Phosphatidylserine exposure in living cells

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Abstract

P4-ATPases, a subfamily of P-type ATPases, translocate cell membrane phospholipids from the exoplasmic/luminal leaflet to the cytoplasmic leaflet to generate and maintain membrane lipid asymmetry. Exposure of phosphatidylserine (PS) in the exoplasmic leaflet is well known to transduce critical signals for apoptotic cell clearance and platelet coagulation. PS exposure is also involved in many other biological processes, including myoblast and osteoclast fusion, and the immune response. Moreover, mounting evidence suggest that PS exposure is critical for neuronal regeneration and degeneration. In apoptotic cells, PS exposure is induced by irreversible activation of scramblases and inactivation of P4-ATPases. However, how PS is reversibly exposed and restored in viable cells during other biological processes remains poorly understood. In the present review, we discuss the physiological significance of reversible PS exposure in living cells, and the putative roles of flippases, floppases, and scramblases.

Introduction

Cell membrane lipid bilayers exhibit asymmetric lipid distribution. In mammalian cells, the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are restricted to the cytoplasmic leaflet, while phosphatidylcholine (PC), sphingomyelin, and glycosphingolipids are enriched in the exoplasmic leaflet (Devaux, 1991; Falzone et al., 2018; Murate et al., 2015; Nagata et al., 2016; Zachowski, 1993). This transbilayer lipid asymmetry is generated and regulated by lipid flippases, floppases, and scramblases.

In normal physiological conditions, PS is generally absent from the exoplasmic leaflet of the plasma membrane. Exposure of PS at the cell surface is atypical and activates important biological processes that can determine the cell's fate. PS exposure has been classically observed in cells fated for disposal, such as apoptotic cells and platelets during blood aggregation. However, PS exposure also occurs in non-apoptotic cells. The physiological significance and molecular mechanisms of PS exposure in living cells are elusive and represent an emerging subject in the field of biology. In the present review, we discuss several contexts in which PS is exposed by living cells, their physiological significance, and potential reversible mechanisms by which PS can be transiently exposed.

Flippases, floppases, and scramblases

PS is removed from the extracellular surface by ATP-dependent active transport, which is regulated by members of the P4-ATPase family (Figure 1). The P4-ATPase family is a subfamily of the P-type ATPase superfamily. All P-type ATPases form an acid-stable aspartyl phosphate intermediate during the reaction cycle and are so named "P-type." Although P-type ATPases primarily facilitate the transport of cations across biological membranes, the P4-ATPase subfamily vectorially translocates lipids from the exoplasmic/luminal to the cytosolic leaflet.

Mammalian P4-ATPases exhibit strict substrate specificities (Table 1), and PS-specific P4-ATPases are responsible for the removal of cell surface PS (Figure 1). Among the P4-ATPases summarized in Table 1, ATP11A and ATP11C are major plasma membrane PS flippases in many cell types (Liou et al., 2019; Segawa et al., 2014; Siggs et al., 2011a; Takada et al., 2015; Tsuchiya et al., 2018; Wang et al., 2018; Yabas et al., 2011) and ATP8A1 and ATP8A2 also flip PS (Coleman et al., 2009; Paterson et al., 2006). ATP8B1, ATP8B2, and ATP10A preferentially flip PC at the plasma membrane (Naito et al., 2015; Takada et al., 2015; Takatsu et al., 2014), and ATP10D translocates a glycosphingolipid, glucosylceramide, but not any other phospholipids (Roland et al., 2019). Recently, ATP10B appeared to translocate PC and glucosylceramide (Martin et al., 2020). Most P4-ATPases, with the exception of ATP9A and ATP9B, interact with CDC50A or B, a chaperone-like protein that is required for their transport from the endoplasmic reticulum (ER) to the plasma membrane and/or intracellular compartments (Figure 1 and Table 1) (Shin and Takatsu, 2019; Takatsu et al., 2011).

Cryo-EM structures of ATP8A1 and Drs2p (a *saccharomyces cerevisiae* P4-ATPase) with CDC50 protein have published (Bai et al., 2019; Hiraizumi et al., 2019; Timcenko et al., 2019) and the crystal structure of ATP11C with CDC50A is disclosed (doi: 10.1101/2019.12.23.881698). In all structures, the substrate PS is thought to transport along the transmembrane (TM) 4 and the transporting crevice is mainly composed of TM2, 4, and 6. The C-terminal regions of ATP8A1 and Drs2p play regulatory roles for transporting of PS (Bai et al., 2019; Hiraizumi et al., 2019; Timcenko et al., 2019). The putative PS entry site of ATP8A1 would be between TM1-TM2 and TM3-TM4 (Hiraizumi et al., 2019) and that of ATP11C would be a cavity between CDC50A exoplasmic domain and the ATP11C TM3-TM4 loop (doi: 10.1101/2019.12.23.881698).

In mice, ATP11C mutations are associated with abnormal B-cell development and anemia secondary to abnormal erythrocyte shape (Siggs et al., 2011a; Yabas et al., 2014; Yabas et al., 2011). *ATP11C* is located on the X chromosome, and a missense mutation in this locus occurs in a male patient with congenital hemolytic anemia (Arashiki et al.,

2016), suggesting that ATP11C activity is important for hematopoietic cell homeostasis. In addition, both ATP11A and ATP11C are implicated in PS exposure on apoptotic cells, and are cleaved by caspases (Segawa et al., 2016). ATP8A1, another PS-flippase, plays a key role in hippocampal cells, as PS externalization is increased in the hippocampal cells of mice lacking *Atp8a1*, which exhibit deficient hippocampus-dependent learning (Levano et al., 2012). ATP8A2 is a causative gene for cerebellar ataxia, mental retardation and disequilibrium syndrome (CAMRQ) (Onat et al., 2013) and other ATP8A2 mutations are related to intellectual disability and severe neurological phenotypes (Cacciagli et al., 2010; Martin-Hernandez et al., 2016), suggesting that ATP8A2 is critical for the development of the nervous system. *Atp8a2* deficient mice exhibit the degeneration of photoreceptor cells (Coleman et al., 2014; Zhu et al., 2012).

