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Interdomain Chaperoning between PSD-95, Dlg, and Zo-1 (PDZ) Domains of Glutamate Receptor-interacting Proteins*

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The multiple PSD-95, Dlg, and Zo-1 (PDZ) domain protein, glutamate receptor-interacting protein (GRIP), is involved in the clustering and trafficking of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor by directly binding to the cytoplasmic tail of the receptor's GluR2 subunit. Both the forth and fifth PDZ domains (PDZ4 and PDZ5) of GRIP are required for effective binding to the receptor. Using NMR and circular dichroism spectroscopic techniques, we show that PDZ5 is completely unstructured in solution. Freshly prepared PDZ4 is largely folded, but the domain can spontaneously unfold. Neither PDZ4 nor PDZ5 binds to GluR2 in solution. Unexpectedly, when PDZ4 and PDZ5 are covalently connected (*i.e.* PDZ45), both PDZ domains become well folded and stable in solution. The covalent linkage of the two PDZ domains is essential for proper folding of the tandem PDZ domains and its effective binding to GluR2. The interdomain chaperoning effect observed in the PDZ domains of GRIP represents a previously uncharacterized function of PDZ domains.

Ion channels and receptors are typically clustered and localized at the specific subcellular sites of neurons such as synapses. Clustering of ion channels is important for concentrating current flux across a small area of membrane and for maximizing responses to local neurotransmitters. Moreover, changes in the distribution and density of channels and receptors may play a role in long term synaptic plasticity and information storage in the brain. In the excitatory synapses, signal transmission is mediated primarily by *N*-methyl-D-aspartate and AMPA[1] glutamate receptors.

PDZ (PSD-95, Disc-large, and ZO-1) homologous domain-containing proteins play fundamental roles in clustering ion channels/receptors as well as assembling their downstream signaling components (1–5). A typical PDZ domain contains ~90 amino acid residues (6) and folds into a compact globular structure comprising a six-stranded β-barrel flanked by two α-helices (7, 8). A common PDZ domain-mediated interaction involves the binding of a short peptide fragment located at the extreme termini of target proteins to a groove formed by the second α-helix (αB) and the second β-strand (βB) of the PDZ domain (8–11). The carboxyl peptide pairs with the βB strand of the PDZ domain in an anti-parallel fashion. In addition to binding to COOH-terminal peptides, PDZ domains can also interact with other PDZ domains, forming homo- or hetero-PDZ dimers (12–17). Formation of PDZ/PDZ dimer is particularly interesting, since this may allow formation of super-molecular weight complexes mediated by multiple PDZ domain-containing scaffold proteins.

Interaction between AMPA receptor and a pair of multiple PDZ proteins, GRIP and ABP, is mediated by the GluR2 subunit of the receptor (13, 18). The interaction between the AMPA receptor and GRIP/ABP may serve a major role in the receptor insertion to the synaptic membrane, a process directly linked to the synaptic plasticity (19–22). Effective binding of the cytoplasmic tail of GluR2 to GRIP requires both the fourth and the fifth PDZ domains of GRIP (18). The requirement of two PDZ domains for proper function is certainly not unique to GRIP proteins. Both *in vitro* syntenin-syndecan binding and proper membrane localization of syntenin require the paired PDZ domains of syntenin (23, 24). The first and second PDZ domains of hDlg together are essential for its binding to cytoskeletal protein 4.1 (25). These emerging observations suggest that tandemly arranged PDZ repeats possess functional features that differ from the simple sum of the properties of the individual PDZ domains. However, current biochemical and structural data cannot provide mechanistic insights into the unique biological functions presented by tandem PDZ domains.

In this work, we report that PDZ4 of GRIP induces *de novo* folding of PDZ5, which, by itself, is unstructured in solution. The mutual chaperoning of PDZ4 and PDZ5 requires the two domains to be covalently linked to each other. The well folded PDZ45 can interact with the GluR2 subunit of the AMPA receptor. The results presented in this work expand our current knowledge of the functional roles of PDZ domains.

MATERIALS AND METHODS

A synthetic peptide (NVYGIESVKI) corresponding to the last 10 amino acid residues of GluR2 (Asn^{874}–Ile^{883}) was synthesized by Research Genetics (Huntsville, VA). The peptide was purified using reverse phase high pressure liquid chromatography.

