Domain-swapped Dimerization of the Second PDZ Domain of ZO2 May Provide a Structural Basis for the Polymerization of Claudins*\(\text{**}\)

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Zonula occludens proteins (ZO), including ZO1/2/3, are tight junction-associated proteins. Each of them contains three PDZ domains. It has been demonstrated that ZO1 can form either homodimers or heterodimers with ZO2 or ZO3 through the second PDZ domain. However, the underlying structural basis is not well understood. In this study, the solution structure of the second PDZ domain of ZO2 (ZO2-PDZ2) was determined using NMR spectroscopy. The results revealed a novel dimerization mode for PDZ domains via three-dimensional domain swapping, which can be generalized to homodimers of ZO1-PDZ2 or ZO3-PDZ2 and heterodimers of ZO1-PDZ2/ZO2-PDZ2 or ZO1-PDZ2/ZO3-PDZ2 due to high conservation between PDZ2 domains in ZO proteins. Furthermore, GST pull-down experiments and immunoprecipitation studies demonstrated that interactions between ZO1-PDZ2 and ZO2-PDZ2 and their self-associations indeed exist both \textit{in vitro} and \textit{in vivo}. Chemical cross-linking and dynamic laser light scattering experiments revealed that both ZO1-PDZ2 and ZO2-PDZ2 can form oligomers in solution. This PDZ domain-mediated oligomerization of ZO proteins may provide a structural basis for the polymerization of claudins, namely the formation of tight junctions.

Epithelial and endothelial cells can form selective barriers between tissues and different body compartments. They polarize and adhere to each other through intercellular complexes, including tight junctions (TJs),\(^3\) adherens junctions, and desmosomes (1). TJs, the most apical component of the junction complex, separate the apical from the lateral plasma membrane through forming a continuous belt-like attachment at the outer end of the intercellular space between cells. TJs play important roles in regulating the passage of ions and molecule through the paracellular pathway (2–5). TJs are also crucial for correct function of blood-brain barrier. Disruption of the TJs in blood-brain barrier is a hallmark of many central nervous system pathologies, including stroke, human immunodeficiency virus encephalitis, Alzheimer disease, multiple sclerosis, and bacterial meningitis. Systemic-derived inflammation has been shown to cause disruption of TJs and increased paracellular permeability (6).

TJs consist of different types of transmembrane proteins, including occludin, claudin, and JAMs (7–9). These transmembrane protein components connect to the cytoskeleton via adaptor/scaffold proteins such as zonula occludens (ZO)s. ZO1, ZO2, and ZO3 (10–12) belong to a membrane-associated guanylate kinase protein family (MAGUKs) (13–15). These proteins contain several protein–protein interaction domains, including three PDZ (for PSD-95, DlgA, and ZO1 homology) domains, one SH3 domain, and one GK domain (16–19). They interact with their cytoplasmic tail to different receptor tyrosine kinases, including PDGF, VEGF, and PDGF receptor (20). Recent studies have provided accumulated evidences to unveil domains that are responsible for the interactions between ZO proteins. The association between ZO1 and ZO2/ZO3 is through their second PDZ domains (20, 21). Utep-

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\(^3\) The abbreviations used are: TJ, tight junction; ZO, zonula occludens; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; r.m.s.d., root mean square deviation; ZO1-PDZ2, the second PDZ domain of ZO1; ZO2-PDZ2, the second PDZ domain of ZO2; ZO3-PDZ2, the second PDZ domain of ZO3; MAGUK, membrane-associated guanylate kinase protein family; mAb, monoclonal antibody; GFP, green fluorescent protein; CMW, cytomegalovirus; GST, glutathione S-transferase; Tricine, N\(^2\)-hydroxy-1,1-bis[hydroxymethyl]ethyl]glycine; PBS, phosphate-buffered saline; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DSS, disuccinimidyl suberate; DLS, dynamic laser light scattering.

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bergenov’s group showed significant amount of ZO1 homodimers in MDCK cells and demonstrated that the second PDZ domain is both necessary and sufficient for the dimerization (22). The SH3/GK domain was also reported to be important in ZO1 dimerization, similarly as of in other MAGUK proteins, such as PSD-95 and Dlg/SAP90/SAP102 (23–29).

Dimerization or oligomerization of ZO proteins plays a pivotal role in determining the activity of their binding partners. Claudins are thought to constitute the backbone of TJ strands (30). They can form homo- and hetero-oligomers during their engagement in the formation of paracellular channels or pores (31–35). ZOs bind to claudin YV C termini through their first PDZ domain (36). The dimerization of ZO1 (probably also ZO2) not only initiates the polymerization of claudins, namely the formation of TJ strands, but also directs the correct localization of TJ strands (23).

To understand the molecular basis of the PDZ domain-mediated dimerization/oligomerization of ZO family proteins, we have determined the solution structure of ZO2-PDZ2 using NMR spectroscopy. Unexpectedly, ZO2-PDZ2 forms very tight 2-fold symmetric homodimers via a three-dimensional domain swapping assembly mode (37). To the best of our knowledge, this ZO2-PDZ2 dimer is the first example for dimerization of PDZ domain via three-dimensional domain swapping. Further chemical cross-linking and dynamic laser light scattering experiments demonstrated that both ZO1-PDZ2 and ZO2-PDZ2 can form oligomers in solution. The ZO2-PDZ2 dimer structure is ideally suited for the tight assembly of ZOs in TJs.