CDC50A (*Tmem30a*) is essential for early embryonic development in mice and neuronal knockout of *Tmem30a* also led to embryonic lethality (Yang et al., 2018), Because CDC50A is required for folding and transporting of multiple P4-ATPases to their cellular destinations (Table 1), CDC50A knockout disrupts P4-ATPases which may function in broad range of tissues at various developmental stages. Accordingly, several conditional *Tmem30a* knockout mice have been produced. Purkinje cell-specific *Tmem30a* knockout mice show the degeneration of the Purkinje cells in cerebellum via apoptotic cell death (Yang et al., 2018). Moreover, conditional *Tmem30a* knockout mice led to loss of cone cells (Zhang et al., 2017), degeneration of rod bipolar cells probably via apoptosis (Yang et al., 2019), and retarded retinal vascular development (Zhang et al., 2019). These phenotypes of the specific *Tmem30a* knockout might be due to the functional loss of P4-ATPases, probably ATP8A1 and/or ATP8A2 since the P4-ATPases are involved in the development of nerve cells and retinal function (Coleman et al., 2014; Levano et al., 2012; Zhu et al., 2012). Moreover, liver-specific *Tmem30a* knockout causes hepatic disorder (Liu et al., 2017). The phenotype might be related to the defect in ATP8B1 or ATP11C because mutations in ATP8B1 and defect in *Atp11c* cause hepatic cholestasis (Bull et al., 1998; Siggs et al., 2011b). In addition, conditional *Tmem30a* knockout mice resulted in depletion of hematopoietic cell lineage (Li et al., 2018). Since all phenotypes observed in the *Tmem30a* knockout mice are most likely due to the functional loss of multiple P4-ATPases, the pathophysiological mechanisms remain unknown.

Some ATP-binding cassette (ABC) transporters facilitate membrane lipid transport in the opposite direction, from the cytosolic to the exoplasmic/luminal leaflet, in an ATP-dependent manner (Figure 1 and Table 2). However, it is presently unclear whether this transport is associated with flopping, efflux, or both. Many ABC transporters do not

exhibit strict substrate specificities (Table 2) (Coleman et al., 2013; Neumann et al., 2017 ; van Meer et al., 2008), but a specific member of this family could be responsible for signal-dependent PS exposure (Hisamoto et al., 2018). Mutations in PC-flopping ABCB4 and PC-flipping ATP8B1 cause progressive familial intrahepatic cholestasis (Bull et al., 1998; Jacquemin et al., 2001; Smit et al., 1993; Takatsu et al., 2014) indicating that the flip-flop of PC in bile canaliculi is important for homeostasis of bile secretion.

The rapid collapse of lipid asymmetry of the plasma membrane, which results in PS exposure, is accomplished by phospholipid scrambling. Lipid scramblases translocate lipids rapidly and bidirectionally down their concentration gradients, but are generally not selective for lipids (Figure 1) (Bevers and Williamson, 2016; Sahu et al., 2007). Scramblases facilitate PS exposure in activated platelets and apoptotic cells (Suzuki et al., 2013a; Suzuki et al., 2010). Two major families of regulated scramblases have thus far been identified, the TMEM16 family and the Xk-related (Xkr) family (Table 3).

There are 10 human TMEM16 proteins, including TMEM16A-H and TMEM16 J-K (Figure 1). TMEM16A and B were initially identified as Ca^{2+}-activated chloride channels (Table 3) (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Mammalian TMEM16C, D, E, F, G, J, and K are primarily associated with scramblase activity (Gyobu et al., 2016; Suzuki et al., 2013b; Tsuji et al., 2019). A recent study suggests that TMEM16J could be a cation channel (Kim et al., 2018). TMEM16F and fungal nhTMEM16 and afTMEM16 contain dual-function channels/scramblases (Brunner et al., 2014; Malvezzi et al., 2013; Scudieri et al., 2015).

Members of the Xkr family of membrane proteins are caspase-activated scramblases involved in PS exposure during apoptosis (Nagata et al., 2016; Suzuki et al., 2014). The Xkr family is comprised of nine human members (Table 3), each of which contains 6–10 predicted transmembrane domains. Xkr8 protein was recently identified as being activated by either caspase cleavage or phosphorylation (Sakuragi et al., 2019). Moreover, some G protein-coupled receptors (GPCRs) are potential nonregulated scramblases (Goren et al., 2014; Menon et al., 2011).

PS Exposure in cellular processes

PS exposure in apoptotic cells

PS is exposed on the surface of apoptotic cells and serves as an ‘eat me’ signal for clearance of the cells by macrophages (Fadok et al., 1992; Nagata, 2018). Suzuki et al. demonstrated that members of the Xkr family mediate Ca^{2+}-independent PS exposure during apoptosis (Suzuki et al., 2014; Suzuki et al., 2016). Deletion of the *Xkr8* gene in cultured cells abolishes PS exposure in response to apoptotic stimuli, and transient

expression of Xkr4, Xkr8, or Xkr9 rescues this response (Suzuki et al., 2014; Suzuki et al., 2016). These proteins contain a caspase recognition site at the C-terminal domain, which is cleaved by caspases 3 and 7 for activation (Figure 2) (Suzuki et al., 2013a; Suzuki et al., 2014). The *C. elegans* Xkr ortholog CED-8 is involved in caspase-dependent PS externalization in apoptotic cells (Chen et al., 2013; Li et al., 2015), suggesting an evolutionarily conserved scramblase function for these proteins.