Cloning, Expression, and Purification of PDZ Domains of GRIP—DNA fragments encoding PDZ4 (amino acids 462–567), PDZ5 (amino acids 564–663), and PDZ45 (amino acids 462–663) of rat GRIP1 were polymerase chain reaction-amplified from the full-length cDNA of the protein (provided by Dr. Morgan Sheng) with specific primers. The amplified DNA fragments of PDZ4, PDZ5, and PDZ45 were inserted into the *Bam*HI and *Xho*I sites of a modified version of pET32a (Novagen) in which the DNA sequences encoding the S-tag and thioredoxin were removed. The recombinant plasmids harboring the respective target genes were transformed into *Escherichia coli* BL21 (DE3) host cells individually for large scale protein preparation. Uniformly ^{15}N-

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[1] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; GRIP, glutamate receptor-interacting protein; PDZ, PSD-95, Dlg, and Zo-1; GST, glutathione *S*-transferase; HSQC, heteronuclear single quantum coherence.

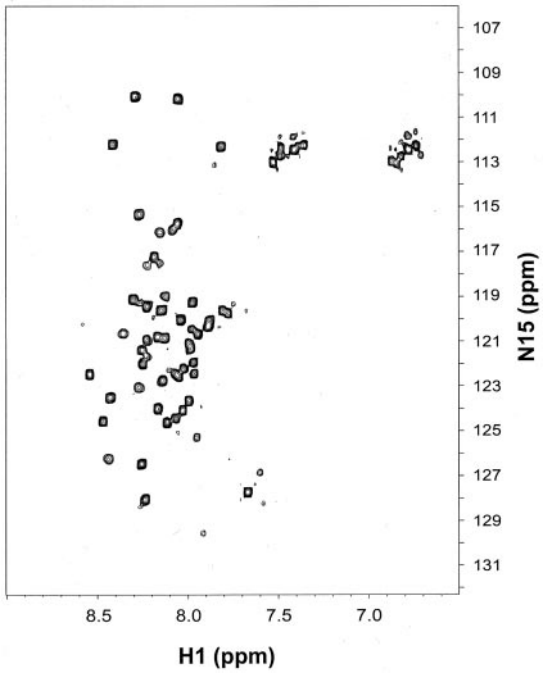

FIG. 1. **The cytoplasmic tail of GluR2 is unstructured in aqueous solution.** The ^{1}H-^{15}N HSQC spectrum of the COOH-terminal 50 amino acid residues of GluR2 was recorded with a 0.5 mM ^{15}N-labeled GluR2 sample dissolved in 100 mM potassium phosphate buffer at pH 6.0.

labeled proteins were prepared by growing bacteria in M9 minimal medium using $^{15}NH_4Cl$ (1 g/liter) as the sole nitrogen source.

His-tagged PDZ4, PDZ5, and PDZ45 were purified by a combination of a Ni^{2+}-nitrilotriacetic acid affinity (Qiagen), gel filtration, and DEAE-Sepharose ion-exchange chromatographic techniques.

Expression and Purification of the COOH-terminal Tail of GluR2—The cDNA encoding the COOH-terminal 50 residues of the GluR2 cytoplasmic tail was polymerase chain reaction-amplified from the full-length clone (provided by Dr. Morgan Sheng) and inserted into a modified version of pET32a vector. The His-tagged, thioredoxin-fused GluR2 was expressed as described for the PDZ domains of GRIP. The fusion protein was purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography. The His tag and thioredoxin parts of the fusion protein were cleaved by thrombin digestion and removed by a second round of Ni^{2+}-nitrilotriacetic acid affinity chromatography. The remaining contaminating proteins were removed by passing the GluR2 peptide containing mixture through a Sephacryl S-100 gel filtration column. The purified GluR2 fragment was dialyzed in the NMR sample buffer and subsequently concentrated by ultrafiltration for NMR studies.

Analytical Gel Filtration Chromatography—The molecular masses of various PDZ domains of GRIP were determined by analytical gel filtration chromatography using a Superose 12 column (Amersham Pharmacia Biotech). The column buffer was 0.1 M sodium phosphate, pH 6.0, containing 0.15 M NaCl, 1 mM dithiothreitol, and 1 mM EDTA.

