Glutamate receptor interacting protein 1 (GRIP1) is a scaffold protein composed of seven PDZ (Postsynaptic density-95/Discs large/Zona occludens-1) domains. The protein plays important roles in the synaptic targeting of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The interaction between GRIP1 PDZ7 and a Ras guanine nucleotide exchange factor, GRASP-1, regulates synaptic distribution of AMPA receptors. Here, we describe the three-dimensional structure of GRIP1 PDZ7 determined by NMR spectroscopy. GRIP1 PDZ7 contains a closed carboxyl group-binding pocket and a narrow αB/β-groove that is not likely to bind to classical PDZ ligands. Unexpectedly, GRIP1 PDZ7 contains a large solvent-exposed hydrophobic surface at a site distinct from the conventional ligand-binding αB/β-groove. NMR titration experiments show that GRIP1 PDZ7 binds to GRASP-1 via this hydrophobic surface. Our data uncover a novel PDZ domain-mediated protein interaction mode that may be responsible for multimerization of other PDZ domain-containing scaffold proteins.

Postsynaptic density-95/Discs large/Zona occludens-1 (PDZ) domains are among the most abundant protein-interacting modules in the genomes of metazoans (1). PDZ domain-containing proteins have been shown to play important roles in diverse cellular processes including receptor/ion channel clustering, signaling complex assembly, and protein targeting (2–4). A typical PDZ domain contains ~90 amino acid residues folded into a compact globular structure comprising a six-stranded β-barrel flanked by two α-helices (5, 6). The majority of PDZ domain-mediated interactions involves the binding of short peptide fragments located at the extreme carboxyl termini of target proteins to grooves formed by the second α-helix (αB) and the second β-strand (βB) of the PDZ domains (6–9). The carboxyl peptide augments the βB strand in an anti-parallel fashion. Amino acid residues at the 0 and −2 positions of the carboxyl peptide play dominant roles in the peptide binding to a cognate PDZ domain, although residues at the −1 and −3 positions and those at further upstream positions also contribute to the binding (10). In addition to binding to carboxyl peptides, PDZ domains can also use the αB/β-target-binding groove to interact with internal short peptide fragments with defined structures (9, 11, 12).

Glutamate receptor-interacting protein 1 (GRIP1) contains seven PDZ domains and interacts through PDZ4 and PDZ5 with the COOH-terminal “ESVKI” sequence of the GluR2/3 subunits of AMPA receptors (13, 14). GRIP1 has several closely related gene products, GRIP2 and AMPA receptor binding protein (ABP), and they are all able to bind to AMPA receptors (15, 16). In addition, the GRIP family proteins can interact through PDZ6 with EphB2/EphA7 receptor tyrosine kinase and the ephrinB1 ligand (17–19). Genetic studies have shown that GRIP1+/− mice are embryonic lethal, and the mutant embryos develop abnormalities in the dermo-epidermal junction (20). GRIP1 additionally interacts through its PDZ7 with GRASP-1, a Ras guanine-nucleotide exchange factor that regulates the synaptic distribution of AMPA receptors (21). The interaction between GRIP1 PDZ7 and GRASP-1 is rather unusual, as GRASP-1 does not contain obvious PDZ-interacting sequence at its carboxyl terminus. Biochemical analysis has indicated that a 100-residue fragment located at the COOH-terminal end of GRASP-1 is responsible for the interaction of the protein with GRIP1 (21).

The solution structure of the PDZ7 of GRIP1 determined in this study shows that the conventional target-binding groove formed by the αB helix and the βB strand adopts a closed conformation that is not likely to be capable of interacting with carboxyl peptides. Unexpectedly, GRIP1 PDZ7 contains a solvent-exposed hydrophobic surface formed by the βE strand, the αB helix, and the loop connecting the two secondary structural elements. We show that GRIP1 PDZ7 interacts with GRASP-1 via this hydrophobic surface. The data presented in this study uncover a novel mode of PDZ domain-mediated protein-protein interaction.

MATERIALS AND METHODS

Expression and Purification of GRIP1 PDZ7 and GRIP-binding Domain of GRASP-1—The seventh PDZ domain of rat GRIP1 (amino acid residues 980–1070) and the COOH-terminal 100-residue fragment of rat GRASP-1 were polymerase chain reaction-amplified from the respective full-length cDNAs of the proteins with specific primers. The amplified DNA fragments were inserted into a modified version of pET32a (Novagen) in which the DNA sequences encoding the S-tag and thioredoxin were removed. The resulting recombinant plasmid harboring the respective target genes were individually transformed into Escherichia coli BL21(DE3) host cells for large scale protein productions. Uniformly 15N- and 15N/13C-labeled proteins were prepared by

Received for publication, July 18, 2002, and in revised form, August 19, 2002
Published, JBC Papers in Press, August 23, 2002, DOI 10.1074/jbc.M207206200

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growing bacteria in M9 minimal medium using \( ^{13}\text{NH}_4\text{Cl} \) (1 g/liter) and \( ^{13}\text{C}_2\text{H}_2\) (1 g/liter) as stable isotope sources.