**MATERIALS AND METHODS**

**Oligonucleotides**—The sequences of the oligonucleotides for target cDNA PCR amplification used in this study are listed as follows: P1, 5′-CATATGACTAAAGTCACTGTGAAATC-3′; P2, 5′-CTCGAGTTTATCTTCTTTGAAC-TACC-3′; P3, 5′-CATATGATCGGGGTCTCTGATGAAAG-3′; P4, 5′-CTCGAGGCTGTCTCTCAACACTAGCTG-3′; P5, 5′-GCGAATTCATGGATGATCACT-GTG-3′; and P6, 5′-GCGAATTCATGGATGATCACTCTATGGTAT-3′.

**Reagents and Antibodies**—Monoclonal antibodies used in this study are mAb-GFP (TaKaRa, Japan) and mAb-FLAG (Sigma). EZview™ Red ANTI-FLAG® M2 affinity gel was a kind gift from Prof. Xuebiao Yao. Restriction enzymes were purchased mostly from New England Biolabs (Beverly, MA). Medium compounds were obtained from Oxid (Basingstoke Hampshire, UK).

**Plasmids Construction**—The DNA fragments coding for human ZO1-PDZ2 domain (Q07157 amino acid residues Thr-185 to Glu-264) and ZO2-PDZ2 domain (Q9UDY2 amino acid residues Ile-306 to Ser-385) were amplified, respectively, from the human brain cDNA library (Clontech) using primer pair P1/P2 or P3/P4 (see “Oligonucleotides”). The amplified DNA fragments were inserted into the Ndel/Xhol-cleaved plasmid pET22b (+) (Novagen) to generate His-ZO1-PDZ2 or His-ZO2-PDZ2 with His$_{6}$ tag fusion at their C termini. They were subsequently subcloned into Ndel/Xhol sites of modified pGEX-4T-1 vector for the preparation of GST-ZO1-PDZ2 and GST-ZO2-PDZ2. In addition, the gene fragments coding for ZO1-PDZ2 and ZO2-PDZ2 domain generated from PCR reactions using primer pair P2/P5 or P4/P6 were digested using restriction enzymes EcoRI and Xhol and subcloned, respectively, into the EcoRI/Sall-cleaved pEGFP-C1 vector for the preparation of GFP-ZO1-PDZ2 and GFP-ZO2-PDZ2 or EcoRI/Xhol sites of p3×-FLAG-MyclCMV-24 expression vector for the preparation of FLAG-ZO1-PDZ2 and FLAG-ZO2-PDZ2. All constructs were verified by DNA sequencing.

**Expression and Purification of Fusion Proteins**—The recombinant plasmids harboring the respective target genes were transformed into *Escherichia coli* BL21(DE3) host cells for large scale protein production. The purified recombinant protein His-ZO2-PDZ2 contains an N-terminal Met and a C-terminal His tag (-LEHHHHHHH), whereas the purified recombinant His-ZO1-PDZ2 protein contains a C-terminal His$_{6}$ tag, with the N-terminal Met being cleaved during expression (data from unpublished mass spectrum).

The GST and GST fusion proteins were purified through the glutathione-Sepharose 4B beads (Amersham Biosciences). The soluble His$_{6}$-tagged fusion proteins were purified using HiTrap Chelating columns (Amersham Biosciences) according to the procedures specified by manufacturer. The purity of recombinant proteins were confirmed by Tricine-SDS-PAGE (15%, w/v), and the concentration was measured using BCA kits (Pierce). Uniformly $^{15}$N- and $^{15}$N/$^{13}$C-labeled His$_{6}$-tagged proteins were prepared through growing bacteria in SV40 medium using $^{15}$NH$_{4}$Cl (0.5g/liter) and $^{13}$C$_{6}$-glucose (2.5g/liter) as stable isotope sources.

**In Vitro Binding Assay using GST Fusion Proteins**—For in vitro binding studies, the bacterially purified His-ZO1-PDZ2 was equally divided into three parts. Then they were mixed with GST alone, GST-ZO2-PDZ2 or GST-ZO1-PDZ2 fusion protein coupled to the glutathione beads individually. His-ZO2-PDZ2 protein was also equally divided and mixed with GST, GST-ZO1-PDZ2, or GST-ZO2-PDZ2 fusion protein coupled to the glutathione beads individually. The GST-conjugated Glutathione-Sepharose beads were used as a negative control. Samples were then centrifuged at 4000 rpm at 4 °C for 4 min and washed ten times using 1× phosphate-buffered saline (PBS). Proteins were eluted from beads by 5-min boiling in sample buffer (Bio-Rad) and separated on 15% denatured SDS-PAGE gel, followed by Coomassie Brilliant Blue staining.

**Western Blot Analysis and Co-immunoprecipitation**—H1299 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO$_{2}$. Transfection of cells with mammalian expression vectors (see “Plasmids Construction”) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s specifications.

