THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 34, pp. 24671–24677, August 25, 2006

Dimerization of the Scaffolding Protein ZO-1 through the Second PDZ Domain*

Received for publication, November 30, 2005, and in revised form, May 1, 2006 Published, JBC Papers in Press, June 21, 2006, DOI 10.1074/jbc.M512820200

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The tight junction protein ZO-1 is known to link the transmembrane proteins occludin, claudins, and JAMs to many cytoplasmic proteins and the actin cytoskeleton. Although specific roles for ZO-1 at the tight junction are unknown, it is widely assumed that ZO-1, together with its homologs ZO-2 and ZO-3, serves as a platform to scaffold various transmembrane and cytoplasmic tight junction proteins. Thus the manner in which the zonula occludens (ZO) proteins multimerize has implications for the protein networks they can coordinate. The purpose of our study was to determine whether ZO-1 forms homodimers and to determine the protein interaction region. Using laser light scattering and analytical centrifugation, we show that protein sequences corresponding to the NH_2-terminal half of ZO-1 form stable homodimers with a submicromolar equilibrium dissociation constant. Analysis of the molecular weight of different truncated forms of ZO-1 revealed that the second PDZ domain is both necessary and sufficient for dimerization. This interaction does not use the β-finger motif described for other PDZ dimers. Furthermore, ZO-1 does not dimerize via an Src homology 3 to Guk domain interaction as was demonstrated previously for MAGUKs, like PSD-95. Results from immunoprecipitation experiments with polarized Madin-Darby canine kidney epithelial cells stably transfected with full-length GFP-ZO-1 indicate that a substantial portion of ZO-1 forms homodimers *in vivo*. As described previously, ZO-1 also forms heterodimers with ZO-2 and ZO-3. We conclude that the dimerization of ZO proteins is unlike that of other MAGUKs and that the previously unrecognized ZO-1 homodimers may allow formation of protein networks distinct from those of heterodimers with ZO-2 and ZO-3.

The tight junction (TJ)[2] is the apical-most element of the junctional complex in epithelial and endothelial cells. This complex includes the TJ, adherens junctions, desmosomes, and gap junctions. The TJ forms a major barrier to paracellular movement of substances preserving unique chemical composition of apical and basolateral fluid compartments on opposite sides of the epithelial cell layer and coordinates transmembrane signaling (1, 2). The TJ is known to contain at least four classes of transmembrane proteins and >30 cytoplasmic signaling, scaffolding, and cytoskeletal proteins (3). To understand how the barrier and signals are coordinated, more information is required on the specific molecular interactions of TJ proteins and specifically the proteins that coordinate interaction among transmembrane and cytosolic proteins.

Peripheral membrane proteins of the tight junction are believed to be highly organized within a complicated network interconnecting cytoskeleton with transmembrane proteins. The three homologous zonula occludens (ZO) MAGUK proteins (ZO-1, ZO-2, and ZO-3) are among the best characterized peripheral membrane proteins of the tight junction. They are composed of three PDZ domains, an SH3-GUK unit, and an actin-binding region; these proteins are believed to cluster different components of tight junctions together through the direct binding to specific domains and/or regions (3). Each has multiple known binding partners; some are shared among all three, such as binding to actin and claudins, whereas others are unique, such as binding of E4-ORF1 protein from type 9 adenovirus to ZO-2 (4). In tissues lacking tight junctions, ZO proteins are localized to adherence junction where they are presumed to interact with cadherin-catenin complexes. Furthermore, ZO-1 was shown to bind many different connexins, structural components of gap junction, in a number of reports (5–7).

Much efforts has been made to define the function of ZO proteins, for example through exogenous expression of truncated ZO-1 (3, 8, 9) and ZO-3 (10). A high degree of sequence conservation between ZO proteins suggests that these proteins may have a significant functional redundancy, making it difficult to elucidate their function. Strong evidence that ZO proteins are indeed functionally redundant came from a recent report demonstrating that in ZO-1-deficient cells, cytoplasmic ZO-2 was recruited into tight junctions to compensate for the loss of ZO-1 (11). ZO proteins are known to hetero-oligomerize via their second PDZ domains. ZO-1 binds to both ZO-2 and ZO-3, whereas ZO-2 and ZO-3 do not bind each other (12). Because no endogenous ZO-1 from MDCK cells was detected in immunoprecipitates of transiently transfected human ZO-1, it was concluded that ZO-1 does not form homo-oligomers (3). Hence, ZO proteins are thought to exist exclusively as ZO-1/ZO-2 and ZO-1/ZO-3 heterodimers (12). Determining whether homodimers exist is critical for understanding the complexity of protein networks that ZO proteins could create.

