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STRUCTURE NOTE

Solution Structure of Human Erythroid p55 PDZ Domain

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Introduction. p55 is a member of the membrane-associated guanylate kinase family, which includes PDZ, SH3, HOOK, and guanylate kinase–like domains.[1,2] This protein forms a ternary complex with protein 4.1 (4.1R) and glycophorin C (GPC) at the cytoplasmic face of the plasma membrane, and the p55–4.1R–GPC ternary complex plays an important role in stabilizing the cell shape.[3–5] In the ternary complex, the HOOK domain of p55 interacts with the FERM domain of 4.1R, the FERM domain of 4.1R interacts with the cytoplasmic loop of GPC, and the C-terminus of GPC interacts with the PDZ domain of p55. Here we focus on the p55 PDZ domain and its interaction with the C-terminus of GPC, as a first attempt to obtain structural insights into the p55–4.1R–GPC ternary complex.

PDZ domains[6,7] are protein–protein interaction modules consisting of 80–100 amino acid residues, and are composed of five or six β-strands (βA to βF) and two α-helices (αA and αB).[8–11] PDZ domains usually bind preferentially to the C-terminal four to five residues of their target proteins and are categorized into several classes according to their specificity for the C-terminal target sequences.[12] For example, class I PDZ domains bind to the motif X-S/T-X-ϕ (with X representing any amino acid residue and ϕ representing a hydrophobic residue), whereas class II PDZ domains bind to the motif X-ϕ-X-ϕ. The residues at the positions 0 (P_0) and −2 (P_{-2}), numbered from the C-terminus of the peptide ligands, are crucial for the specific interaction with PDZ domains.[12] GPC contains a class II type motif (E-Y-F-I) in its C-terminus, and two hydrophobic residues, Ile128 (P_0) and Tyr126 (P_{-2}), are responsible for binding to the p55 PDZ domain, since the mutagenesis of these residues abolished GPC binding to the p55 PDZ domain.[13] Here we report the first NMR-derived structure of the human erythroid p55 PDZ domain and propose a possible interaction mode with the C-terminus of GPC.

Materials and Methods. *Sample preparation:* DNA encoding the human erythroid p55 PDZ domain (SWISS-PROT entry Q00013, residues 69–153) was amplified from a human universal cDNA library (Clontech). The resulting polymerase chain reaction (PCR) product was cloned with *Nde*I and *BamH*I sites into a modified pET-15b vector (Novagen) that contains 12 additional amino acids (MGH-HHHHHHSGHM) at the N-terminus. The hexahistidine-tagged p55 PDZ domain was overproduced in *Escherichia coli* strain Rosetta (DE3) and was purified by a TALON Superflow (Clontech), Q Sepharose (Amersham), and Resource S columns (Amersham). M9 minimal medium containing $^{15}NH_4Cl$ or $^{15}NH_4Cl/^{13}C_6$-D-glucose was used to prepare ^{15}N- or $^{13}C/^{15}N$-labeled protein, respectively. The 12 C-terminal residue GPC peptide (residues 117–128, DAGDSSRKEYFI), referred as to GPC(117–128) in this article, was purchased from ANYGEN Co., Ltd.

NMR spectroscopy: All NMR samples contained 1.1–1.5 m*M* protein in 20 m*M* sodium phosphate (pH 6.8), 41 m*M* NaCl, 0.8 m*M* dithiothreitol (DTT), 9% glycerol-d_5, and 90% H_2O/10% D_2O. NMR spectra were acquired on a Bruker AVANCE 500 spectrometer equipped with a $^1H/^{13}C/^{15}N$ cryogenic probe at 25°C and 35°C. Backbone and side-chain ^1H, ^{13}C, and ^{15}N resonances were assigned using standard triple-resonance NMR experiments.[14,15] All data were processed with the program NMRPipe[16] and analyzed with the program PIPP.[17]