Blot Overlay Assay of the Interaction between GluR2 and GRIP PDZ Domains—The purified COOH-terminal 50-amino acid residue fragment of GluR2 fused to the COOH-terminal end of GST was first resolved on a 12.5% SDS-polyacrylamide gel. GST-GluR2 was then transferred to a polyvinylidene difluoride membrane. The membrane was subsequently blocked with 10% skim milk in 50 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and 0.1% Tween 20. The polyvinylidene difluoride membrane strips were then incubated with purified, His-tagged PDZ4, PDZ5, and PDZ45, respectively, in the same buffer for 1 h at room temperature. After extensive washing, the bound His-tagged PDZ proteins on the strips were immunodetected by anti-His tag monoclonal antibody (Amersham Pharmacia Biotech).

Circular Dichroism—CD spectra of PDZ proteins were measured on a JASCO J-720 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 dithiothreitol. The protein concentration used in the CD experiment was 15 μM.

NMR Spectroscopy—All NMR samples were dissolved in 100 mM sodium phosphate buffer, pH 6.0, containing 10 mM d_{10}-dithiothreitol in 90% H_2O, 10% D_2O. NMR spectra were acquired at 30 °C on a four-channel Varian Inova 750-MHz spectrometer equipped with an actively *z*-gradient shielded triple resonance probe.

RESULTS

The Cytoplasmic Tail of GluR2 Adopts a Random Coil Structure in Solution—To better understand the functional role of the cytoplasmic tail of GluR2, we characterized its structure by NMR spectroscopy. Large quantities of ^{15}N-labeled whole cytoplasmic tail domain of GluR2 (amino acids 834–883) were obtained using the protein expression and purification scheme developed in this work. Fig. 1 shows the ^{1}H-^{15}N HSQC spectrum of the domain in 100 mM sodium phosphate buffer, pH 6.0. The narrow ^{1}H and ^{15}N chemical shift dispersions in Fig. 1 clearly indicated that the cytoplasmic tail of GluR2 adopts a random coil structure in aqueous solution. A ^{15}N-separated three-dimensional nuclear Overhauser effect spectroscopy experiment of the same sample further confirmed the random coil structure of the domain (data not shown).

Individual PDZ Domains of PDZ4 and PDZ5 Are Unstable and Do Not Interact with GluR2—To dissect functional roles of PDZ4 and PDZ5 of GRIP in binding to GluR2, we studied the structure of the individual PDZ domains in solution. The ^{1}H-^{15}N HSQC spectrum of the freshly prepared PDZ4 displayed good chemical shift dispersions in both ^{1}H and ^{15}N dimensions typical for structured proteins (Fig. 2A, *red*). Detailed inspection of the NMR spectrum of the freshly prepared PDZ4 (Fig. 2*A*, *red*) revealed a second set of weak resonances with very narrow line width and narrow ^{1}H chemical shift dispersion (7.8–8.7 ppm), suggesting the existence of a random coil-like conformation in PDZ4. The CD spectrum of the freshly prepared PDZ4 was also somewhat anomalous (Fig. 3*A*), since the α-helix content of the domain estimated from the spectrum was far more than that expected for the conventional PDZ domains (~15% of amino acids adopt α-helical structure in PDZ domains). We further observed that the intensities of peaks in the ^{1}H-^{15}N HSQC spectrum of PDZ4 corresponding to the random coil conformation continuously increased as a function of time (both at 30 and 4 °C). Fig. 2*B* shows the ^{1}H-^{15}N HSQC spectrum of PDZ4 after the sample had been stored in a 4 °C refrigerator for 5 days. In this spectrum, the resonances corresponding to the random coil conformation of the protein dominated the spectrum. Although still detectable, the resonances for the folded PDZ4 became much weaker. CD spectra of PDZ4 also experienced dramatic changes with time. The CD spectrum of a 5-day-old PDZ4 sample resembled that of a random coil protein (Fig. 3*A*). Possible proteolytic degradation of the aged PDZ4 samples was ruled out by checking the integrity of the protein using SDS-polyacrylamide gel electrophoresis (data not shown).

Unexpectedly, the ^{1}H-^{15}N HSQC spectrum of GRIP PDZ5 is typical for a completely unstructured protein (Fig. 2*C*). The CD spectrum of the protein at a much lower concentration also indicated that PDZ5 adopts a random coil structure in solution (Fig. 3*A*). Analytical gel filtration chromatography experiment showed that PDZ5 did not form large molecular weight aggregates at concentrations used for both CD and NMR experiments (data not shown).

