Characterization of the scrambling domain of the TMEM16 family

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The TMEM16 protein family has 10 members, each of which carries 10 transmembrane segments. TMEM16A and 16B are Ca^{2+}-activated Cl^- channels. Several other members, including TMEM16F, promote phospholipid scrambling between the inner and outer leaflets of a cell membrane in response to intracellular Ca^{2+}. However, the mechanism by which TMEM16 proteins translocate phospholipids in plasma membranes remains elusive. Here we show that Ca^{2+}-activated, TMEM16F-supported phospholipid scrambling proceeds at 4 °C. Similar to TMEM16F and 16E, seven TMEM16 family members were found to carry a domain (SCRD; scrambling domain) spanning the fourth and fifth transmembrane segments that conferred scrambling ability to TMEM16A. By introducing point mutations into TMEM16F, we found that a lysine in the fourth transmembrane segment of the SCRD as well as an arginine in the third and a glutamic acid in the sixth transmembrane segment were important for exposing phosphatidylserine from the inner to the outer leaflet. However, their role in internalizing phospholipids was limited. Our results suggest that TMEM16 provides a cleft containing hydrophilic "stepping stones" for the outward translocation of phospholipids.

scramblase | phospholipids | point mutation | TMEM16

The plasma membrane of eukaryotic cells is composed of two layers, an outer and an inner leaflet; phospholipids, the major components of the plasma membranes, are asymmetrically distributed between these leaflets (1, 2). That is, 70 to 80% of phosphatidylcholine (PtdCho) and sphingomyelin (SM) in the plasma membrane are in the outer leaflet, whereas phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) are exclusively localized to the inner leaflet (2). This asymmetrical distribution of phospholipids, in particular of PtdSer and PtdEtn, is maintained by phospholipid flippases, which translocate PtdSer and PtdEtn from the outer to the inner leaflet in an ATP-dependent manner (3, 4). We recently showed that ATP11A and ATP11C, members of the type IV P-type ATPase family, play a major role in flipping PtdSer and PtdEtn at the plasma membrane (5–7).

The asymmetrical distribution of phospholipids is disrupted in various biological processes. For example, PtdSer exposed to the cell surface of activated platelets acts as a scaffold for blood clotting factors, and PtdSer exposed on apoptotic cells serves as an "eat me" signal for phagocytes (8). Phospholipid scramblases translocate phospholipids bidirectionally between the outer and inner leaflets, and are necessary for efficiently exposing PtdSer on activated platelets and apoptotic cells (1, 8). We previously identified two families of membrane proteins (TMEM16 and Xkr) that function as Ca^{2+}- or caspase-dependent phospholipid scramblases (8).

The mouse TMEM16 family, also called the Ano family, has 10 members (TMEM16A to H, J, and K), each of which carries 10 transmembrane segments with cytosolic N- and C-terminal tails (9). Except for TMEM16E, 16H, and 16K, the TMEM16 members are localized to the plasma membrane (8, 10), among which TMEM16A and 16B are Ca^{2+}-dependent chloride channels (11–14) whereas TMEM16C, 16D, 16F, 16G, and 16J act as Ca^{2+}-dependent phospholipid scramblases (14, 15). By establishing chimeric molecules between TMEM16A and 16F and between 16A and 16E, Yu et al. (16) and we (10) showed that a 35-amino acid domain (SCRD; scrambling domain) in TMEM16F and 16E is necessary to support the Ca^{2+}-dependent scrambling of phospholipids. Based on the X-ray structure analysis of *Nectria hematococca* (nh)TMEM16 (9), a "cleft" model was proposed, in which phospholipids translocate through membranes via a hydrophilic residue. Consistent with this model, TMEM16F's SCRD carries hydrophilic residues (16). A recent computational analysis of TMEM16-mediated phospholipid scrambling indicated that the hydrophilic residues in the transmembrane segments distort the membrane structure to facilitate the movement of phospholipids (17).

In this report, we found that the TMEM16F-supported Ca^{2+}-induced scrambling of phospholipids proceeds at 4 °C. We then analyzed the scrambling activity of the SCRDs from various TMEM16s at 4 °C, using chimeric molecules in which the 35-amino acid SCRD was swapped with its corresponding region in TMEM16A. These analyses indicated that SCRDs could be divided into three groups: strong, weak, and no ability to support PtdSer exposure. Alignment of the SCRDs of TMEM16 members and mutational analysis identified a basic residue in the transmembrane segment of SCRD that was critical for exposing PtdSer. In addition, two hydrophilic amino acids in transmembrane segments 3 and 6 that form a cleft were found to be indispensable for outward translocation of PtdSer, supporting the idea that the cleft of TMEM16 provides a pathway for phospholipid translocation.

Results

Ca^{2+}-Dependent Phospholipid Scrambling by TMEM16F. The spontaneous or nonenzymatic translocation of phospholipids between the two leaflets of a plasma membrane is extremely slow, with a

Significance

Plasma membrane in eukaryotic cells is composed of two layers, and phospholipids are asymmetrically distributed between them by an action of flippases. Ca^{2+}-dependent scramblases (TMEM16 family members) disrupt the asymmetry, and phosphatidylserine (PtdSer) exposed to the cell surface functions as a signaling molecule in various biological systems. We show that TMEM16F-supported phospholipid scrambling proceeds at 4 °C, independent of phospholipid flipping. Seven TMEM16 members carry a scrambling domain that confers scrambling ability to TMEM16A, a Ca^{2+}-dependent Cl^- channel. Mutational analysis identified hydrophilic residues in the transmembrane segments of TMEM16F that are important for exposing PtdSer. Our results may support a model in which TMEM16 proteins provide a cleft containing hydrophilic "stepping stones" in the transmembrane segments for the outward translocation of phospholipids.

