Crystal structures of Mmm1 and Mdm12–Mmm1 reveal mechanistic insight into phospholipid trafficking at ER-mitochondria contact sites

Hanbin Jeong,a,b Jumi Park,a,b Youngsoo Jun,b,c and Changwook Leea,b,d,1

*Department of Biological Sciences, School of Life Sciences, Ulsan National Institute of Science and Technology, Ulsan 44919, Republic of Korea; aCell Logistics Research Center, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea; bSchool of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea; and cCenter for Genome Integrity, Institute for Basic Science, Ulsan 44919, Republic of Korea

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The endoplasmic reticulum (ER)-mitochondria encounter structure (ERMES) comprises mitochondrial distribution and morphology 12 (Mdm12), maintenance of mitochondrial morphology 1 (Mmm1), Mdm34, and Mdm10 and mediates physical membrane contact sites and nonvesicular lipid trafficking between the ER and mitochondria in yeast. Herein, we report two crystal structures of the synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain of Mmm1 and the Mdm12–Mmm1 complex at 2.8 Å and 3.8 Å resolution, respectively. Mmm1 adopts a dimeric SMP structure augmented with two extra structural elements at the N and C termini that are involved in tight self-association and phospholipid coordination. Mmm1 binds two phospholipids inside the hydrophobic cavity, and the phosphate ion of the distal phospholipid is specifically recognized through extensive H-bonds. A positively charged concave surface on the SMP domain not only mediates ER membrane docking but also results in preferential binding to glycerophospholipids such as phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylserine (PS), some of which are substrates for lipid-modifying enzymes in mitochondria. The Mdm12–Mmm1 structure reveals two Mdm12s binding to the SMP domains of the Mmm1 dimer in a pairwise head-to-tail manner. Direct association of Mmm1 and Mdm12 generates a 210-Å-long continuous hydrophobic tunnel that facilitates phospholipid transport. The Mdm12–Mmm1 complex binds all glycerophospholipids except for phosphatidylethanolamine (PE) in vitro.

Mmm1 | Mdm12–Mmm1 complex | ERMES | phospholipid trafficking | membrane contact site

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Data deposition: The atomic coordinates and crystallographic structure factors have been deposited in the Protein Data Bank (PDB ID codes 5YK6 and 5SYK).

1To whom correspondence should be addressed. Email: changwook@unist.ac.kr.

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Significance

The endoplasmic reticulum (ER) forms membrane contact sites (MCSs) with other organelles such as mitochondria, endosomes, and peroxisomes in eukaryotic cells. The MCS plays a pivotal role in exchanging cellular materials such as ions and lipids. More importantly, nonvesicular lipid trafficking occurring at the ER-mitochondria MCS is essential for the biogenesis of the mitochondrial membrane. In yeast, the ER-mitochondria encounter structure (ERMES) complex comprising the ER proteins Mmm1 and cytosolic Mdm12 and the mitochondria proteins Mdm34 and Mdm10 provides a tethering force between the ER and the mitochondria and mediates lipid trafficking. Here, we present two crystal structures of Mmm1 and the Mdm12–Mmm1 complex. Based on these structures, we propose the model by which the Mdm12–Mmm1 complex contributes to phospholipid trafficking at the ER-mitochondria MCS.
Previously, we determined the crystal structure of *Saccharomyces cerevisiae* Mdm12 at 3.1 Å resolution and revealed that Mdm12 forms a dimeric SMP structure that binds phospholipids inside a hydrophobic channel, with a preference for glycerophospholipids harboring a positively charged head group (20). Another study determined a 17 Å resolution electron microscopy (EM) structure of the Mdm12–Mmm1 (SMP domain) complex, revealing an elongated tubular structure with an Mdm12–Mmm1–Mdm12 arrangement (19, 35). Despite these structure studies, the molecular-level mechanism by which the SMP domains of Mdm12, Mmm1, and Mdm34 are directly organized and facilitate phospholipid trafficking without consuming energy at the ER-mitochondria contact site remains unknown. Additionally, exactly how Mmm1, an ER component of the ERMES complex, recognizes specific phospholipids in the ER membrane remains elusive, as does the mechanism by which phospholipids selected by Mmm1 are transported into Mdm12, as a direct binding partner of the ERMES complex.

In the present study, we determined crystal structures of the Mmm1 SMP domain and the Mdm12–Mmm1 binary complex, and discuss the resultant molecular-level insight into how the Mmm1 SMP domain contributes to the organization of the ERMES components, as well as phospholipid trafficking.