At the same time, caspases 3 and 7 cleave and inactivate ATP11A and ATP11C, major PS flippases that regulate transport of PS to the cytosolic leaflet at the plasma membrane (Figure 2) (Segawa et al., 2016; Segawa et al., 2014). Cells expressing caspase-resistant ATP11C do not expose PS during apoptosis and are not phagocytosed by macrophages, suggesting that inactivation of PS flippases is required for PS exposure in apoptotic cells (Segawa et al., 2014). Thus, caspase-dependent activation of Xkr8 and inactivation of P4-ATPases leads to sustained PS exposure in apoptotic cells (Figure 2).

PS is also exposed during non-apoptotic cell death such as necroptosis, which is important in host immune defense against pathogens, although the key players have not yet been elucidated. Although the non-apoptotic cell death would not be discussed in this review, recent review can be refereed has been published about non-apoptotic cell death (Shlomovitz et al., 2019).

PS exposure in platelets

PS is critical for blood coagulation and is exposed on the cell surface of activated platelets in a Ca^{2+}-dependent manner. PS exposure creates scaffolds that allow aggregation of various coagulation factors (Bevers et al., 1983) (Bevers and Williamson, 2016). Scott syndrome, a mild autosomal bleeding disorder, is caused by deficient Ca^{2+}-dependent PS exposure in activated platelets (Wielders et al., 2009). Patients carrying Scott syndrome exhibited a homozygous null mutation or complex heterozygous mutations of the *TMEM16F* gene (Castoldi et al., 2011; Suzuki et al., 2013a). Moreover, dogs carrying Canine Scott syndrome, a naturally occurring bleeding disorder, lack procoagulant activity in activated platelets and carry a point mutation in the *TMEM16F* gene causing an absence of platelet TMEM16F (Brooks et al., 2015). The phenotypes of platelet-specific *TMEM16F*-null mice resemble those of patients with Scott syndrome (Fujii et al., 2015). These findings suggest that the TMEM16F scramblase is responsible for PS exposure in activated platelets.

Platelet activation leads to dramatic changes in platelet morphology. Microparticles are generated from platelet plasma membrane shedding during platelet activation (Dachary-Prigent et al., 1995). Microparticles express platelet membrane receptors, expose PS on

their surfaces, and play an important role in hemostasis and thrombosis (Bevers et al., 1991). Microparticle release is severely reduced in the absence of TMEM16F (Fujii et al., 2015), and platelets from Scott syndrome patients exhibit defective microparticle release (Sims et al., 1989), suggesting that PS exposure and/or scrambling activity is involved in membrane shape changes and microparticle formation. Because greater PC flipping activity at the plasma membrane can drive inward membrane curvature (Takada et al., 2018), lipid translocating activity could affect these membrane shape changes.

PS exposure in living cells

PS exposure in apoptotic cells and in activated platelets occurs irreversibly. However, it also occurs reversibly in living cells. Here, we discuss several contexts in which PS is exposed by living cells, their physiological significance, and potential reversible mechanisms by which PS can be transiently exposed and recovered.

Viral infection The presence of PS in the target membrane promotes fusion of many enveloped viruses (Coil and Miller, 2005). Human immunodeficiency virus 1 (HIV-1) entry into host cells is initiated by interaction of the viral envelope glycoprotein (Env), and cellular CD4 receptors and coreceptors. Ca^{2+} signaling is triggered by engagement of the coreceptors with Env (Melar et al., 2007; Wilen et al., 2012). HIV-1-induced PS redistribution depends on Ca^{2+} signaling and involves the lipid scramblase TMEM16F (Zaitseva et al., 2017). Externalized PS strongly promotes Env-mediated membrane fusion and HIV-1 infection. Blocking PS externalization with PS binding proteins or suppression of TMEM16F function inhibits Env-mediated fusion (Zaitseva et al., 2017). Therefore, cell surface PS acts as an important cofactor that promotes fusogenic complex formation (Zaitseva et al., 2017). Similar to HIV-1, binding of the alpha-herpesvirus envelope glycoprotein to the viral receptor induces Ca^{2+} signaling and PS exposure on the plasma membrane, facilitating fusion (Azab et al., 2015). Moreover, vesicular stomatitis virus (VSV)-mediated fusion also requires an interaction between a VSV glycoprotein and PS at the plasma membrane (Carneiro et al., 2002). Other anionic lipids also appear to be required for VSV particle fusion (Matos et al., 2013).

Myoblast fusion Transient exposure of PS occurs in mouse embryonic myotubes on embryonic day 13 (E13), a stage of development when primary myotubes are formed. Mammalian skeletal muscles are formed by fusion of mononucleated precursor cells (myoblasts) into unusually elongated multinucleated cells known as myotubes, whose formation relies on complexed cell-to-cell fusion and elongation of multinucleated

syncytia (Abmayr and Pavlath, 2012). During myotube formation, PS transiently translocates to the outer leaflet of the plasma membrane, and recognition of PS on the cell surface by PS receptors induces contact-dependent signaling to promote fusion with neighboring myoblasts (Hochreiter-Hufford et al., 2013; Leikina et al., 2013; Park et al., 2016; van den Eijnde et al., 2001).

Annexins A1 and A5, which interact with many cell surface proteins (Moss and Morgan, 2004) and PS, facilitate myotube formation by direct or indirect regulation of membrane fusion (Leikina et al., 2013). The PS receptor BAI-1 recognizes PS-exposing apoptotic cells, and a subsequent signaling cascade induces myoblast fusion (Hochreiter-Hufford et al., 2013). Another PS receptor, stabilin-2, contributes to myoblast fusion during muscle growth and regeneration in a PS-dependent manner (Park et al., 2016). Because caspase inhibitors fail to block myotube formation, PS exposure (van den Eijnde et al., 2001), and stabilin-2-mediated myoblast fusion (Park et al., 2016), apoptosis might not be essential for myotube formation although the requirement of apoptotic cells was previously reported (Hochreiter-Hufford et al., 2013). Recently, Tsuchiya et al. demonstrated that recovery of exposed PS is critical for the regulation of the Ca^{2+} channel PIEZO1, which is indispensable for proper morphogenesis during myotube formation. The phospholipid flippase ATP11A is responsible for the recovery of exposed PS (Tsuchiya et al., 2018), which could be a prerequisite for PIEZO1 activation.