For Western blot analysis, cells were washed with 1×PBS and resuspended using 5 volumes of cold lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% sodium deoxycholate) supplemented with protease inhibitor mixture (Roche Applied Science). The cell lysates were incubated on ice for 30 min and were then centrifuged for 10 min at 4 °C. Equal amounts of total cellular proteins were separated by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membrane. After blocking with 5% nonfat milk...
in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for overnight at 4 °C, the blot was incubated with primary antibody for 2 h at room temperature, and followed by incubating with a alkaline phosphatase-conjugated secondary antibody for 1 h. After washing three times with TBST, the blots were developed using the chemiluminescence detection system (Amersham Biosciences) according to the manufacturer's protocol.

To perform co-immunoprecipitation, we co-transfected H1299 cells with pEGFP-ZO1-PDZ2 and p3×FLAG, p3×FLAG-ZO1-PDZ2 or p3×FLAG-ZO2-PDZ2, or with pEGFP-ZO2-PDZ2 and p3×FLAG, p3×FLAG-ZO1-PDZ2 or p3×FLAG-ZO2-PDZ2. 24 h later, cells were harvested and resuspended with lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Science). After incubated on ice for 2 h, the cell lysates were centrifuged for 10 min at 4 °C. Successfully, the supernatants were collected and incubated with EZview™ Red ANTI-FLAG® M2 affinity gel for 4 h at 4 °C, bound proteins were recovered by boiling the washed beads in SDS sample buffer and analyzed by immunoblot.

Fast Protein Liquid Chromatography Size-exclusion Chromatography—The purified His6-tagged proteins were concentrated and then gel-filtered with Superdex 75 10/300 GL column (Amersham Biosciences), which was equilibrated with 20 mM phosphate buffer (pH 6.5) and 200 mM NaCl, at 25 °C. Elution was performed using the same buffer at a flow rate of 0.5 ml/min, at 25 °C. Previously, the column was calibrated using known molecular mass proteins: bovine albumin (67 kDa, monomer), albumin egg (45 kDa), chymotrypsinogen (25 kDa), myoglobin (17.8 kDa), and lysozyme (14.4 kDa).

Sedimentation Velocity Analysis—Sedimentation velocity studies were carried out with a Beckman Optima XL-A analytical ultracentrifuge with an An60Ti rotor at 20 °C and 56,000 rpm. The partial specific volumes (0.7389 ml/g for ZO1-PDZ2 and 0.7448 ml/g for ZO2-PDZ2), and molecular masses of the monomers (10010 Da for ZO1-PDZ2 and 10081Da for ZO2-PDZ2) were calculated based on their amino acid composition using the program SEDFIT. Protein samples (at approximate concentrations of 5 mg/ml and 0.5 mg/ml) were prepared in sedimentation buffer (PBS buffer including 1 mM EDTA, pH 7.2). 380-µl samples and 400-µl reference volumes were loaded into cells. Absorbance scans at 280 or 235 nm were collected at a time interval of 2 min. Sedimentation profiles were analyzed with the software SEDFIT (version 8.9) using the continuous distribution c(s) Lamm equation model (38, 39).

NMR Experiments—The purified proteins were dissolved to a final concentration of 0.8 mM in 500 µl of 20 mM phosphate buffer (pH 6.5), containing 200 mM NaCl and 1 mM EDTA. Unless otherwise specified, all NMR spectra were acquired at 99.96% D2O. The 15N-separated NOESY and 13C-separated NOESY were recorded separately for samples of ZO1-PDZ2 and ZO2-PDZ2 (40). These samples were made from a 1:1 mixture of uniformly

**FIGURE 1.** The interactions between ZO1-PDZ2 and ZO2-PDZ2 and their self-associations. A, in vitro binding assay. The interactions in vitro were examined by GST pulldown assay. Coomassie Brilliant Blue staining was used to ensure the protein loads. GST alone was used as a negative control. His-ZO1-PDZ2 was retained by GST-ZO1-PDZ2 or GST-ZO2-PDZ2 (lanes 2 and 3), but not by GST alone (lane 1). His-ZO2-PDZ2 was captured by GST-ZO2-PDZ2 or GST-ZO1-PDZ2 but not by GST alone (lanes 4 – 6). Samples from GST, GST-ZO1-PDZ2, or GST-ZO2-PDZ2, incubating with no His-tagged proteins, were also shown as a control (lanes 7–9). The mobility of molecular mass markers (in kilodaltons) is indicated on the right of the gels. B, in vivo binding assay. The immunoprecipitates were analyzed by Western blot using anti-GFP antibody (upper panel). The presence of GFP-ZO1-PDZ2 or GFP-ZO2-PDZ2 in each immunoprecipitate precipitated was confirmed by Western blot analysis (middle panel) with an anti-GFP antibody. The expression of respective transfected FLAG-tagged constructs was also detected by anti-FLAG antibody (bottom panel). GFP-ZO1-PDZ2 interacted with both FLAG-ZO1-PDZ2 and FLAG-ZO2-PDZ2 (lanes 2 and 6) but not by FLAG alone (lane 1). GFP-ZO2-PDZ2 interacted with both FLAG-ZO2-PDZ2 and FLAG-ZO1-PDZ2 (lanes 3 and 4) but not by FLAG alone (lane 5). The mobility of molecular mass markers (in kilodaltons) is indicated on the left of the gels.
Solution Structure of ZO2-PDZ2 Homodimer

FIGURE 2. Comparison of fast-protein liquid chromatography size-exclusion chromatography and analytical ultracentrifugation results. A, shown are Superdex 75 (Amersham Biosciences) gel-filtration profiles of ZO1-PDZ2 (upper panel) and ZO2-PDZ2 (lower panel). B, shown are the distributions of sedimentation coefficients (cs) versus s, where s is plotted in Svedberg units, for ZO1-PDZ2 (upper panel) and ZO2-PDZ2 (lower panel) at concentrations of 250 µM (solid line) and 25 µM (dotted line). The c(M) analysis shows the possible molecular weight distributions (inset). c(M), distribution of molecular weight.