We next assayed possible interactions between GluR2 and PDZ4 and PDZ5, respectively. The addition of excess molar ratio amount of a 10-residue GluR2 tail peptide (NVYGIESVKI) to ^{15}N-labeled PDZ4 or PDZ5 did not induce chemical shift changes in the ^{1}H-^{15}N HSQC spectra of both PDZ domains (data not shown), indicating that the GluR2 peptide does not bind to either PDZ domain. We also used a blot overlay assay to detect potential binding of GluR2 to PDZ4 and PDZ5. In this assay, the entire

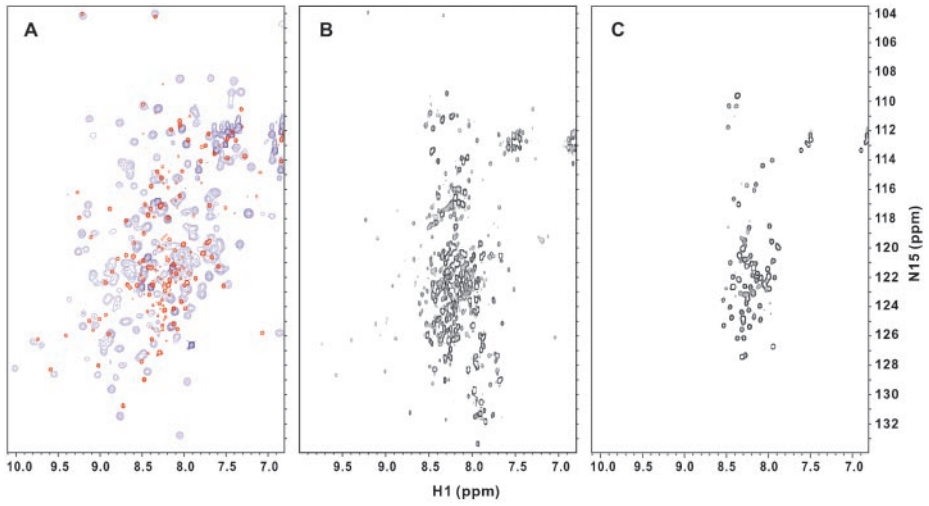

FIG. 2. **PDZ4 and PDZ5 in GRIP mutually chaperone each other.** *A*, an overlay plot of the ^{1}H-^{15}N HSQC spectra of the freshly prepared PDZ4 (*red*) and PDZ45 (*blue*). *B*, ^{1}H-^{15}N HSQC spectrum of a PDZ4 sample that was left at 4 °C for 5 days. The resonances corresponding to the folded PDZ4 (*A*, *red*) is much weaker but still observable. *C*, ^{1}H-^{15}N HSQC spectrum of PDZ5. All of the NMR spectra were recorded at 30 °C with protein concentrations of ~0.5 mM. The NMR samples were dissolved in 100 mM potassium phosphate buffer at pH 6.0.