TMEM16E is mutated in humans with myopathies associated with abnormal muscle regeneration (Bolduc et al., 2010; Whitlock and Hartzell, 2017). Mice lacking TMEM16E are defective in myoblast fusion necessary for the proper repair and regeneration of myotubes (Griffin et al., 2016) suggesting that scramblase activity is required for the fusion of myoblasts to regenerate myotubes. Moreover, the mice exhibit the compromised recovery of muscle fibers following laser damage, which can induce membrane pores. Similarly, TMEM16F is critical for PS exposure and the plasma membrane repair of the damaged membranes by treatment with a pore-forming reagent (Wu et al., 2020). Therefore, the scrambling activity and PS exposure would be critical for the membrane repair process.

Osteoclast fusion Similar to myotube formation by myoblast fusion, PS is exposed during the formation of inflammatory giant cells from macrophage fusion (Helming and Gordon, 2009) and placental syncytiotrophoblast formation from trophoblast fusion (Lyden et al., 1993). Cell surface PS influences differentiation processes in osteoclasts (Wu et al., 2010), and myoblasts require PS exposure for pre-fusion steps. Bone-resorbing multinucleated osteoclasts, which play central roles in bone maintenance and repair, arise

from the fusion of mononuclear osteoclast precursors from the monocyte or macrophage lineage. Cell–cell fusion is not essential for bone resorption, but bone-resorbing ability is greatly decreased in mononuclear osteoclasts (Helming and Gordon, 2009; Yagi et al., 2005). Osteoclast deficiencies or dysfunction lead to osteopetrosis, a rare congenital disease characterized by high bone density and impaired bone marrow cavity formation. Contrastingly, increased osteoclast activity leads to osteoporosis and mediates joint destruction in rheumatoid arthritis (Helming and Gordon, 2009). Osteoclast fusion requires dendrocyte-expressed seven transmembrane protein (DC-STAMP)-dependent non-apoptotic PS exposure at the surface of fusion-committed cells (Verma et al., 2018; Yagi et al., 2005). This fusion is also dependent on extracellular annexins and PS binding proteins, which, together with the annexin-binding protein S100A4, regulate the fusogenic activity of syncytin 1 (Verma et al., 2018). Thus, the cell fusion step of osteoclastogenesis is controlled by the PS-mediated activity of several proteins.

Neuronal degeneration/regeneration Recent evidence has linked PS exposure to neurite degeneration (Sapar et al., 2018; Wakatsuki and Araki, 2017). Phagocytic clearance of degenerating dendrites or axons is critical for maintaining tissue homeostasis and preventing neuroinflammation (Nagata, 2010; Salter and Stevens, 2017). Exposed PS is postulated to be an 'eat-me' signal, allowing phagocyte recognition of degenerating neurites. Either the scramblase Xkr8 or the lipid translocase ABC1 (a member of the ABC transporter family) is involved in PS exposure during axon degeneration in cultured murine dorsal root ganglion neurons (Wakatsuki and Araki, 2017). In *D. melanogaster*, PS is also exposed on degenerating dendrites during developmental pruning and after physical injury. PS exposure can also be induced by either knockout of the phospholipid flippase ATP8A or overexpression of the scramblase TMEM16, which causes phagocytes to break down PS-exposing neurites (Sapar et al., 2018). Moreover, knockout of CDC50 and overexpression of scramblase can lead to dendrite reduction. Mammalian orthologs of ATP8A, including ATP8A1 and 2, flip PS (Table 1) (Coleman et al., 2009; Lee et al., 2015), suggesting ATP8A could be responsible for PS asymmetry in *drosophila* dendrites. Therefore, both the inhibition of flippases and the activation of scramblases appear to be associated with PS exposure and neurite degeneration.

Following axon injury, a cascade of signaling events initiates axon regeneration. Neumann et al. demonstrated that axon injury in the nervous system of *C. elegans* results in PS exposure, which functions as a signal to promote axon reconnection and fusion, consequently re-establishing axonal integrity (Neumann et al., 2015). Axon injury induces PS accumulation around injured axons, which is dependent on the caspase-

activated ABC transporter CED-7 in *C. elegans* (Hisamoto et al., 2018). PS also functions as a critical signal for triggering the initiation of axon regeneration through the apoptotic clearance molecules CED-7 and CED-3 after injury. PS-associated thyretin (TTR)-11 may then activate integrin, thereby initiating axon regeneration (Hisamoto et al., 2018). Thus, components of the apoptotic cascade can also play protective roles in axonal regeneration.

Fertilization The plasma membrane of spermatozoa, like that of other mammalian cells, exhibit transbilayer phospholipid asymmetry (Gadella et al., 1999). Upon capacitation, in which sperm cells are prepared for binding and penetration of the zona pellucida, phospholipid scrambling appears to occur (Gadella and Harrison, 2000). A recent study identified that PS is exposed on the head region of viable and motile sperm, and that masking sperm PS inhibits fertilization (Rival et al., 2019). On oocytes, the PS receptors BAI1/3, CD36, Tim-4, and Mer-TK contribute to fertilization. Therefore, PS exposure in viable sperm and PS receptors on oocytes are critical for sperm-egg fusion.