15N/13C-labeled and unlabeled proteins by unfolding and refolding with 8 M urea, which therefore contained 25% labeled-labeled, 50% labeled-unlabeled, and 25% unlabeled-unlabeled dimers. The appropriate processing of such spectra yields a sub-spectrum where predominantly only NOESY cross-peaks resulting from intermolecular interactions are observed. All of NMR spectra were processed with NMRPipe (41) and analyzed using Sparky3 software.

Structure Calculations of ZO2-PDZ2 Domain—NMR distance restraints were collected from two different NOESY spectra: three-dimensional 15N-separated NOESY in H2O for amide protons and three-dimensional 13C-separated NOESY in D2O for aliphatic protons. NOEs were grouped into four distance ranges according to their relative intensity: strong, 1.8–3.0 Å; medium, 1.8–4.0 Å; weak, 1.8–5.0 Å; very weak, 1.8–7.0 Å. The 1.8–Å lower limits were imposed only implicitly by the van der Waals repulsion force. The NOEs were specified as intermolecular and intramolecular according to domain swapped topology inferred from the 13C F1-filtered, F2-edited two-dimensional NOESY spectrum. Dihedral restraints (ϕ and ψ) were determined using the 1Hα, 15N, 13Cα, 13Cβ, and 13CO chemical shifts and the program TALOS (43). Hydrogen bond (H-bond) restraints were generated by a combination of the H/D exchange experiment and the standard secondary structure of the protein based on NOE patterns. Structures were calculated using the program CNS version 1.1 (44), employing a simulated annealing protocol for torsion angle dynamics. The calculated structures were analyzed by the programs PROCHECK (45) and MOLMOL (46).

Cross-linking of His Fusion Proteins—Purified His fusion proteins were diluted into indicated concentrations and centrifuged at 13,200 rpm for 5 min. The supernatants were then collected and treated with Me2SO alone or disuccinimidyl suberate (DSS) in Me2SO from a 10-fold stock solution to a final concentration of 5 mM. After incubating at 37 °C for 30 min, the cross-linker was quenched by the adding of 1 M Tris-HCl (pH7.5) to a final concentration of 20 mM at room temperature for 15 min. Samples were then solubilized in sample buffer (Bio-Rad), boiled, and centrifuged at 13,200 rpm for 5 min. His fusion proteins were separated on 15% SDS-PAGE gel, followed by Coomassie Brilliant Blue staining.

Dynamic Laser Light Scattering Measurements—DLS measurements (47) were conducted on an ALV/DLS/SLS-5022F spectrometer with a multi-7 digital time correlation (ALV5000) and a cylindrical 22-milliwatt UNIPHASE He–Ne laser (λ0 = 632 nm) as the light source. The intensity-intensity time correlation function G(1)(t) was measured to determine the line-width distribution G(1)(Γ). For diffusive relaxation, Γ is related to the translational diffusion coefficient (D) of the scattering object (macromolecules or colloid particles) in dilute solution or dispersion by D = (1/4πη)(c,0,q)−1 and further to hydrodynamic radius (Rh) from the Stokes-Einstein equation: Rh = kBT/(6πηD), where η, k, and T are the solvent viscosity, the Boltzmann constant, and the absolute temperature, respectively. Hydrodynamic radius distribution f(Rh) was calculated from the Laplace inversion of a corresponding measured G(1)(t) using the CONTIN program.

All DLS measurements were conducted at a scattering angle (θ) of 45° at 37.0 °C. Protein samples (at approximate concentrations of 3.0 × 10−3 g/ml) were prepared in physiological buffer conditions (PBS buffer including 1 mM EDTA, pH 7.2).

RESULTS

The Interactions between ZO1-PDZ2 and ZO2-PDZ2 and Their Self-associations—It has been demonstrated that ZO1 interacts with both ZO2 and ZO3. Deletion of any PDZ2

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The striking homology (67.5%) between ZO1-PDZ2 and ZO2-PDZ2 suggests a great possibility of their self-associations. To test this possibility, we transformed *E. coli* BL21(DE3) with the plasmids encoding GST-ZO1-PDZ2, GST-ZO2-PDZ2, or GST alone. The lysate from each transformant was incubated with glutathione-Sepharose 4B beads and mixed with bacterially purified His-ZO1-PDZ2 or His-ZO2-PDZ2 proteins in pull-down buffer. As shown in Fig. 1A, His-ZO1-PDZ2 was retained by both GST-ZO2-PDZ2 and GST-ZO1-PDZ2; His-ZO2-PDZ2 was retained by both GST-ZO1-PDZ2 and GST-ZO2-PDZ2, indicating that ZO1-PDZ2 and ZO2-PDZ2 were able to bind not only to each other, but also to themselves. Neither His-ZO1-PDZ2 nor His-ZO2-PDZ2 was retained by GST alone. To further confirm the physical interactions between PDZ2 domains of ZO1 and ZO2 in vivo, co-