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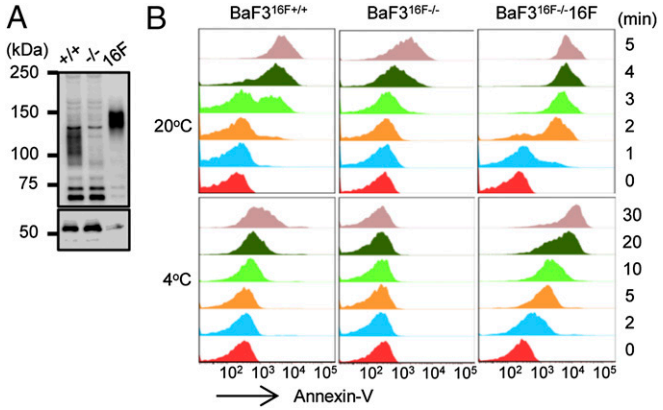

Fig. 1. Ca^{2+}-dependent phospholipid scrambling. (*A*) Expression of TMEM16F. Cell lysates were prepared from wild-type BaF3 cells (+/+), *TMEM16F*$^{-/-}$ BaF3 cells (–/–), and *TMEM16F*$^{-/-}$ BaF3 cells transformed with TMEM16F (16F). Proteins (5 μg for +/+ and –/–; 0.1 μg for 16F) were separated by 7.5% SDS/PAGE and analyzed by Western blotting with anti-TMEM16F (*Upper*) or anti–α-tubulin (*Lower*) antibodies. (*B*) Ca^{2+}-dependent PtdSer exposure by TMEM16F. Wild-type BaF3 cells (BaF3$^{16F+/+}$), *TMEM16F*$^{-/-}$ BaF3 cells (BaF3$^{16F-/-}$), and TMEM16F transformants (BaF3$^{16F-/-}$16F) were incubated at 20 or 4 °C with 3 μM A23187 and 1 mM $CaCl_2$ in the presence of Cy5-Annexin V and PI. Annexin V-staining profiles in the PI-negative population at the indicated time after A23187 addition are shown.

half-time of 6.5 h at 30 °C (18). One of the models for phospholipid scramblases, based on the X-ray structure analysis of nhTMEM16, is that the TMEM16 protein provides a cleft for phospholipid translocation (9). We speculated that this cleft might reduce the activation energy needed to translocate phospholipids between two leaflets. To examine this possibility, we assayed TMEM16F-mediated Ca^{2+}-induced phospholipid scrambling, specifically the PtdSer exposure, at different temperatures using TMEM16F-competent or -deficient mouse BaF3 cells (Fig. 1*A*). As shown in Fig. 1*B*, wild-type BaF3 cells treated at 20 °C with the Ca^{2+} ionophore A23187 and 1 mM Ca^{2+} rapidly and biphasically exposed PtdSer, which was detected by Cy5-labeled Annexin V. This PtdSer exposure was severely decreased in TMEM16F-deficient cells, indicating that the Ca^{2+}-induced PtdSer exposure in BaF3 cells mostly depends on TMEM16F. When BaF3 cells were treated at 4 °C with A23187, a weak but significant gradual PtdSer exposure was observed in wild-type but not *TMEM16F*$^{-/-}$ BaF3 cells (Fig. 1*B*). Propidium iodide (PI)-positive necrotic cells generated during the 30-min incubation at 4 °C were marginal (Fig. S1). To confirm that the phospholipid scrambling at 4 °C was TMEM16F-dependent, *TMEM16F*$^{-/-}$ cells were transformed with C-terminally EGFP-tagged mouse TMEM16F, and transformants expressing TMEM16F at least 50 times more abundantly than the parental BaF3 cells were established (Fig. 1*A*). Treatment of the transformants at 4 °C with A23187 promoted the gradual PtdSer exposure but with much faster kinetics than the parental cells, with the exposed PtdSer reaching its near-maximum level within 30 min (Fig. 1*B*).

Flippase-Independent Scrambling of PtdSer by TMEM16F. Flippases translocate PtdSer and PtdEtn from the outer to inner leaflet in an ATP-dependent manner (1, 2), and therefore might affect our assay system for phospholipid scrambling. To examine this possibility, two major flippase genes (*ATP11A* and *11C*) were knocked out by CRISPR-Cas9 technology (19) in W3 cells (Fig. S2*A*) with oligonucleotides shown in Table S1. As shown in Fig. S3, *ATP11A*$^{-/-}$ *ATP11C*$^{-/-}$ (double knockout; DKO) W3 cells almost completely lost their flippase activity, as assayed by the incorporation of a fluorescently labeled phospholipid, nitrobenzoxadiazol (NBD)-PS. On the other hand, Ca^{2+}-induced PtdSer exposure was not affected by the loss of flippase, but this activity was lost by further deleting the *TMEM16F* gene in *ATP11A*$^{-/-}$*ATP11C*$^{-/-}$*TMEM16F*$^{-/-}$ (triple knockout; TKO) W3 cells. To confirm that the flippase and scramblase function independently, EGFP-tagged TMEM16F or EGFP-tagged ATP11C was introduced into the TKO W3 cells, and stable transformants were established (Fig. S2*B*). The flippase activity was fully rescued by the transformation of TKO cells with ATP11C but not TMEM16F, whereas the Ca^{2+}-induced PtdSer scrambling was rescued by their transformation with TMEM16F but not ATP11C (Fig. S3). As found with the TMEM16F-transformed BaF3 cells, the Ca^{2+}-induced PtdSer exposure in TMEM16F-transformed TKO W3 cells occurred at 4 °C, with a similar time course as in TMEM16F-transformed BaF3 cells.

