asp99–Arg167–Tyr134) are strictly conserved in all known GRIP proteins across difference species (Fig. 5). Therefore, it is likely that the supramodular structural nature seen in the rat GRIP1 PDZ12 tandem is a common property of PDZ12 in other isoforms of GRIP or GRIP proteins from other species. Supporting this prediction, a very recent study demonstrated that both PDZ1 and PDZ2 of *Drosophila* GRIP are required for the protein to interact with a transmembrane cell adhesion molecule called Echinoid.[25]

Next, we directly tested the role of the salt bridges formed by the two Asp–Arg pairs and the cation–π interaction between Tyr134 and Arg167 observed from the PDZ12/Fras1 complex structure. We substituted Arg167 and Arg186 in PDZ2 with Ala individually to probe the role of the salt bridges. We chose to mutate Arg residues from PDZ2 as isolated

PDZ2 is folded (Fig. 1b), and substitution of either of the two Arg with Ala did not disrupt the overall folding of the isolated PDZ2 (data not shown; Fig. 4e). In contrast, the isolated PDZ1 is largely unfolded, and we did not change residues from PDZ1 in order to avoid potential changes to the folding property of the domain. Additionally, since PDZ1 is responsible for binding to the Fras1 peptide, we chose not to alter the two Asp residues to avoid potential interference to the Fras1 binding that might be induced by the amino acid changes. The stability changes of the two Arg to Ala mutants of GRIP1 PDZ12 were evaluated by partial trypsin digestion (Fig. 4c). Mutation of either Arg167 or Arg186 led to accelerated degradations of PDZ12 by trypsin compared to the wild-type protein (Fig. 4c), indicating that disruption of either Asp–Arg pair of the charge–charge interactions decreased the stability of PDZ12. Additionally, the Arg167Ala mutation has a larger impact on the stability of PDZ12 than the Arg186Ala mutation, as the Arg167Ala mutant of PDZ12 was completely degraded within 1 min