Recombinant GRIP1 PDZ7 and GRASP-1 fragment were purified by a combination of nickel-nitrotriacetic acid affinity column (Qiagen) followed by gel filtration chromatography. The purified recombinant proteins contained an NH\(_2\)-terminal His tag carried over from the cloning vector.

**NMR Experiments**—Four NMR samples were prepared for structural determination of GRIP1 PDZ7 with a protein concentration of \( \sim 1.0 \) mM (unlabeled PDZ7 in 99.9% D\(_2\)O, \( ^{15}\text{N}\)-labeled protein in 90% H\(_2\)O/10% D\(_2\)O, two \( ^{15}\text{N}/^{13}\text{C}\)-labeled samples in 99.9% D\(_2\)O and in 90% H\(_2\)O/10% D\(_2\)O). The protein was dissolved in 100 mM potassium phosphate buffer at pH 6.5 (direct meter reading). The \( ^{15}\text{N}\)-labeled sample in 90% H\(_2\)O/10% D\(_2\)O was also used for backbone relaxation data measurements.

All of the NMR experiments were carried out at 25 °C on Varian Inova 500 and 750 spectrometers equipped with 5 mm shielded triple resonance probes. NMR spectra were processed with the nmrPipe software package (22) and analyzed using PIP (23) and Sparky (www.cgl.ucsf.edu/home/sparky). Sequential backbone resonance assignments of the protein were obtained by standard heteronuclear correlation experiments including HNCO, HNCCAB, and CB-CA(CO)NH and confirmed by a three-dimensional \( ^{15}\text{N}\)-separated NOESY experiment (24, 25). The non-aromatic non-exchangeable side-chain assignments were obtained using an HCC-TOCSY experiment. The side chains of the aromatics were assigned by \( ^{1}H\) two-dimensional TOCSY and NOESY experiments with an unlabeled protein sample in D\(_2\)O (26). The \( \beta\)-NH\(_2\) side chains of Asn and Gln residues were assigned by a three-dimensional \( ^{15}\text{N}\)-separated NOESY experiment with the \( ^{15}\text{N}\)-labeled protein dissolved in H\(_2\)O.

The pulse sequences used to obtain the \( ^{1}H\)-longitudinal relaxation times, \( T_1\), the \( ^{15}\text{N}\) spin-lattice relaxation times, \( T_2\), and the \( ^{1}H\)/\( ^{15}\text{N}\) steady-state NOE values were described previously (27). All of the data were processed using nmrPipe and nmrDraw software (22), and the peak intensities were characterized as peak heights using the Sparky software. The relaxation data were fitted using the Modelfree 4.0 program (provided by A.G. Palmer).

**Structural Calculations**—Approximate interproton distance restraints were derived from three NOE spectra (a \( ^{1}H\) two-dimensional homonuclear NOE spectrum, a \( ^{15}\text{N}\)-separated NOE spectrum, and a \( ^{13}\text{C}\)-separated NOE spectrum). NOEs were grouped into three distance ranges of 1.8–2.7 Å (1.8–2.9 Å for NOEs involving NH protons), 1.8–3.3 Å (1.8–3.5 Å for NOEs involving NH protons), and 1.8–5.0 Å, respectively, corresponding to strong, medium, and weak NOEs. Hydrogen-bonding restraints (two per hydrogen bond where \( r_{\text{NH-proton}} = 1.8–2.2\) Å and \( r_{\text{C=O-proton}} = 2.2–3.3\) Å) were derived from the standard secondary structure libraries of the protein based on the NOE patterns and backbone secondary chemical shifts. Backbone dihedral angle restraints (\( \phi\) and \( \psi\) angles) were derived from \( J_{\text{HNCO}}\) coupling constants measured using an HNHA experiment and the backbone chemical shift analysis program TALOS (28). Structures were calculated using the program CNS (29).

**Results and Discussion**

**Structural Determination**—To investigate the mechanism of the interaction between GRIP1 and GRASP-1, we determined the three-dimensional structure of GRIP1 PDZ7 by NMR spectroscopy. A total of 1826 experimentally derived distance and dihedral angle restraints were calculated with force constants of 50 kcal mol\(^{-1}\) Å\(^{-2}\) and 200 kcal mol\(^{-1}\) rad\(^{-2}\), respectively.

