**Structural basis for tandem L27 domain-mediated polymerization**

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**ABSTRACT** The establishment of epithelial cell polarity requires the assembly of multiprotein complexes and is crucial during epithelial morphogenesis. Three scaffolding proteins, Dlg1, MPP7, and Mals3, can be assembled to form a complex that functions in the establishment and maintenance of apicobasal polarity in epithelial tissues through their L27 domains. Here we report the crystal structure of a 4-L27-domain complex derived from the human tripartite complex Dlg1-MPP7-Mals3 in combination with paramagnetic relaxation enhancement measurements. The heterotrimer consists of 2 pairs of heterodimeric L27 domains. These 2 dimers are asymmetric due to the large difference between the N- and C-terminal tandem L27 domain of MPP7. Structural analysis combined with biochemical experiments further reveals that the loop αα-αβ and helix αβ of the C-terminal L27 domain of MPP7 play a critical role in assembling the entire tripartite complex, suggesting a synergistic tandem L27-mediated assembling event.—Yang, X., Xie, X., Chen, L., Zhou, H., Wang, Z., Zhao, W., Tian, R., Zhang, R., Tian, C., Long, J., Shen, Y. Structural basis for tandem L27 domain-mediated polymerization. *FASEB J.* 24, 4806–4815 (2010). www.fasebj.org

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**Cell polarity is a fundamental property of most eukaryotic cells and is required to distribute cellular constituents asymmetrically (1, 2). The establishment of epithelial cell polarity requires the assembly of multiprotein complexes and is crucial during epithelial morphogenesis (3). Membrane-associated guanylate kinases (MAGUKs) contain multiple domains and act as adaptors to assemble supramolecular signaling protein complexes, therefore playing crucial roles at cell polarity (4). The assemblies of MAGUKs with themselves or with other MAGUKs-like proteins are mediated by a L27 domain, which is initially identified in the *Caenorhabditis elegans* Lin-2 and Lin-7 (5). The L27 domain exists in a large family of scaffolding proteins and is indispensable for cell polarization (6–9). Membrane-associated guanylate kinases p55 subfamily member 7 (MPP7) contains tandem L27 domains followed by a PDZ, SH3, and GUK domain (Fig. 1A). The MPP7 gene is potentially involved in maturity onset diabetes of the young (10) and is also a candidate gene for human ophthalmo-acromelic syndrome with clinical anophthalmia and limb anomalies (11). Discs large homologue 1 (Dlg1) is another MAGUK protein that contains 1 L27 domain, 3 PDZ domains, 1 SH3 domain, and 1 guanylate kinase (GUK) domain (Fig. 1A; ref. 12). *Drosophila* Dlg was identified as a tumor suppressor gene (13), and loss of the mammalian Dlg1 gene results in developmental defects (14). Furthermore, Mals3, or lin-7 homologue C (Lin-7C), is a small protein composed of an L27 domain and a PDZ domain (Fig. 1A). The *C. elegans* Lin7 is necessary for vulva development (15). The complex consisting of MPP7 and Dlg1 has been shown to promote epithelial cell polarity and tight junction formation (16). The dissociation of Dlg1 and Mals3 leads to defects in epithelial cell polarization, causing cystic and fibrotic renal diseases (17). Dlg1, MPP7, and Mals3 have been shown to assemble together to form a complex through L27 domains that functions in the establishment and maintenance of apicobasal polarity in epithelial tissues (6).

Structural studies of L27-domain heterodimers have been studied extensively, and it is generally accepted that L27 domains have assembled as symmetric dimer of tandem L27-mediated supracomplexes are seen more often (6, 17). The molecular mechanism of assembling tandem L27-mediated protein complexes remains elusive. In this study, we present the structure of an entire assembling unit, a L27 heterotrimer, of tandem L27-mediated human tripartite complex Dlg1-MPP7-Mals3 using X-ray crystallography combined with para-

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magnetic relaxation enhancement measurements. Our structure reveals an intrinsic asymmetry existing in tandem L27, which results in an asymmetric dimer of heterodimer.