The mouse ortholog of ATP8B3, a putative sperm aminophospholipid transporter (SAPLT), is expressed in the acrosomal region of the spermatozoal head and has been implicated in capacitation (Wang et al., 2004). In SAPLT-null spermatozoa, PS is exposed on the outer leaflet prior to sperm capacitation, suggesting that SAPLT is required for PS asymmetry. Fertilization rates are normal when the null spermatozoa are added to zona pellucida-free eggs, but in the presence of the extracellular matrix, fewer null spermatozoa bind or penetrate the zona pellucida, and fewer undergo acrosome reactions, indicating that regulated PS externalization is critical for sperm capacitation.

The murine P4-ATPase ATP8B5, originally identified as flippase expressed in testis splicing form A (FetA), is absent in humans. ATP8B5 is expressed in gamete cells, and its localization is tightly associated with acrosome biogenesis, a process that involves intensive intracellular vesicle formation and fusion. ATP8B5 depletion perturbs Golgi organization and secretion, suggesting that ATP8B5 plays a role in spermatogenesis (Xu et al., 2009). It is currently unknown whether CDC50 is a partner of ATP8B3 and ATP8B5. CDC50C is specifically expressed in mouse testis, and thus is presumed to be an ATP8B3 and/or ATP8B5 partner. However, CDC50C is not present in the human gene database, and human ATP8B3 remained in the ER when coexpressed with either CDC50A or CDC50B in HeLa cells (Shin and Takatsu, 2019) (our unpublished data). This suggested that the two CDC50s might not be partners of ATP8B3, or that ATP8B3 could be an ER-localizing human P4-ATPase.

TMEM16E is expressed in germ cells during early spermatogenesis and is localized to

the sperm tail. TMEM16E localizes to the ER and appears to function as a lipid scramblase (Gyobu et al., 2016). TMEM16E-null sperm exhibit no apparent defects in morphology, capacitation, PS exposure during capacitation, or binding to the zona pellucida. However, the null sperm exhibit low motility, particularly after capacitation, although the mechanism of TMEM16E function remains unknown.

Immune response Activated immune cells were observed to expose PS nearly two decades ago, but the physiological relevance of PS exposure has still been obscure. We here summarize several examples of PS exposure in immune cells in order to provide insight into the relationship between PS exposure and immune response. Neutrophils stimulated with the chemotactic peptide formylated Met-Leu-Phe (fMLP) transiently expose PS on the cell surface (Frasch et al., 2004). Moreover, PS exposure is accompanied by enhanced uptake of several lipid probes, including NBD-PS and NBD-PC during fMLP stimulation, suggesting that lipid scrambling might be activated. PS exposure is not associated with apoptosis in activated neutrophils (Frasch et al., 2004). Moreover, several members of the human galectin family of glycan binding proteins, galectins-1, -2, and -4, induce PS exposure in activated but not resting human neutrophils and in several leukocyte cell lines (Stowell et al., 2007). Although PS exposure is evidently important for the immune response in activated leukocytes, its mechanism and physiological relevance in this context remain unclear.

PS is exposed at high levels on a subpopulation of T-lymphocytes, where it is required for activation of the P2X7 cation channel. Changes in the intracellular ion homeostasis by P2X receptor stimulation trigger inflammation (Di Virgilio et al., 2018). High PS levels in the inner leaflet of the plasma membrane appear to inhibit P2X7 and prevent channel opening (Elliott et al., 2005). On the other hand, in myoblasts, high levels of PS in the outer leaflet of the plasma membrane inhibit activation of the PIEZO1 ion channel, perturbing normal myotube formation (Tsuchiya et al., 2018). Therefore, PS distribution in the lipid bilayer is important for the regulation of membrane transporters, including ion channels.

IgE receptor stimulation rapidly induces reversible PS exposure in viable mast cells (Martin et al., 2000). Ionomycin, Ca^{2+} ionophore, treatment induces both PS exposure and degranulation in mast cells, suggesting that PS exposure depends on factors downstream of Ca^{2+}, and could thus be related to the exocytic event. However, how PS exposure is associated with the degranulation process remains unknown.

In addition, cell surface PS appears at the exocytic sites in activated chromaffin cells (Ory et al., 2013). PS exposure is required for compensatory endocytosis, but is not

required for exocytosis or constitutive endocytosis. Compensatory endocytosis is triggered subsequently to the supply of secretory granule membranes after calcium-regulated exocytosis. Because calcium-regulated exocytosis and compensatory endocytosis are tightly coupled, PS reorganization is critical in the endocytic phase of regulated neuroendocrine secretion (Ory et al., 2013). However, the mechanisms of PS reorganization during exocytosis and compensatory endocytosis remain elusive.

Perspectives: ***How is transient PS exposure regulated?***

As described above, transient PS exposure occurs in many cell types and biological processes. However, the physiological relevance and molecular mechanisms of transient PS exposure remain unclear. Apoptotic PS exposure requires not only the activation of scramblases, but also the inhibition of P4-ATPase flippases (Segawa et al., 2014). Moreover, increased cytosolic Ca^{2+} in human erythrocytes inhibits incorporation of aminophospholipids (Bitbol et al., 1987), and Ca^{2+}-dependent protein kinase C (PKC)α activation mediates scramblase activation, flippase inhibition, and subsequent PS exposure (Noh et al., 2010; Wesseling et al., 2016). Therefore, transient PS exposure could be potentiated by inhibition of PS flippases upon Ca^{2+}-dependent signaling, as well as by activation of scramblases. Indeed, the ATPase activities of ATP8A1, ATP8A2, ATP11A, and ATP11C are inhibited by high calcium concentrations (Segawa et al., 2016) (Chalat et al., 2017). The C-terminal region of ATP8A2 appear to play a complexed regulatory role for its ATPase activity via phosphorylation of the C-terminal region by CaMKII (calcium/calmodulin-dependent protein kinase II) (Chalat et al., 2017). The interaction between an autoinhibitory and an anti-autoinhibitory domain in the C-terminal region of ATP8A2 would be dissociated by the phosphorylation resulting in an inhibition of ATP8A2 activity (Chalat et al., 2017). Importantly, ATP11C is endocytosed by Ca^{2+} signaling-dependent phosphorylation of its C-terminal cytoplasmic tail (Figure 3) (Takatsu et al., 2017). ATP11C is endocytosed and sequestered from the plasma membrane in response to signal transduction of the Gq-coupled GPCR, which increases cytosolic Ca^{2+}. Notably, ATP11C can be recycled back to the plasma membrane after switching off the signal, allowing PS flipping and recovery of asymmetry (Figure 3) (Takatsu et al., 2017). Therefore, this Ca^{2+}-dependent downregulation and recycling mechanism of ATP11C is likely responsible for signal-dependent PS exposure and recovery, in the various cell types and biological processes described above.