**Precision of the atomic coordinates is defined as the average r.m.s. difference between the 20 final structures and the mean coordinates of the protein.**

Throughout the evolution (Fig. 2), in contrast, the rest of PDZ domains in the data base have rather limited amino acid sequence identities with that of PDZ7 (the PDZ1 of Calliphora vicina InAD has the highest sequence identity with GRIP1 PDZ7 of 37%). We further note that the amino acid residue in the first residue of the \( \alpha\)B helix (\( \alpha\)B1) of GRIP PDZ7 is an absolutely conserved Cys residue (Cys-1048). A database search revealed that no other known PDZ domains contain a Cys residue in the \( \alpha\)B1 position (1). The amino acid residue in the \( \alpha\)B1 position is known to play a critical role in recognizing the −2 residue in target carboxyl peptides (6, 10). We analyzed the structural features of this residue as well as the \( \alpha\)B/\( \beta\) groove in detail, hoping to gain insight into the potential target-binding mechanism of this PDZ domain. Fig. 3A compares the conformation of the \( \alpha\)B/\( \beta\) grooves of PDZ7 and PSD-95 PDZ2. The side chain of Cys-1048 in GRIP1 PDZ7 is packed with hydrophobic residues from both \( \alpha\)B and \( \beta\) (see an example of NOE contacts between Cys-1048 and Pro-1049), resulting in a significantly smaller \( \alpha\)B/\( \beta\) groove. The size of the \( \alpha\)B/\( \beta\) groove in PDZ7 is not likely to accommo-
date a target carboxyl peptide, which is supposed to assume a \( \beta \)/H9252-strand conformation when binding to the PDZ domain. Additionally, the conformation of the carboxyl group-binding pocket of PDZ7 is not suited to interact with negatively charged carboxyl groups in the target peptides. The \( \beta \)/H9252/beta/loop of PDZ7 (corresponding to the carboxyl group-binding

Fig. 1. Three-dimensional structure of GRIP1 PDZ7 determined by NMR spectroscopy. A, stereoview showing the best-fit superposition of the backbone atoms (N, Ca, and C') of the final 20 structures of PDZ7. The structures are superimposed against the average structure using the residues 985–1007 and 1012–1068. The NH\(_2\) and COOH termini of the PDZ domain are labeled. The program MOLMOL (34) was used to generate the figure. B, ribbon diagram of the GRIP1 PDZ7 structure. The secondary structural elements are labeled following the scheme used in the crystal structure of PSD-95 PDZ2 (6). The figure is generated using MOLESCRIPT (35) and Raster3D (36).

Fig. 2. Amino acid sequence alignment of the PDZ7 domains of the GRIP family proteins. The secondary structure of GRIP1 PDZ7 determined from this work is indicated at the top of the sequences. For comparison, the amino acid sequence of PSD-95 PDZ2 is also included in the alignment. The putative carboxyl group-binding residues are highlighted with stars underneath the PSD-95 PDZ2 sequence, and the residue at the \( \beta \)/H9252 position is indicated with a black dot. The amino acid residues of PDZ7 that form the GRASP-1-binding hydrophobic surface are shaded with gray boxes (see Fig. 5). In the nomenclature of the sequences, the labeling \( r \), \( m \), \( h \), \( p \), and \( f \) represent "rat", "mouse", "human", "mosquito", and "fruitfly", respectively.