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[2] The abbreviations used are: TJ, tight junction; MDCK, Madin-Darby canine kidney cells; SH, Src homology; PBS, phosphate-buffered saline; ZO, zonula occludens; SEC-MALS, size exclusion chromatography with multiangle light scattering; GFP, green fluorescent protein; LS, light scattering; RI, refractive index.

In this work we demonstrate that, contrary to the previous beliefs, ZO-1 forms a stable dimer via its second PDZ domain and partially characterizes biophysical properties of the dimer. We also show that significant amounts of ZO-1 in MDCK cells exist as a homodimer.

EXPERIMENTAL PROCEDURES

Antibodies and Affinity Media—Mouse monoclonal antibodies were purchased from Molecular Probes Inc. (Eugene, OR). R40.76 antibody was obtained from Bruce Stevenson (Salk Institute, La Jolla, CA). All other antibodies and goat anti-rat IgG immobilized on Sepharose 4B were purchased from Zymed Laboratories Inc. Protein G-Sepharose was purchased from GE Healthcare.

Molecular Cloning—A NotI-XhoI fragment of plasmid pSKZO-1 encoding about 1000 amino acid residues of ZO-1 was cloned into the pFastBacHTa vector. The resulting plasmid was digested with NheI and HindIII and ligated by PCR with a fragment of ZO-1 (~720 bp) amplified with primers AAT CAT AAG GAG GTA GAA CGA GGC and TTT AA GCT TCT AAT CCG CCT TTC CCT CGG AAA CCC to introduce a stop codon after amino acid 806. To modify the NH_2 terminus, the construct was digested with NotI and KasI and ligated with annealed oligonucleotides GCG CCA TGT CCG CCA GAG CTG C and GGC CGC AGC TCT GGC GGA CAT G. The resulting construct encodes amino acids 1–806 of human ZO-1 with an NH_2-terminal His_6 tag. Other constructs were obtained by similar procedures (details are available upon request). These constructs and their amino acid sequence ranges are presented in Fig. 4. GFP-ZO-1 was obtained by subcloning of ZO-1 into pEGFP-C1 vector, followed by subcloning of GFP-ZO-1 into pIRES vector.

Expression and Purification of Recombinant ZO-1 Fragments—Recombinant bacmids and baculoviruses were obtained using Bac-to-Bac kit (Invitrogen). High Five cells were infected at a multiplicity of infection of 3–5, harvested 48–60 h after infection, and kept frozen at −80 °C. Cell pellets were thawed in 6 volumes of lysis buffer (20 mM Tris-Cl, pH 8, 150–500 mM NaCl, 10% glycerol, 0.8% Igepal CA-630, 12.5 mM imidazole, 5 mM 2-mercaptoethanol, and Complete™ protease inhibitor mixture (Roche Applied Science)), homogenized in a Dounce homogenizer, and centrifuged for 10 min at 20,000 × *g*. Protein was purified from the supernatant using EZview™ Red His-Select HC nickel affinity gel from Sigma and eluted into PBS containing 5 mM β-mercaptoethanol and 125 mM of imidazole.

MDCK Cell Culture, Transfection, and Stable Clone Selection—MDCK cells were cultivated in DMEM containing 10% fetal bovine serum using 25-cm^2 tissue culture flasks or 6-well plates. Cells in 6-well plates were transfected with 3 μg of GFP-ZO-1 plasmid and 5 μg of Cellfectin (Invitrogen) according to the manufacturer's instructions. Transfected cells were cultivated in media containing 0.8 mg/ml G-418, and cell clones were isolated with cloning rings.

Immunoprecipitation and Immunoblotting—MDCK cells grown on 50-cm^2 Petri dishes were washed twice with PBS and lysed in 0.6 ml of RIPA buffer (20 mM Tris-Cl, 150 mM NaCl, 5 mM 2-mercaptoethanol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). The lysate was clarified by centrifugation at 15,000 × *g* for 10 min and added to 30 μl of protein G beads preloaded with 5 μg of antibodies. Beads were incubated with the lysates for 2 h, washed five times with PBS containing 0.2% of Triton X-100, and boiled for 5 min in 100 μl of electrophoresis sample buffer to elute bound proteins. Protein samples were resolved on 4–12% gradient polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were blocked in PBS containing 5% nonfat dry milk, incubated with primary and fluorophore-labeled secondary antibodies, and scanned with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

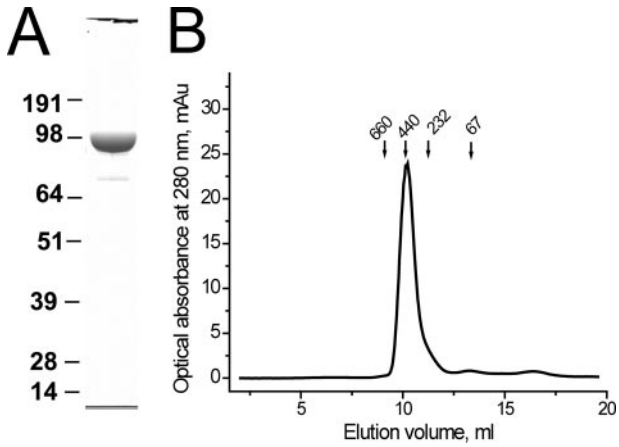

FIGURE 1. **Evidence for oligomerization of NZO-1.** NZO-1, expressed in insect cells and purified over Ni^{2+}-Sepharose, shows a single band of the expected molecular weight with no major impurities (*A*). Gel filtration profile suggests that NZO-1 may form a stable oligomer in solution (*B*).