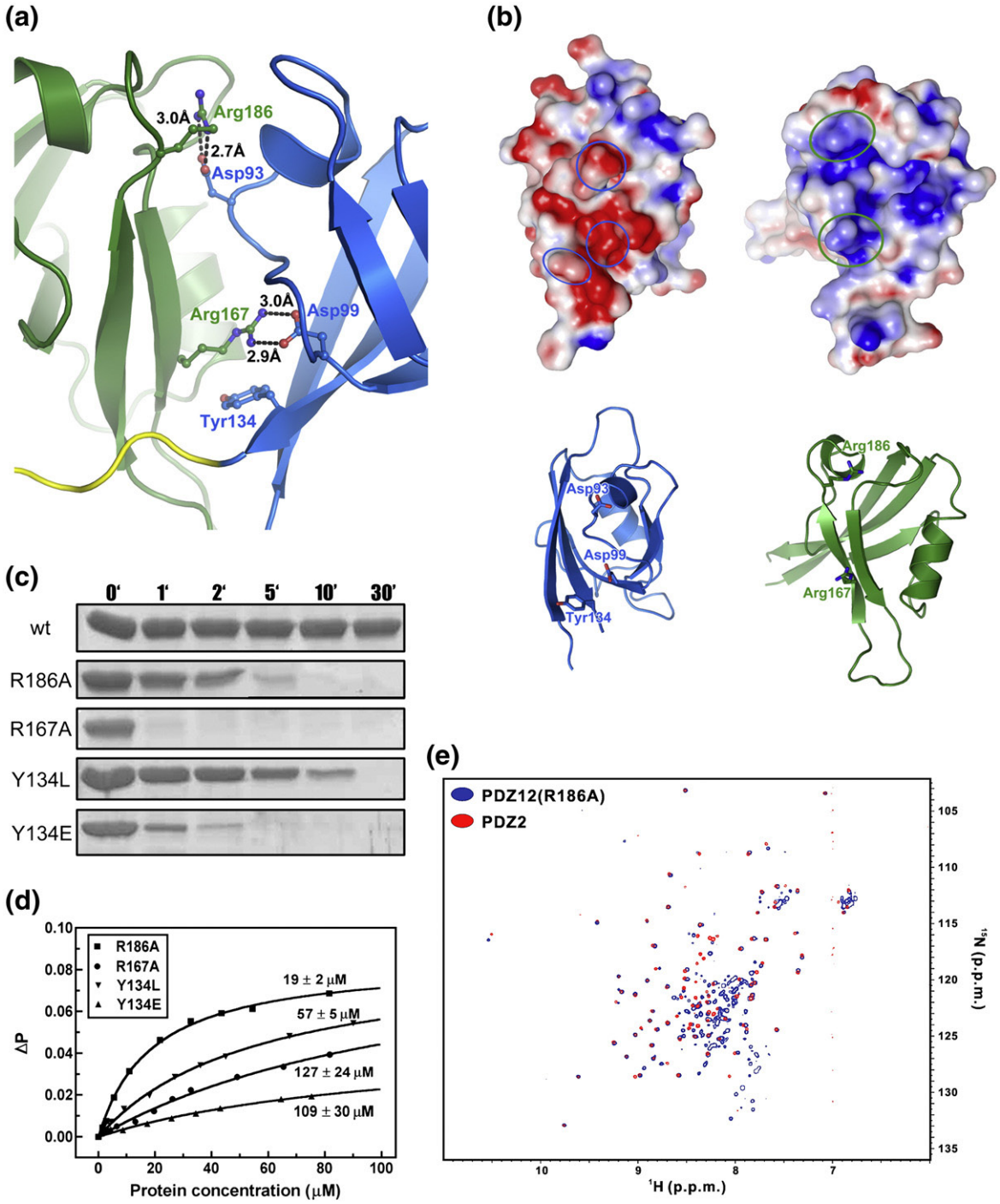

Fig. 4 (*legend on next page*)

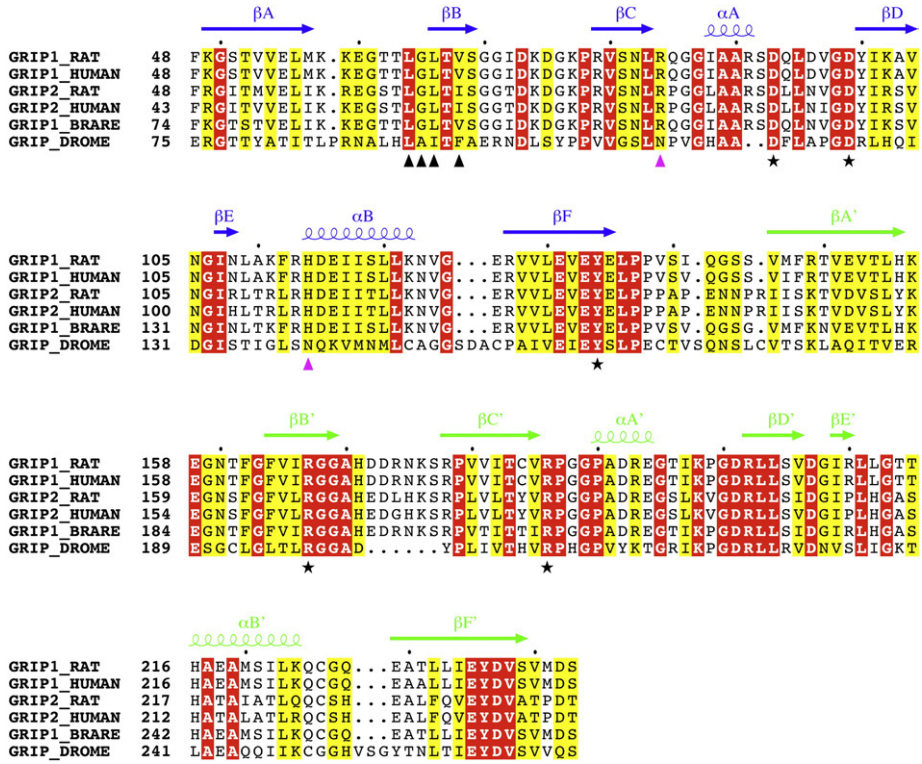

Fig. 5. Multiple sequence alignment of the PDZ12 tandem of GRIP from different species. The protein sequences were from rat, human, zebrafish, and fruit fly. In this diagram, residues that are identical and similar are shown in red and yellow boxes, respectively. The secondary structural elements are indicated above the alignment and are colored blue (for PDZ1) and green (for PDZ2). The amino acid residues in PDZ1 that are directly involved in the Fras1 peptide binding are highlighted with triangles, and the residues labeled with asterisks are critical for the interdomain interaction between PDZ1 and PDZ2.