Future directions include the regulatory mechanisms of flippases and floppases/scramblases in PS exposure, and the contribution of PS translocation to diverse biological processes, including those discussed in the present review.

Disclosure statement

There are no conflicts of interest to declare.

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Figure legends

Figure 1 Lipid translocases (flippase, floppase, and scramblase).
Cryo-EM structures of P4-ATPase (human ATP8A1, PDB: 6k7m) with CDC50A (gray) (Hiraizumi et al., 2019), ABC transporter (human ABCB1, PDB: 6c0v) (Kim and Chen, 2018), and scramblase (mouse TMEM16F dimer, PDB: 6p46) (Feng et al., 2019). The enzymatic activity of P4-ATPase allows the aminophospholipids PS and PE to translocate to the cytosolic leaflet of the plasma membrane.

Figure 2 Mechanism of PS exposure in apoptotic cells.
Following the apoptotic cascade, activated caspases cleave between the N- and P-domains of the PS flippases ATP11A and ATP11C and inactivate them, and cleave and activate the scramblase Xkr8. As a result of flippase inactivation and scramblase activation, PS is persistently exposed on the apoptotic cell surface, allowing phagocytosis by macrophages. A, actuator domain; P, phosphorylation domain; N, nucleotide binding domain.

Figure 3 Model for transient PS exposure and recovery by regulation of ATP11C.
① Cytosolic Ca^{2+} influx is induced by Gq-coupled receptor-mediated signal transduction. ② Phospholipase C hydrolyzes phosphatidyl 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). ③④ IP3 stimulates increased cytosolic Ca^{2+} concentration. ⑤ PKCα is activated by binding with Ca^{2+} and DAG. ⑥ PKCα phosphorylates the C-terminus of ATP11C, resulting in generation of a di-leucine motif, which recruits the AP-2 clathrin adaptor protein complex. ⑦ ATP11C is sequestered from the plasma membrane via clathrin-mediated endocytosis, ⑧ decreasing plasma membrane PS flipping activity. ⑨⑩ Increased cytosolic Ca^{2+} could activate Ca^{2+}-dependent scramblases (such as TMEM16 members), resulting in local PS exposure. ⑪ ATP11C is recycled back to the plasma membrane after the signaling is switched off. ⑫ Exposed PS is recovered to the cytosolic leaflet.

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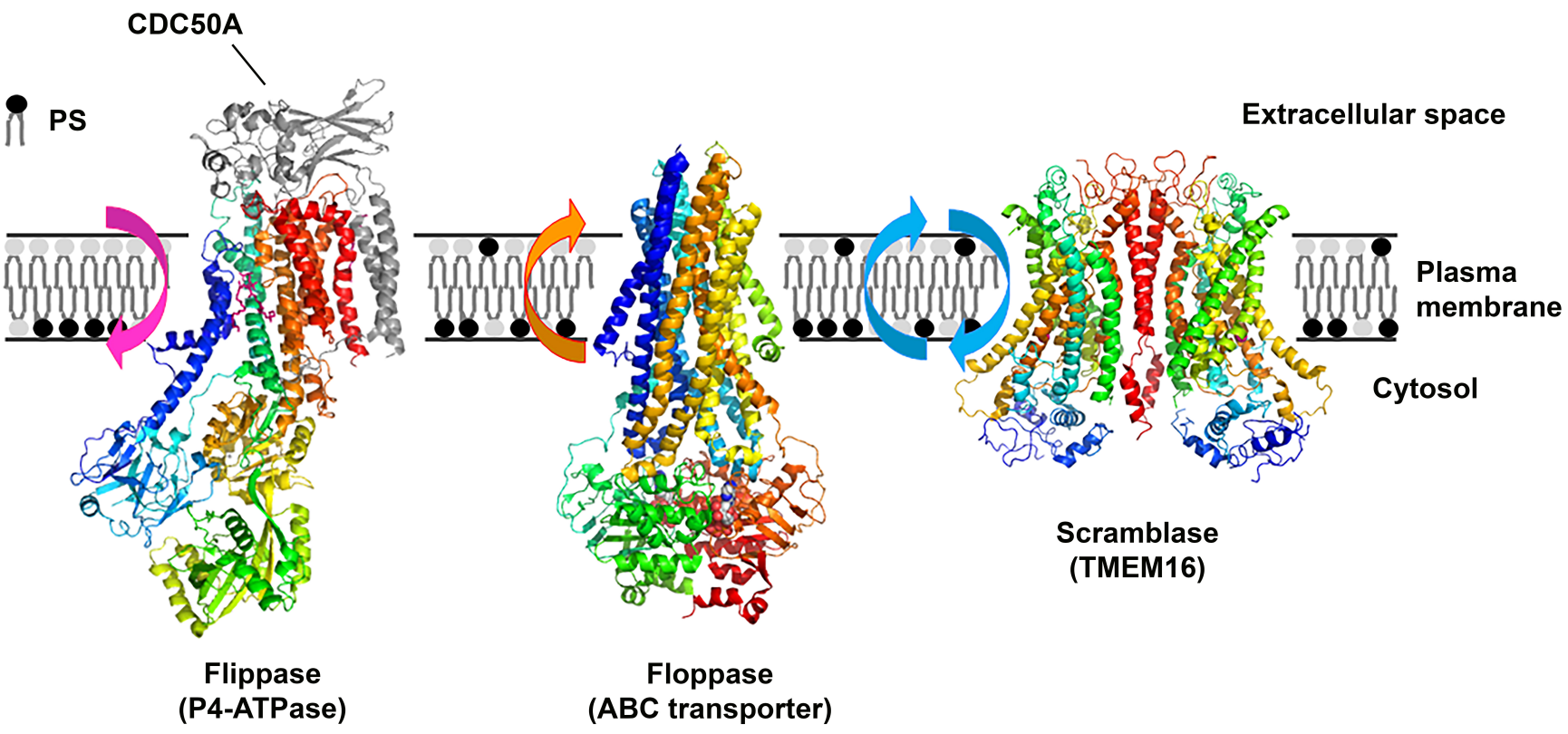