Analytical Centrifugation—Sedimentation equilibrium experiments were performed with a Beckman Optima XL-A analytical centrifuge. Partial specific volume of NZO-1 was estimated to be 0.727 using SEDNTERP software. NZO-1 at 2–6 μM was analyzed in PBS containing 1 mM of 2-mercaptoethanol at 20 °C at speeds ranging between 6,000 and 20,000 rpm. Samples were spun in a 6-channel 12-mm external fill equilibrium centerpiece in an An-60 Ti rotor. Scans (280 nm) were collected at equilibrium in radial step mode with 0.001-cm steps and 5-point averaging. Data fitting was performed with one-component or monomer-dimer equilibrium models using XL-A/XL-I data analysis software (Beckman Instruments, Rockville, MD).

Size Exclusion Chromatography with Multiangle Light Scattering (SEC-MALS)—SEC-MALS experiments were performed with an Akta FPLC (GE Healthcare) coupled to a Wyatt DAWN EOS light scattering instrument, Wyatt Optilab refractometer, and Wyatt dynamic light scattering module (Wyatt Technology Corp., Santa Barbara, CA). For chromatographic separations, a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with phosphate-buffered saline containing 5 mM 2-mercaptoethanol was used at an eluent flow rate of 0.5 ml/min. Molecular weight calculations were made by ASTRA software using a d*n*/d*c* value of 0.19 ml/g.

RESULTS

The NH_2-terminal Half of ZO-1 Forms a Dimer in Solution—The protein corresponding to the NH_2-terminal half of ZO-1 (NZO-1, amino-acids 1–888) comprising three PDZ

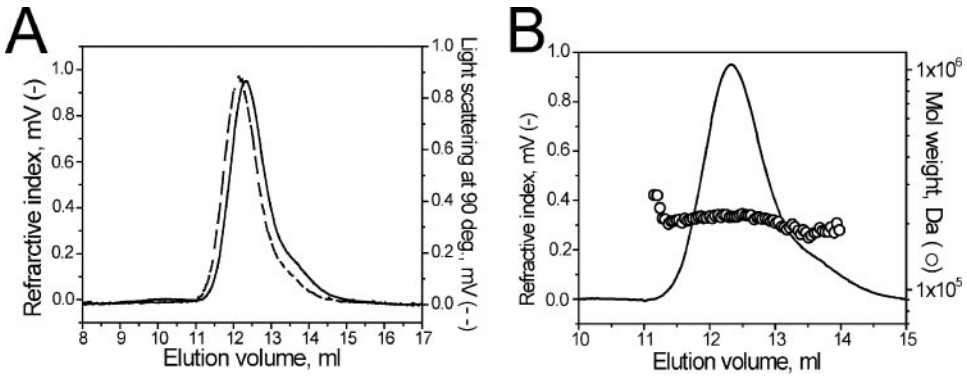

FIGURE 2. **NZO-1 is a dimer.** *A*, refractive index (*solid line*) and light scattering (*dashed line*) profiles of NZO-1; *B*, the dependence of calculated molecular weight on elution volume. The profiles appear displaced because the detectors are positioned in series in the flow path.

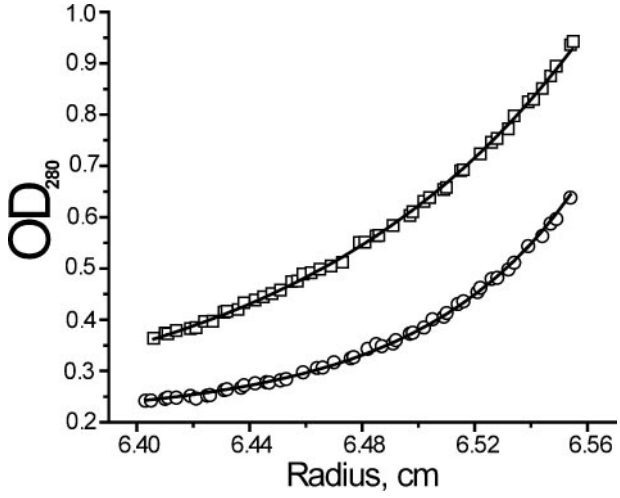

FIGURE 3. **Sedimentation equilibrium analysis of NZO-1.** Sedimentation equilibrium data for NZO-1 at 5 μM are shown for 8000 (□) and 1100 rpm (○). Fits corresponding to the monomer-dimer model are shown as *solid lines*.

