Ion selectivity mechanism of the MgtE channel for Mg$^{2+}$ over Ca$^{2+}$

**Highlights**

- The ion selectivity filter of MgtE is highly selective for Mg$^{2+}$ over Ca$^{2+}$
- The higher resolution Ca$^{2+}$-bound structure of the MgtE TM domain was determined
- The water molecules associated with Ca$^{2+}$ at the selectivity filter were visualized
- The comparison with the previous Mg$^{2+}$-bound structure provided mechanistic insights

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SUMMARY

MgtE is a Mg$^{2+}$-selective ion channel whose orthologs are widely distributed from prokaryotes to eukaryotes, including humans, and are important participants in the maintenance of cellular Mg$^{2+}$ homeostasis. The previous high-resolution structure determination of the MgtE transmembrane (TM) domain in complex with Mg$^{2+}$ ions revealed a recognition mechanism of MgtE for Mg$^{2+}$ ions. In contrast, the previous Ca$^{2+}$-bound structure of the MgtE TM domain was determined only at moderate resolution (3.2 Å resolution), which was insufficient to visualize the water molecules coordinated to Ca$^{2+}$ ions. Here, we showed that the metal-binding site of the MgtE TM domain binds to Mg$^{2+}$ ~500-fold more strongly than to Ca$^{2+}$. We then determined the crystal structure of the MgtE TM domain in complex with Ca$^{2+}$ ions at a higher resolution (2.5 Å resolution), revealing hexahydrated Ca$^{2+}$. These results provide mechanistic insights into the ion selectivity of MgtE for Mg$^{2+}$ over Ca$^{2+}$.

INTRODUCTION

Mg$^{2+}$ ion is a fundamental biological cation implicated in various physiological functions, such as genomic stability, DNA and RNA folding, and catalysis by hundreds of enzymes. Therefore, cellular Mg$^{2+}$ homeostasis is vital to all domains of life and is therefore strictly controlled by Mg$^{2+}$ channels and transporters.

MgtE is a bacterial member of the MgtE/SLC41 superfamily of Mg$^{2+}$ channels and transporters, whose orthologs are widely conserved from bacteria to eukaryotes, including humans. MgtE is a Mg$^{2+}$-selective ion channel implicated in cellular Mg$^{2+}$ homeostasis and is involved in bacterial survival upon exposure to antibiotics. The previous single-channel recording of MgtE from Thermus thermophilus showed high Mg$^{2+}$ conductance, independent of both the pH and the Na$^{+}$ gradient, which is consistent with the role of MgtE as a passive ion channel.

The first full-length MgtE structure showed the homodimeric architecture of MgtE, where each chain consists of the transmembrane (TM) and cytoplasmic domains and a long amphipathic “plug” helix connecting these two domains (Figure 1A). The MgtE cytoplasmic domain possesses regulatory Mg$^{2+}$-binding sites to stabilize the closed state in the Mg$^{2+}$-bound form. In other words, the MgtE cytoplasmic domain acts as an intracellular Mg$^{2+}$ sensor to maintain cellular Mg$^{2+}$ homeostasis.

The subsequent crystal structures of the MgtE TM domain, in particular the one in complex with Mg$^{2+}$ ions at high resolution (2.3 Å resolution), revealed the binding of the fully hydrated Mg$^{2+}$ ion to the ion selectivity filter in the ion-conducting pore. Nevertheless, because the previously reported MgtE TM domain structure in the presence of Ca$^{2+}$ ions was determined only at moderate resolution (3.2 Å resolution), the mechanism of ion selectivity by MgtE, particularly the specificity for Mg$^{2+}$ over Ca$^{2+}$, another essential biological divalent cation, was not yet fully understood.