Structure calculation: The 12 additional amino acid residues at the N-terminus were not used in the structure calculation, because these residues are unstructured. Distance restraints were obtained from two-dimensional (2D) nuclear Overhauser effect spectroscopy (NOESY) and 3D ^{13}C- and ^{15}N-edited NOESY experiments (mixing time of 100 ms). Distance restraints were grouped into four distance ranges, 1.8–3.0 Å, 1.8–4.0 Å, 1.8–5.0 Å, and 1.8–6.0 Å, according to the peak intensity. Dihedral ϕ and ψ angle restraints were determined from the chemical shift data using the program TALOS.[18] The χ1 angle restraints were estimated from HNHB,[19] HN(CO)HB,[20] and $^{13}C'$-{$^{13}C^{\gamma}$} and ^{15}N-{$^{13}C^{\gamma}$} spin-echo difference ^1H-^{15}N HSQC[21] experiments. Hydrogen bond restraints were generated from the secondary structure of the protein, based on the backbone chemical shift data and nuclear Overhauser effect (NOE) patterns. Structures were calculated using a torsion angle

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TABLE I. Structural Statistics of the Final 20 Structures of the p55 PDZ Domain

Distance restraints	1271
Intraresidue	437
Sequential ($\|i-j\|=1$)	354
Medium range ($1<\|i-j\|\leq 4$)	153
Long range ($\|i-j\|>4$)	279
Hydrogen bonds	24×2
Dihedral angle restraints	127
Φ/Ψ/χ1	
57/57/13	
Restraint satisfaction	
RMSDs for distance restraints (Å)	0.0152 ± 0.0006
RMSDs for torsion angle restraints (°)	0.1817 ± 0.0325
Deviations from ideal covalent geometry	
Bond lengths (Å)	0.0020 ± 0.00007
Bond angles (°)	0.3952 ± 0.0083
Impropers (°)	0.2784 ± 0.0148
Average RMSDs (Å)[a]	
Residues 69–153	$0.78\pm 0.13\,(1.34\pm 0.18)$
Residues in secondary structure elements	$0.35\pm 0.08\,(1.37\pm 0.18)$
Ramachandran Plot[b]	
Most favored (%)	70.4
Additional allowed (%)	24.3
Generously allowed (%)	4.5
Disallowed (%)	0.8

[a]The average RMSDs from the energy-minimized average structure are given for selected residues. The value for the backbone atoms (N, Cα, and C′) is followed by that for all heavy atoms in parentheses.
[b]The values were obtained by PROCHECK-NMR.[23]

simulated annealing protocol with the program CNS.[22] From the 100 trial structures, the final 20 lowest energy structures with no distance violations greater than 0.2 Å and no torsion angle violations greater than 2° were selected. The final structures were analyzed with the program PROCHECK-NMR.[23] Coordinates have been deposited in the Protein Data Bank (PDB), with the accession code 2EV8. Structure figures were generated with the programs MOLMOL,[24] MOLSCRIPT,[25] and RASTER3D.[26]

Identification of the GPC-binding site of the p55 PDZ domain: To identify the GPC-binding site of the p55 PDZ domain, GPC(117–128) was added to approximately 0.3 m*M* of the $^{13}C/^{15}N$-labeled protein. The 2D ^{1}H-^{15}N HSQC spectra of the protein with 0, 0.24, 0.5, 1, 1.4. 2, 4, 6, and 10 equivalents of peptide were recorded at 35°C. The combined chemical shift perturbation was calculated with the equation

$$\Delta\delta_{ppm} = \sqrt{(\Delta\delta_{HN})^2 + (\Delta\delta_N * \alpha_N)^2}, \quad (1)$$

with a scaling factor (α_N) of 0.17. The backbone assignments of the p55 PDZ domain of the peptide-free and peptide-bound states in the presence of a 10-fold molar excess of peptide were accomplished by HNCO, HN(CA)CO, CBCA(CO)NH, and HNCACB experiments at 35°C.

Results and Discussion. The 3D structure of the p55 PDZ domain has been determined on the basis of 1271 distance restraints and 127 dihedral angle restraints. The NMR structural statistics are summarized in Table I. The structure of the p55 PDZ domain consists of five β-strands (βA to βF) and two α-helices (αA and αB) [Fig. 1(A, B)], and is similar to those of other PDZ domains determined by X-ray crystallography and NMR spectroscopy [Fig. 1(C)].[8–11,27–29] For example, a comparison of the p55 PDZ domain with a class I PDZ domain [PSD95-PDZ3[8] (PDB entry code: 1BE9; root-mean-square difference (RMSD) of 2.27 Å; sequence identity: 23.3%; red)] and four class II PDZ domains [hCASK[10] (1KWA; 2.38 Å; 62.4%; green); InaD[27] (1IHJ; 1.83 Å; 23.1%; yellow); GRIP1-PDZ6[28] (1N7E; cyan; 2.03 Å; 20.7%); AF-6[29] (1T2M; 2.19 Å; 20.0%; purple)] reveals good superposition of these structures, with the exception of helix αB and loops βA/βB, βB/βC, and αB/βF [Fig. 1(C)]. The RMSDs range from 1.83 to 2.38 Å for 83–85 Cα atoms, as calculated by FATCAT.[30]