domains, an SH3 domain, a GuK domain, and an acidic domain was purified to homogeneity from baculovirus-infected cells. NZO-1 resolves as a 100-kDa band on polyacrylamide gel in the presence of SDS (predicted molecular mass of NZO-1 is 102.7 kDa), showing no significant impurities (Fig. 1*A*). Size exclusion chromatography revealed that NZO-1 elutes as a single peak with an apparent molecular mass of 400 kDa, suggesting that NZO-1 may form a stable oligomer or be very highly asymmetric (Fig. 1*B*). Because size exclusion chromatography data are dependent on the shape of the protein, the determination of the exact molecular weight of the protein is difficult. To characterize the oligomeric state of NZO-1 in solution, we performed size exclusion chromatography with on-line detection of light scattering (LS) and refractive index (RI) signals, because this approach allows an accurate measurement of the molecular weight of the protein independently of its shape and/or amino acid composition (13). Representative chromatographic profiles of NZO-1 obtained from LS and RI detectors are shown in Fig. 2*A*. The molecular mass of the NZO-1 calculated from LS and RI data was found to be 209 kDa indicating that NZO-1 is a dimer. The molecular mass is independent of the elution volume (Fig. 2*B*) indicating the absence of significant amounts of monomer and higher order oligomers under these conditions.

In an attempt to determine the K_d value of the dimer, the NZO-1 was further characterized by analytical centrifugation. In two independent experiments, the single ideal species fit of sedimentation equilibrium data returned values close to 200 kDa indicating the predominance of a dimer in solution. The analysis of sedimentation equilibrium data shows that good fits can be obtained using a monomer-dimer model (Fig. 3). However, because comparable fits were obtained with a single species model using the theoretical molecular weight of the dimer (not shown), we concluded that monomer concentrations are sufficiently low that they cannot be determined accurately. This precluded the determination of K_d values of monomer-dimer equilibrium, still allowing us a theoretic analysis of simulated binding isoterms, which suggested that the K_d must be below 0.7 μM.

Identification of the Dimerization Domain—To define which part of NZO-1 is responsible for dimerization, we purified truncated forms of NZO-1 using the insect cell/baculovirus expression system, and we investigated their oligomerization status by SEC-MALS. We created a truncated form lacking the seven NH_2-terminal amino acid residues of ZO-1 because another MAGUK, hDlg, was reported to oligomerize via their NH_2 termini (14). Other constructs lacking different domains are shown on Fig. 4*A*. All constructs could be expressed and purified to apparent homogeneity (Fig. 4*B*). Molecular masses of all proteins determined from the positions of visualized bands on denaturing SDS-polyacrylamide gels are close to the corresponding theoretical values except for PDZ2+I2 (amino acids 183–349), which has an apparent molecular mass 10 kDa higher than predicted.

All purified truncation forms of ZO-1 were subjected to SEC-MALS in order to determine their molecular weight. Similarly to NZO-1, all investigated proteins eluted as single peaks with little or no dependence of calculated molecular weight on elution volume (data not shown). The results of SEC-MALS experiments are summarized in Table 1. Absolute molecular masses of all truncated forms of ZO-1 except PDZ3-acidic (this construct was found to be a monomer) are close to the theoretical masses of corresponding dimers (Table 1). Because all constructs except PDZ3-acidic include the second PDZ domain, these results clearly indicate that PDZ2 is necessary and sufficient for dimerization.

Dimerization of ZO-1 in Vivo—Because ZO-1 was not previously thought to dimerize, we next sought to determine whether dimers exist *in vivo*. We established stable lines of MDCK cells expressing either GFP or GFP-tagged ZO-1. As reported previously (15), the distribution of GFP-ZO-1 was similar to endogenous ZO-1, both being found almost exclusively in tight junctions (Fig. 5*A*). The localization of ZO-1 in control cells expressing GFP remained unchanged (Fig. 5*A*). Endogenous ZO-1 was immunoprecipitated from GFP and

GFP-ZO-1 expressing cell lines using the R40.76 monoclonal antibody (this antibody recognizes canine but not exogenous human GFP-ZO-1). A significant fraction of GFP-ZO-1 was found to co-immunoprecipitate with endogenous ZO-1, indicating the presence of ZO-1 dimers or higher order oligomers *in vivo* (Fig. 5*B*). In converse experiments, when GFP-ZO-1 was immunoprecipitated with anti-GFP antibodies endogenous ZO-1 was also found to co-immunoprecipitate with GFP-ZO-1. No binding of GFP to ZO-1 was observed in control experiments (Fig. 5*B*).