Scrambling Domain in TMEM16 Family Members. In addition to TMEM16F, TMEM16C, 16D, 16G, and 16J are localized to the plasma membrane and support Ca^{2+}-induced scrambling activity (14). Referring to the work of Yu et al. (16), who showed that the SCRD of TMEM16F confers the scrambling activity to TMEM16A, we previously showed that the SCRD of TMEM16E, a protein localized to intracellular membranes, also has the ability to scramble phospholipids when inserted into the corresponding region of TMEM16A (10). Here, to examine whether TMEM16C, 16D, 16G, and 16J use a similar SCRD as TMEM16E and 16F, and to examine whether the other intracellularly localized TMEM16 members (TMEM16H and 16K) carry a similar scrambling domain, the 35-amino acid domain corresponding to TMEM16F's SCRD of each TMEM16 protein was used to replace the domain of TMEM16A (Fig. 2*A*). The chimeric molecules were tagged with EGFP or DsRed and expressed in *TMEM16F*$^{-/-}$ BaF3 cells. Western blot with anti-GFP confirmed that the transformants expressed the chimeric proteins (Fig. 2*B*). Observation of the cells by confocal microscopy indicated that the TMEM16-EGFP chimeric proteins were localized to the plasma membrane in most cases (Fig. 2*C*).

Characterization of SCRD-Mediated Phospholipid Scrambling. Transformants expressing each TMEM16-EGFP chimera were then

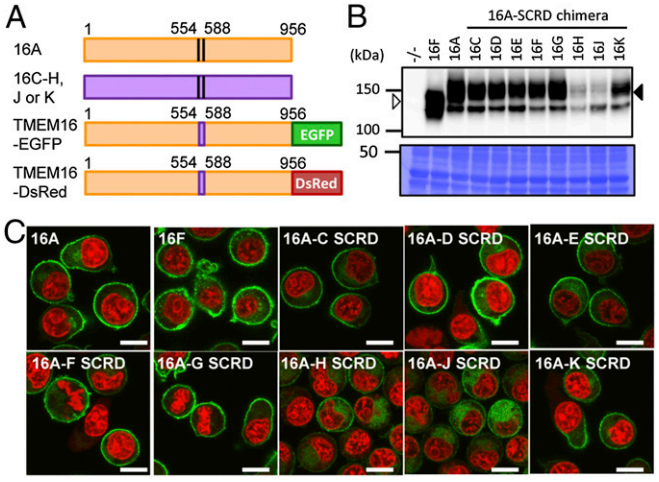

Fig. 2. Expression of TMEM16A-SCRD chimeric proteins. (*A*) Schematic representation of the TMEM16A-SCRD chimeras. The numbers above each diagram indicate the amino acid positions in TMEM16A. In the chimeric constructions, the 35-amino acid segment in TMEM16A (positions 554 to 588) was replaced with the corresponding sequence of other TMEM16 family members (TMEM16C–H, J, or K). The chimeric proteins were fused with EGFP or DsRed at the C terminus. (*B* and *C*) Expression of EGFP-tagged TMEM16A-SCRD chimeras. *TMEM16F*$^{-/-}$ BaF3 cells were stably transformed with the indicated TMEM16A-SCRD chimera tagged with EGFP. (*B*) The cell lysates were analyzed by Western blotting with anti-GFP Ab. After blotting, the membrane was stained by Coomassie brilliant blue (*Lower*). Open arrowhead indicates TMEM16F, while closed arrowhead indicate TMEM16A or its chimera with SCRD. (*C*) The transformants were observed by fluorescence confocal microscopy. (Scale bars, 10 μm.)

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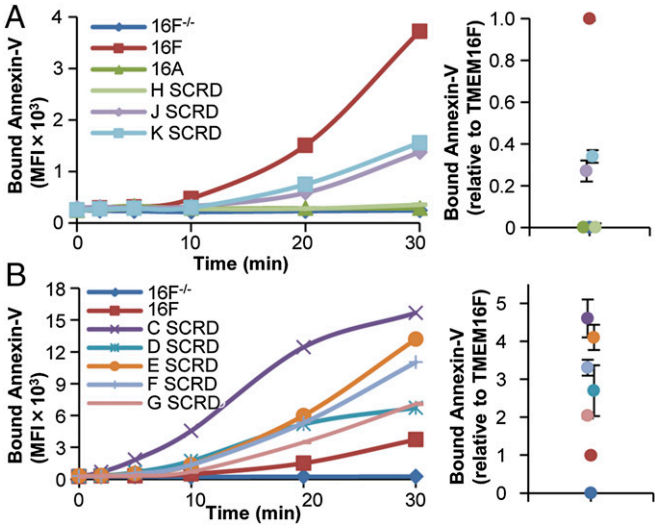