after addition of trypsin (Fig. 4c). The above biochemical data support our structure-based prediction that the salt bridge formed by the Asp99–Arg167 pair plays a more significant role in the interdomain stability of PDZ12 than the Asp93–Arg186 pair. We then evaluated the role of the cation–π interaction involving Arg167 and Tyr134 in the stability of PDZ12. In the first mutation, we substituted Tyr134 with a bulky hydrophobic Leu. The Tyr134Leu mutation should disrupt the cation–π interaction but largely retain the hydrophobic environment at the amino acid position 134. In the second mutation, we substituted Tyr134 with Glu, aiming to eliminate both the cation–π and the hydrophobic interactions of Try134. As expected, both Tyr134 mutations decreased the stability of PDZ12. Importantly, the

Fig. 4. Critical interactions in the interface of PDZ1 and PDZ2 that stabilize the overall folding of the PDZ12 tandem. (a) An enlarged view of the PDZ1 and PDZ2 interface highlighting the two buried Arg–Asp salt bridges that play critical roles in the folding of the PDZ12 tandem. The aromatic ring of Tyr134 from the βF-strand of PDZ1 that forms a cation–π interaction with the guanidium group of Arg167 from PDZ2 is also drawn in explicit atomic representation. (b) An open-book surface model showing the charge–charge complementation between PDZ1 and PDZ2 in the PDZ12 tandem. The orientation of each of the PDZ domains is shown beneath their surface diagram. The negative charge potential is shown in red, and the positive charge potential is shown in blue. The positions of the critical amino acid residues in the domain interface are also indicated in the ribbon diagrams and highlighted with circles in the surface diagram. (c) Partial trypsin digestion comparing the stabilities of the wild-type PDZ12 and its mutants. "wt," "R186A," "R167A," "Y134L," and "Y134E" denote the wild-type protein and the Arg184Ala, Arg167Ala, Tyr134Leu, and Tyr134Glu mutants, respectively. In this experiment, a total of 200 μl of each protein sample with the same protein concentration was started for the trypsin digestion. At each indicated time point after addition of trypsin, an aliquot of 10 μl was withdrawn from each digestion mixture and mixed with equal volume of 2× sample loading buffer for SDS-PAGE. The amount of the remaining wild-type or mutant PDZ12 in the digestion mixtures was evaluated by the Coomassie blue staining of proteins separated by SDS-PAGE. (d) Mutations that decreased the stability of the PDZ12 tandem weaken their Fras1 binding affinity in parallel. The Fras1 peptide bindings to the mutant PDZ12 were assayed using the same fluorescence polarization assay described in Fig. 2. (e) The overlay plot of the $^{1}H,^{15}N$ HSQC spectrum of the isolated PDZ2 with that of the R186A-PDZ12. The peaks in the HSQC spectrum of isolated PDZ2 overlap with a subset of the peaks in the HSQC spectrum R186A-PDZ12, indicating that PDZ1 no longer interacts with PDZ2 in R186A-PDZ12. The appearance of an additional set of peaks in the random coil region in R186A-PDZ12 when compared to the spectrum of the wild-type PDZ12 further indicates that PDZ1 in the R186A-PDZ12 mutant is unfolded. The integrity of the R186A-PDZ12 mutant sample was checked by SDS-PAGE after recording the NMR spectrum.

Tyr134Glu mutation has a significantly larger impact on the stability of PDZ12 than the Tyr134Leu mutation does (Fig. 4c).