To understand how ZO-1 is distributed among homo- and hetero-oligomers with ZO-2 and ZO-3, we performed a series of repeated immunoprecipitations trying to completely deplete either ZO-1, ZO-2, or ZO-3 from MDCK cell lysates with subsequent quantification of the proteins in bound and unbound fractions. The NH_2-terminal part of ZO-1 (amino acids 1–888) and the full-length ZO-2 expressed in insect cells were used as standards to perform quantitative immunoblotting (Fig. 6*A*); ZO-3 from insect cells could not be obtained in sufficient purity. ZO-2 and ZO-3 were depleted from the MDCK cell lysate to find out what fraction of ZO-1 is not bound to ZO-2 and ZO-3. A representative experiment and quantification as shown in Fig. 6*B* show that over 85% of ZO-2 and over 95% of ZO-3 could be immunoprecipitated with antibodies directed against them. Quantitative analysis of ZO-1 revealed that about 107 fmol (50%) of solubilized ZO-1 is not bound to either ZO-2 or ZO-3 and remains in the supernatant. Accordingly, we found that about 50% of ZO-1 is bound to ZO-2 and ZO-3.

Finally, we used MDCK cells stably expressing GFP-ZO-1 to quantify the amount of ZO-2 and GFP-ZO-1 that co-precipitate with endogenous ZO-1. Fig. 6*C* shows that endogenous ZO-1 could be quantitatively precipitated from MDCK cells stably expressing GFP-ZO-1. Immunoblotting with anti-GFP antibodies (Fig. 6*C*) revealed that about 40% of GFP-ZO-1 co-precipitated with endogenous ZO-1. Immunoblot with rabbit antibodies recognizing both GFP-ZO-1 and endogenous ZO-1 allowed us quantify the amounts of these proteins. Endogenous ZO-1 (*arrowhead*) and GFP-ZO-1 (*arrow*) were found to be expressed at comparable levels (Fig. 6*C*). Analysis of the protein sample obtained from R40.76 immunoprecipitates revealed that it contains 90 fmol of endogenous ZO-1 and about 65 fmol of GFP-ZO-1. Analysis with anti-ZO-2 antibodies revealed that 19 fmol of ZO-2 co-precipitated with endogenous ZO-1 (Fig. 6*C*) in MDCK cells stably expressing GFP-ZO-1 (Fig. 6*C*).

DISCUSSION

ZO-1 was the first tight junction protein to be discovered and is thought to be one of the major scaffolds at tight junctions. The biochemical and biophysical properties of ZO-1 were poorly characterized so far, probably because of the difficulties associated with the expression of large constructs in bacteria. Using insect cells/baculovirus expression system, we were able to obtain milligram amounts of the NH_2-terminal part of ZO-1 (the basic core structure ZO-1 shares with other MAGUKs like PSD-95 and *disc large*), which permitted the first detailed characterization of its biophysical properties. Because of anomalous gel filtration behavior of NZO-1, we used multiangle light scattering to characterize its oligomeric state. Unexpectedly, NZO-1 was found to exist exclusively as a stable dimer. Analytical centrifugation confirmed that NZO-1

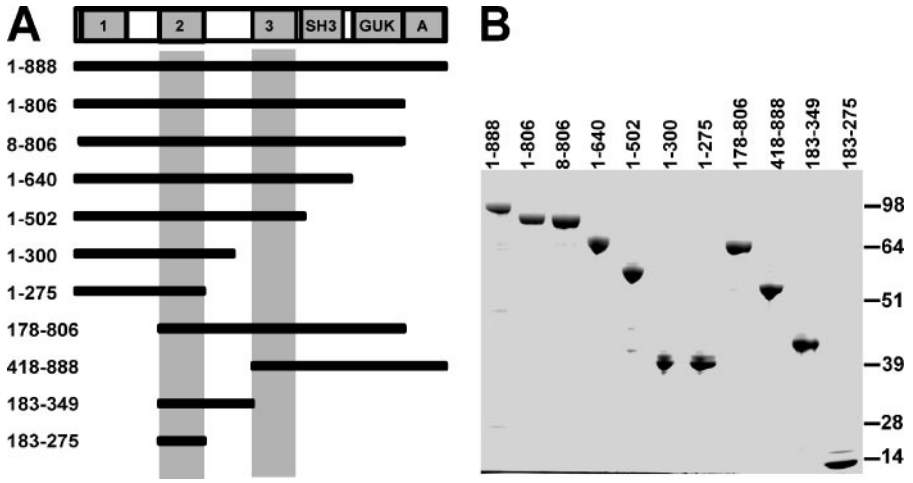

FIGURE 4. **Truncated ZO-1 constructs expressed in insect cells.** *A*, different truncated mutants of NZO-1 designed for expression. *B*, Coomassie-stained polyacrylamide gel showing truncated ZO-1 proteins expressed in baculovirus-infected insect cells and purified to apparent homogeneity with Ni^{2+}-Sepharose and used for study of their oligomeric state.