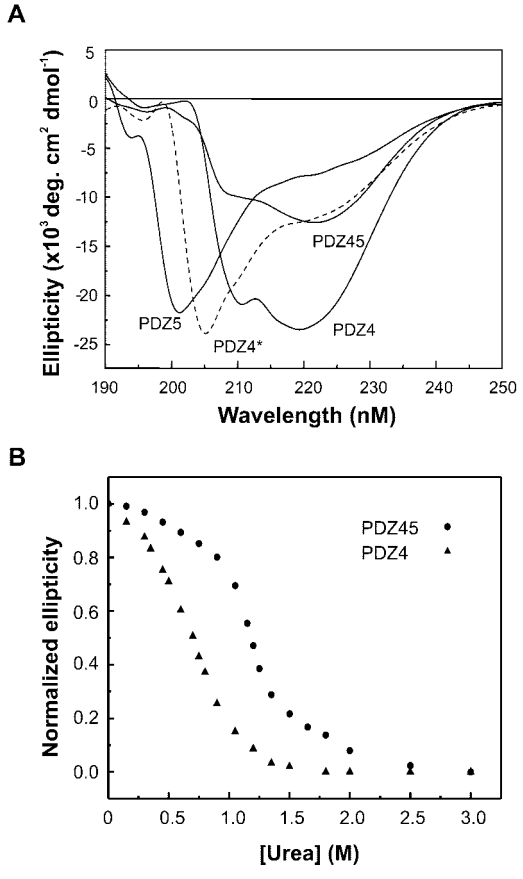

FIG. 3. **Stability of PDZ4, PDZ5, and PDZ45 of GRIP.** *A*, characterization of the folding of PDZ4, PDZ5, and PDZ45 by far-UV-CD spectroscopy. The freshly prepared PDZ4 spectrum was shown using a *solid line* (labeled *PDZ4*), and the spectrum for the "aged" PDZ4 was drawn as a *dashed line* (labeled *PDZ4**). The CD spectra of PDZ5 and PDZ45 were labeled with their respective names. *B*, denaturation profiles of PDZ4 (freshly prepared) and PDZ45 as a concentration of urea. The CD spectra of the proteins at different concentrations of urea were measured. The ellipticities of each spectrum at 222 nm were used to construct the denaturation curves shown in the *figure*.

COOH-terminal tail of GluR2 was fused to GST. GST-GluR2 was chosen as the immobilized phase in the overlay assay, since GluR2 adopts a random coil structure in solution (Fig. 1), and proper refolding of GluR2 was not a concern in the experiment. Consistent with the NMR titration experiment, neither PDZ4 nor PDZ5 showed detectable interaction with GST-GluR2 (Fig. 4*A*).

PDZ4 and PDZ5 Mutually Chaperone Each Other in Covalently Linked PDZ45—To compare structural difference of PDZ45 with individual PDZ domains of PDZ4 and PDZ5, a ^{1}H-^{15}N HSQC spectrum of PDZ45 was recorded under the identical condition used for the individual PDZ domains (Fig. 2*A*, *blue*). The chemical shift dispersion and line width of the spectrum indicated that both domains of PDZ45 were well folded with a uniform conformation in solution. The CD spectrum of PDZ45 also showed that the protein was well folded (Fig. 3*A*). The NMR and CD spectra of PDZ45 remained identical when the protein samples were "aged" for 2 weeks, suggesting that the folded PDZ45 conformation is stable in solution. We compared denaturation profiles of PDZ4 and PDZ45 as a concentration of urea (Fig. 3*B*). Consistent with NMR studies, the freshly prepared PDZ4 readily denatured in the presence of low concentrations of urea, whereas PDZ45 displayed a clear transition from folded to denatured states when the concentration of urea increased.

The overlay plot of the ^{1}H-^{15}N HSQC spectra of PDZ4 (*red*) and PDZ45 (*blue*) showed that the majority of resonances of PDZ4 did not overlap with the peaks of PDZ45, suggesting that PDZ4 undergoes a significant structural change when it is linked with PDZ5. Such structural changes presumably resulted from direct contacts between PDZ4 and PDZ5. The *de novo* folding of PDZ5 induced by PDZ4 was obvious from the complete disappearance of the random coil peaks in the PDZ45 ^{1}H-^{15}N HSQC spectrum. Taken together, we conclude that PDZ4 and PDZ5 mutually promote each other's folding and stability.

We then investigated whether the chaperoning effect between PDZ4 and PDZ5 was intra- or intermolecular in nature. Mixing of equal or excess molar ratio amount of unlabeled PDZ4 with ^{15}N-labeled PDZ5 did not change the NMR spectrum of PDZ5. In a reverse experiment, the addition of unlabeled PDZ5 did not change the NMR spectrum of ^{15}N-labeled PDZ4 (data not shown). The above data strongly indicated that the chaperoning effect requires both PDZ domains to be covalently connected to each other and that the two PDZ domains stabilize each other in an intramolecular fashion.

To assess functional consequence of the mutual chaperoning effect between PDZ4 and PDZ5, we assayed interaction between GluR2 and PDZ45. A number of backbone amide resonances of PDZ45 experienced peptide-induced chemical shift changes in the overlay plot of the ^{1}H-^{15}N HSQC spectra of free PDZ45 (*black*) and PDZ45 saturated with the 10-residue GluR2 peptide (*red*), indicating that PDZ45 binds to the GluR2 peptide (Fig. 4*B*). The interaction between GluR2 and PDZ45 was also confirmed in the blot overlay assay (Fig. 4*A*).