Here, we determined the crystal structure of the MgtE TM domain in complex with Ca$^{2+}$ at a higher resolution of 2.5 Å, enabling the visualization of water molecules coordinated to Ca$^{2+}$ at the ion selectivity filter of MgtE. A combination of structural, biochemical, and computational analyses provided structural insights into the ion selectivity of MgtE for Mg$^{2+}$ ions over Ca$^{2+}$ ions.
RESULTS

Biochemical cross-linking experiments of MgtE with Mg²⁺ and Ca²⁺

The previously reported MgtE structures in the presence and absence of Mg²⁺ ions showed Mg²⁺-dependent conformational changes in the TM domain, including the TM2 and TM5 helices, which form an ion-conducting pore (Figure 1B). The close inspection shows that the conformational changes originate from the selectivity filter (Figure 1C). We verified these structural changes by cross-linking experiments with the cysteine-substituted mutant of MgtE at Leu421 and Thr336, where the intersubunit distances of Cα atoms between Leu421 and Thr336 were 5.2 and 14.0 Å in the presence and absence of Mg²⁺ ions, respectively (Figure 1D).

To estimate the selectivity of the selectivity filter of MgtE in the TM domain for Mg²⁺ over Ca²⁺, using the MgtE T336C/L421C mutant lacking the N domain (MgtE ΔN T336C/L421C) and Cu²⁺ phenanthroline as a
**A** 

Mg\(^{2+}\), D432D

**B** 

Ca\(^{2+}\), D432D

**C** 

Mg\(^{2+}\), D432A

**D** 

Ca\(^{2+}\), D432A

**E** 

Mg\(^{2+}\), D432D

**F** 

Ca\(^{2+}\), D432D

**G** 

Mg\(^{2+}\), D432A

**H** 

Ca\(^{2+}\), D432A
also showed high selectivity for Mg$^{2+}$ over Ca$^{2+}$. The replacement of Asp432 with alanine in the TM5 helix, is consistent with the previous proteoliposome-based transport assay using a fluorescent indicator, which yielded a resolution of 2.5 Å, higher than before (3.2 Å) (Table 1). The newly determined structure of the MgtE TM domain is essentially the same as the previously determined MgtE TM domain (PDB ID: 4U9L), with an RMSD of 0.34 Å for C$\alpha$MgtE TM domain.

As the concentrations of Mg$^{2+}$ and Ca$^{2+}$ increased, the MgtE ΔN T336C/L421C mutant exhibited a stronger band corresponding to the MgtE dimer (Figure 2). The estimated EC$_{50}$ values of 27.7 ± 3.7 μM and 12.2 ± 1.9 mM for Mg$^{2+}$ and Ca$^{2+}$, respectively, indicated high selectivity for Mg$^{2+}$ over Ca$^{2+}$. This result is consistent with the previous proteoliposome-based transport assay using a fluorescent indicator, which also showed high selectivity for Mg$^{2+}$ over Ca$^{2+}$. The replacement of Asp432 with alanine in the TM5 helix, which forms the ion selectivity filter called the M1 site (Figure 3A), abolished the divalent cation-dependent cross-linking (Figure 2), indicating that the divalent cation binding to the M1 site indeed induces chemical cross-linking. Notably, there was a certain proportion of the dimer fraction even in the presence of 5 mM EDTA and water (control) (Figure 2), indicating the flexibility of the TM domain in the absence of divalent cations. This is consistent with the previous results of the biochemical cross-linking of MgtE as well as the patch clamp recording of MgtE, showing the structural equilibrium of MgtE between open and closed conformations under Mg$^{2+}$-free conditions. Furthermore, the previous $^{57}$Co$^{2+}$ uptake assay with Escherichia coli cells expressing MgtE showed the half-maximal inhibitory concentration (K$_{50}$) value in the sub-100 μM order, presumably corresponding to the affinity of the MgtE pore region for Mg$^{2+}$ ions, because of the coordination chemistry of Mg$^{2+}$ similar to that of Co$^{2+}$. Thus, our result is consistent with the previous experiments. Overall, these results indicate that the ion selectivity filter of MgtE is highly selective for Mg$^{2+}$ binding to the cytoplasmic domain.