TABLE 1
Identification of dimerization motif of ZO-1 by light scattering

Protein	Amino acids	Theoretical mass of monomer	Theoretical mass of dimer	Mass by light scattering	Oligomerization state
		kDa	*kDa*	*kDa*	
NZO1	1–888	102.7	205.4	209	Dimer
Δacidic	1–806	93.8	187.6	185	Dimer
ΔN7	8–806	92.9	185.8	183	Dimer
ΔGuK	1–640	74.8	149.6	147	Dimer
PDZ1-3	1–502	58.8	117.6	126	Dimer
PDZ1-2+I2a	1–300	38.9	77.8	78.9	Dimer
PDZ1-2	1–275	33.4	66.8	71.0	Dimer
ΔPDZ1+I1	178–806	74.2	148.4	145	Dimer
PDZ3-acidic	414–888	56.3	112.6	53.6	Monomer
PDZ2+I2	183–349	30.3	60.6	70.8	Dimer
PDZ2	183–275	14.0	28.0	28.3	Dimer

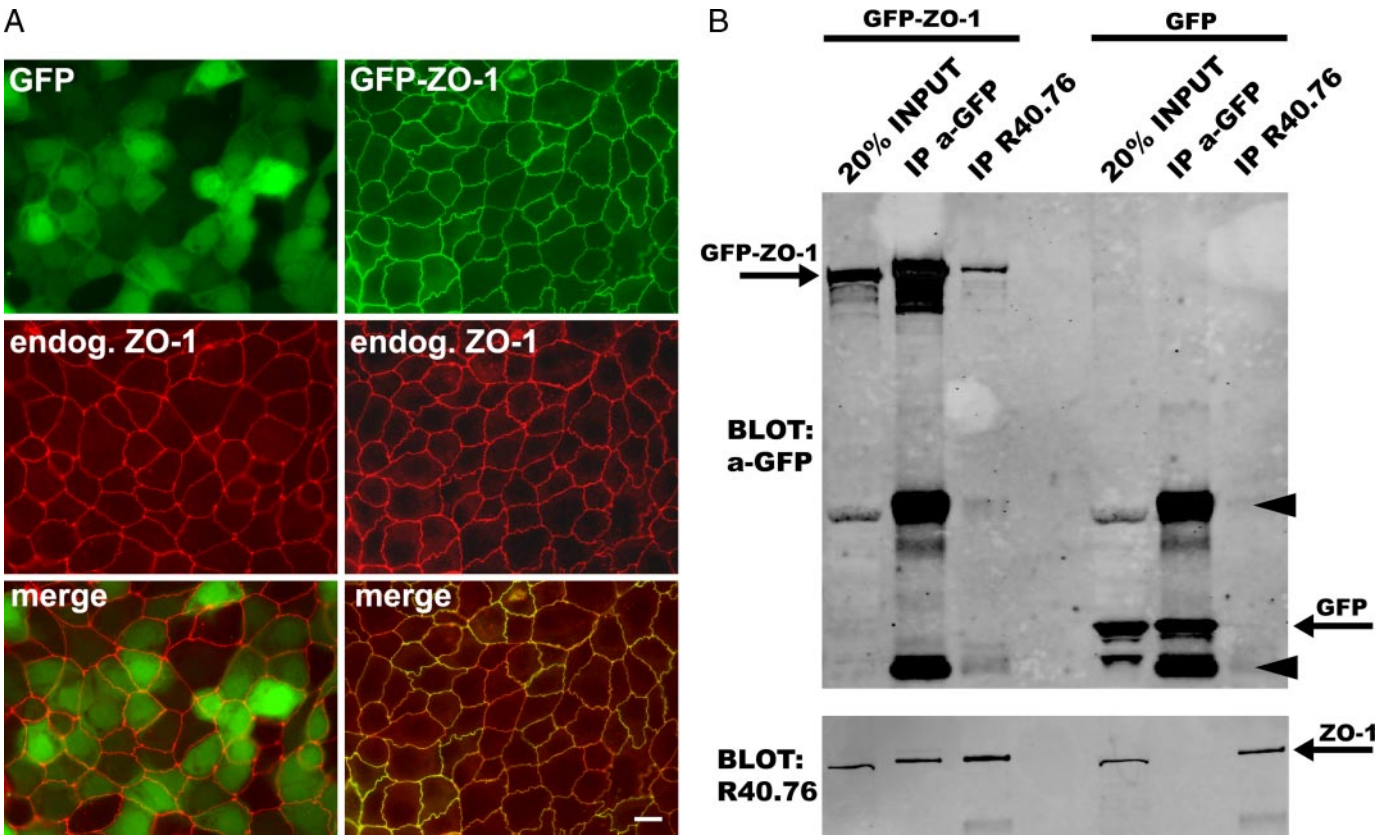

FIGURE 5. **Oligomerization of ZO-1 *in vivo*.** *A*, the distribution of GFP-ZO-1 is similar to endogenous (*endog.*) ZO-1 localizing at tight junctions, whereas GFP in control cells localizes to the cytoplasm (*bar,* 10 μm). *B*, in cells expressing GFP-ZO-1 (*left*) or GFP alone (*right*), transgenic constructs were precipitated using anti-GFP antibodies, whereas endogenous ZO-1 was precipitated using R40.76 antibody. *IP*, immunoprecipitate. Immunoblot analysis with anti-GFP antibodies shows that GFP-ZO-1 but not GFP co-precipitates with endogenous ZO-1 (*upper panel*). Heavy and light chains of immunoglobulins are marked with *arrowheads*. *Lower panel* shows that endogenous ZO-1 co-precipitates with GFP-ZO-1 but not GFP control.

forms a dimer with a submicromolar equilibrium dissociation constant. Such a low K_d value implies that NZO-1 would readily form homodimers *in vivo*.

By analyzing the oligomerization status of various truncation mutants of ZO-1, we could unambiguously identify PDZ2 as a dimerization motif. The dimerization does not seem to occur via the recognition of internal "β-finger" by PDZ2 as in the case of neuronal nitric-oxide synthase-syntrophin (16), because the deletion of the regions flanking PDZ2 did not disrupt dimer formation. Rather, PDZ2 itself is likely to create a specific dimerization interface as reported for PDZ domains of Shank (17) and GRIP1 (18). The SH3-GuK unit of ZO-1 does not seem to contribute to oligomerization by domain swapping as suggested previously (19), because a construct containing PDZ3, SH3, GuK, and acidic domain was found to exist exclusively as a monomer.