**Results**

**Structure Determination of Mmm1.** The Mmm1 protein is predicted to comprise a single transmembrane domain near its N terminus that anchors it to the ER membrane, an unstructured region consisting of around 50 residues, and an SMP domain at the C terminus (Fig. 1A and Fig. S1). The N-terminal region of Mmm1 is located in the ER lumen, while the SMP domain is localized in the cytosol and directly interacts with Mdm12, a cytosolic component of the ERMES complex. Despite significant effort to purify Mmm1 proteins, size-exclusion chromatography (SEC) experiments revealed that the SMP domain of *S. cerevisiae* Mmm1 (scMmm1) aggregated in solution unless in a complex with Mdm12 (20). Extensive screening for solubility and homogeneous dispersal in solution for Mmm1 orthologs, together with limited proteolysis analysis, revealed that the Mmm1 SMP domain of *Zygosaccharomyces rouxii* (zrMmm1, residues 190–444) was soluble even when not complexed with Mdm12 (Fig. 1B). The SMP domain of zrMmm1 shares 76% sequence identity with that of scMmm1. The zrMmm1 proteins eluted from the gel-filtration column at a volume corresponding to the molecular weight of a dimer, suggesting that the recombinantly expressed zrMmm1 SMP domain forms a homodimer in solution. Interestingly, the SEC experiment confirmed that zrMmm1 was able to interact with scMdm12 when coexpressed in *Escherichia coli* cells despite the organismal disorganization (Fig. 1B). Diffraction-quality crystals of zrMmm1 were grown in the P3_2121 space group at 4 °C over a period of 1 wk, and the structure was solved using selenomethionine-substituted crystals by the single-wavelength anomalous dispersion method (Fig. S2). The final model of zrMmm1 was refined with data from native crystals to 2.8 Å resolution.

**Structure of the zrMmm1 SMP Domain.** Crystals of zrMmm1 contained one zrMmm1 molecule in the asymmetric unit. However, zrMmm1 forms a tight dimer with a crystal symmetry-related molecule via a twofold rotation arrangement. The dimeric organization of zrMmm1 was confirmed by previous biochemical experiments, and is consistent with other SMP domain structures (20, 36–38). Overall, the dimeric zrMmm1 SMP structure resembles a compact diamond with dimensions of 50 × 60 × 120 Å, and each component consists of four helices and six extended and twisted antiparallel β-strands that assemble into a typical SMP structure with an extended hydrophobic channel (Fig. 2A and Figs. S1 and S2). In a previous study, we suggested that the N terminus (residues 198–214) of the Mmm1 SMP domain dimer might be involved in the twofold interface and might be structurally similar to that of E-SYT2 based on sequence similarity (20). Consistent with our prediction, the twofold interface of the zrMmm1 dimer is composed of two helices in a face-to-face arrangement reminiscent of that in the E-SYT2 structure (Fig. 2B, interface I and Fig. S3A). In particular, three hydrophobic residues (Leu219, Trp221, and Phe222) stabilize the twofold axis through van der Waals interactions.

Upon comparing the SMP domains of E-SYT2 and Mdm12, it was immediately apparent that two extra structural elements absent in the Mdm12 and E-SYT2 domains are present at the N and C termini of zrMmm1 (Fig. 2B and Fig. S3). These structural elements presumably make an important contribution to the tight association between subunits of the zrMmm1 dimer, since over 3,400 Å² of solvent-accessible surface area is buried upon self-association. The N terminus of zrMmm1 adopts an α-helix (α1) and a well-ordered loop that contacts the head region of the other molecule of the dimer (interface II). In particular, the N-terminal helix comprising residues 196–207 wraps around the twofold axis helix of the opposing molecule in an antiparallel domain-swapped manner (Fig. 2B, interface II). The highly conserved C terminus of zrMmm1 exhibits a long, extended loop that crosses over the two molecules and essentially mediates the self-association of the zrMmm1 dimer, as well as phospholipid binding (Fig. 2B and C, interface III). In more detail, the extended loop consisting of residues 425–432 forms an antiparallel β-strand–like strap structure that zips up the opposing twofold central helices, and eventually
covers the concave surface at the center of the dimeric SMP domain (Fig. 2B, interface III). This loop also contains the absolutely conserved Trp430 and Arg432 residues that are essential for the recognition of phospholipids, as discussed below. Additionally, the C terminus of zrMmm1 adopts a short 3_10 helix (residues 433–435), followed by antiparallel β-strands, and is incorporated between β5 and an 11-residue loop (residues 347–357) from the opposing molecule of the dimer through the formation of an extensive hydrogen-bonding network (Fig. 2B, interface IV).