MATERIALS AND METHODS

Expression and purification of the L27 heterotrimer

Gene fragments corresponding to the L27 domain of human Dlg1 (residues 1–65), the L27 domain tandem of human MPP7 (residues 9–120), and the L27 domain of human MALS3 (residues 3–66) were PCR amplified from the cDNAs of full-length proteins and then connected with 2 PreScission protease-cleavable segments (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro). The single frame was cloned into an in-house-modified version of the pET32a vector (Novagen, San Diego, CA, USA) in which the S-tag and the thrombin recognition site were replaced by a sequence encoding a TEV protease cleavage site (Glu-Asn-Leu-Tyr-Phe-Gln-Ser). All point mutations described here were created using a standard PCR-based mutagenesis method and confirmed by DNA sequencing. BL21(DE3) Escherichia coli cells harboring the fusion protein expression plasmid were grown at 37°C to an OD_{600} of >0.6 and then induced with 0.25 mM isopropyl-β-D-thiogalactoside at 16°C for 16–18 h. E. coli cells were resuspended in T_{50}N_{500}I_{5} buffer (50 mM Tris-HCl, pH 7.5; 500 mM NaCl; and 5 mM imidazole) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml antipain before cell lysis using
sonication. After the lysate was spun at 20,000 g for 30 min, the supernatant of the E. coli lysate was loaded directly onto a Ni-NTA agarose column (Qiagen, Valencia, CA, USA) that was equilibrated with T50N500I5 buffer. The Ni-NTA column was then washed with 3 column volumes of T50N500I5 buffer. The His6-tagged protein was then eluted with T50N500I5 buffer containing 1 M imidazole. The eluted protein was digested with TEV protease to remove His6-tag and then loaded on a High Load 26/60 Superdex-200 size-exclusion column (GE Healthcare, Piscataway, NJ, USA) and eluted with T50N500E5D1 buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; and 1 mM DTT) at a flow rate of 2.5 ml/min. Each fraction of the column elute was 5 ml. The small amount of contaminant protein was further removed from the target protein by a Q-Sepharose column (GE Healthcare) eluted with a NaCl gradient of 50–500 mM in T50N500E5D1 buffer. The final target protein was loaded onto a Superdex 200 size-exclusion column (GE Healthcare) and eluted with T50N500E5D1 buffer. The protein peak was identified by SDS-PAGE gel and harvested and concentrated by Centricon (Millipore, Bedford, MA, USA).

The SE-Met derivative protein was produced by following the same protocol as that of the wild-type protein, with the exception that methionine auxotroph E. coli B834(DE3) was used in the purification. The triple-mutant (C206S/C238M/S43C) and tetra-mutant (C206S/C238M/S43C/Y184F) of hMPP7 were created using a standard PCR-based mutagenesis method and confirmed by DNA sequencing. All of these mutants were expressed in the same way as the wild-type protein.

Expression and purification of full-length protein

DNA fragments encoding hemagglutinin (HA), the B1 domain of streptococcal protein GB1, and the PreScission protease-cleavable segment (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) were fused in frame via a PCR-based strategy and inserted into a pET32a vector to replace the nucleotides encoding the S-tag thioredoxin, and the thrombin-cleavable segment. The main of streptococcal protein GB1, and the PreScission protease-cleavable segment (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) was treated by PreScission protease to cleave the HA-GB1 tag and passed through a Superdex 200 10/300 GL column (GE Healthcare). The protein peak was identified by SDS-PAGE gel and harvested and concentrated by Centricon (Millipore).

Expression and purification of 15N-tyrosine-labeled mutants

The triple-mutant (C206S/C238M/S43C) and tetra-mutant (C206S/C238M/S43C/Y184F) of hMPP7 were created using a standard PCR-based mutagenesis method and confirmed by DNA sequencing. E. coli strains BL21 (DE3) transformed with MPP7-expression vectors were incubated at 37°C in modified M9 medium with ammonium chloride, 15N-Tyrosine, and 19 other unlabeled amino acids as the nitrogen source. Expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside once the OD600 reached 0.80, followed by another 5 h of incubation at 37°C. The desired proteins were purified using a Ni-NTA agarose affinity column and a Superdex 200 size-exclusion column.