Higher-resolution crystal structure of MgtE in the Ca$^{2+}$-bound form

To obtain a higher-resolution structure of MgtE in complex with Ca$^{2+}$ ions, we crystallized the MgtE TM domain in the presence of Ca$^{2+}$ ions using the lipidic cubic phase (LCP) technique, collected the datasets from a number of microcrystals using the ZO automated data collection system, and merged the datasets from 209 microcrystals using the KAMO automated data processing system. The final datasets yielded a resolution of 2.5 Å, higher than before (3.2 Å) (Table 1). The newly determined structure of the MgtE TM domain is essentially the same as the previously determined MgtE TM domain (PDB ID: 4U9L), with an RMSD of 0.34 Å for C$\alpha$ atoms (Figure 3).

In this structure, we observed more detailed features in the electron density maps at the ion selectivity filter (Figure 3C) than could be observed in the previous, lower-resolution Ca$^{2+}$-bound structure (Figure 3B). Among the possible coordination geometries of Ca$^{2+}$ ions in biology, with a range of water coordination numbers from six to eight, the electron densities in our structure adopted an octahedral coordination geometry of Ca$^{2+}$ ions with six water molecules in the first hydration shell (Figure 3C). To further verify the origin of the electron densities, in particular to rule out the possibility of Mg$^{2+}$ as an origin of the electron densities, we performed inductively coupled plasma atomic emission spectroscopy (ICP–AES) with purified MgtE TM domain proteins, and the results showed no detection of Mg$^{2+}$ contamination (Table 2). It is also unlikely that Na$^{+}$ is an origin of the electron densities for the following reasons. First, without adding Ca$^{2+}$ ions, it was impossible to obtain crystals in the current crystallization condition. Furthermore, two acidic residues of Asp432 from both subunits face each other at the M1 site (Figure 3). Such an environment would be unfavorable to accommodate a monovalent cation, considering the reported structures of monovalent cation transport proteins. The side chains of Asp432 residues interact with four of six water molecules in the first hydration shell (Figure 3E). In addition, two extra water molecules in the second hydration shell form hydrogen bonds with the side chain of Asp432 residues and main chain carbonyl oxygen atoms of Ala428 residues (Figure 3E). The bonding distances between Ca$^{2+}$ ions and coordinated water molecules...
Figure 3. Ion selectivity filter

(A) The Mg$^{2+}$-bound MgtE TM domain structure (PDB ID: 4U9L). The location of the ion selectivity filter (M1 site) is marked.

(B–E) A close-up view of the M1 site in the Ca$^{2+}$-bound MgtE TM domain structures, previously determined at 3.2 Å resolution (PDB ID: 4WIB) (B) and determined at 2.5 Å resolution in this study (C, E).

(D and F) A close-up view of the M1 site in the Mg$^{2+}$-bound MgtE TM domain structure (PDB ID: 4U9L). The POLDER-OMIT maps and associated water molecules are shown in blue mesh (contoured at 3.0 σ) (C, D). The coloring scheme is the same as in Figure 1. Amino acid residues at the metal binding site are shown in stick representation. Mg$^{2+}$, Ca$^{2+}$ and water molecules are shown as magenta, green and red spheres, respectively. Dashed lines indicate hydrogen bonds, and associated numbers show the water-metal distances (Å).
are 2.4–2.6 Å (Figure 3E), which is consistent with the range of Ca-O distances (2.2–2.7 Å) in previously reported crystal structures.

Notably, in the previously reported Mg$^{2+}$-bound structure (Figures 3D and 3F), Mg$^{2+}$ also adopts a very similar octahedral coordination geometry to that observed in the present Ca$^{2+}$-bound structure (Figure 3E) but with shorter bonding distances of 2.0–2.2 Å between Mg$^{2+}$ and water molecules, which is also consistent with the typical Mg-O distances in previously reported crystal structures.