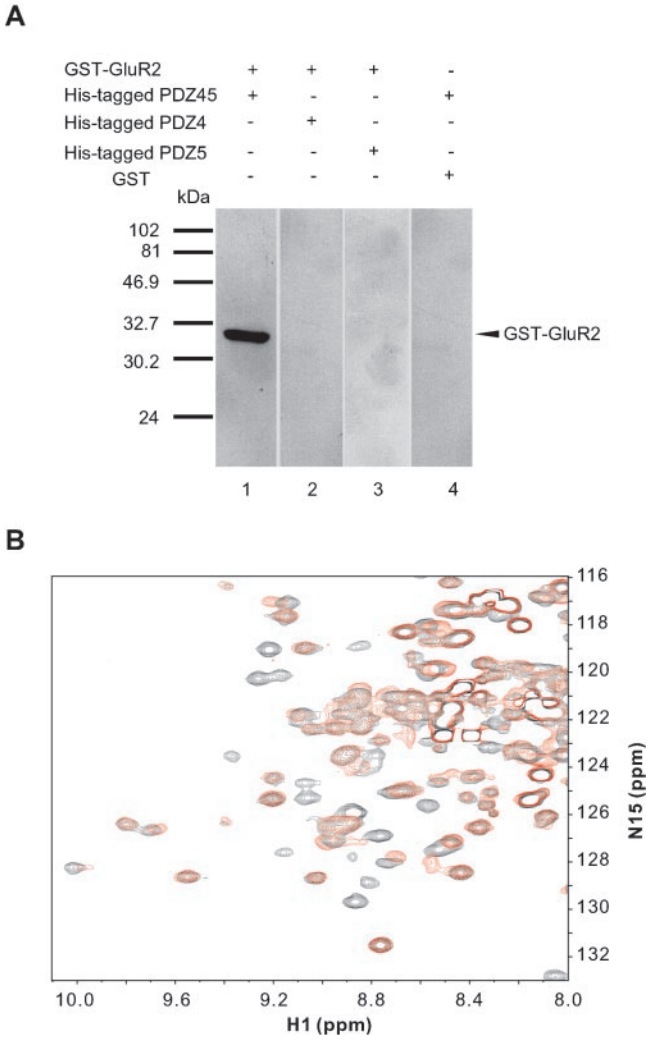

FIG. 4. **Interaction of PDZ45 with GluR2.** *A*, blot overlay assay of the binding of GluR2 to PDZ45 (*lane 1*), PDZ4 (*lane 2*), and PDZ5 (*lane 3*). Purified GST was used as a negative control of the interaction between PDZ45 and GluR2 (*lane 4*). About ~3 μg of GST-GluR2 was loaded to the each lane of the SDS-polyacrylamide gel, and the protein was transferred to a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was cut into strips, and each strip was overlaid with various PDZ domains. The concentration of the PDZ domains used in the overlay solution was 15 μg/ml. *B*, a superposition plot of the ^{1}H-^{15}N HSQC spectra of free PDZ45 (*black*) and its complex with the GluR2 peptide (*red*). For clarity, only a selected region of the NMR spectra was shown.

PDZ4 and PDZ45 Form Dimer in Solution—PDZ domain-mediated multimerization represents an important property of the GRIP/ABP family multi-PDZ domain proteins (13, 26). To understand how PDZ domains of GRIP1 might associate with each other, we measured molecular masses of various PDZ domains by analytical gel filtration chromatography (Fig. 5). His-tagged PDZ4 and PDZ45 were eluted at molecular masses of 23.9 and 46.8 kDa, respectively, which matched the dimer molecular weight of both proteins. The data indicated that PDZ4 forms homodimer in solution. The result also suggested that PDZ5 is not likely to be involved in the dimerization of PDZ45.

DISCUSSION

Increasing experimental evidence suggests that PDZ domains in multi-PDZ domain-containing proteins are grouped into functional units. Two or more PDZ domains are often found to be necessary to mediate specific interactions with the COOH-terminal peptides of target proteins. We chose to study

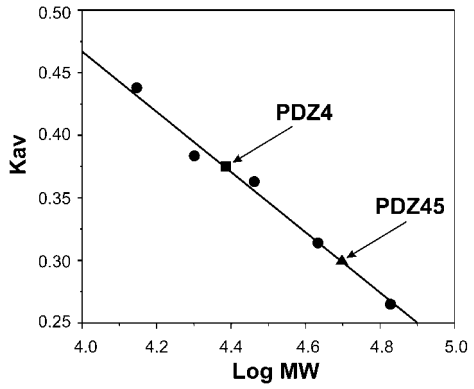

FIG. 5. **Dimerization of the PDZ4 domain in GRIP.** The molecular masses of PDZ4 and PDZ45 were determined by analytical gel filtration chromatography. One hundred microliters of protein samples (0.5 mg/ml) was injected into the gel filtration column. The column was calibrated using ribonuclease A (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), bovine serum albumin (66 kDa), and blue dextran 2000 (2000 kDa). K_{av} is defined as $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of a protein, and V_0 and V_t are the void and total volume of the column, respectively.