In summary, the extensive interfaces that are lacking in E-SYT2 and Mdm12 provide the driving force for the tight self-association observed in the zrMmm1 dimer. Consistently, SEC and native PAGE revealed that the dynamic distribution between monomer and dimer observed for Mdm12 and the SMP domain of E-SYT2 was not a feature of zrMmm1 (20).

**The zrMmm1 Dimer Binds Glycerophospholipids.** The crystal structure revealed that recombinant zrMmm1 expressed in bacteria contained glycerophospholipids bound in the hydrophobic channel formed from the SMP domain (Fig. 3A). Based on the observed electron density, we concluded that two glycerophospholipids were bound to each zrMmm1 molecule in two distinct regions: One phospholipid binds at the dimeric interface (proximal), and the other molecule is located in the middle (distal) part of the SMP channel. As mentioned above, the zrMmm1 dimer formed from symmetry-related molecules in the crystal, and the two phospholipids superimposed precisely over the two molecules of the zrMmm1 dimer, suggesting that the phospholipids are specifically recognized by zrMmm1 and were not the result of nonspecific binding. The head groups of two glycerophospholipids are located within a concave surface generated by helices α2–α4, and are solvent-exposed and disordered in the structure, suggesting that zrMmm1 does not possess clear selectivity for particular phospholipids, consistent with Mdm12 and E-SYT2 (20, 38) (Fig. 3B and C and Fig. S3). However, unlike in other SMP domain proteins, the phosphate group and carboxyl oxygen of the distal phospholipid can be clearly seen in the structure, and are systematically coordinated by the conserved Arg253, Arg415, Trp411, Trp430, Arg432, and Ser433 through an extensive hydrogen-bonding network (Fig. 3C). Among these, three residues (Trp430,
Arg432, and Ser433) are from the opposing molecule in the dimer, suggesting that lipid coordination in zrMmm1 requires homodimerization.

To examine if zrMmm1 shows preferential binding to certain phospholipids in solution, we performed lipid displacement experiments using 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,3-benzoxadiazol-4-yl) (NBD)-PE, as reported in our previous study (20). First, we confirmed the binding between NBD-PE and purified zrMmm1 using native PAGE and fluorescence detection (Fig. 3D), and found that NBD-PE bound to zrMmm1 could be easily displaced by phosphatidylglycerol (PG), phosphatidic acid (PA), PS, or phosphatidylcholine (PC), but only relatively weakly by PE, even at high concentrations (Fig. 3D). However, the NBD-PE on Mmm1 could not be displaced by the nonphospholipid cholesterol, ergosterol, or ceramide, even at high concentrations (Fig. 3D). Based on these results, we conclude that zrMmm1 can bind efficiently to any glycerophospholipid. A previous structural study suggested that Mdm12 binds preferentially to PC or PE, both of which have a positively charged head group in common, via their negatively charged surfaces (20). Analysis of
the electrostatic surface potential of zrMmm1 using the Adaptive Poisson–Boltzmann Solver (APBS) program (39) revealed a strong positively charged region in the vicinity of the bound phospholipid head group (Fig. 3B). Unlike Mdm12, the positively charged residues of zrMmm1 might be critically responsible for screening phospholipids themselves, not for the selection of certain head groups of phospholipids.

Next, we mutated key residues involved in lipid coordination and measured binding between zrMmm1 mutants and NBD-PE using blue native PAGE and fluorescence methods. As shown in Fig. 3E, R415E, W411A, and W430A variants completely lost the ability to bind NBD-PE, while the negative control R379E could still bind NBD-PE. Interestingly, two bands consistent with the monomer and dimer of zrMmm1 were observed with the R415E and W430A mutants, supporting our structural analysis and conclusion that self-association of zrMmm1 is required for lipid conjugation, and suggesting that lipid binding might enhance the stability of the dimeric form.