Crystallization and data collection

The wild-type protein (130 mg/ml in 50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; and 1 mM DTT) was crystallized using the sitting-drop vapor-diffusion method equilibrated against a reservoir solution of 10% PEG8000, 0.2 M MgCl2, 0.1 M guanidine HCl, and 0.1 M Tris-HCl, pH 7.0. Crystals were grown over 1 wk at 20°C and frozen in cryoprotectant consisting of the reservoir solution supplemented with 25% glycerol. The crystals had to be dehydrated in the cryoprotectant solution for ~30 min before use. The annealing procedure was required to get good quality data. The SE-Met substituted crystals were produced as the wild type. Diffraction data were collected on beamline 3W1A of the Beijing Synchrotron Radiation Facility (Beijing, China) and then processed using the software HKL2000 (21). Single anomalous data were collected for SE-Met substituted crystals at element SE peak wavelength on beamline 19-1D at the Advanced Photon Source of Argonne National Laboratory (Argonne, IL, USA). All crystals belong to the space group of P6,22, with unit cell dimensions a = 153.19 Å and c = 58.90 Å, and diffract to ~3.0 Å.

Structure determination and refinement

The program HKL2MAP (22) was used to search a single SE site, and initial phases were then calculated by Phenix software (23). The electron density map modification was done by the program Solomon (24). Model building and refinement were performed using Coot (25) and CNS (26). After the initial main-chain model was built by the Resolve program in Phenix software, the wild-type data were applied to carry out an iterative refinement to assign all side chains. During the model building, previously solved structures of a single L27 domain from rat Dlg1 [Protein Data Bank (PDB) code: 1RSO] and mouse Mals2 (PDB code: 1Y74) were superimposed onto our model as a guide. For the cross-validation, a total of 472 (~5%) reflections was selected randomly by the program CNS (26) to calculate the Rfree value and to monitor the process of the structure refinement. The final structure had an Rcrystal value of 25.5% and an Rfree value of 28.4%. The Ramachandran plot created by the program PROCHECK (27) shows that 88.2% of the residues are in the most favored regions, 11.8% of the residues are in the additional allowed regions, and none of the residues are in the generously allowed regions or disallowed regions. Detailed data collection and refinement statistics are summarized in Table 1. All figures were calculated using the program PyMOL (28). To calculate the buried surface area and shape complementarity between the 2 interacting molecules, the corresponding atomic coordinates were input into the programs AREAIMOL (29) and SC (30), respectively, and the default parameters for the programs were used in the calculations.

NMR sample preparation and methanethiosulfonate (MTSL) labeling

A 0.1 mM protein solution in 50 mM NaH2PO4/Na2HPO4, pH 7.0, and 0.2 mM DTT was mixed with MTSL solution (Toronto Research Chemicals, Toronto, ON, Canada) at a final concentration of 2 mM, and then the reaction mixture was put in a 37°C incubator for 3 h as described previously.

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Two-dimensional $^{1}$H-$^{15}$N correlated heteronuclear single acid (Sigma-Aldrich, St. Louis, MO, USA). The sample was reduced with 6 protein equivalents of ascorbic acid/H$_{2}$O. The resulting solution was divided into two 500–ml samples. EDTA; 2 mM DTT) at room temperature. The digestion reactions were terminated by adding sample loading dye buffer for SDS-PAGE at different time points.