Because ZO-1/ZO-2 interaction was shown to involve the second PDZ domains of ZO-1 and ZO-2 (3, 20), it appears that second PDZ domains of all ZO proteins possess an intrinsic ability to dimerize. This idea is supported by the fact that the second PDZ domains of ZO proteins are very well conserved (70% identity and 94% similarity between PDZ2 of human ZO-1 and ZO-2). Noteworthy, previous reports suggest that various deletion mutants of ZO-2 bound to ZO-1 only if co-expressed in fibroblasts; no binding was detected using separately purified proteins for *in vitro* binding assays (20). Based on our results, this is most likely due to the inability of ZO-2 to disrupt preformed dimers of ZO-1 and/or vice versa. In this regard, *in vitro* binding assays with separately purified ZO proteins seem to be less reliable compared with experiments utilizing co-expression of proteins in the same cell.

The previous report of Fanning *et al.* (3) suggests that ZO-1 does not form homo-oligomers in MDCK cells because transiently transfected human ZO-1 and its deletion mutants did not precipitate endogenous canine ZO-1. This conclusion differs from that of the current study. A possible explanation for the previous failure to detect the ZO-1/ZO-1 interaction is that low levels of *transient* transfection efficiency and/or the low expression levels of transfected ZO-1 precluded the detection of the binding. To overcome the problem of low transfection rate and low expression levels, we cloned GFP-ZO-1 into the pIRES vector that is designed to select cells with high expressions of transgene polypeptides. Stably expressing GFP-ZO-1 correctly targets to the tight junction. Using R40.76 antibody to precipitate endogenous ZO-1 and anti-GFP antibody to precipitate exogenous GFP-ZO-1, we could show that homodimers (or homo-oligomers) of ZO-1 exist *in vivo*.

In another experiment we could precipitate about 85% of ZO-2 and over 95% of ZO-3 from MDCK cell lysates. Even if one assumes that the remaining 15% of ZO-2 (~32 fmol) is bound to ZO-1, about one-third of the total ZO-1 appears to be free of ZO-2 or ZO-3. In addition, in contrast to ZO-2 and