GRIP PDZ45 in this work, hoping to gain insights into the structural interplay of the two PDZ domains and to provide a mechanistic understanding of grouped PDZ domain-mediated target recognition.

The most important discovery of this work is that PDZ domains in GRIP mutually chaperone each other, enabling the tandem PDZ domains (PDZ45 of GRIP) to interact with its target (GluR2). We showed that PDZ5 alone is completely unstructured in solution. Although it is largely folded, the freshly prepared PDZ4 domain can spontaneously unfold. In PDZ45, both PDZ domains are well folded and stable. It is interesting to note that the mutual chaperoning effect between PDZ4 and PDZ5 occurs only when the two PDZ domains are covalently connected, since simple mixing of the two individual domains does not change their respective structures. This result also implies that the biological function of tandem PDZ domain may not be attained by association of two PDZ domains from different proteins. Since the folded PDZ4 cannot bind to GluR2, we suggest that PDZ5 in the GRIP is directly involved in binding to GluR2. Consistent with this interpretation, PDZ5, but not PDZ4, of ABP robustly interacts with GluR2 (13). Although PDZ4 in GRIP is not likely to be involved in binding to GluR2, this domain may bind to other neuronal proteins. In a recent yeast two-hybrid screening, a number of proteins were found to interact with PDZ456 of GRIP (26). Further work is required to determine whether PDZ4 is direct involved in binding to these targets. We further note that PDZ4 domain can dimerize. The dimerization of PDZ4 is somewhat intriguing, since the construct of PDZ4 domain used in this work contains only a canonical PDZ domain sequence. It is unlikely that PDZ4 can dimerize using a β-finger-mediated mechanism observed in the PSD-95/neuronal nitric-oxide synthase PDZ dimmer (11, 16, 17). Detailed structural studies will be required to elucidate how two canonical PDZ domains might interact with each other to form a dimmer. The data in this study suggested that the function of PDZ4 in GRIP is likely to promote folding of PDZ5 and to assemble GRIP into a multimeric complex (27). The binding of PDZ5 to PDZ4 in PDZ45 in turn stabilizes the structure of PDZ4. The mutual chaperoning function of PDZ domains represents a previously uncharacterized property of PDZ domains. The work provides a mechanistic understanding of why both PDZ4 and PDZ5 are required for the binding between GRIP and GluR2. Further work is necessary to determine whether chaperoning effect between PDZ domains also

occurs in other PDZ domain-containing proteins. What we do know is that tandemly arranged PDZ domains (*e.g.* PDZ1 and PDZ2 in PSD-95) may use different mechanisms in ensuring their targeting specificity.[2] It is clear that combinations of protein modules often render new biological functions in addition to the ones provided by the individual domains in multidomain proteins. The much more complicated protein domain organizations found in human proteins with respect to yeast and *Caenorhabditis elegans* partially explain why we only need ~30,000 genes in our genome (28, 29).

Functional AMPA receptors contain at least one GluR2 subunit. The cytoplasmic tail of GluR2 contains 50 amino acid residues. This 50-amino acid segment plays central roles in regulating AMPA receptor-mediated signaling events by binding to a number of regulatory proteins. For example, *N*-ethylmaleimide-sensitive fusion protein binds to a short segment of the GluR2 tail (30–32). Interaction between protein-tyrosine kinase Lyn and GluR2 was also confined to the GluR2 tail (33). A number of PDZ domain proteins including GRIP, ABP, and PICK1 interact with the extreme carboxyl terminus of GluR2 (13, 18, 34). In addition, the GluR2 tail can be phosphorylated by protein kinase C (19, 22). It is rather mysterious how this short stretch of peptide fragment encodes such an incredible number of functions of the receptor. We show by NMR spectroscopy that the GluR2 tail is completely unstructured in aqueous solution, suggesting that the linear amino acid sequence rather than its higher order structure defines the function of the receptor tails. However, it is possible that the GluR2 tail may adopt a different conformation when it is covalently linked to the fourth transmembrane segment of the receptor.

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[2] M. Zhang, unpublished data.