**Glutathione S-transferase (GST) pulldown assay**

GST-hMals3 fusion proteins were expressed in E. coli BL21(DE3) cells and purified by a glutathione-Sepharose 4B column (GE Healthcare) followed by a Superdex S200 size-exclusion column (GE Healthcare), according to the instructions provided by the manufacturer. The MPP7 gene was cloned into a pCMV-Myc vector and expressed in transfected HEK293T cells. The MPP7 chimeras were created using a standard PCR-based mutagenesis method and confirmed by DNA sequencing. The GST fusion protein, the HA-GB1 fusion protein, and 500 µl of cell lysate containing the myc-MPP7 or chimeras was mixed and incubated at 4°C for 2 h. Glutathione-Sepharose 4B beads (GE Healthcare) were then added, and binding was allowed to proceed at 4°C with rocking for another 2 h. The bound proteins were washed 3 times with PBS buffer, boiled in sample loading dye buffer, separated by SDS-PAGE, and subsequently analyzed by immunoblotting with the appropriate antibodies.

**Analytical ultracentrifugation**

Sedimentation equilibrium measurements were made on a Beckman Coulter ProteomeLab XL-I equipped with an An60 Ti 4-hole rotor (Beckman Coulter, Brea, CA, USA). The sedimentation equilibrium data were collected at the rotation speed of 8000 rpm and scanned by absorbance at 280 nm at 20°C. The samples were in a buffer containing 50 mM NaH$_{2}$PO$_{4}$/Na$_{2}$HPO$_{4}$, pH 7.0, and concentrated to a volume of 900 µl. D$_{2}$O (Cambridge Isotope Laboratory, Andover, MA, USA) was added to the samples at 10% (v/v). The digestion reactions were terminated by adding sample loading dye buffer for SDS-PAGE at different time points.

**Partial trypsin digestion experiment**

Partial trypsin digestion was carried out by mixing 5 mg/ml of each protein with 0.125 mg/ml of trypsin in 200 µl of digestion buffer (50 mM Tris-HCl, pH 8.0; 500 mM NaCl; 2 mM EDTA; and 2 mM DTT) at room temperature. The digestion reactions were terminated by adding sample loading dye buffer for SDS-PAGE at different time points.
Tris-HCl, pH 8; 500 mM NaCl; and 2 mM EDTA. A protein concentration of ~0.4 mg/ml and volume of 400 μl were used in the experiment. The data were analyzed by a self-associating multimerization model using the manufacturer’s software.

Accession number

Atomic coordinates and structure factors for the reported crystal structures have been deposited in the Protein Data Bank with accession code 3LRA.

RESULTS

Structure of the L27 heterotrimer

The L27 domains of humanDlg1 (L27hDlg1), human MPP7 (L27N and L27C), and human Mals3 (L27hMals3) were covalently linked via 2 PreScission protease-cleavable segments to form 1 single polypeptide (Supplemental Fig. S1). This technique has been extensively used in previously determined dimeric L27 domain structures and did not alter the global structure of the complex (9, 18). The structure was solved by the single anomalous dispersion (SAD) method. The Fourier map calculated from the initial SAD phases showed 2 alternative conformations (closed vs. open) of this molecule (Supplemental Fig. S2). To further ascertain the conformation of the L27 heterotrimer in solution, we took advantage of paramagnetic relaxation enhancement measurements (34). On the basis of our crystal structure of the L27 heterotrimer, the distance between Ser43 and Tyr184 (all residue numbers refer to a single polypeptide chain as shown in Supplemental Fig. S1) is ~51 Å in the open conformation but ~10 Å in the closed conformation (Supplemental Fig. S3). We first built a cysteine-free mutant of the L27 heterotrimer by mutating Cys206 and Cys238 to serine and methionine, respectively. After that, we constructed 2 other mutants mutating Cys206 and Cys238 to serine and methionine, built a cysteine-free mutant of the L27 heterotrimer by closed conformation (Supplemental Fig. S3). We first used the anomalous dispersion (SAD) method. The Fourier map complex (9, 18). The structure was solved by the single measurement (34). On the basis of our crystal structures have been deposited in the Protein Data Bank with accession code 3LRA.