Fig. 3. Ca^{2+}-dependent PtdSer exposure by TMEM16A-SCRD chimeras. *TMEM16F*$^{-/-}$ BaF3 transformants expressing the chimera of TMEM16A with 16H's SCRD (H SCRD), 16J's SCRD (J SCRD), or 16K's SCRD (K SCRD) (*A*) or the chimera of TMEM16A with 16C's SCRD (C SCRD), 16D's SCRD (D SCRD), 16E's SCRD (E SCRD), 16F's SCRD (F SCRD), or 16G's SCRD (G SCRD) (*B*) were treated at 4 °C with 3 μM A23187 in the presence of 1 mM $CaCl_2$. At the indicated times, the exposed PtdSer, assessed by the binding of Cy5-labeled Annexin V in the PI-negative population, was determined by flow cytometry, and is expressed as the mean fluorescence intensity (MFI). The experiments were also carried out with the parental *TMEM16F*$^{-/-}$ BaF3 cell line (16F$^{-/-}$) and its transformants expressing TMEM16A (16A) or TMEM16F (16F). (*Right*) The scramblase activity of each chimera determined at 30 min is plotted relative to that of the intact TMEM16F. The experiments were performed three times, and the average values are plotted with SD (bars).

treated at 4 °C with the Ca^{2+} ionophore in the presence of 1 mM $CaCl_2$, and the PtdSer exposure, assayed by Cy5-Annexin V binding, was followed by flow cytometry. As shown in Fig. 3*A* and Table 1, TMEM16A did not respond to the Ca^{2+} ionophore to expose PtdSer, but its chimera carrying TMEM16F's SCRD responded well and exposed PtdSer in a time-dependent manner. The other chimeras could be divided into three groups: no, weak, and strong responders to the Ca^{2+} ionophore for PtdSer exposure. Of these, the chimera carrying TMEM16H's SCRD did not expose PtdSer in response to the Ca^{2+} ionophore (Fig. 3*A*). The cells carrying TMEM16J's or 16K's SCRD weakly responded, with about 30% of the efficiency of the intact TMEM16F (Fig. 3*A*), whereas the cells carrying TMEM16C's, 16D's, 16E's, 16F's, or 16G's SCRD responded to the Ca^{2+} ionophore for PtdSer exposure with an efficiency similar to or better than that of TMEM16F (Fig. 3*B*). These results indicated that the PtdSer-exposing activity of TMEM16D, 16F, 16G, and 16J at the plasma membrane (14) is mediated at least in part by their SCRD, and that TMEM16K may act as a phospholipid scramblase at intracellular membranes. Regarding TMEM16C, the PtdSer-exposing activity of the intact molecule was weak compared with TMEM16F (14) (Table 1) but its SCRD in the context of TMEM16A was very strong (Table 1), suggesting that another domain(s) in TMEM16C may inhibit the externalization of PtdSer.

SCRD's Scrambling Activity for Other Lipids. The phospholipid scrambling activity of the TMEM16s' SCRDs for other lipids was then examined at 4 °C using cells expressing TMEM16-DsRed chimeras. As shown in Fig. S4 and Table 1, the chimera carrying TMEM16H's SCRD did not support the Ca^{2+}-induced incorporation of any of the NBD-labeled phospholipids examined (NBD-PS, NBD-PC, NBD-SM, and NBD-GalCer; see definitions in *Materials and Methods*), consistent with its inability to expose PtdSer. The chimeras carrying the SCRD from other TMEM16s showed significantly different scrambling activities for these lipids. That is, the chimeras carrying TMEM16C's, 16D's, 16E's, 16F's, 16G's, 16J's, and 16K's SCRD responded similar to the Ca^{2+} ionophore and internalized NBD-PS and NBD-PC with efficiencies similar to that of TMEM16F's SCRD; their ability to internalize these lipids was 0.6 to 1.3 times that of the intact TMEM16F. NBD-SM was efficiently internalized by cells expressing the chimeras carrying TMEM16C's, 16D's, and 16F's SCRD (4 to 5 times the efficiency of the intact TMEM16F) but not by the cells expressing 16J's SCRD. TMEM16C's and 16D's chimera, but not TMEM16J's or 16K's, supported the incorporation of NBD-GalCer with 2.5 to 3.0 times the efficiency of TMEM16F. These results suggest that the SCRDs of different TMEM16s have different substrate specificities for scrambling phospholipids and sphingolipids.

Point Mutations That Affect SCRDs' Scrambling Activity. The above results indicated that the SCRDs of the TMEM16 family can be divided into three groups. TMEM16A and 16H, which do not support phospholipid scrambling, belong to the first group. The SCRDs of TMEM16C, 16D, 16E, 16F, and 16G strongly mediate the Ca^{2+}-induced PtdSer exposure, and form the second group.

Table 1. Scramblase activity of TMEM16's SCRD

	TMEM16 SCRD					Intact TMEM16	
TMEM	PtdSer exposure	NBD-PS incorporation	NBD-PC incorporation	NBD-SM incorporation	NBD-GalCer incorporation	PtdSer exposure	Cellular localization
16A	0.0 ± 0.01	0.0 ± 0.02	0.0 ± 0.04	0.0 ± 0.04	0.0 ± 0.06	0.0 ± 0.06	PM
16C	4.6 ± 0.50	1.3 ± 0.10	1.2 ± 0.18	5.2 ± 0.48	2.9 ± 0.71	0.1 ± 0.74	PM
16D	2.7 ± 0.67	1.2 ± 0.07	1.3 ± 0.01	4.0 ± 0.33	2.5 ± 0.46	0.8 ± 0.06	PM
16E	4.1 ± 0.33	0.7 ± 0.01	0.5 ± 0.12	1.1 ± 0.01	0.6 ± 0.20	0.0 ± 0.18	Intracellular
16F	3.3 ± 0.21	1.0 ± 0.15	1.2 ± 0.03	3.8 ± 0.49	1.2 ± 0.21	1.0	PM
16G	2.0 ± 0.07	0.8 ± 0.14	0.8 ± 0.06	1.7 ± 0.39	0.7 ± 0.07	0.7 ± 0.57	PM
16H	0.0 ± 0.02	0.0 ± 0.07	0.0 ± 0.04	0.0 ± 0.03	0.0 ± 0.14	0.2 ± 0.26	Intracellular
16J	0.3 ± 0.05	0.7 ± 0.04	0.7 ± 0.12	0.0 ± 0.01	0.1 ± 0.06	0.7 ± 0.19	PM
16K	0.3 ± 0.03	0.6 ± 0.62	0.7 ± 0.04	1.0 ± 0.08	0.1 ± 0.03	0.0 ± 0.01	Intracellular
Int16F	1.0	1.0	1.0	1.0	1.0	—	—