The protein stability data in Fig. 4c correlate well with the Fras1 peptide-binding affinities of each mutant (i.e., the order of decreasing stability of the mutants matches well with the order of weakening binding of PDZ12 to the Fras1 peptide, Fig. 4c and d). The residual bindings to the Fras1 peptide seen for the Arg167Ala and Tyr134Glu mutants of PDZ12 are essentially the same as the isolated PDZ2 binding to the peptide, suggesting that PDZ1 is nonfunctional in these mutants and is likely to be unfolded. Indeed, both of the purified Arg167Ala and Tyr134Glu mutants were highly unstable and prone to self-degradation after overnight incubation at room temperature without any addition of trypsin. The highly unstable and degradable nature of the Arg167Ala and Tyr134Glu mutants prevented us from performing a detailed NMR-based study. As an alternative approach, we recorded an overnight ^{1}H–^{15}N HSQC spectrum of the Arg186Ala mutant of PDZ12 (the mutant sample readily precipitated under the NMR condition when the concentration of protein exceeded 0.1 mM). Under our NMR condition (at 30 °C in Tris–HCl buffer, pH 7.5), only folded PDZ2 resonances were retained in the HSQC spectrum of Arg186Ala-PDZ12 (Fig. 4e). A number of sharp peaks with narrow chemical shift dispersions (i.e., in the ^{1}H shift range of 7.5–8.5 ppm) in the spectrum are presumably from the unfolded PDZ1 in the mutant PDZ12 tandem (Fig. 4e). Taken together, our above biochemical data support the structural observation that the salt bridges formed by the both pairs of Asp–Arg interactions play critical roles in the folding and stability of the GRIP1 PDZ12 supramodule. Our data further support that the Asp99, Arg167, and Tyr134 triad plays a more critical role in the stability of the PDZ12 supramodule than the Asp93–Arg186 salt bridge. We noticed that substitution of Leu193 in PDZ2 of *Drosophila* GRIP with Ala (corresponding residue Phe162 in the βB′-strand of PDZ2 of rat GRIP1, which is critical for the proper folding of PDZ2) disrupted GRIP PDZ12-mediated ligand binding.[25] According to the structure of GRIP1 PDZ12 presented in our study, we predict that the substitution of buried Leu193 with Ala is expected to impair the folding of PDZ2 and, thereby, indirectly disrupt the PDZ1-medaited binding of dGRIP to Echinoid.

Fras1 recognition mechanism of the GRIP1 PDZ12 supramodule

Consistent with the peptide-binding data shown in Figs. 1 and 2, the Fras1 peptide was found to bind to the canonical peptide-binding groove of PDZ1 in the PDZ12 tandem crystal structure (Fig. 3a). In the crystal structure of the complex, the electron densities of only the last four residues (GTEV) of the Fras1 peptide could be assigned. Similar to the majority of PDZ/target peptide complexes, the Fras1 peptide binds to the βB/αB-groove of PDZ1 by augmenting the βB-strand in an antiparallel fashion (Figs. 3a and 6a). The carboxylate of the Fras1 peptide forms hydrogen bonds with the backbones of the "TLGL" sequence of PDZ1 (equivalent to the "GLGF" motif in PDZ domains, Fig. 5). The side chain of the 0-position Val inserts into the conserved hydrophobic pocket situated at one end of the PDZ1's target-binding groove. Substitution of this Val with a Gly completely abolished Fras1's binding to the PDZ12 tandem (Table 2). The hydroxyl group of Thr at the −2-position of the Fras1 peptide forms a strong hydrogen bond with the N-3 nitrogen of His114 at the αB1 position of PDZ1, explaining that GRIP1 PDZ1 is a type I PDZ domain and specifically recognizes a Thr/Ser at the −2-position of its ligands. In addition to the expected interactions between the side chains at the 0- and −2-positions of the Fras1 peptide and PDZ1, the negatively charged Glu at the −1-position of the Fras1 peptide forms a salt bridge with the conserved Arg84 located at the end of βC of PDZ1, indicating that the Glu(−1) of the Fras1 peptide also plays a direct role in binding to GRIP1 PDZ12. Substitution of Glu(−1) with a neutral Asn decreased the binding affinity of the Fras1 peptide to PDZ12 by ~20-fold, and replacing Glu(−1) with a positively charged Arg eliminated Fras1's binding to PDZ12 (Table 2). Consistent with the above structure-based observation, the only other known GRIP1 PDZ1–3 ligand, Frem2, contains the "SEV" sequence at its C-terminus. Finally, we probed the role of Gly(−3) of the Fras1 peptide in binding to GRIP1 PDZ12. Substitution of Gly(−3) with Ser, which contains a small polar side chain, only mildly decreased the binding affinity between Fras1 and PDZ1. Substitutions of Gly(−3) with any amino acid residues with bulkier side chains (e.g., Asn and Glu in Table 2) led to more severe decreases in the binding between Fras1 and PDZ12. Therefore, we conclude that the GRIP1 PDZ12 tandem prefers a small residue at the −3-position of its ligands. The preference for a small amino acid residue at the −3-position of the GRIP1 PDZ12's ligands is likely due to the unique conformation of the βB/βC-loop of the PDZ1 domain (Fig. 3a and c). In the crystal structure of the complex, the βB/βC-loop of PDZ1 is well defined and acts as a "stopper" at the bottom of the αB/βB-groove of the domain. The side chains of bulky residues at the −3-position of peptide ligands would be sterically unfavorable with the side chains from the βB/βC-loop of PDZ1. Taken together, our structural and biochemical data indicate that the interaction between GRIP1 PDZ12 and Fras1 is quite specific. Each of the four C-terminal residues of Fras1 is directly involved in binding to GRIP1 PDZ12. It is unique to GRIP1 PDZ12 that it requires a negatively charged residue at the −1-position and a small residue at the −3-position, in addition to the expected Val(0) and Ser/Thr(−2), in its binding peptides.