### MD simulations

To further examine Mg$^{2+}$ and Ca$^{2+}$ recognition by the M1 site of MgtE, we performed MD simulations based on the current Ca$^{2+}$-bound crystal structure together with the previously reported Mg$^{2+}$-bound structure (Figure 4). The overall structures were mostly stable during the 1-μs simulations starting from the MgtE structure embedded in the POPC lipid bilayer (Figure 4A). Both the Mg$^{2+}$ ion and Ca$^{2+}$ ion were stably bound to the M1 site with all six water molecules in the first hydration shell. Importantly, the distances between Mg$^{2+}$ and water molecules and between Ca$^{2+}$ and water molecules were stable during

### Table 1. X-ray data collection and refinement statistics

| Data collection | MgtE TM with Ca$^{2+}$ |
|----------------|------------------------|
| Wavelength (Å) | 1.000 |
| Space group | P21;2;2; |
| Cell dimensions |  |
| a, b, c (Å) | 64.8, 70.3, 104.0 |
| α, β, γ (°) | 90.0, 90.0, 90.0 |
| Resolution (Å)* | 47.62–2.50 (2.65–2.50) |
| R<sub>merge</sub>* | 0.448 (3.690) |
| I<sub>ref</sub>* | 10.82 (1.02) |
| Completeness (%)* | 99.9 (100.0) |
| Redundancy* | 35.3 (34.0) |
| CC<sub>1/2</sub> (%)* | 99.5 (57.4) |
| Refinement |  |
| Resolution (Å) | 2.5 |
| No. reflections | 16972 |
| R<sub>work</sub>/R<sub>free</sub> | 0.232/0.259 |
| No. atoms |  |
| Protein | 2671 |
| Ligand/ion | 65 |
| Water | 91 |
| B-factors |  |
| Protein | 69.21 |
| Ligand/ion | 82.96 |
| Water | 71.27 |
| R.m.s deviations |  |
| Bond lengths (Å) | 0.005 |
| Bond angles (°) | 1.194 |
| Ramachandran plot |  |
| Favored (%) | 100.0 |
| Allowed (%) | 0.0 |
| Outliers (%) | 0.0 |

*Highest resolution shell is shown in parenthesis.
the simulations, and Ca\(^{2+}\) maintained a longer distance from the water molecules than Mg\(^{2+}\) (Figures 4B–4E).

These results further support the insights from the crystal structures that the M1 site of MgtE can accommodate both Mg\(^{2+}\) and Ca\(^{2+}\) in the octahedral coordination geometry with six water molecules on the experimental timescale.

**DISCUSSION**

Mg\(^{2+}\) and Ca\(^{2+}\) are essential divalent cations for life. In the case of soluble proteins, various Ca\(^{2+}\)-binding proteins are known to be capable of binding to both Mg\(^{2+}\) and Ca\(^{2+}\) but with a higher affinity to Mg\(^{2+}\), apart from the low physiological Ca\(^{2+}\) concentration, which is 3–5 orders of magnitude lower than that of Mg\(^{2+}\) in the cytosol.\(^{27}\) For example, EF-hand is a common motif in many Ca\(^{2+}\)-binding proteins, which typically seem to have a 2–4 orders of magnitude higher affinity for Mg\(^{2+}\) than Ca\(^{2+}\).\(^{28}\) Nevertheless, only binding to Ca\(^{2+}\) can induce the conformational changes necessary to perform the physiological functions of these proteins.\(^{29}\) Likewise, many Mg\(^{2+}\)-binding proteins, including the Mg\(^{2+}\)-dependent enzyme ribonuclease H1, have a Ca\(^{2+}\)-binding preference *in vitro*, whereas only Mg\(^{2+}\) can activate the enzyme.\(^{30}\) Although there is no simple rule to explain such selectivity bias, several contributing factors have been characterized, such as ion net charge, binding cavity size and binding site geometry.\(^{27,31}\) However, relatively little is known about the selectivity mechanisms by which ion channels and transporters discriminate them compared to those for soluble proteins.