In PDZ–peptide ligand complexes, the ligand usually fits into a groove between strand βB and helix αB, oriented antiparallel to strand βB as an additional β-strand. In the ligand, the C-terminal oxygen atoms (residue P_0) form hydrogen bonds with the carboxylate-binding loop (also referred to as the GLGF repeat) of the PDZ domain. In addition, the side-chain of the residue P_0 dips into the hydrophobic pocket in the vicinity of the carboxylate-binding loop, while the side-chain of the residue P_{-2} interacts with the first (αB1 position) and/or fifth (αB5 position) amino acid residue of helix αB. In the p55 PDZ domain, such a groove is also present and is composed of Met82, Ile84, Thr85, Leu86, Leu88, Val130, Gln134, and Met137, which are mainly hydrophobic residues [Fig. 1(D)]. The carboxylate-binding loop has the sequence Pro-Met-Gly-Ile (residues 81–84), and a hydrophobic pocket (Met82, Ile84, and Met137) resides in the vicinity of the carboxylate-binding loop. In addition, the first and fifth amino acid residues of helix αB are valine (Val130) and glutamine (Gln134), respectively.

To identify the ligand-binding site on the p55 PDZ domain, we performed an NMR chemical shift perturbation experiment by the addition of GPC(117–128), which mimics the C-terminus of GPC, as a ligand. Resonances with small perturbations remain visible throughout the titration, while resonances with large perturbations are broadened at the same protein:peptide ratio, probably due to intermediate exchange under this NMR condition. This feature is occasionally observed with other PDZ–peptide ligand complexes.[29,31] At a protein:peptide ratio of 1:10, all of resonances of the p55 PDZ domain (residues 69–153), with the exception of Met82 [cyan in Fig. 1(E)], were completely assigned, and the molar ratio indicated the nearly completed saturation of the protein with the peptide. As expected, large chemical shift changes of the backbone amide resonances were observed upon peptide binding, mainly on the surface of the hydrophobic groove including the carboxylate-binding loop [Fig. 1(E)]. The affected residues were Gly83, Thr85, Leu86, Leu88, Val125, Ser129, Val130, Met137, and Glu139. In addition, on the same surface, the side-chain amide resonance of Gln134 was also greatly perturbed upon peptide binding [Fig. 1(E)]. These results strongly suggest that the p55 PDZ domain uses a ligand-binding mode similar to those of other PDZ domains, and the hydrophobic groove is involved in the interaction with the C-terminus of GPC