PDZ2 adopts a highly similar overall fold to PDZ1 (Fig. 3c). Additionally, the peptide-binding grooves of the two PDZ domains are also highly similar (Fig. 6a and b). Specifically, the amino acid residues in PDZ1 (e.g., Arg84 and His114) that are responsible

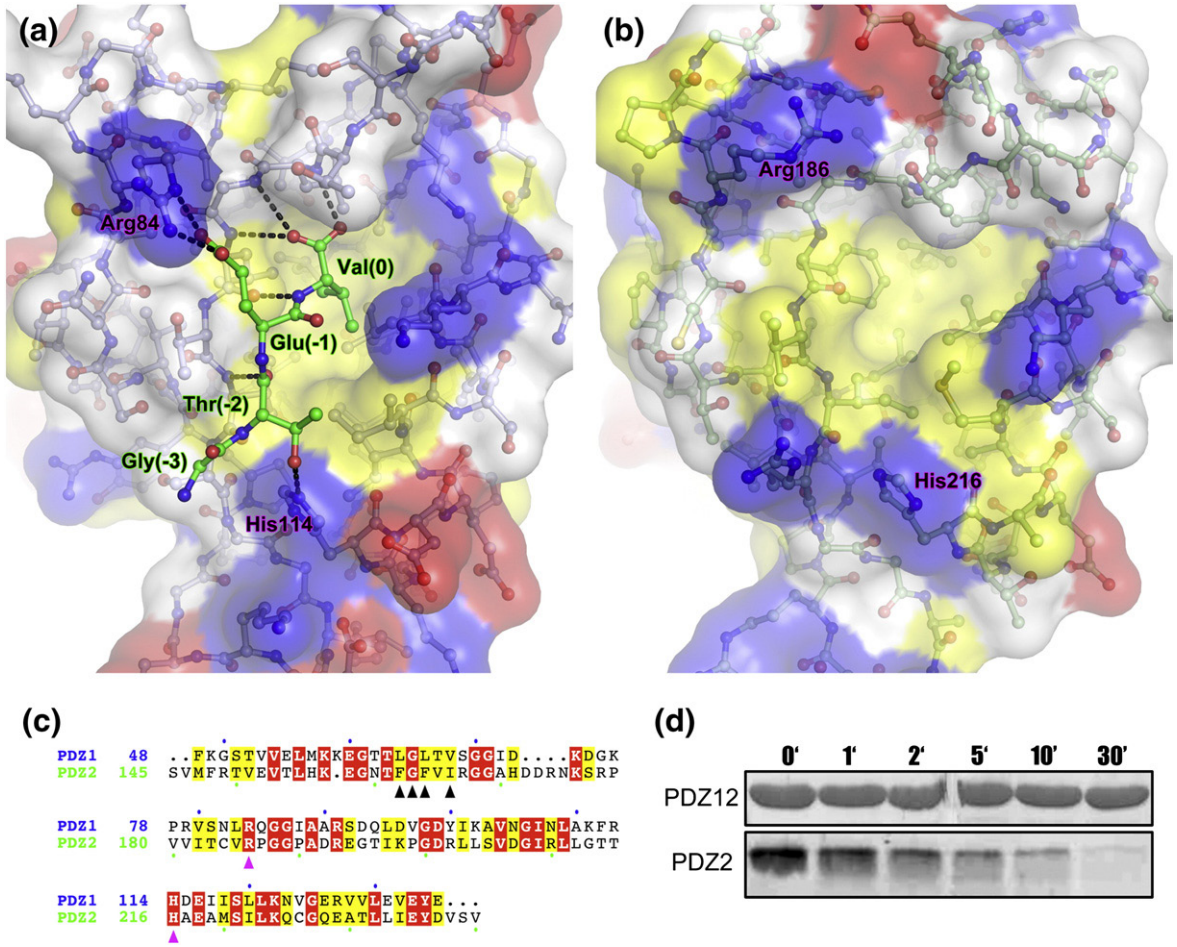

Fig. 6. Structural basis of the Fras1 peptide recognition by the GRIP1 PDZ12 tandem. (a,b) Combined stick–ball model and surface representation showing the detailed interactions of the Fras1 peptide with the residues from the target recognition groove of PDZ1 (a) and the hypothetical target-binding groove of PDZ2 (after removing the contacting PDZ1 domain) in the PDZ12 tandem (b). (c) Sequence alignment showing high amino acid sequence similarity between PDZ1 and PDZ2 of GRIP1. (d) Partial trypsin digestion showing that PDZ2 in the PDZ12 tandem is more stable than the isolated PDZ2. The trypsin digestion experiment was carried out at a similar fashion as described in Fig. 4c, except that the trypsin concentration in the digestion mixtures was further reduced.

for binding to the Fras1 peptide are found at the same positions in PDZ2 (e.g., Arg186 and His216). The conformational similarity between the two PDZ domains is correlated with their high amino acid sequence identity (Fig. 6c). However, the isolated PDZ2 binds to the Fras1 peptide with a significantly lower affinity than PDZ1 in PDZ12 does. We discovered, by partial trypsin digestion, that the isolated PDZ2 is considerably less stable than PDZ2 in the PDZ12 tandem, as the majority of PDZ2 was degraded within 30 min of trypsin digestion (Fig. 6d). In contrast, little degradation was observed for PDZ12 under the same conditions. The partial trypsin digestion data also indicate that the inter-domain interaction between PDZ1 and PDZ2 also enhances the stability of both PDZ domains. It is likely that the lower stability of the isolated PDZ2 largely accounts for its weaker target peptide binding. Consistent with this hypothesis, it has been recently demonstrated that the thermodynamic stability of a PDZ domain is directly correlated with its target-binding affinity.[26] Since the peptide-binding surface of PDZ2 is completely occluded by PDZ1 in the PDZ12 tandem (Fig. 3b), we believe that

Table 2. The binding affinities between GRIP1 PDZ12 and various peptides with different residues in the last four residues

Peptides	K_d (μM)
GTEV	9.4±0.3
GTE**G**	Not detectable[a]
G**D**E**L**	Not detectable
GT**N**V	170±34
ET**R**V	Not detectable
STEV	26±9
NTEV	114±15
ETEV	72±10
ESDV	100±26
NT**N**V	182±20
IT**K**V	Not detectable
QT**S**V	Not detectable

[a] See Ref. 10.

PDZ2 likely plays an auxiliary role by facilitating the folding of PDZ1.