In this work, we showed by biochemical cross-linking that the M1 site of the MgtE TM domain is highly selective for Mg\(^{2+}\) over Ca\(^{2+}\) (Figure 2). The improved crystal structure of the MgtE TM domain in complex with Ca\(^{2+}\) together with the MD simulations suggested that the M1 site recognizes Ca\(^{2+}\) in an octahedral coordination geometry with six water molecules, similar to that observed in the previously determined Mg\(^{2+}\)-bound structure, but with longer metal–water bond lengths (Figures 3 and 4).

Based on these results, we can discuss the ion selectivity mechanism of MgtE for Mg\(^{2+}\) over Ca\(^{2+}\). First, the coordination number can range from six to eight for Ca\(^{2+}\), but seven is most common in aqueous solution.\(^{23–25,32}\) Consistently, the Ca\(^{2+}\) ATPase pump and Na\(^{+}/Ca\(^{2+}\) exchanger also recognize Ca\(^{2+}\) ions with a coordination number of seven,\(^{33,34}\) which directly explains their ion selectivity for Ca\(^{2+}\) over Mg\(^{2+}\) because Mg\(^{2+}\) ions have a strict octahedral coordination with six water molecules. On the other hand, in MgtE, the M1 site recognizes Ca\(^{2+}\) ions in the octahedral coordination geometry with six water molecules (Figure 3). Because Ca\(^{2+}\) is estimated to have a coordination number from six to eight and because the coordination number of seven is preferred, a loss of coordination would occur when Ca\(^{2+}\) takes on an octahedral coordination number in the M1 site of MgtE, according to our structure. During this process, Ca\(^{2+}\) must be forced into a lower coordination mode when bound to the M1 site of MgtE, where it requires more energy for dehydration.

Next, whereas the size of the fully hydrated Mg\(^{2+}\) fits well into the M1 site through electrostatic interactions between Mg\(^{2+}\) and the negative charges of Asp432 and hydrogen bonds between the first and second hydrated water shells,\(^{16}\) the hexahydrated Ca\(^{2+}\) is larger than the hexahydrated Mg\(^{2+}\), owing to the longer bond distance and the larger ion radius (Figures 3E and 3F). Moreover, the charge density of Mg\(^{2+}\) is three times that of Ca\(^{2+}\), providing a stronger electrostatic attraction affinity to oxygen in biological systems.\(^{35}\) Therefore, the differences in both the hydrated size and charge density between Mg\(^{2+}\) and Ca\(^{2+}\) may also account for the hindrance of the Ca\(^{2+}\) permeation by MgtE compared with that of Mg\(^{2+}\).

To summarize, these findings may explain the selectivity of MgtE for Mg\(^{2+}\) over Ca\(^{2+}\), and our structural, biochemical and computational analyses provided insights into the selectivity of MgtE.

**Table 2. Inductively coupled plasma atomic emission spectroscopy**

| Sample | Elem | Reported Conc (Samp) | Samp Units |
|--------|------|----------------------|------------|
| Buffer | Mg   | ND                   | mg/L       |
|        | Ca   | 0.099                | mg/L       |
| MgtE   | Mg   | ND                   | mg/L       |
|        | Ca   | 0.124                | mg/L       |
Limitations of the study

Notably, Grotz et al. recently proposed that water exchange as well as the concerted motions of two exchanging water molecules occurred on the microsecond timescale with divalent ions in MD simulations.36 Therefore, the exchange of the divalent cations at the M1 site would need a long timescale once some divalent cations are bound. Thus, further MD simulations are required to complete the conclusion in this study.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105565.