Asymmetric L27 heterotrimer

In our crystal structure, with the exceptions of the N-terminal first methionine of L27hDlg1, a region (residues 108–110) of L27N, and the last 2 C-terminal serines of L27hMals3, the L27 heterotrimer, is well defined. The entire molecule consists of 2 pairs of heterodimeric L27 domains (Fig. 1B). The L27hDlg1 dimerizes with L27N, as does L27hMals3 with L27C. Unexpectedly, the 2 heterodimers do not follow the symmetric “dimer of dimer” assembling mode shown in earlier studies (18–20). This symmetry is broken mainly due to the unusual conformation of L27N. First, the L27hDlg1, L27hMals3- and L27C domains contain 3 α-helices (αA-αC), while L27N has 4, because the long αA helix of L27N breaks into 2 short helices (Fig. 2A). Second, helix αB of L27N is 1 turn of the helix shorter, while helix αC of L27N is at least 1 turn of the helix longer than the other 3 L27 domains. Third, the orientation of helix αC of L27N relative to helices αA and αB is very different from the other 3 L27 domains (Fig. 2A, B). Moreover, the superposition of heterodimeric L27hDlg1-L27N onto L27hMals3-L27C shows that the 3 α-helices section of L27hDlg1 and L27hMals3 can be superimposed well with a root-mean-square deviation (RMSD) of 1.1 Å for 44 Ca atoms, with the exception of the loop αA-αB region. However, the L27N domain does not match with the L27C domain (Fig. 2C). An additional 35° of rotation was required to align helices αA and αB of these 2 domains. If the L27N domain is superimposed onto the L27C domain, the hydrophobic patches recognized as L27hDlg1 and L27hMals3 are also positioned differently (Fig. 2D). The calculation of a surface complementary (Sc) value (30) shows that the L27N and L27C domains can be complementary to the L27hDlg1 and L27hMals3 domains, respectively, but they cannot be exchanged (Fig. 2E). The buried surface area calculation (29) is 1,366 Å² for the heterodimer of L27hDlg1-L27N and 2,554 Å² for the heterodimer of L27hMals3-L27C, indicating that the association of the L27hMals3-L27C dimer is stronger than that of the L27hDlg1-L27N dimer. This is consistent with the conclusion drawn by Bohl et al. (6).

We also compared our L27 tandem-mediated heterodimers with previously reported L27 heterodimers (9, 18–20), L27CASK-L27Mals2i, L27Patj-L27Npals1, and L27SAP97-L27NCASK (Supplemental Fig. S8). The superposition of our heterodimer of L27hDlg1-L27N onto the L27 heterodimer of L27Patj-L27Npals1 and L27SAP97-L27NCASK gives an RMSD of 2.5 Å for 76 Ca atoms and 1.9 Å for 84 Ca atoms, respectively. The superposition of our heterodimer of L27C-L27hMals3 onto L27CASK-L27Mals3 gives an RMSD of 1.06 Å for 87 Ca atoms. However, the L27N-mediated heterodimer and L27C-
mediated heterodimer cannot be superimposed onto each other (RMSD > 4.0 Å).