The PtdSer-exposing activities of TMEM16A carrying the SCRD of the indicated TMEM16 family members are from Fig. 3. For the incorporation of NBD-PS, NBD-PC, NBD-SM, and NBD-GalCer, the cells expressing the chimera were treated at 4 °C for 20 min with A23187 in the presence of NBD-PS, NBD-PC, NBD-SM, or NBD-GalCer as described in Fig. S4. The experiments were carried out in triplicate, and average ratios to those obtained with the cells expressing the full-length TMEM16F (Int16F) are shown with SD. The PtdSer-exposing activities and cellular localization of the intact TMEM16 members are from Suzuki et al. (14) and Gyobu et al. (10). PM, plasma membrane.

The SCRD activity of TMEM16J and 16K for PtdSer exposure is weak, and they form the third group. An alignment of the SCRDs' amino acid sequences (Fig. 4*A*) revealed several residues in the SCRDs (E525, K526, S546, and M551 in the numbering of TMEM16E) that were well-conserved in the second group (TMEM16C, 16D, 16E, and 16F). To examine the contribution of these residues to the strong scrambling activity, we mutated E525, K526, S546, and M551 in TMEM16E's SCRD to the residues that are found in the corresponding positions of TMEM16A. These mutated SCRDs of TMEM16E were introduced into TMEM16A, and the chimeras were expressed in *TMEM16F*$^{-/-}$ BaF3 cells (Fig. S5). As shown in Fig. 4*B*, the ability to support the Ca^{2+}-induced PtdSer exposure was severely affected by mutation at either K526 or S546 but not by mutation at E525 or M551. On the other hand, the mutation of these residues had little effect on the incorporation of NBD-PS or NBD-PC (Fig. 4*C*), suggesting that these residues are specifically involved in the process of PtdSer externalization.

Point Mutations in Hydrophilic Residues in TMEM16F Transmembrane Segments. A recent computational analysis of the scrambling activity of TMEM16 indicated two lipid head-group interaction sites, *Sc* (cytoplasmic site) and *Se* (extracellular site) (17). Because the K526 identified as a critical residue for the PtdSer exposure of TMEM16E is at the *Sc* site, we analyzed the hydrophilic residues in the *Se* site localized in the third and sixth transmembrane segments of TMEM16 proteins. Point mutations were introduced into R478 and E604 at the *Se* site of TMEM16F as well as K530 in the *Sc* site (Fig. 5*A*). Each mutant was fused to EGFP and introduced into *TMEM16F*$^{-/-}$ BaF3 cells, and stable transformants expressing a comparable level of mutant protein were established (Fig. S6). As shown in Fig. 5 *B* and *C*, the Ca^{2+}-induced PtdSer-exposing activity was abolished by R478A, but its phospholipid-internalizing activity with NBD-PC or NBD-PS was not affected by this mutation. Similarly, the K530C and E604A mutations reduced TMEM16F's PtdSer-exposing activity to a level of 40 to 20% and the activity incorporating NBD-PS to 70 to 50%. However, these mutations had no effect on internalizing NBD-PC. These results indicated that the three hydrophilic amino acid residues at the *Se* and *Sc* sites of TMEM16F are critical for PtdSer exposure but that their role in internalizing phospholipids is limited.

Discussion

Various membrane proteins transport ions and chemicals from extracellular to intracellular or from intracellular to extracellular regions. However, how phospholipid scramblases and flippases translocate phospholipids, which consist of hydrophobic tails and a hydrophilic head, through hydrophobic membrane bilayers has been a particular challenge in cell biology (20–22). We previously identified mouse and human TMEM16F as a phospholipid scramblase (14, 15). There was a possibility that the scrambling of phospholipids, in particular the PtdSer exposure, could be counterbalanced by the action of flippases, which translocate PtdSer from the outer to the inner leaflet (1, 23). We show here that the Ca^{2+}-activated TMEM16F-mediated PtdSer exposure was not affected by the loss of ATP11A and ATP11C (5, 6),