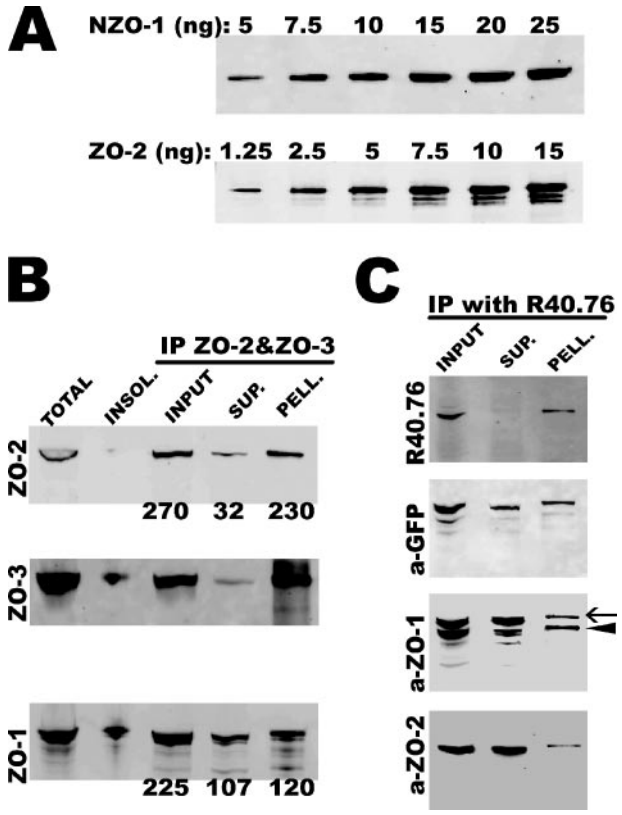

FIGURE 6. **A substantial fraction of ZO-1 forms homodimers.** *A*, defined amounts of purified NZO-1 and ZO-2 were immunoblotted to establish a standard curve and to quantify ZO-1 and ZO-2 in samples derived from MDCK cells. *B*, antibodies against ZO-2 and ZO-3 were mixed and used to deplete ZO-2 and ZO-3 proteins from soluble fraction of MDCK cells. Over 85% of ZO-2 and over 95% of ZO-3 could be depleted. About 50% of ZO-1 remains in the supernatant (*sup.*), and the remaining 50% is bound to ZO-2 and ZO-3. Numbers correspond to the amounts of proteins in femtomoles for this representative experiment. *Insol.*, insoluble; *pell.*, pellet; *IP*, immunoprecipitate. *C*, endogenous ZO-1 was depleted from the MDCK cell clone stably expressing GFP-ZO-1. Immunoblotting analysis with anti-GFP antibodies suggests that substantial amounts of GFP-ZO-1 co-precipitate with endogenous ZO-1. Immunoblot with rabbit anti-ZO-1 antibody was made to quantify both endogenous (*arrowhead,* 90 fmol) and transgenic (*arrow,* 65 fmol) ZO-1 in the pellet sample. Substantially smaller amounts of ZO-2 (~19 fmol) co-precipitated with endogenous ZO-1 in this experiment.

ZO-3, a substantial amount of ZO-1 is not soluble in RIPA buffer and is therefore likely not to be in a complex with ZO-2 and ZO-3. We do not have ultimate proof that ZO-1 is free from ZO-2/ZO-3 and exists in a cell as a homodimer or homo-oligomer. However, experiments with cell lines expressing GFP-ZO-1 indicate that ZO-1/ZO-1 dimers are at least as abundant as ZO-1/ZO-2 heterodimers. Given that purified recombinant NZO-1 exists exclusively as a dimer with submicromolar K_d, it is likely that ZO-1 free of ZO-2 and ZO-3 dimerizes in the cell via its second PDZ domain.

The relative efficiencies of homo- or hetero-oligomerizations between all ZO proteins have yet to be determined, but it is likely that the percentage of all kinds of homodimers and heterodimers will be dependent on the relative level of expression of all ZO proteins. In this regard, it is interesting that the expression profiles of ZO proteins differ from each other in various tissues indicating that various types of mono- and heterodimers of ZO proteins may form in different tissues. For example, ZO-3 is absent in ZO-1/ZO-2-positive endothelial tight junctions (21), and the expression levels of ZO-1 and ZO-2 show different patterns along the nephron (22). Furthermore, a significant part of ZO-3 and especially of ZO-2 is localized to the cytoplasm of highly polarized cells, whereas ZO-1 is localized almost exclusively to the tight junction (Fig. 5*A*). This implies that particular homo- and hetero-oligomers of ZO proteins may also have differential subcellular distribution.

ZO-1 and other MAGUKs are implicated in scaffolding of different proteins at plasma membranes through the direct binding to PDZ, SH3, and GuK domains. To enhance clustering, MAGUKs oligomerize via different mechanisms. For example, neuronal MAGUK PSD-95 was found to oligomerize through palmitoylation of two NH_2-terminal cysteines (23). The clustering of K^+ channels was found to be palmitoylation-dependent, pointing to palmitoylation as a potential regulation mechanism (23). ZO proteins, which are normally associated with tight junction in epithelial cells, are unique among MAGUKs in their ability to form highly stable dimers via the second PDZ domain. It is likely that the dimerization is highly important for the function of ZO proteins as molecular scaffolds, and our future studies will be aimed at understanding the role of PDZ2-dependent dimerization for the function of ZO proteins.

Acknowledgments—We thank Dr. Ashutosh Tripathy (Macromolecular Interaction Facility, University of North Carolina) for the assistance with the analytical centrifugation and light scattering experiments.

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