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AUTHOR CONTRIBUTIONS

X.T. and D.S. purified and crystallized the MgtE TM domain and determined the Ca\(^{2+}\)-bound structure of MgtE. X.T. performed the biochemical cross-linking experiments. J.W. and Y.Y. performed the MD simulations. X.T., D.S., and M.H. wrote the manuscript. M.H. supervised the research. All authors discussed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *Escherichia coli*, Rosetta (DE3) | Sigma-Aldrich | Cat# 70954 |
| **Chemicals, peptides, and recombinant proteins** | | |
| TtMgtE 268-450 | This paper | N/A |
| TtMgtE 130-450, T336C/L421C | This paper | N/A |
| TtMgtE 130-450, T336C/L421C/D432A | This paper | N/A |
| Polyethylene glycol 400 | Molecular Dimensions | Cat# MD2-250-3 |
| CuSO₄ | Sangon Biotech | Cat# A100330 |
| Bis-1,10-phenanthroline | Sangon Biotech | Cat# A600693 |
| **Deposited data** | | |
| TtMgtE TM domain in complex with Ca²⁺ ions | This paper | PDB: 8H5E |
| **Recombinant DNA** | | |
| pET28a-TtMgtE 130-267, HRV3C, 268-450 | This paper | N/A |
| pET28a-TtMgtE 130-450, T336C/L421C | This paper | N/A |
| pET28a-TtMgtE 130-450, T336C/L421C/D432A | This paper | N/A |
| **Software and algorithms** | | |
| Desmond | Shaw, 2005 | https://www.schrodinger.com/products/desmond |
| PyMOL | Schrodinger, LLC. | https://pymol.org/ |
| XDS | Kabsch, 2010 | http://xds.mpimf-heidelberg.mpg.de/ |
| ZOO | Hirata et al., 2019 | https://github.com/keitaroyam/yamtbx |
| KAMO | Yamashita et al., 2018 | https://github.com/keitaroyam/yamtbx |
| Phenix | Liebschner et al., 2019 | http://www.phenix-online.org/ |
| Coot | Emsley et al., 2010 | https://www.ccp4.ac.uk/ |

### RESOURCE AVAILABILITY

#### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Motoyuki Hattori (hattorim@fudan.edu.cn).

#### Materials availability
Plasmids and other materials generated in this study can be requested from the lead contact, Motoyuki Hattori (hattorim@fudan.edu.cn).

#### Data and code availability
The atomic coordinates and structure factors of MgtE were deposited in the Protein Data Bank (PDB ID: 8H5E). All SDS–PAGE gel images were deposited to Mendeley Data (https://doi.org/10.17632/8xtr9pn6h.1). This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS
*T. thermophilus* MgtE proteins were expressed in the in *E. coli* Rosetta (DE3) strain.
### METHOD DETAILS

#### Expression and purification

The MgtE \( \Delta N \) domain mutant gene from *T. thermophilus* (residues 130-450) was subcloned into a pET28a vector containing an N-terminal hexahistidine tag and a thrombin cleavage site. The human rhinovirus 3C (HRV3C) protease cleavage site was inserted between residues Asp267 and Val268 at the loop region between the cytoplasmic domain and TM domain. MgtE protein was overexpressed in *E. coli* Rosetta (DE3) cells in LB medium containing 30 \( \mu \)g/mL kanamycin at 37°C by adding 0.5 mM isopropyl-\( \beta \)-D-thiogalactoside (IPTG) at an OD\(_{600}\) of ≈0.5, and then *E. coli* cells were further cultured at 18°C for 16 h. The *E. coli* cells were harvested by centrifugation (6,000 \( \times \) g, 15 min) and then resuspended in buffer H [150 mM NaCl, 50 mM HEPES (pH 7.0) and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)]. All purification procedures were performed at 4°C. The *E. coli* cells were disrupted with a microfluidizer. After centrifugation (20,000 \( \times \) g, 30 min), the supernatants were collected and subjected to ultracentrifugation (200,000 \( \times \) g, 1 h). The membrane fraction from ultracentrifugation was then solubilized with buffer S [300 mM NaCl, 50 mM HEPES (pH 7.0), 2% (w/v) n-dodecyl-beta-D-maltopyranoside (DDM) (Anatrace, USA) and 0.5 mM PMSF] for 2 h. The solubilization fraction was loaded onto a Ni-NTA column preequilibrated with buffer A [300 mM NaCl, 50 mM HEPES (pH 7.0) and 0.05% (w/v) DDM] containing 20 mM imidazole, mixed, and incubated for 1 h. The Ni-NTA column was washed with buffer A containing 50 mM imidazole, and the MgtE protein was eluted with buffer A containing 300 mM imidazole.