Structure Determination of the Mdm12–Mmm1 Complex. Mmm1 specifically interacts with the Mdm12 component of the ERMES complex (19, 20). In our previous study, we proposed a dimeric model for the Mdm12–Mmm1 complex involving dimerization via the SMP domains in a tail-to-tail manner. In this model, the conserved long C-terminal helices of the SMP domains lie adjacent to each other in a twofold rotation arrangement, resulting in an extended arch-shaped structure (20). However, one of the concerns raised from this model was the lack of direct evidence for the tail-to-tail junction, and contacts between the self-associated Mdm12 molecules could be an artifact of crystallization (i.e., the result of crystal contacts rather than physiologically relevant molecular interfaces). Additionally, the potential interface between Mdm12 and Mmm1 in this model is exposed to solvent, suggesting that it is energetically unfavorable for hydrophobic glycerophospholipids to cross the solvent region in the Mdm12 and Mmm1 interface.

To further investigate how phospholipids could be transferred through the SMP domains of Mdm12 and Mmm1, we determined the crystal structure of the Mdm12–Mmm1 complex. Initially, we obtained crystals of the S. cerevisiae Mdm12–Mmm1 complex and hybrid complex of scMdm12–zrMmm1, but all were of low crystallographic quality. Through extensive screening, we eventually obtained diffraction-quality crystals of truncated scMdm12Δ, in which both the unstructured loop (residues 74–114) and proline-rich region (residues 184–211) were excluded, in complex with zrMmm1 (Fig. 4A). The ability of scMdm12Δ to interact with zrMmm1 was assessed by SEC experiments (Fig. 1B). However, crystals only diffracted to low resolution (~5 Å). To overcome this, we attempted dehydration of crystals using a higher percentage of precipitant, and the diffraction quality was dramatically improved (details are provided in Materials and Methods). Dehydrated crystals of the scMdm12Δ–zrMmm1 complex diffracted to 3.8 Å synchrotron radiation, and the structure was determined by the molecular replacement method. Crystals contained one heterotetramer organized in an scMdm12Δ–zrMmm1–scMdm12Δ arrangement in the asymmetric unit (Fig. 4I). The Mdm12 modification needed for crystallization did not affect the overall structure or binding to Mmm1 compared with wild-type Mdm12 (rmsd of 1.5 Å for all Ca atoms). The overall conformation of zrMmm1 and scMdm12Δ was not significantly changed upon formation of the complex (rmsd of 0.9 Å and rmsd of 1.5 Å, respectively). No apparent electron density corresponding to the hydrocarbon chain of glycerophospholipids was observed in the complex structure except for the phosphate group of phospholipids, but this might be due to the relatively low resolution of the complex structure or to treatments such as crystal dehydration.

Architecture and Organization of the scMdm12Δ–zrMmm1 Complex. The overall structure of the scMdm12Δ–zrMmm1 complex closely resembles the EM structure described in a previous study (19) (Fig. S4A). The scMdm12Δ–zrMmm1 complex adopts an elongated curved and tubular structure with dimensions of 60 × 65 × 210 Å. The zrMmm1 dimer is located at the center, with scMdm12Δ monomers bound at each end (Fig. 4I and Fig. S4A). Consistent with the previously reported model (19), scMdm12Δ and zrMmm1 are organized in a head-to-tail manner, with the N terminus of scMdm12Δ (referred to as the head) that is proximal to the dimeric interface in the scMdm12 dimer associating with the distal end (referred to as the tail) of the homodimeric interface of the zrMmm1 SMP domain. The interaction between scMdm12Δ and zrMmm1 appears to be strong, and buries 1,012 Å² of surface-accessible surface area. The truncated residues of the unstructured loop and proline-rich region of Mdm12 are not involved in the interaction. In the crystal structure of Mdm12 alone, the N terminus (residues 1–7) adopts a β-strand that is involved in self-association by forming a domain-swapped structure with the opposing molecule of the dimer (20). However, no such conformation of Mdm12 was observed in the complex structure. Rather, the N terminus of scMdm12Δ forms an extended loop structure and lies adjacent to the β2 strand of scMdm12Δ itself.