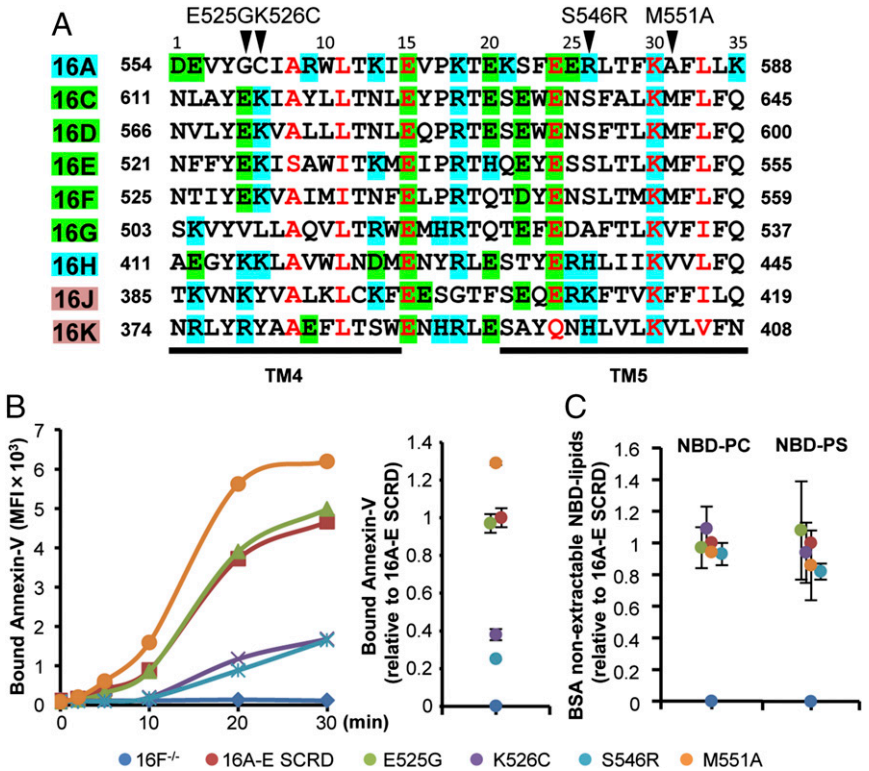

Fig. 4. Effect of SCRD point mutations on lipid scrambling activity. (*A*) Alignment of the SCRDs of mouse TMEM16 family members. The amino acid residues that are homologous among all members are in red. Basic and acidic amino acid residues are highlighted in blue and green, respectively. The fourth and fifth transmembrane segments (TM4 and TM5) are underlined. The four mutated residues are indicated above the top line. (*B*) *TMEM16F*$^{-/-}$ BaF3 (16F) transformants expressing the EGFP-tagged TMEM16A-chimera carrying 16E's SCRD or its mutants were treated at 4 °C with 3 μM A23187 and 1 mM $CaCl_2$ in the presence of Cy5-Annexin V and PI. At the indicated times, the bound Annexin V in the PI-negative population was determined by flow cytometry, and is expressed as the MFI. (*B*, *Right*) The amount of bound Annexin V for each mutant was determined at 30 min, and is expressed relative to the value observed with the chimera of TMEM16A the wild-type 16E SCRD. (*C*) The cells were treated at 4 °C for 20 min with 3 μM A23187 and 1 mM $CaCl_2$ in the presence of NBD-PC or NBD-PS. The incorporated NBD-phospholipids were determined by flow cytometry, and are expressed relative to the value obtained with the chimera of TMEM16A with 16E's SCRD. The experiments in *B* and *C* were carried out three times, and the average values are plotted with SD (bars).