Fig. 3. The GRIP1 PDZ7 contains a closed \( \alpha \)/beta/\( \beta \)/gamma/groove. A, comparison of the \( \alpha \)/beta/\( \beta \)/gamma-groove conformation of GRIP1 PDZ7 to that of PSD-95 PDZ2. In this comparison, the two PDZ domains were superimposed with each other using their respective \( \beta \)/beta/strand. The amino acid residue in the \( \alpha \)/gamma1 position of each PDZ domain is drawn in explicit atom representations. \( B \) and \( C \), surface representation of the \( \alpha \)/beta/\( \beta \)/gamma-grooves of GRIP1 PDZ7 (\( B \)) and PSD-95 PDZ2 (\( C \)). In this presentation, the hydrophobic amino acid residues are drawn in yellow, the positively charged residues in blue, the negatively charged residues in red, and the uncharged polar residues are shown in gray. The orientations of the molecules in \( B \) and \( C \) are the same as shown in \( A \). The surface diagram is generated using the program GRASP (37).
Fig. 4. Characterization of the $\alpha B/\beta B$-groove. A, representative NOE contacts between Cys-1048 in the $\alpha B$1 with the residues from $\beta B$ and $\alpha B$. The data is taken from a three-dimensional $^{13}C$-separated NOESY spectrum of the protein at the C$\beta$ chemical shift of Cys-1048. B, an $^{15}N$-NOESY strip showing NOE contacts between the backbone amide proton of Phe-1000 in the DFGF-loop with Ile-1055 at the end of $\alpha B$ of PDZ7. C, backbone amide-order parameters ($S^2$) of GRIP1 PDZ7 determined by NMR relaxation experiments. The $S^2$ values were derived from $T_1$, $T_2$, and $^1H-^{15}N$ NOE values of GRIP1 PDZ7 measured on field strength of 500 MHz. For easy comparison, the secondary structure of the protein is included in the figure.
loop of conventional PDZ domains) contains two additional residues (Fig. 2). The conformation of this loop is largely defined (Fig. 1A) by a number of NOE contacts between amino acid residues from this loop with those from the COOH-terminal end of the αB helix (see Fig. 4B for an example). The limited conformational freedom of the βA/βB-loop is also supported by the order parameter values ($S^2$) of the backbone amides in this region measured from the NMR relaxation experiments (Fig. 4C). Fig. 3, B and C, compares the surface structures of the αB/βB-grooves of PDZ7 and PSD-95 PDZ2. Because of the packing of the βA/βB-loop with the αB helix, PDZ7 does not have a hydrophobic pocket to accommodate the side chain of the target peptide residue at the 0 position (Fig. 3B). Additionally, a highly conserved positively charged residue that functions to stabilize the negative charge of the carboxyl group of target peptide (Lys-233 in PSD-95 PDZ2) (Fig. 3C) is missing in PDZ7. Instead, several negatively charged residues (Glu-998, Asp-999, and Glu-1057) surround the remaining small hydrophobic surface in PDZ7 (Fig. 3B). Taken together, we conclude that the GRIP1 PDZ7/βA-groove adopts a closed conformation. We predict that GRIP1 PDZ7 is not likely to be able to interact with carboxyl peptides using its αB/βB-groove. Consistent with our prediction, no proteins with classical PDZ-binding
carboxyl termini were found to interact with GRIP1 PDZ7 in a recent yeast two-hybrid screening (21). However, we cannot rule out the possibility that GRIP1 PDZ7 may undergo a large conformational change in the αB/β region, rendering an interaction of the domain with carboxyl peptides.

**GRIP PDZ7 Contains a Distinct Hydrophobic Surface**

**Formed by βE/αB—Structural analysis shows that the space between the backbones of αB and βE of PDZ7 is larger than that of PSD-95 PDZ2 (as well as other PDZ domains with known structures) (Fig. 5A).** A prominent hydrophobic surface formed by amino acid residues from βE to αB can be observed in PDZ7 (Fig. 5B). We term this hydrophobic surface as the βE/αB-surface. The entire αB helix of GRIP PDZ7 is composed of hydrophobic amino acid residues, and it contributes four residues (Cys-1049, Leu-1050, Pro-1053, and Leu-1054) to the βE/αB-hydrophobic surface (Figs. 2 and 5B). Furthermore, the amino acid residues forming the βE/αB-hydrophobic surface are highly conserved in both GRIP1 and GRIP2 (Fig. 2). Such hydrophobic surface is not observed in other PDZ domains including PSD-95 PDZ2 (Fig. 5C). It is possible that GRIP1 PDZ7 may use the βE/αB-hydrophobic surface rather than the αB/β-groove to interact with its binding partner(s).

**GRASP-1 Binds to PDZ7 via the βE/αB-hydrophobic Surface**

The binding of GRASP-1 to GRIP1 PDZ requires a ~100-residue fragment at the COOH-terminal end of GRASP-1, and this 100-residue PDZ7-binding domain of GRASP-1 contains neither a classical PDZ-binding motif at its carboxyl terminus nor a PDZ-like fold (21). Preliminary NMR and circular dichroism spectroscopic studies of the purified COOH-terminal 100-residue PDZ7-binding domain of GRASP-1 indicated an α-helix-rich structure, and the domain forms a large molecular multimer in solution (data not shown). We titrated 15N-labeled PDZ7 with the unlabeled PDZ7-binding domain of GRASP-1. The region that formed by amino acid residues from D/E1, indicating that GRASP-1 binds to PDZ7 via the disulfide bond (21). The data in Fig. 6A shows that the PDZ7 determined in this work shows that the PDZ domain has a deformed βE/αB-groove that is not likely to be capable of binding to carboxyl peptide ligands. Instead, GRIP1 PDZ7 contains a distinct hydrophobic surface largely composed of the residues from the βE to αB regions of the protein. Unexpectedly, GRIP1 PDZ7 binds to its target GRASP-1 via the βE/αB-hydrophobic surface instead of the conventional target-binding βE/αB-groove. The novel protein-binding surface revealed in this work may help us to understand the molecular basis of frequently observed PDZ domain-mediated multimerization of multimodular scaffold proteins.

**Acknowledgments**

We thank Drs. Richard Huganir and Morgan Sheng for providing cDNA constructs of GRASP-1 and GRIP1 and Dr. Virginia Unkefer for critical reading of the paper.

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Novel Target Binding Site of GRIP1 PDZ7

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