![Image](https://example.com/image1.png)

**FIGURE 3.** Evidence of three-dimensional domain swapping for ZO2-PDZ2 domain. A, a region from a two-dimensional NOESY spectrum of unlabeled sample showing the $\text{H}_\text{n}/\text{H}_\text{o}$ connectivities characteristic of antiparallel $\beta$-sheets for ZO2-PDZ2. A total of twelve such NOEs, both intramolecular and intermolecular, is indicated by paired residue numbers. Single numbers identify intra-residue NOEs for Ser21 and Gly3. B, the same region as in A from a $^{13}$C F1-filtered, F2-edited two-dimensional NOESY spectrum showing seven intermolecular NOEs involving in the domain-swapping interfaces. C, the secondary structures of the domain-swapping dimer inferred from the NMR data. The observed intermonomer NOEs are indicated by dashed lines. Carbon atoms from different chains are colored in green and cyan; oxygen, nitrogen, and $\text{H}_\text{n}$ atoms are colored red, blue, and white, respectively.

![Image](https://example.com/image2.png)

**FIGURE 4.** ZO1-PDZ2 and ZO2-PDZ2 can form heterodimer in solution. Superposition of $^{1}H$-$^{15}N$ HSQC spectra of a mixed sample consisting of $^{15}$N-labeled ZO2-PDZ2 and unlabeled ZO1-PDZ2 before unfolding and after refolding. The $^{15}$N-labeled ZO2-PDZ2 and unlabeled ZO1-PDZ2 ratio is 1:3. The cross-peaks of ZO2-PDZ2 before unfolding are shown in magenta, and the cross-peaks of ZO2-PDZ2 after refolding are shown in blue.

The domain of these proteins can disrupt the complex formation (20, 21).
FIGURE 5. Structure of ZO2-PDZ2. A, backbone overlay of 20 NMR structures with the lowest energy from the final CNS v1.1 calculation. This figure was produced with MOLMOL. B, a ribbon representation of the energy-minimized average structure with the secondary structure elements labeled. This figure was produced with PyMOL (W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA); for clarity, the two chains A and B are colored in green and cyan, with their N and C termini labeled with NAs, CAs, Ns, and Cs, respectively. C, comparison of the three-dimensional structure of the GRIP2PDZ3 monomer (PDB entry: 1V62) to that of one subunit in the ZO2-PDZ2 homodimer. The backbones of some secondary structured regions of the GRIP2PDZ3 monomer (yellow, α1, α2, β3, β4, β5, and β6) and a subunit of the ZO2-PDZ2 homodimer (green, α1, α2, β2a, β3, β4, and β5) are superimposed to each other. This figure was produced with PyMOL. D, sequence alignment of the second PDZ domains of ZO proteins, GRIP2PDZ3, GRIP1PDZ6, and Shank1PDZ. The sequences were aligned using the program ESPript (Easy Sequencing in PostScript) (42). Residues identical in all sequences are colored white in the red column; conserved residues are colored red, whereas the others are in black. Secondary structure elements for ZO2-PDZ2 and Shank1PDZ are shown above or below the sequences, respectively, with helices as squiggles, strands as arrows, and turns as TT.
immunoprecipitation was performed as described under “Materials and Methods.” As expected, GFP-ZO1-PDZ2 could be co-precipitated by both FLAG-ZO2-PDZ2 and FLAG-ZO1-PDZ2, and GFP-ZO2-PDZ2 could be co-precipitated by both FLAG-ZO1-PDZ2 and FLAG-ZO2-PDZ2. Neither GFP-ZO1-PDZ2 nor GFP-ZO2-PDZ2 was co-precipitated by FLAG only (Fig. 1B). The above results strongly suggest that interactions between ZO1-PDZ2 and ZO2-PDZ2 as well as their self-associations indeed exist in mammalian cells.

ZO1-PDZ2 and ZO2-PDZ2 Form Both Homodimer and Heterodimer Tightly in Solution—As shown in Fig. 2A, ZO1-PDZ2 was eluted out at a molecular mass of 28 kDa, and ZO2-PDZ2 was eluted out as a 25-kDa molecule, suggesting that they both exist as dimers. Their association states were further examined by analytical ultracentrifugation. Only one major peak was detected for each of these two proteins, indicating that they are structurally homogeneous in solution. The results of data analysis with Sedfit version 8.9d showed that ZO1-PDZ2 and ZO2-PDZ2 sediment at 1.95 S and 1.83 S, corresponding to a molecular masses of 21.2 and 20.6 kDa, respectively (Fig. 2B). These results confirmed that ZO1-PDZ2 and ZO2-PDZ2 both exist as dimers, in agreement with the results of gel-filtration chromatography. The two dimers are very tight, because no monomeric fraction was detected by sedimentation velocity analysis.

To characterize the dimeric states of ZO1-PDZ2 and ZO2-PDZ2 by NMR spectroscopy, equivalent amounts of $^{15}$N and $^{13}$C labeled and unlabeled proteins were mixed in a buffer (containing 20 mM PBS, 200 mM NaCl, and pH 6.5) to reach a heterolabeled dimer. The mixture of proteins was treated with 8 M urea to make the protein unfolded and then refolded through dialysis. Two-dimensional $^{13}$C F1-filtered, F2-edited NOESY, HSQC and 2D HSQC spectra showed the H$_{\alpha}$N, H$_{\alpha}$C$_{\alpha}$ protons were found in close proximity. Moreover, the N-terminal residues (1–22), including B1 (2–7) and half of B2 (17–22) of one monomer demonstrate other NOEs with residues beyond Gln22 of another monomer, suggesting that the dimeric ZO2-PDZ2 adopted a domain-swapping structure.