To cleave the HRV3C protease cleavage site, the eluate was mixed with Ni-NTA beads preequilibrated in buffer B and His-tagged HRV3C protease and then dialyzed against buffer B overnight. The sample was reloaded on a column, and the flow-through fractions containing the MgtE TM domain protein were concentrated using an Amicon Ultra 50 K filter (Merck Millipore, USA). After concentration, the sample was injected into a Superdex 200 10/300 size-exclusion column (GE Healthcare, USA) equilibrated with buffer C [25 mM HEPES (pH 7.0), 150 mM NaCl and 0.025% (w/v) DDM] for size-exclusion chromatography (SEC). The peak fractions containing the MgtE TM domain protein were collected and concentrated to 10 mg/mL using an Amicon Ultra 50 K filter (Merck Millipore, USA) for crystallization.

For the MgtE \( \Delta N \) T336C/L421C and MgtE \( \Delta N \) T336C/L421C/D432A cysteine mutants, the protein expression and preparation of the membrane fractions were performed similarly to the methods described above. The membrane fractions were solubilized with buffer D [50 mM HEPES (pH 7.0), 150 mM NaCl, 2% DDM, 20 mM imidazole, 1 mM PMFS, 1 mM \( \beta \)-mercaptoethanol (\( \beta \)-ME)] for 2 h. Then, insoluble materials were removed by ultracentrifugation (200,000 \( \times \) g, 1 h). The supernatant was mixed with Ni-NTA resin preequilibrated with buffer D, incubated for 1 h, washed with buffer E [50 mM HEPES (pH 7.0), 150 mM NaCl, 0.05% DDM, 50 mM imidazole, 1 mM \( \beta \)-ME], and then eluted with buffer F [50 mM HEPES (pH 7.0), 150 mM NaCl, 0.05% DDM, 300 mM imidazole, 1 mM \( \beta \)-ME]. The eluted MgtE proteins were dialyzed overnight in buffer G [50 mM HEPES (pH 7.0), 150 mM NaCl, 0.05% DDM, 20 mM dithiothreitol (DTT)] and applied to a Superdex 200 10/300 size-exclusion column equilibrated with buffer H [20 mM HEPES (pH 7.0), 150 mM NaCl, 0.03% DDM] for SEC. The peak fractions were concentrated to 0.5 mg/mL using an Amicon Ultra 50 K filter.

#### Crystallization

Before crystallization, the purified MgtE TM domain protein was mixed with CaCl\(_2\) at a final concentration of 100 mM and incubated on ice for 30 min. The protein was then mixed with monoolein (NU-CHEK, USA) at a ratio of 2:3 (w:w) in a twin syringe to generate the lipidic cubic phase (LCP). For crystallization, a Gryphon LCP crystallization robot (Art Robbins Instruments, USA) was employed to dispense 50 nL drops of LCP onto a 96-well sandwich plate and to overlay 700 nL reservoir solutions. Crystals appeared at 18°C after one week in the reservoir solution containing 30% (w/v) polyethylene glycol (PEG) 400, 100 mM HEPES (pH 7.5), and 100 mM NaSCN.

#### X-ray data collection and structure determination

X-ray diffraction data were collected at the BL32XU beamline at SPring-8 (Harima, Japan) using the ZO automatic data collection system\(^{21}\) and processed with KAMO\(^{22}\) and XDS.\(^{38}\) The structure of the MgtE TM domain in complex with Ca\(^{2+}\) (residues 271-448 for chains A and B) was determined by molecular replacement with Phaser\(^{23}\) using the Mg\(^{2+}\)-bound MgtE TM domain structure (PDB ID: 4U9L). The atomic model was then manually built using COOT\(^{40}\) and refined with PHENIX.\(^{39}\) The Ramachandran plots were...
calculated using MolProbity. The X-ray data collection and refinement statistics are summarized in Table 1. All structure figures were generated using PyMOL (https://pymol.org/).