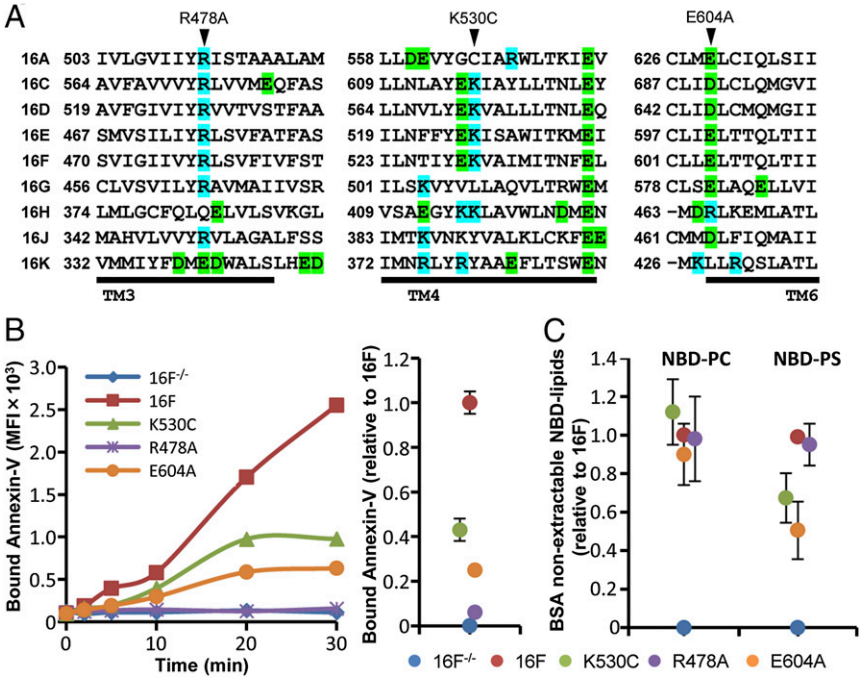

Fig. 5. Effect of TMEM16F point mutations on phospholipid scrambling activity. (*A*) Alignment of mouse TMEM16 family members. Basic and acidic amino acid residues are highlighted in blue and green, respectively. The third, fourth, and sixth transmembrane segments are underlined, and the position of the first amino acid residue of each segment is indicated. The TMEM16F mutants analyzed are indicated above the top line. (*B*) *TMEM16F*$^{-/-}$ BaF3 (16F$^{-/-}$) transformants expressing EGFP-tagged TMEM16F (16F) or its mutant (K530C, R478A, or E604A) were treated at 4 °C with 3 μM A23187 and 1 mM $CaCl_2$ in the presence of Cy5-Annexin V and PI. The bound Annexin V in the PI-negative population was analyzed by flow cytometry at the indicated times, and is expressed as the MFI. (*B*, *Right*) The amount of bound Annexin V at 30 min is expressed relative to the value observed with TMEM16F. (*C*) The cells were treated with 3 μM A23187 and 1 mM $CaCl_2$ in the presence of NBD-PC or NBD-PS, and incubated at 4 °C for 20 min. The incorporated NBD-phospholipids were determined by flow cytometry, and are expressed relative to the value obtained with TMEM16F. The experiments in *B* and *C* were carried out three times, and the average values are plotted with SD (bars).

confirming that scramblases and flippases are independent systems for regulating the phospholipid distribution in plasma membranes.

We previously showed that TMEM16F has a different conformation in the presence versus the absence of Ca^{2+} (24). The TMEM16F-supported scrambling at 20 °C proceeded biphasically, whereas it proceeded in a monophasic manner at 4 °C, suggesting that the initial reaction (Ca^{2+}-binding and Ca^{2+}-induced conformational change of the TMEM16 protein) requires higher activation energy than the second step (scrambling). Once the TMEM16F is activated, or Ca^{2+} is bound to the protein, the scrambling reaction goes very fast. It seems that the scrambling reaction is slowed down at 4 °C, which gives the synchronous PtdSer exposure. These results also suggest that, different from Ca^{2+}-ATPase, which cycles different conformations to transport Ca^{2+} (25), TMEM16F may not change its conformation during lipid conduction.

The gradual synchronous scrambling at 4 °C provided us with a reliable assay procedure with which to compare the Ca^{2+}-activated scramblases. We found that similar to TMEM16F and 16E (10, 16), TMEM16C, 16D, 16G, and 16J carry a SCRD. The scrambling activity of the SCRD from TMEM16D, 16G, and 16J agreed with the results obtained with the respective intact molecules (Table 1) (15). By computationally simulating the interaction between TMEM16K and lipids, Bethel and Grabe (17) predicted that TMEM16K has scrambling activity. In fact, we found that the SCRD of TMEM16K has a weak but significant phospholipid scrambling activity (Table 1), suggesting that TMEM16K is a scramblase that functions at intracellular membranes. This finding also indicates that the computational simulation of the interaction of TMEM16 with lipids (17, 26) is a reliable method for predicting the function of membrane proteins.

The ability of the SCRD to expose PtdSer differed significantly among TMEM16 members. On the other hand, the ability to incorporate NBD-PS did not differ among the members. This finding suggests that the PtdSer scrambling activity of the TMEM16 SCRDs, in particular those of 16C, 16D, 16E, and 16F, may differ for inward versus outward translocations. Among the conserved residues in SCRDs, the mutations in K526 or S546, but neither E525 nor M551, severely decreased its ability to expose PtdSer but not its ability to incorporate phospholipids, which supports this idea.

The X-ray crystal analysis of nhTMEM16 by Brunner et al. (9) and a subsequent computational simulation of TMEM16-mediated lipid scrambling (17) indicated that TMEM16 distorts the membrane and provides a cleft or gate for phospholipid transport. The cleft consists of three transmembrane segments in which charged amino acids are present. A high density of phospholipids was found at two sites (*Sc* and *Se*) of the cleft. The *Sc* site is at the fourth transmembrane segment in SCRD, where two neighboring charged amino acids, glutamic acid and lysine, are present. Bethel and Grabe postulated that the *Sc* site organizes a dipole stack to support phospholipid movement from inside to outside (17). Our mutational study indicated that K526, but not E525, is essential for the PtdSer exposure, suggesting that the "bipolar stack" model may not apply to mouse TMEM16F scramblase. The *Sc* site in mouse TMEM16F is shifted one helix turn into membrane compared with nhTMEM16; we prefer that K526 at the *Sc* site serves as a stepping stone, as discussed below for the *Se* site.

The charged amino acids in the *Se* site were postulated by Bethel and Grabe (17) to serve as "stepping stones" by which phospholipids bind to the "stones" via their hydrophilic head group and move stepwise through the membrane bilayer. In fact, we found that mutations of the charged residues (R478 and E604) in the *Se* site of TMEM16F severely inactivate the PtdSer-exposing activity, which strongly supports the stepping stone model. The requirement of these residues to internalize NBD-PC or NBD-PS varied greatly. That is, the R478A mutation did not affect the internalization of NBD-PC or NBD-PS, whereas the E604A mutation significantly affected the internalization of NBD-PS but not NBD-PC. Whether this is because the inward and outward scrambling of phospholipids take different pathways as discussed above remains to be studied. The region where the *Se* site is localized is well-conserved between a Cl^--channel TMEM16A and phospholipid-scramblase TMEM16F. The arginine residue in the third transmembrane segment of TMEM16A was shown to be critical for the Cl^--channel activity of mouse TMEM16A (27), indicating that the Cl^- ion as well as PtdSer transports via the *Se* site. Thus, the ability of TMEM16 proteins to translocate phospholipids or Cl^- seems to be determined by the *Sc* site or the SCRD. Comparison of the tertiary structures of TMEM16A and 16F will be necessary to understand their transport mechanism.