To further verify if ZO1-PDZ2 and ZO2-PDZ2 can form heterodimer in solution, we mixed $^{15}$N-labeled ZO2-PDZ2 with unlabeled ZO1-PDZ2. The mixture was treated with 8 M urea, and subsequently dialyzed against refolding buffer. The $^1$H-$^{15}$N HSQC spectrum of the refolded mixture showed two sets of resonances (Fig. 4, blue), one completely overlaps with the ZO2-PDZ2 homodimer (Fig. 4, magenta), and the other has its distinct pattern presumably representing ZO1-PDZ2/ZO2-PDZ2 heterodimer. Taken together, the biochemical and NMR spectroscopic data conclusively demonstrate that ZO1-PDZ2 and ZO2-PDZ2 can form not only homodimers, but also heterodimers in solution.

**TABLE 1**

| Distance restraints | 2202 |
|--------------------|------|
| Intraresidue (i−j=0)| 720  |
| Sequential (i−j=1) | 684  |
| Medium range (2≤i−j≤4) | 452 |
| Long range (i−j≥5)  | 346  |
| Intermolecular      | 590  |
| Hydrogen bonds      | 62   |
| Intramolecular      | 38   |
| Intermolecular      | 24   |

**Table Mean r.m.s. deviations from the experimental restraints**

| Distance (Å) | 0.0093 ± 0.0004 |
| Dihedral (°) | 0.1608 ± 0.0235 |

**Table Mean r.m.s. deviations from idealized covalent geometry**

| Bond (Å) | 0.0026 ± 0.00009 |
| Angle (°) | 0.3748 ± 0.0048 |
| Improper (°) | 0.1830 ± 0.0060 |

**Mean energies (kcal mol$^{-1}$)**

| Enoe | 19.05 ± 1.74 |
| Ecdih | 0.11 ± 0.03 |
| E$_{ij}$ | −481.76 ± 15.59 |

**Ramachandran plot (%) (residues)**

| Residues in the most favorable regions | 83.7 |
| Additional allowed regions | 13.8 |
| Generously allowed regions | 1.6 |
| Residues in disallowed regions | 0.8 |

**Atomic r.m.s. differences (Å)**

| Backbone heavy atom (N, C$_\alpha$, and C') | 0.485 |
| Heavy atoms | 1.016 |

*The program Procheck was used to assess the overall quality of the structures.*

*The 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.*
Finally the three-dimensional structure of the dimeric ZO2-PDZ2 was determined using a total of 2854 experimental restraints derived from NMR spectroscopy, including 590 intermolecular NOEs and 24 intermolecular H-bonds. Intermolecular NOEs were obtained from the three-dimensional 15N-edited NOESY-HSQC spectrum and three-dimensional 13C-edited NOESY-HSQC spectrum and further confirmed by the 13C F1-filtered, F2-edited two-dimensional NOESY spectrum. There are 20 intermolecular H-bonds and 26 intramolecular H-bonds determined experimentally from H/D exchange experiments, and 4 intermolecular H-bonds and 12 intramolecular H-bonds inferred from the standard secondary structure of the protein based on NOE patterns.

Fig. 5A shows an ensemble of 20 lowest energy NMR structures. It was selected from 200 accepted structures by requiring no NOE violation >0.5 Å and no dihedral angle violations >5°. A ribbon representation of the energy-minimized average structure of ZO2-PDZ2 domain was shown in Fig. 5B. The coordinates of these 20 NMR structures have been deposited into the Protein Data Bank (code 2OSG).

The structural statistics are listed in Table 1. The root mean square deviation (r.m.s.d.) of the well defined regions (Ile2–Lys8 and Tyr15–Leu78 of both subunits) of the 20 structures to the average structure was 0.485 Å for the backbone and 1.016 Å for the heavy atoms. In contrast, the regions of Ser9–Glu14 of two subunits are disordered because of fewer medium and long range NOEs. PROCHECK analysis showed that >97% of the residues lied in the most favored and additional allowed regions of the Ramachandran plot.

Three-dimensional Domain-swapping Structure of ZO2-PDZ2 Homodimer—ZO2-PDZ2 homodimer consists of two identical polypeptide chains. Each chain adopts the βαβαβ topology, with two strands from the other subunit (Fig. 5B). Domain swapping of the homodimer occurs through exchanging their N-terminal β1- and β2-strands. The β1-strand (residues 2–7) from one subunit forms an antiparallel β-sheet with β5 from the other subunit (residues 77–72) in canonical PDZ domains, strands 2 and 3 are linked by a loop with various lengths, and the two strands form an intramolecular antiparallel β-sheet. In the domain-swapped ZO2-PDZ2 dimer, the cor...
responding antiparallel \( \beta \)-sheet is formed by \( \beta_{2a} \) (residues 17–22) from one subunit and \( \beta_{2b} \) (residues 28'–22') from the other subunit (Fig. 5B). Except for the domain swapping, the overall fold of the PDZ subunits in the dimer is highly similar to canonical PDZ domains (Fig. 5C). The backbone r.m.s.d. value for the core structure of one subunit of ZO2-PDZ2 homodimer (residues L19–R79) and GRIP2PDZ3 (residues N40–P100) was 1.4 Å (calculated using the Combinatorial Extension method (48)). Three interchain antiparallel \( \beta \)-sheets, \( \beta_{1}–\beta_{5}' \), \( \beta_{1}'–\beta_{5} \), and \( \beta_{2}–\beta_{2}' \) are shown in Fig. 6.