Figure 1 Shin and Takatsu

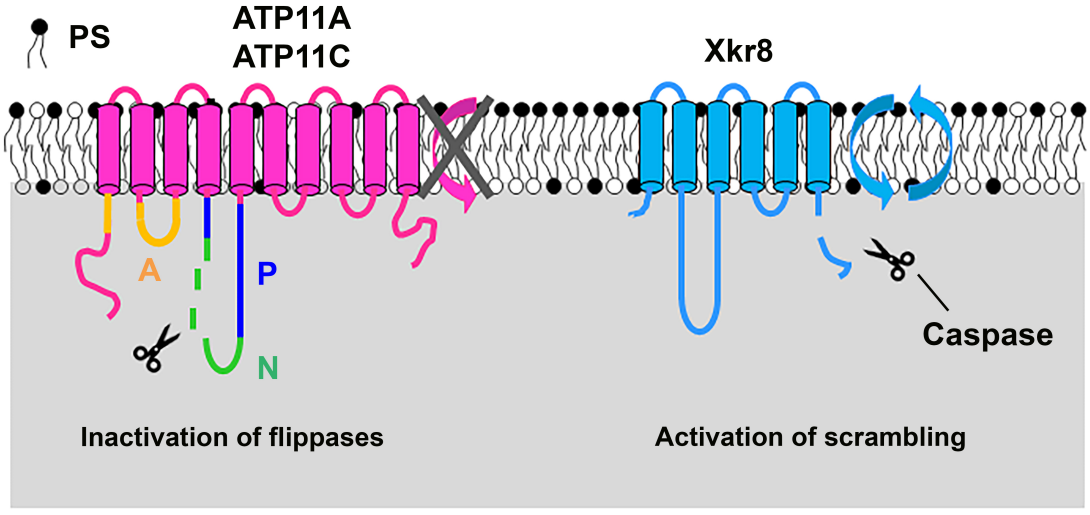

Figure 2 Shin and Takatsu

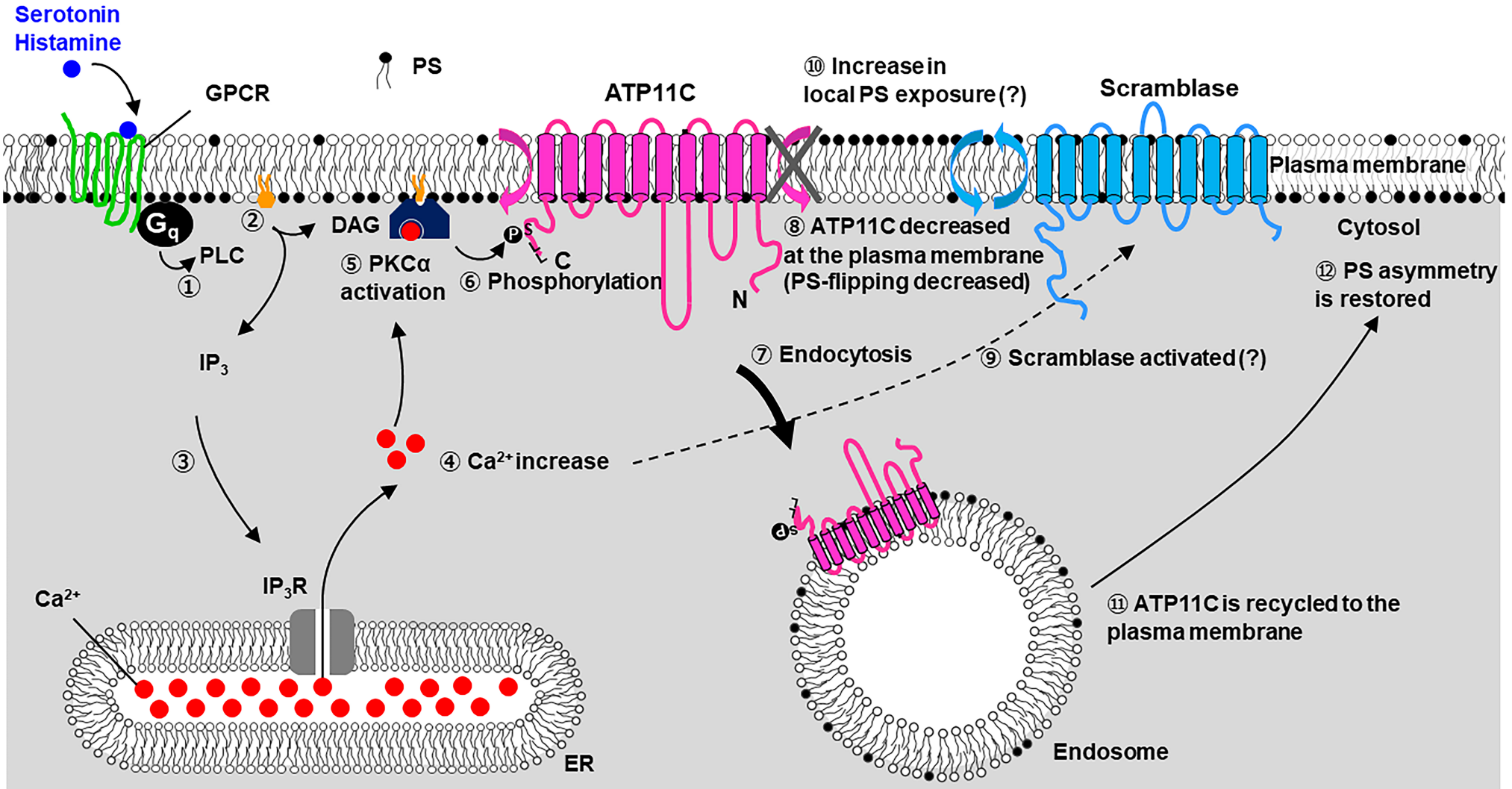

Figure 3 Shin & Takatsu

Table 1 P4-ATPase family

P4-ATPase	CDC50 partner[a]	Substrate[b]	Cellular localization[c]	Tissue distribution[d]
ATP8A1	CDC50A	PS>PE	LE, RE, Golgi, PM	Most tissues
ATP8A2	CDC50A	PS>PE	Golgi, RE, PM	Retina, brain, testis
ATP8B1	CDC50A>B	PC (PS?)	PM	Ubiquitous
ATP8B2	CDC50A, B	PC	PM	
ATP8B3		PS?		Testis
ATP8B4	CDC50A, B		PM	
ATP8B5 (mouse only)			PM?	Testis
ATP9A	-		EE, RE, Golgi	
ATP9B	-		Golgi	
ATP10A	CDC50A	PC	PM	
ATP10B	CDC50A	PC, GlcCer	LE	
ATP10D	CDC50A	GlcCer	PM	
ATP11A	CDC50A	PS, PE	PM	Ubiquitous
ATP11B	CDC50A	PS>PE	EE, RE	
ATP11C	CDC50A	PS>PE	PM	Ubiquitous

PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; GlcCer, glucosylceramide; PM, plasma membrane; LE, late endosome; RE, recycling endosome; EE, early endosome; -, not required

[a] Bryde et al., 2010; van der Velden et al., 2010; Takatsu et al., 2011; Naito et al., 2015; Andersen et al., 2016; Shin and Takatsu, 2019

[b] Coleman et al., 2011; Takatsu et al., 2014; Lee et al., 2015; Andersen et al., 2016; Wang et al., 2018; Roland et al., 2019; Shin and Takatsu, 2019; Martin et al., 2020

[c] Bryde et al., 2010; van der Velden et al., 2010; Coleman et al., 2011; Takatsu et al., 2011; Lee et al., 2015; Andersen et al., 2016; Shin and Takatsu, 2019

[d] Bull et al., 1998; Coleman et al., 2009; Gong et al., 2009; Xu et al., 2009; Cacciagli et al., 2010; Segawa et al., 2016; Andersen et al., 2016

Table 2 Lipid translocases in the ABC transporter family

ABC transporter	Deduced substrate[a]
ABCA1	PC, PS, Chol
ABCA2	Chol, PE, PS