The highly conserved β2 and β3 strands, the extended hairpin loop (referred to as the guide loop (G-loop)) generated between β2 and β3, and the α4 helix of zrMmm1 contribute to interactions with the β2 and β3 strands of scMdm12Δ (Fig. 4B). In particular, the hydrophobic amino acids Leu315, Leu317, Leu327, Ile388, and Ile397 in zrMmm1 form extensive and coordinated nonpolar contacts with the side chains of Phe3, Ile5, Leu56, Ile59, Ile119, Phe121, and Cys170 of scMdm12Δ (Fig. 4B). In addition, Lys399 of zrMmm1 forms a salt bridge and H-bonds with the side chain of Asp61 and the main chain of Asp118 of scMdm12Δ. To confirm whether these residues are involved in the interaction, we generated a series of zrMmm1 mutants and scMdm12Δ proteins (with GST fused at the N terminus of zrMmm1) and examined their binding ability using GST pull-down experiments. Single-residue mutants of scMdm12Δ (L565, I598, I1195, and F1215) lost appreciable affinity for zrMmm1 (Fig. 4C). Likewise, single-site mutants of zrMmm1 (L315S or L327S) interacted with scMdm12Δ in a less stable manner (Fig. 4D). Furthermore, to confirm the effect of the L315S mutation in solution, we titrated purified native and L315S mutant tag-free zrMmm1 proteins with purified scMdm12Δ over a wide protein concentration range and analyzed their interactions using native PAGE. As shown in Fig. S4B, wild-type zrMmm1 interacted with scMdm12Δ and formed a heterotetramer in a concentration-dependent manner, while the L315S mutant did not interact with scMdm12Δ at even higher concentrations, suggesting that the observed hydrophobic contacts are critical for the Mdm12–Mmm1 interaction.

The scMdm12Δ–zrMmm1 Complex Has an Extended Hydrophobic Tunnel Mediating Lipid Trafficking. Structural comparison between zrMmm1 and scMdm12 alone, and as part of the scMdm12Δ–zrMmm1 complex, revealed that the structure of zrMmm1 was changed slightly upon complex formation. Interestingly, the structural changes appear to be functionally relevant regarding phospholipid trafficking between the two distinct SMP domains. First, the G-loop of zrMmm1 undergoes a conformational change to form a more extended form that can plug into the scMdm12Δ head region and completely covers the solvent-exposed concave surface of scMdm12Δ (Fig. 5A). Second, the β4 strand of zrMmm1 is extended by two residues (Leu387 and Ile388) in the complex, and these residues are part of a flexible loop and are solvent-exposed in the structure of zrMmm1 alone. By interacting with scMdm12Δ, Ile388 is projected inward toward the center of the SMP domain and contributes to the formation of a hydrophobic boundary at the junction of the two SMP domains (Fig. 5B).
Third, the conserved loop formed between β4 and α4, which are well ordered in the structure of zrMmm1 alone, becomes disordered upon forming a complex with scMdm12Δ. In particular, three hydrophilic residues (Arg391, Ser392, and Lys393) are not visible in the scMdm12Δ–zrMmm1 complex (Fig. 5B). Finally, the α4 helix of zrMmm1 and the loop formed between α3 and β1 are pushed outward, generating a wider space inside the cavity that might be important for phospholipid trafficking (Fig. 5C and D). Taken together, the formation of the scMdm12Δ–zrMmm1 complex generates a continuous hydrophobic tunnel ~210 Å long through the elongated SMP domains of scMdm12Δ and zrMmm1, which could conceivably translocate phospholipids harboring nonpolar hydrocarbon chains between two components without consuming energy (Fig. 5E). These results strongly indicate that the Mdm12–Mmm1 complex acts as a lipid-transferring vehicle in addition to tethering molecules to physically connect two distinct subcompartments.

**The scMdm12–zrMmm1 Complex Binds All Glycerophospholipids Except for PE in Vitro.** To identify differences in binding priority to phospholipids between the scMdm12–zrMmm1 complex and zrMmm1 or scMdm12 alone, we performed a lipid displacement experiment using the scMdm12–zrMmm1 complex. Interestingly, NBD-PE bound to the scMdm12–zrMmm1 complex could be displaced only by PA, PG, PC, or PS (Fig. 6A). In the case of PA, high concentrations resulted in band shifts above those of the NBD-PE preloaded scMdm12–zrMmm1 complex alone on native PAGE. No such changes have been observed using NBD-PE–preloaded scMdm12 alone (20). However, high concentrations of PA also resulted in similar band shifts of NBD-PE–preloaded zrMmm1 alone, indicating that PA binding to zrMmm1 might affect the overall conformation of zrMmm1 or the scMdm12–zrMmm1 complex. One of the most striking differences between zrMmm1 and the scMdm12–zrMmm1 complex was the absence of scMdm12–zrMmm1...
binding to PE (Fig. 6A). Even though both scMdm12 alone and zrMmm1 alone bound to PE with noticeable efficiency (20) (Figs. 3D and 6B), the scMdm12–zrMmm1 complex did not bind PE at all, suggesting that the association between scMdm12 and zrMmm1 affects the binding preferences of zrMmm1 and scMdm12 to phospholipids. Although the tests were performed using purified proteins in vitro, these results could have important biological implications. The PE component of the mitochondrial membrane might not be directly transferred from the ER but might be synthesized within the mitochondrial matrix via the conversion of PS to PE. Furthermore, the PE generated outside mitochondria via the Kennedy pathway might not be efficiently transferred to mitochondria for unknown reasons (40). Consistent with this, the scMdm12–zrMmm1 complex did not engage in PE binding in vitro.