**Association of L27 dimers**

The efficient association of hDlg1 with L27N is dependent on the binding of hMals3 with L27C (6). To determine the specific region of L27C that is crucial for the assembly of the tripartite complex and the cooperative assembly of the L27 heterotrimer, we first compared the amino acid sequences between L27N and L27C as well as between hDlg1 and hMals3. The major hydrophobic residues responsible for dimer formation in L27N and L27C show a similar distribution in regions of helices αA and αC but not in regions of loop αA-αB and helix αB (Fig. 3A). In addition, the hydrophobic residues involved in dimer packing in L27_hDlg1 and L27_hMals3 show similarity in regions of helices αA and αB. We next analyzed the detailed interactions between the 2 packed L27 domains (Fig. 3B, C). The interactions between helices αA and αC of L27N with L27_hDlg1 have some similarity to those of their counterparts between L27C and L27_hMals3 (Fig. 3B, C, top and middle panels). By contrast, the interactions between the region consisting of loop αA-αB and helix αB of L27N with L27_hDlg1 is different from that of their counterparts between L27C and L27_hMals3 (Fig. 3B, C, bottom panel). Specifically, the amino acid Arg10 of L27_hDlg1 forms 2 hydrogen bonds with amino acids Glu98 and Asp99 of L27N (Fig. 3D). Also, its long side chain forms π-cation interactions with Phe102 of L27N. The corresponding arginine residue (Arg207) of L27_hMals3 forms a salt bridge with residue Glu159 of L27C (Fig. 3E). Moreover, a sequence alignment of helix αB of the proteins in the MAGUK family containing tandem L27 domains revealed that the residues Phe102 of L27N and Glu159 of L27C are highly conserved (Fig. 3F). A timedependent partial trypsin digestion experiment showed that the mutation of these 2 residues in the L27 heterotrimer greatly reduced the stability of the L27 heterotrimer compared with the wild type (Fig. 3G), indicating that these 2 residues indeed contribute to the formation of the L27 heterotrimer. Finally, we analyzed the contacts...
Figure 3. Detailed interactions within an L27 heterotrimer. A) Sequence comparisons of L27N and L27C (top) and of L27hDlg1 and L27hMals3 (bottom). Key residues crucial for contacts are underscored and boxed in red if the 2 residues are conserved. Residue number in a single polypeptide is shown in black. Cartoon representation of helices A, B, and C is displayed. Residues Phe102 and Glu159 are colored green and pointed out by a green arrow. B, C) Schematic diagrams showing detailed interactions within the heterodimer interface of L27hDlg1 and L27N (B) and of L27hMals3 and L27C (C). Residues from L27hDlg1 and L27hMals3 are colored red and orange, respectively. Interactions occurring between 2 residues are represented by dotted lines. Paired interactions that are similar in the 2 heterodimers are shown by residues with a dotted green circle. D, E) Stereoviews of the comparison of residues Phe102 and Glu159 in the heterodimer interface of L27hDlg1 and L27N (D) and that of L27hMals3 and L27C (E). L27 domains from human Dlg1, MPP7, and Mals3 are colored red, cyan, and orange, respectively. Oxygen and nitrogen atoms are colored red and blue, respectively. Hydrogen bonds are shown by magenta dotted lines. F) Sequence comparison of L27N and L27C from various proteins containing tandem L27 domains in the MAGUK family. G) Partial trypsin digestion experiment of wild-type and mutant L27 heterotrimers. Top and bottom bands in the same row represent an undigested and a digested product that will eventually be digested out.
between 2 heterodimers of L27N-L27\textsubscript{hDlg1} and L27C-L27\textsubscript{hMals3}. The C-terminal region (Glu250-Ile254) of L27\textsubscript{hMals3} and the C-terminal region (Lys182-Tyr184) of L27C interact with the entire helix H9251\textsubscript{Co} of L27\textsubscript{hDlg1} (Supplemental Fig. S9), suggesting that the formation of the heterodimer of L27C-L27\textsubscript{hMals3} is able to provide a binding platform for helix H9251\textsubscript{Co} of L27\textsubscript{hDlg1}. Based on the above data, we hypothesize that the region consisting of loop H9251\textsubscript{A}-H9251\textsubscript{B} and helix H9251\textsubscript{B} of L27C plays an important role in assembling the entire tripartite complex.

Assembly of the full-length tripartite complex

To ensure that the full-length tripartite complex could be assembled in vitro, we purified the 3 proteins individually to >95% purity and then mixed them together. The data from the size-exclusion column revealed that the tripartite complex is stable in vitro and is monodispersed with a stoichiometry of 1:1:1 (Fig. 4A). The sedimentation equilibrium measurement (Fig. 4B) confirmed that the tripartite complex is a monomer with a molecular mass of ~189.6 kDa (calculated molecular mass of 185 kDa). Taken together, these results suggest that the tripartite complex can be assembled correctly in vitro. We then evaluated the role of loop αA-αB and helix αB of L27C within the full-length tripartite complex through a GST pulldown assay in vitro. We further expressed another 3 full-length MPP7 chimera proteins to test the assembly. NN or CC denotes that the loop H9251\textsubscript{A}-H9251\textsubscript{B} and helix αB of L27C are replaced by their counterpart of L27N or vice versa. CN switches the loop αA-αB and helix αB of L27C and L27N. Our results show that only the NN chimera
disrupted the tripartite complex; the others did not (Fig. 4D), indicating that the region consisting of loop αAαB and helix αB of L27C is critical for the tripartite complex formation.