**Biochemical cross-linking**

Biochemical cross-linking experiments were performed as described previously. First, 4 μL of the MgtEΔN domain double cysteine mutant T336C/L421C construct protein at 0.5 mg/mL was mixed with 0.5 μL of EDTA at a final concentration of 5 mM and with MgCl₂ or CaCl₂ at appropriate concentrations, incubated on ice for 30 min, and then mixed with 0.5 μL of 10 mM freshly prepared Cu²⁺ bis-1,10-phenanthroline (with a molar ratio of 1:3) and allowed to react on ice for another 30 min. The control samples and other samples were analyzed by SDS–PAGE under reducing conditions and nonreducing conditions, respectively. Experiments were repeated six times. SDS–PAGE gels were quantified by ImageJ (NIH, USA), and the quantified data were fitted to a nonlinear curve by Origin (OriginLab, USA).

**Molecular dynamics simulations**

Molecular dynamics (MD) simulations were performed using ‘Desmond’ software. The initial positioning of MgtE in the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane was obtained from the OPM database. The POPC membrane-bound structures were built using a simple point charged water model (SPC) in an orthorhombic box with dimensions of 10 Å × 10 Å × 10 Å, with 10 Å distances between the box’s edge and the protein atoms. To maintain balance and neutralize the system, counter ions (Na⁺ or Cl⁻) were added. NaCl (150 mM) was added to the simulation box to represent background salt under physiological conditions. Prior to MD simulation, the DESMOND default relaxation protocol was applied to each system: (1) 100 ps simulations in the NVT ensemble with Brownian kinetics using a temperature of 10 K with solute heavy atoms constrained; (2) 12 ps simulations in the NVT ensemble using a Berendsen thermostat with a temperature of 10 K and small-time steps with solute heavy atoms constrained; (3) 12 ps simulations in the NPT ensemble using a Berendsen thermostat and barostat for 12 ps simulations at 10 K and 1 atm, with solute heavy atoms constrained; (4) 12 ps simulations in the NPT ensemble using a Berendsen thermostat and barostat at 300 K and 1 atm, with solute heavy atoms constrained; and (5) 24 ps simulations in the NPT ensemble using a Berendsen thermostat and barostat at 300 K and 1 atm without constraint. After equilibration, the MD simulations were performed for 1000 ns. Long-range electrostatic interactions were computed using a smooth particle mesh Ewald method. The trajectory recording interval was set to 200 ps, and other default parameters of DESMOND were used during MD simulation runs. All simulations used the all-atom OPLS_2005 force field with zero-order bond technology added to improve the handling of metal systems. All simulations were run on a DELL T7920 graphic working station (with an NVIDIA Tesla K40C-GPU). Analysis and visualization were performed on a 12-CPU CORE DELL T3610 graphic working station.

**Inductively coupled plasma atomic emission spectroscopy**

The metal (Mg²⁺/Ca²⁺) amounts of the purified MgtETM domain protein were measured by ICP–AES using Optima8000DV (PerkinElmer, USA) (Table 2). Proteins were purified as described above. Purified MgtETM domain and buffer C (control) were heated at 65°C for 20 min after adding HNO₃ at a final concentration of 1% (w/v) and incubated at RT overnight. The samples were then centrifuged (14,000 × g, 20 min). The supernatants were harvested, and the Mg²⁺ and Ca²⁺ concentrations were measured by ICP–AES.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

In the biochemical cross-linking experiments in Figure 2, the relative intensity of the bands was quantified by ImageJ software. Experiments were repeated six times. Error bars represent the standard error of the mean. The X-ray data collection and refinement statistics are summarized in Table 1.