ABCA3	PC, PG, PE, Chol
ABCA4	*N*-retinylidene-PE, PE
ABCA5	Chol
ABCA7	PC, SM, Chol?
ABCA12	Cer, GlcCer
ABCB1	PC, PS, PE, SM, Chol, GlcCer, PAF
ABCB4	PC, PE, SM
ABCB11	Bile salt
ABCC1	PC, PS, SM, GlcCer, Bile salt
ABCG1	Chol, PS, PC, SM
ABCG2	PC, PS
ABCG4	Chol
ABCG5/G8	Chol, sitosterol

PC, phosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SM, sphingomyelin; Cer, ceramide; GlcCer, glucosylceramide; PAF, platelet activating factor

[a] van Meer et al., 2008; Kang et al., 2010, Coleman et al., 2013; Neumann et al., 2017

Table 3 TMEM16 and Xkr family

TMEM16 family	Primary function[a]	Cellular localization[a]	Tissue distribution[a]
TMEM16A	Cl^- channel	PM	Most tissues
TMEM16B	Cl^- channel	PM	Eye
TMEM16C	Lipid scramblase	PM	Brain
TMEM16D	Lipid scramblase	PM	Brain, eye, ovary, uterus
TMEM16E	Lipid scramblase	Intracellular	Muscle, bone, testis
TMEM16F	Lipid scramblase	PM	Ubiquitous
TMEM16G	Lipid scramblase	PM	Intestine
TMEM16H		Intracellular	Ubiquitous
TMEM16J	Lipid scramblase	PM	Intestine, stomach, skin
TMEM16K	Lipid scramblase	Intracellular	Ubiquitous

Xkr family	Primary function[b]	Cellular localization[b]	Tissue distribution[b]
Xkr1		PM	Most tissues
Xkr2		Intracellular, PM	Placenta, adrenal gland
Xkr3			Testis
Xkr4	Lipid scrambling	PM	Brain, eye, skin
Xkr5		PM	
Xkr6		PM	
Xkr7		PM	
Xkr8	Lipid scrambling	PM	Ubiquitous
Xkr9	Lipid scrambling	PM	Intestine, stomach

PM, plasma membrane; Intracellular, intracellular membranes

[a] Yang et al., 2008; Schroeder et al., 2008; Caputo et al., 2008; Suzuki et al., 2013b; Nagata et al., 2016; Whitlock and Hartzell, 2016; Falzone et al., 2018

[b] Calenda et al., 2006; Suzuki et al., 2014; Nagata et al., 2016