Discussion

Supramodular nature of the GRIP1 PDZ12 tandem

The biochemical and structural data presented in this work clearly demonstrate that the first two PDZ domains in GRIP1 form a structurally and functionally distinct supramodule. In this PDZ12 supramodule, the folding of PDZ1 depends on the covalently linked PDZ2. Interestingly, the target-binding site of PDZ2 is completely occluded by the back side (i.e., the side opposite to the target-binding groove) of PDZ1, and therefore, only the target-binding site of PDZ1 is accessible in the GRIP1 PDZ12 tandem. Since the residues mediating PDZ1/PDZ2 interactions are completely conserved among GRIP proteins throughout the evolution (Fig. 5), it is likely that the supramodular nature of the GRIP1 PDZ12 tandem is a conserved feature in other GRIP isoforms. It is puzzling that nature "dedicates" PDZ2 of the GRIP PDZ12 tandem to facilitate the folding of PDZ1 at the "expense" of its own target-binding capacity, rather than economically building two autonomously folded and functional PDZ domains. One possible explanation is that the PDZ2-facilitated folding of PDZ1 allows the building of a regulatory switch into the PDZ12 tandem to control its ligand interaction. For example, the partially solvent-accessible Tyr134 in the PDZ1 and PDZ2 interface plays a critical role in the stability as well as the Fras1 peptide binding of PDZ12 (Fig. 4). Phosphorylation of Tyr134 is likely to be detrimental for the folding and the Fras1 peptide binding of PDZ12, as the substitution of Tyr134 with a Glu in PDZ12 is (Fig. 4). The GRIP1/Fras1 interaction is believed to be important for the trafficking of Fras1 and Frem2 to membranes and organization of cell adhesion complexes at the basement membranes of epidermal cells.[10,11] During the GRIP1-mediated trafficking, Fras1 is expected to be released from the GRIP1 scaffold once Fras1 has reached its destination. One plausible mechanism to release Fras1 from GRIP1 is simply by unfolding PDZ1 (e.g., by destabilizing PDZ1/PDZ2 interaction via Try134 phosphorylation). Although speculative at this stage, our biochemical and structural studies point to the feasibility of such target-releasing mechanism and serve as a working model for future experimental testing of this hypothesis.

The supramodular nature of GRIP1 PDZ12 bears similarity to that of PDZ45 in the same protein.[23] In GRIP1 PDZ45, the folding PDZ5 and, thus, its target binding similarly require the covalent attachment of PDZ4. However, the overall packing and the interdomain interactions of the two PDZ domains in PDZ12 and PDZ45 are significantly different. In the PDZ45 tandem, the two PDZ domains interact in a back-to-back fashion; hence, both target-binding grooves of the tandem are accessible to its potential binders. Additionally, the interdomain linker and a short stretch of amino acid residues N-terminal to PDZ4 play the most critical roles in stabilizing the PDZ45 tandem structure.

Implications to the Fraser syndrome

Fraser syndrome is a recessive human genetic disorder characterized by embryonic epidermal blistering, cryptophthalmos, syndactyly, renal defects, and mental retardation. To date, mutations of two cellular matrix proteins, Fras1 and Frem2, are known to be directly associated with this genetic disorder. Both Fras1 and Frem2 are cell matrix proteins critical for proper dermal–epidermal interactions during development. It is noted that a significant portion of Fraser syndrome patients do not contain mutations in either Fras1 or Frem2,[19,27] indicating that mutations in other genes are also likely to be involved in the disease. Since mutations of GRIP1 in mice caused phenotypes remarkably similar to the human Fraser syndrome,[10,16] *GRIP1* is likely a disease-associated gene. The association of *GRIP1* with the disease is reinforced by the finding that both Fras1 and Frem2 can directly bind to GRIP1.[10,11] It will be interesting to screen for possible GRIP1 mutation(s) in Fraser syndrome patients who do not contain mutations in Fras1 or Frem2. The biochemical characterization of the interaction between GRIP1 and the Fras1 peptide and the structure of the GRIP1 PDZ12/Fras1 peptide complex presented in this work clearly depicts the molecular basis governing the interaction of these two proteins. Additionally, the GRIP1 PDZ12/Fras1 peptide complex structure provides a molecular basis to predict whether mutations in GRIP1, if found in Fraser syndrome patients, will affect the interactions between GRIP1 and Fras1/Frem2.