**DISCUSSION**

The L27 domain mediates various signaling and cell polarity complexes and plays an important role in the events that cause cell polarity (5, 35–37). Under physiological conditions, the L27 domain often forms an L27 tandem-mediated heterotrimer to achieve its biological functions (4, 6, 17, 38). Previous studies (18, 19) of a single L27 domain-mediated heterodimer showed that the heterodimer can further assemble a L27 homotetramer and display an arrangement of a symmetric dimer of heterodimers. Our reported structure of the L27 heterotrimer, the first of its kind, reveals the molecular basis of the L27 tandem-mediated assembly. It shows that different conformations of the tandem L27 domains exist, which reflects the binding specificity of tandem L27 domains. This property may be favorable for the correct assembly of protein complexes and may prevent promiscuous binding. Interestingly, our structural comparison of 2 L27 heterodimers (L27N-L27hDlg1 and L27C-L27hMals3) to other previously reported L27 heterodimers showed that for those L27 tandem-containing proteins, all of the N-terminal L27 domain-mediated heterodimers are similar to each other but are not similar to any C-terminal L27 domain-mediated heterodimers or vice versa. This infers that the asymmetric L27 heterotrimer in our structure may represent a general framework of other L27 tandem-mediated heterotrimer.

In our structure of the L27 heterotrimer, we identified a region consisting of loop αAαB and helix αB of L27C that is crucial for the assembly of the tripartite protein complexes (Fig. 4C). More important, the heterodimer of L27C-L27hMals3 has multiple contacts, including several hydrogen bonds with helix αC of L27hDlg1 (Supplemental Fig. S9), suggesting that the assembly of the L27C-L27hMals3 promotes the recruitment of L27hDlg1 and further facilitates the association of L27N-L27hDlg1. This result is consistent with the fact that the association of Dlg1 with the L27N domain of MPP7 required the prior formation of a complex between the L27C domain of MPP7 and Mals3 (6). Because the L27 domain was largely unfolded unless associated with its cognate L27 domain (18, 39), our results suggest that Mals3 presumably binds to the L27C domain of MPP7 first by forming a heterodimer of L27C-L27hMals3, which may then recruit the Dlg1 molecule, put the L27N domain of MPP7 and L27hDlg1 together, and induce the folded conformation of the heterodimer of L27N-L27hDlg1. The formation of heterodimeric L27N-L27hDlg1 may also be favorable to packing the heterodimer L27C-L27hMals3 and the final assembly of the tripartite complex. Therefore, such an assembly procedure may be a synergistic event instead of random binding.

The above model provides another possibility that post-translational modifications could occur to dynamically regulate the transition from the L27 heterodimer to the L27 heterotrimer in the L27 tandem-containing tripartite complex. We searched the entire sequence of the L27 heterotrimer (Supplemental Fig. S1) for currently known kinase recognition motifs and found several candidates, including Ser43 by cyclin-dependent kinase 1, Tyr249 by proto-oncogene tyrosine-protein kinase, Thr251 by protein kinase C, and Ser256 by casein kinase II. Interestingly, these residues are within the interface of L27hDlg1 and the heterodimer of L27C-L27hMals3 in our structure, as mentioned above. The modifications of these residues will presumably interfere with the transition process. More experiments are required to test this hypothesis in the near future.

In all, our results provide a structural basis for the correct assembly of the tripartite complex Dlg1-MPP7-Mals3 and may represent a general assembly mode for multiple L27 tandem-mediated suprasignaling complexes. Such a synergistic tandem L27-mediated assembling mechanism is a very unique mode to ensure signaling specificity in the establishment and maintenance of epithelial cell polarity.

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