PS transfer to mitochondria is required for the synthesis of PE in mitochondria. Because scMdm12 alone could not bind PS (20) (Fig. 6B), we inferred that the PS that displaced NBD-PE from scMdm12 in the scMdm12–zrMmm1 complex might have been directly transferred from zrMmm1. To verify this, we generated an Y261W mutant of zrMmm1. The Y261 residue is located at the interface between zrMmm1 and scMdm12 and is involved in

Fig. 5. Direct association of zrMmm1 and scMdm12Δ generates a hydrophobic tunnel for phospholipid trafficking. (A) Ribbon diagram showing superposition of zrMmm1 (yellow) and the scMdm12Δ (green)–zrMmm1 (blue) complex. To analyze structural changes in zrMmm1 upon association with scMdm12Δ, the structure of zrMmm1 was aligned with the zrMmm1 structure in the scMdm12Δ–zrMmm1 complex. The scMdm12Δ is shown in surface representation. The G-loop of zrMmm1 undergoes conformational changes following interaction with scMdm12Δ, forming an extended structure that covers the solvent-exposed region of scMdm12Δ. Residues of zrMmm1 undergoing this structural reorganization are shown, and their directions are indicated with red arrows. (B) Structural changes in zrMmm1 occurring upon association with scMdm12Δ further highlighted (more information is provided in the main text) in a diagram colored the same as in A. The dotted line indicates zrMmm1 residues that become disordered upon forming the complex. (C, Right) Direct association of zrMmm1 and scMdm12Δ moves the α4 helix of zrMmm1 by ∼10° outward, vacating enough space to accommodate and transfer phospholipids. Phospholipids bound to zrMmm1 are shown in surface-filling representation. The red arrow indicates the putative pathway of phospholipids from zrMmm1 to scMdm12Δ. (C, Left) Ribbon diagrams compare the overall structure of zrMmm1 in the apo (yellow) and complexed (blue) forms viewed from the left side of the figure (C, Right). Loops, including Tyr261, in the complexed form are shifted outward, generating an open space in the process. The scMdm12Δ is omitted for clarity. The overall color scheme is the same as in A. (D) Structures of zrMmm1 in the apo (yellow) and complexed (blue) forms viewed from the right side of the picture (C, Right). (E) Overall structure of the scMdm12Δ–zrMmm1 complex shown in meshed line (Top) and ribbon (Bottom) representations. (Top) Red mesh representing hydrophobic amino acids inside the tunnel was superimposed on the figure. (Bottom) Channel (cavity) through the scMdm12Δ–zrMmm1 complex was analyzed by Mole 2.0 (43), and is shown in black tubule representation. Black arrows indicate the putative pathway for phospholipid trafficking.

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generating a hydrophobic channel. However, the residue does not directly contribute to the interaction between scMdm12 and zrMmm1 (Figs. 5C and 6B). We hypothesized that the conversion of Tyr to Trp would sterically hinder the transfer of phospholipids between zrMmm1 and scMdm12. As expected, the mutation did not affect the association between scMdm12 and zrMmm1 (Figs. 6C and 6B), and PS binding by the zrMmm1 (Y261W) mutant was similar to that of wild-type zrMmm1 (Fig. S5). However, in contrast to the wild type, the NBD-PE bound to the zrMmm1(Y261W)–scMdm12 complex was slowly displaced by PS (Fig. 6D), suggesting that the bulky side chain of Trp sterically impeded PS transfer from zrMmm1 to scMdm12 (Fig. 6E). We also tested whether the mutation affected the displacement of NBD-PE from the zrMmm1(Y261W)–scMdm12 complex by PC and PG, and observed that PC, but not PG, resulted in slightly slow displacement (Fig. 6D). Since scMdm12 alone could efficiently bind to PC and PG unlike PS (20), the effect of the mutation might not be significant in vitro. In summary, from these observations, we confirmed that the direct association of SMP domains in the scMdm12–zrMmm1 complex generates a hydrophobic tunnel for lipid trafficking.