Dimer Interface of ZO2-PDZ2—A great part of the dimer interface is involved in extended \( \beta \)-strands matching most swapped domains (Fig. 6A). The protruding \( \beta_{1} \), \( \beta_{2} \), and \( \beta_{5} \) strands of one subunit are paired off with \( \beta_{5}' \), \( \beta_{2}' \), and \( \beta_{1}' \) strands of the other subunit, respectively, in an antiparallel manner. Intermolecular hydrogen bonds are deduced from the geometry of the structure. 24 H-bonds are found in the three antiparallel \( \beta \)-sheets (Fig. 6B). The interactions of some residues in the interface between the two monomeric subunits, such as Phe\(^{24} \)/Ile\(^{45}' \), Phe\(^{24} \)/Glu\(^{55}' \), and Asn\(^{56} \)/Leu\(^{59}' \), steady the hinge region of the dimer (Fig. 6C). The protein complex with abundant domain-swapping structure also shows abundant hydrophobic residues in the dimeric interface, suggesting that the hydrophobic interactions might play an important role in maintaining the stabilization of dimer structure.

Evidence for Oligomerization of ZO1-PDZ2 and ZO2-PDZ2—The oligomeric state of ZO1-PDZ2 or ZO2-PDZ2 in solution was probed by chemical cross-linking experiments. DSS cross-linking of ZO1-PDZ2 generated a dimeric and some oligomeric species. ZO2-PDZ2 yielded less dimer and more oligomers when compared with ZO1-PDZ2 treated by DSS under the similar cross-linking condition (Fig. 7A, lanes 1–4 versus 5–8). This suggests that ZO2-PDZ2 might have more oligomeric species in solution than ZO1-PDZ2, or alternatively, ZO2-PDZ2 might contain more favorably positioned lysine residues to allow DSS cross-linking than ZO1-PDZ2. Without DSS treatment, both ZO1-PDZ2 and ZO2-PDZ2 appeared as monomers, despite trace amounts of dimers on SDS-PAGE gel (Fig. 7B), which indicate no covalent bond was formed between the two subunits of the dimers.

The oligomeric state of ZO1-PDZ2 and ZO2-PDZ2 was further examined by DLS. The DLS experiments were performed under physiological buffer conditions at 37 °C (Fig. 7, C and D). For ZO1-PDZ2 and ZO2-PDZ2, the hydrodynamic radius distributions \( f(R_h) \) exhibit single peak at 1–10 nm at 0 h. As time increases, another peak at \(~210\) nm appears, indicating the
occurrence of aggregation. Actually, the aggregation should already occur before the DLS measurements. However, the aggregates were filtered out by a 0.2-μm filter, namely, no aggregation at the starting point we chose. Note that $f(R_m)$ is $z$-averaged here. Actually only a small amount of ZO1-PDZ2 or ZO2-PDZ2 molecules form aggregates or oligomers in terms of weight. After a period of time (28 h for ZO1-PDZ2 and 16 h for ZO2-PDZ2), the oligomerization was nearly finished and the system reached an equilibrium. Therefore, either ZO1-PDZ2 or ZO2-PDZ2 mainly consists of their dimers in solution.

To get an insight into the possible molecular mechanism of oligomerization, the electrostatic potential at the solvent-accessible surface for ZO2-PDZ2 dimer was calculated. Apparently, complementary surface electrostatic potential might contribute to oligomerization of ZO2-PDZ2 (Fig. 8). In our experiments, the ion strength of the solution does affect the processes of aggregation, the higher the salt concentration, the narrower the line width of signals in the $^1$H-$^1$N HSQC spectrum of ZO2-PDZ2 or ZO1-PDZ2 (data not shown). Based on these results, we propose that the tight ZO2-PDZ2 dimers are oligomerized by the charge interactions in solution.