Materials and Methods

Protein expression and purification

DNA fragments encoding rat PDZ1 (residues 48–142), PDZ2 (residues 143–243), PDZ3 (residues 144–336), PDZ12 (residues 48–243), and PDZ123 (residues 48–336) were amplified by PCR using the full-length GRIP1 cDNA as the template and individually cloned into an in-house modified version of the pET32a vector. All point mutations of GRIP1 PDZ domains described in this study were created using the standard PCR-based mutagenesis method and confirmed by DNA sequencing. Recombinant proteins were expressed in BL21 (DE3) *Escherichia coli* cells at 16 °C. Thioredoxin-fused or His_6-tagged GRIP1 PDZ domains expressed in bacterial cells were purified by Ni^{2+}–NTA agarose (Qiagen) affinity chromatography followed by size-exclusion chromatography. After protease 3C digestion, the N-terminal thioredoxin- or His_6-tag of each recombinant protein was cleaved by protease 3C, and the cleaved tag was removed by passing the digested mixture through a diethylaminoethyl–Sepharose anion-

exchange column. Uniformly ^{15}N-labeled PDZ domains were prepared by growing bacteria in M9 minimal medium using $^{15}NH_4Cl$ as the sole nitrogen source.

NMR spectroscopy

All NMR samples (0.2–0.4 mM) were dissolved in 100 mM potassium phosphate, pH 6.5, containing 10 mM d_{10}-dithiothreitol in 90% H_2O and 10% D_2O, unless otherwise indicated. NMR spectra were acquired at 30 °C on Varian Inova 500- or 750-MHz spectrometers.

Crystallography

Crystals of GRIP1 PDZ12 in complex with the Fras1 peptide were obtained by the hanging drop vapor diffusion technique at 16 °C. Freshly purified PDZ12 was concentrated to 30 mg/ml before a saturating amount of the Fras1 peptide (up to 4 molar ratio of peptide to PDZ12) was added. The Fras1 peptide containing the last 10 amino acid residues of rat Fras1 was commercially synthesized (GenScript Corp., New Jersey). The PDZ12/Fras1 peptide mixture was set up in hanging drops with an equal volume of 0.2 M magnesium acetate tetrahydrate and 0.1 M sodium cacodylate trihydrate, pH 6.5, containing 20% (w/v) polyethylene glycol 8000. A 2.3-Å-resolution X-ray data set was collected at 100 K by a Rigaku R-AXIS IV++ imaging-plate system with a Rigaku MicroMax-007 copper rotating-anode generator. The crystallization solution plus 15% glycerol was used as a cryoprotectant. The diffraction data were processed and scaled using the MOSFLM[28] and SCALA[29] modules in the CCP4 suite.

Molecular replacement was employed to solve the phase problem by using the second PDZ domain structures of Dlg2 [Protein Data Bank (PDB) ID: 2BYG] and Dlg3 (PDB ID: 2FE5) as the search models with PHASER.[30] The phase improvement and the initial model building were performed by RESOLVE.[31] The initial model was rebuilt manually and then refined using REFMAC[32] against the 2.3-Å-resolution data set. Further manual model building and adjustment were completed using COOT.[33] Noncrystallographic symmetry restraints were applied at the beginning of the refinement and were released at the final step. The stereochemical quality of the final model was validated by PROCHECK.[34] The final refinement statistics are listed in Table 1.

Fluorescence anisotropy measurements

Fluorescence anisotropy binding assays were performed on a PerkinElmer LS-55 fluorimeter equipped with an automated polarizer at 25 °C. Fluorescein isothiocyanate (FITC)-labeled Fras1 carboxyl peptide (FITC-NNLQDGTEV) was commercially synthesized (GenScript Corp.), and the purity of the peptide was >95%. Fluorescence titration was performed with an increasing amount of unlabeled PDZ proteins and a constant amount of FITC-labeled peptide (1 μM). The titration curves were fitted with the MicroCal Origin software package.

Circular Dichroism

CD spectra of PDZ proteins were measured on a JASCO J-815 CD spectropolarimeter at room temperature using a cell path length of 1 mm. Each spectrum was collected with 10 scans spanning a spectral window of 190–250 nm. The proteins were dissolved in 10 mM Tris–HCl, pH 7.5, containing 1 mM DTT. The protein concentration used in the CD experiment was 15 μM.

Partial trypsin digestion

Partial trypsin digestion was carried out by mixing ~1 mg of each PDZ protein with 0.05 mg of trypsin in 200 μl digestion buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 1 mM DDT) at room temperature. The digestion reactions were terminated by adding the sample loading dye buffer for SDS-PAGE.

PDB accession numbers

The atomic coordinates and structural factors for the structure of the GRIP1 PDZ12 tandem in complex with the Fras1 peptide have been deposited in the PDB‡ with the accession code 2QT5.

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