**Discussion**

SMP domains in ERMES and tubular lipid-binding superfamily complexes are believed to have a common role in binding and transferring lipids (41). However, molecular recognition of specific phospholipids by SMP domains is not conserved among SMP-containing proteins. For example, scMdm12 has a binding preference for phospholipids harboring positively charged head groups, while the SMP domain of zrMmm1 broadly binds to most phospholipids, although zrMmm1 preferentially binds to PS, PA, PG, and PC. In addition, our previous work revealed that scMdm12 binds one molecule of phospholipid (20), while the zrMmm1 SMP domain binds two phospholipids in distinct regions (Fig. S3B). Interestingly, the phosphate group of the distal phospholipid is specifically coordinated by conserved residues in zrMmm1 (Fig. 3C). Specifically, two pairs of Arg–Trp residues (Arg415/Trp411 and Arg432/Trp430) from the opposing molecule

![Fig. 6. The scMdm12–zrMmm1 complex does not bind PE in vitro, and acts as a lipid transfer module. (A) In vitro phospholipid displacement experiments using the scMdm12–zrMmm1 complex. NBD-PE–preloaded scMdm12–zrMmm1 complexes were mixed with increasing concentrations of phospholipids (PA, PC, PE, PG, and PS). Decreasing fluorescence was used to measure NBD-PE displacement by each phospholipid. (Left) Fluorescence and Coomassie staining of clear-native PAGE gels are shown side by side. (Right) Graph shows quantification data. Experiments were performed in triplicate, independently. Means ± SD are given. (B, Left) Schematic diagram shows possible routes for phospholipid access to Mdm12 or Mmm1 in the Mdm12–Mmm1 complex. The table below shows a summary of the results of the phospholipid displacement experiment using Mmm1, Mdm12–Mmm1 complex (from this study), and Mdm12 (20). The symbols X, △, and ○ indicate that the fluorescence reduction rate is within the range of 0–35%, 35–70%, and 70–100% at 250 μM, respectively, of each phospholipid. (B, Right) Ribbon diagram highlights the role of the zrMmm1 Y261 residue at the interface between scMdm12Δ and zrMmm1. (C) SEC analysis shows that the Y261W mutant of zrMmm1 can still associate with scMdm12. Molecular weight standards are indicated above the chromatogram. mAu, milliabsorbance unit. (D) In vitro phospholipid displacement experiment with the scMdm12–zrMmm1 complex (wild-type and Y261W mutant). The graph indicates the concentration of a phospholipid required to reduce the NBD-PE fluorescence signal by 50%. The bar graph shows means ± SD (n = 3). (E) Schematic representation highlights the role of the SMP domain in phospholipid transport. The SMP domains in the two distinct subunits directly associate with each other, generating a successive hydrophobic tunnel through which phospholipid transfer can occur from one subunit to the other.

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of the zrMmm1 dimer), which are absolutely conserved among other Mmm1 orthologs, form an extensive H-bonding network with the phosphate ion and carboxyl oxygen of the phospholipid (Fig. 3C). From this observation, we proposed that the Arg and Trp residues act as a filter for screening phospholipids among the pool of cellular lipids. This represents a unique feature of Mmm1 because most SMP domains bind hydrocarbon chains of phospholipids through nonpolar contacts with hydrophobic residues inside the cavity of the SMP domain.

Regarding phospholipid trafficking at the ER-mitochondria contact site, it is well established that PC is synthesized from PS by the action of two enzymes that are distinctly located in the ER and mitochondria. The conversion of PS to PE is catalyzed by enzymes resident in mitochondria, whereas PA, an important intermediate in the formation of PG and cardiolipin in mitochondria, is synthesized in the ER (11). PS, PA, and PG must therefore be transferred from the ER, their site of synthesis, to mitochondria. Furthermore, PC synthesized in the ER must be eventually translocated to mitochondria for maintenance of membrane integrity. Because Mmm1 is the only ER resident protein among ERMES components, and since Mmm1 might be involved in phospholipid selection from the ER, the specific and favored recognition of phospholipids by Mmm1 might help to facilitate efficient lipid trafficking. In this study, we structurally and biochemically demonstrated that zrMmm1 alone and the scMDM12–zrMmm1 complex preferentially bind to phospholipids. This apparent selective extraction of phospholipids, facilitated by the surface charge and phospholipid filter of Mmm1, might be critical to the initiation of cooperative phospholipid synthesis at ER-mitochondria contact sites.