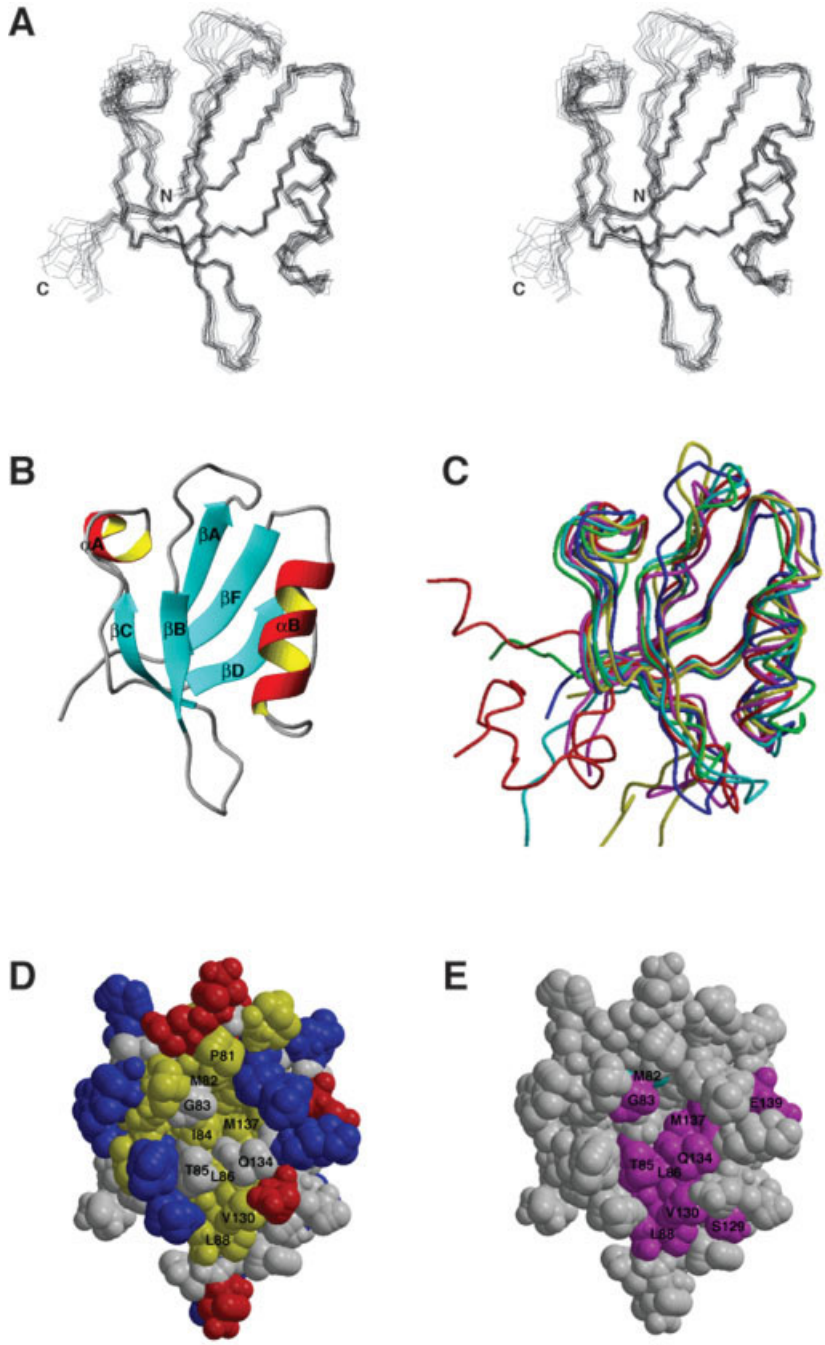

Fig. 1. Solution structure of the p55 PDZ domain. (**A**) Stereo view of the 20 final structures of the p55 PDZ domain (residues 69–153). The superposition of the backbone atoms (N, Cα, and C′) is shown. (**B**) Ribbon diagram of the energy-minimized average structure of the p55 PDZ domain. The α-helices are shown in red, and the β-strands are cyan. The secondary structure elements are labeled according to the scheme used in the crystal structure of PSD95 PDZ3.[8] (**C**) Superposition of the PDZ domain of p55 (blue) with those of PSD95-PDZ3[8] (red), hCASK[10] (green), InaD[27] (yellow), GRIP1-PDZ6[28] (cyan), and AF-6[29] (purple). (**D**) Space-filling model of the groove between strand βB and helix αB of the p55 PDZ domain. Basic, acidic and hydrophobic amino acids are shown in blue, red and yellow, respectively. (**E**) Identification of the GPC-binding site on the p55 PDZ domain by the chemical shift perturbation experiment. The residues (Gly83, Thr85, Leu86, Leu88, Val125, Ser129, Val130, Gln134, Met137, and Glu139) showing large chemical shift changes caused by the addition of a 10-fold molar excess of peptide are labeled and colored purple. With the exception of Val125, all of these residues reside on one side of the protein surface.

containing the two hydrophobic residues, Ile128 and Tyr126. In terms of its binding specificity, it is most likely that the residues in the hydrophobic pocket interact with Ile128, while Val130 and/or Gln134 interact with Tyr126. Additional biochemical and structural studies will help to characterize more fully the specific interaction between

the PDZ domain of p55 and the C-terminus of GPC in the p55–4.1R–GPC ternary complex.

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