Materials and Methods

Cell Lines and Materials. Mouse IL-3–dependent *TMEM16F*$^{-/-}$ BaF3 cells (10) were cultured in RPMI1640 containing 10% FCS, 45 U/mL mouse IL-3, and 50 μM β-mercaptoethanol. Mouse WR19L cells expressing Fas (W3) were maintained in RPMI1640 containing 10% FCS. Plat-E (28) and HEK293T cells were cultured in DMEM containing 10% FCS.

Fluorescently labeled phospholipids, 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC), 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoserine (NBD-PS), *N*-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sphingosine-1-phosphocholine (NBD-SM), and *N*-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-D-galactosyl-β1-1′-sphingosine (NBD-GalCer), were purchased from Avanti Polar Lipids.

cDNAs. Mouse cDNAs for TMEM16A (GenBank accession no. BC062959.1), 16C [National Center for Biotechnology Information (NCBI) accession no. NM_001128103.1], 16D (Ensemble ID code ENSMUST 00000070175), 16E (NCBI accession no. NM_177694.5), 16F (NCBI accession no. NM_175344), 16G (GenBank accession no. BC116706.1), 16H (NCBI accession no. NM_001164679.1), 16J (NCBI accession no. NM_178381.3), and 16K (NCBI accession no. NM_133979.2) were described previously (14). Human cDNA for ATP11C (NCBI accession no. XM_005262405.1) was described previously (6). The cDNAs for mouse TMEM16C, 16D, and 16E and for human ATP11C were edited to enhance the efficiency of their translation, as described (6, 14). TMEM16A cDNAs carrying the SCRD of TMEM16C (amino acids 611 to 645), 16D (amino acids 566 to 600), 16E (amino acids 521 to 555), 16F (amino acids 525 to 559), 16G (amino acids 503 to 537), 16H (amino acids 411 to 445), 16J (amino acids 385 to 419), or 16K (amino acids 374 to 408) were constructed by fusing three fragments using In-Fusion HD Cloning Kits (Takara Clontech). The point mutants of TMEM16E SCRD (E525G, K526C, S546R, and M551A) and TMEM16F (K478A, K530C, and E604A) were prepared by recombinant PCR (29) with the complementary primers carrying mutated nucleotides (Tables S2 and S3).

Western Blotting. Cells were lysed by incubation on ice for 30 min in radioimmunoprecipitation assay buffer [50 mM Tris·HCl buffer, pH 8.0, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and a protease-inhibitor mixture (cOmplete Mini; Roche Diagnostics)], and centrifuged at 4 °C at 16,500 × *g* for 15 min to remove cell debris. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were mixed 1:1 with 2× SDS sample buffer (125 mM Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue), incubated at room temperature for 30 min, separated by electrophoresis on a 7.5% polyacrylamide gel, and transferred to a PVDF membrane. After incubating at room temperature for 1 h in blocking buffer [TBS (25 mM Tris·HCl, pH 7.5, 150 mM NaCl) containing 10% skim milk, 0.01% Tween 20], membranes were incubated at 4 °C overnight with a rabbit anti-mouse TMEM16F antibody (14) in Can Get Signal Solution 1 (Toyobo Life Science) or with a mouse anti–α-tubulin monoclonal antibody (Merck Millipore), rabbit anti-GFP antibody-HRP-DirecT (MBL), or rabbit anti-human ATP11C antibody (5) in blocking buffer. The membrane was incubated at room temperature for 1 h with HRP-conjugated goat anti-rabbit Ig or HRP-conjugated goat anti-mouse Ig (Dako) in Can Get Signal Solution 2 or blocking buffer, followed by incubation with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore).

Assay for Scramblase and Flippase Activities. Ca^{2+}-dependent PtdSer exposure was measured as described previously (10). Briefly, 2.0×10^5 cells at early exponential phase were washed twice with Annexin V binding buffer without $CaCl_2$ (10 mM Hepes·NaOH, pH 7.4, 140 mM NaCl) and preincubated at 4 °C for 20 min or at 20 °C for 5 min in 1.0 mL of Annexin V Binding buffer containing 1 mM $CaCl_2$, 5 μg/mL propidium iodide, and 1,000-fold–diluted Cy5-Annexin V (BioVision). After adding the Ca^{2+} ionophore A23187 (Sigma-Aldrich) to a final concentration of 3 μM, the cells were incubated at 4 °C for 30 min or 20 °C for 200 s, and the Annexin V-positive cells in the PI-negative population were analyzed by FACSAria II (BD Biosciences). Scramblase activity was also assayed by the incorporation of NBD-labeled phospholipids as described (14). In brief, cells (1.2×10^6) were incubated at 4 °C for 3 min with 0.5 μM NBD-PC, 0.5 μM NBD-PS, 0.25 μM NBD-SM, or 0.5 μM NBD-GalCer in Annexin V binding buffer without $CaCl_2$. The mixture was adjusted to 1 mM $CaCl_2$ and 3 μM A23187, and incubated at 4 °C. A 150-μL aliquot was mixed with an equal volume of Annexin V binding buffer containing 5 mg/mL fatty acid-free BSA (Sigma-Aldrich) and 5 nM Sytox red (Thermo Fisher Scientific), and analyzed by FACSAria II.

To assay phospholipid-flippase activity, cells were preincubated at 20 °C for 5 min in Annexin V binding buffer without $CaCl_2$. NBD-PS was added to a final concentration of 0.5 μM, and the mixture was incubated at 20 °C. The cells were mixed with 5 mg/mL fatty acid-free BSA and 5 nM Sytox red, and analyzed by FACSAria II.

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