The proximal surfaces of membrane proteins are often positively charged, and we therefore suggest that the positively charged concave inner surface in the SMP domain of zrMmm1 might interact closely with the ER membrane. The concave structure of zrMmm1 might complement membrane curvature in terms of shape and size. In addition, the adjacent circumference of a positively charged patch composed of hydrophobic residues, including Y245, W238, P354, P357, and Y406, with the side chains of these residues exposed to the surface of zrMmm1, indicates that these residues might play a role in tight docking to the ER membrane (Fig. 5A and B). Interestingly, we observed that unlike the head groups of phospholipids bound to Mdm12, which are exposed to the concave surface of Mdm12, the head groups of phospholipids bound to zrMmm1 project into the concave surface of zrMmm1 (Fig. S6C). Moreover, the concave surface in the scMDM12–zrMmm1 complex precisely conforms to that generated by zrMmm1, strongly supporting the possibility that the concave inner surface of zrMmm1 binds to a convex membrane region.

Mmm1 interacts with Mdm34 through Mdm12 via relatively weak or transient interactions (19, 20). Additionally, we previously demonstrated that zrMmm1 alone and the scMDM12–zrMmm1 complex preferentially bind to phospholipids. This apparent selective extraction of phospholipids, facilitated by the surface charge and phospholipid filter of Mmm1, might be critical to the initiation of cooperative phospholipid synthesis at ER-mitochondria contact sites.

In conclusion, the Mdm12–Mmm1 complex establishes a molecular basis for protein-mediated MCSs between the ER and mitochondria, and for phospholipid trafficking through the ERMES complex.

Materials and Methods

Plasmid Construction. The DNA fragment encoding the SMP domain of Mmm1 (Z. rouxii, residues 190–444) was generated by PCR amplification from genomic DNA and cloned into the pET28b-SMT3 expression vector with BamHI and SalI restriction enzymes. To construct scMDM12Δ, residues 74–114 and residues 183–211 from full-length Mdm12 were substituted to GGGSG (E73-GGGSGG-S115) and G (D182-GE-S212), respectively, and cloned into the pCDF-duet vector with NdeI and Xhol. All mutants were generated by PCR-based mutagenesis, and mutations were confirmed by DNA sequencing.

Protein Expression and Purification. All proteins in this study were expressed by transforming the expression plasmids into E. coli BL21 (DE3) bacterial cells. Cells were grown to an OD600 of ~0.7 at 37 °C with vigorous shaking and induced overnight at 18 °C with 0.3 mM isopropyl-β-D-thiogalactoside. Cells were collected by centrifugation at 3,200 × g for 15 min; resuspended in buffer A containing 25 mM sodium phosphate (pH 7.8), 400 mM sodium chloride, and 10 mM imidazole; and flash-frozen in liquid nitrogen for later use. The zrMmm1 proteins were purified by Ni2+-immobilized metal affinity chromatography (Ni2+-IMAC). His6-SMT3 tags were removed by adding Ulp1 protease at a ratio of 1:1,000 (wt/wt), and proteins were dialyzed overnight against buffer B comprising 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, and 5 mM β-mercaptoethanol at 4 °C. Digested proteins were passed through an Ni2+-chelating column a second time to remove SMT3 tags and undisggested protein, followed by SEC with a Superdex 200 (16/60) column (GE Healthcare) preequilibrated with buffer C comprising 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, and 5 mM DTT.

For the scMDM12–zrMmm1 complex, pET28b-SMT3-zrMmm1 and pCDF-duet-scMDM12 plasmids were simultaneously transformed into E. coli BL21 (DE3) cells. The scMDM12–zrMmm1 complex proteins were purified using Ni2+-IMAC. After Ulp1 digestion, proteins were further purified by HiTrap Q HP (GE Healthcare) and Superdex 200 columns in buffer C. Purified zrMmm1 and scMDM12–zrMmm1 complex proteins were concentrated to 12.5 mg/mL and 5 mg/mL, respectively, using Amicon ultra centrifugal filters (Merck Millipore), and were flash-frozen at ~80 °C for later use.

For selenomethionine-substituted proteins, the zrMmm1 plasmid was transformed and expressed in the E. coli/BL21(DE3) methionine auxotrophic strain. Cells were grown in M9 minimal media supplemented with l-selenomethionine, and proteins were purified as described above. Additional methods are described in SI Materials and Methods.

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