**DISCUSSION**

In this study, we discovered for the first time that a PDZ domain (ZO2-PDZ2 in this case) can form dimer via three-dimensional domain swapping. The unique amino acid sequence within the $\beta$2a and $\beta$2b region of the ZO2-PDZ2 is likely responsible for the formation of the domain swapped dimer. Previous studies have discovered several dimeric patterns for PDZ domains. For example, in Shank1PDZ, its dimer interface involves the $\beta$1 strand and the $\beta$2/3 loop from each monomer (49), as for GRIPIPDZ6, it involves the $\beta$1 strand and the $\alpha$1/$\beta$4 loop (ligand binding state) (50). As revealed by the multiple sequence alignment analysis (Fig. 5D), ZO2-PDZ2 lacks the long loop between $\beta$2 strand and $\beta$3 strand that can be found in shank1PDZ and GRIPIPDZ6. This means that the linker between $\beta$2a and $\beta$2b of ZO2-PDZ2 is not long enough for $\beta$2b to swing back to form an antiparallel $\beta$-sheet with $\beta$2a strand, hence facilitates the swapping structure. There are accumulated evidences supporting that engineering the hinge loop can affect three-dimensional domain swapping (51). With shortened hinge loop, Domain 1 of CD2 (52), staphylococcal nuclease (53), and single chain Fv (54, 55) form domain-swapped dimers. On the other hand, extending the hinge loop in the domain-swapped dimer of cro repressor leads to monomer formation (56). Due to the high conservation of PDZ2 domains in ZO proteins, we suppose that domain-swapping structure also applies for the ZO1-PDZ2 and ZO3-PDZ2 homodimers or ZO1-PDZ2/ZO2-PDZ2 and ZO1-PDZ2/ZO3-PDZ2 heterodimers. The tight dimeric structure of ZO2-PDZ2 (and likely ZO1-PDZ2) is due to extensive intersubunit hydrogen bonds and hydrophobic interactions formed via swapping of the N-terminal two $\beta$-strands of each subunit.

Furthermore, the *in vitro* GST pulldown experiments and *in vivo* immunoprecipitation study have been utilized to define the interactions between ZO1-PDZ2 and ZO2-PDZ2. We dem-

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**FIGURE 8. Electrostatic surface potential representation of the ZO2-PDZ2 dimer.** The molecular surfaces are colored from dark blue (most positive) to deep red (most negative) according to the local electrostatic potential on a relative scale. The orientation of the molecule in panel B is rotated 180° from panel A. The charge topology was calculated and displayed using MOLMOL.
onstrated that ZO1-PDZ2 and ZO2-PDZ2 interact directly both in vitro and in vivo; concomitantly, both PDZ2 domains undergo self-associations. Using chemical cross-linking and the dynamic laser light scattering method, we concluded that both ZO1-PDZ2 and ZO2-PDZ2 exist as a combination of dimers and oligomers in solution. The in vitro self-association of and the interactions between ZO1-PDZ2 and ZO2-PDZ2 should be caused by their homo- and hetero-oligomerization, because both ZO1-PDZ2 and ZO2-PDZ2 are tight dimers. It is likely that the unique surface-charge potential property of the homodimers afford them the ability to aggregate.

The fundamental functions of epithelia and endothelia in multicellular organisms are to separate compositionally distinct compartments and regulate the exchange of small solutes and other substances between them. TJ between adjacent cells constitute the barrier to the passage of ions and molecules through the paracellular pathway. Occludin and claudins are identified as constituents of TJ strands (7, 8), and claudins are thought to constitute the backbone of TJ strands (30). ZOs bind the carboxyl termini of claudin YV through their first PDZ domain (36). Umeda et al. have shown that dimerized ZO1 (and probably also ZO2) not only initiates the polymerization of claudins but also directs the correct localization of TJ strands. Utebergoven et al. found ZO1 forms stable homodimers in solution or higher order oligomers in vivo via its second PDZ domain, whereas PDZ3-acidic (a construct containing PDZ3, SH3, GK, and acidic domain) exists only as monomer. They concluded that it is the PDZ2 of ZO1 instead of SH3/GK domain that functions as a dimerization motif (22). On the contrary, Umeda et al. maintained that the SH3/GK domain, but not PDZ2 domain, is important for ZO1 dimerization, and this is similar to other MAGUK proteins such as PSD-95 and Dlg/SAP90/SAP102 (23). We found that both ZO1-PDZ2 and ZO2-PDZ2 can form tight homodimers, and the homodimers are formed by domain swapping. It has been suggested that either intramolecular or intermolecular domain swapping between SH3 and GK domains exists in PSD-95, and the intermolecular domain swapping may be promoted by regulatory factors in vivo (25, 26). Accordingly, we presume that intramolecular domain swapping kept the PDZ3-acidic of ZO1 in its monomer form in Utebergoven’s study, whereas in Umeda’s study, regulatory factors induced intramolecular domain swapping of SH3/GK module in ZO1. For the oligomerization or multimerization of ZO proteins in vivo, both PDZ2 domain and SH3/GK module may be necessary.

Therefore, we propose a model for the roles of PDZ2 domains as well as SH3/GK modules in regulated oligomerization or multimerization of ZOs (Fig. 9). Fig. 9A presents three-dimensional domain swapping between PDZ2 domains and between SH3-GK modules of closed dimer; Fig. 9B shows three-dimensional domain swapping between PDZ2 domains and between SH3-GK modules of open oligomer. Both cases may promote homo-, hetero-oligomerization or homo-, hetero-multimerization of ZOs, which in turn initiate the polymerization of claudins and consequently the assembly of TJ strands. This model is consistent with the observation that ZO1 or ZO2 was first recruited to the junction area, which initiated and facilitated the polymerization of claudins (23). The three-dimensional domain swapping of ZOs, perhaps directed by sets of regulatory proteins, could provide combinatorial scaffold diversity, resulting in different protein recruitments. Previous studies have provided evidence for three-dimensional domain swapping as a mechanism for allostery and signal sensing in a macromolecule, and therefore regulating biological functions of proteins (51). ZO proteins’ function as scaffolding proteins in the formation of intracellular signaling complexes could be greatly promoted by oligomerization or multimerization via three-dimensional domain swapping.

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