Methods in Enzymology

Phosphalipid scrambling on the plasma membrane

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Abstract

Phospholipids are asymmetrically distributed at plasma membrane in normal cells, and scrambled in various biological situations such as blood clotting and apoptotic cell death. Recent studies revealed that phospholipid scrambling is mediated by at least two independent mechanisms. An eight transmembrane-containing protein TMEM16F Ca^{2+}-dependently promotes the phospholipid scrambling, which is required for the PS exposure in activated platelets during blood clotting. On the other hand, a six transmembrane-containing protein Xk-related family protein 8 (Xkr8) is activated by caspases during apoptosis, and promotes the phospholipid scrambling, thus exposing PS as an "eat-me-signal". In this chapter, we describe the assay procedures for the phospholipid scrambling.

1. Introduction

Programmed cell death or apoptosis is characterized by DNA fragmentation, nuclear condensation, cell shrinkage, blebbing, and phosphatidylserine (PS) exposure (Vaux and Korsmeyer, 1999; Nagata and Golstein, 1995). The anionic phospholipid PS is normally restricted to the inner side of the plasma membrane by an ATP-dependent flippase; P4-type ATPases are candidate flippases that have been demonstrated to have lipid transporting activities in cells (Balasubramanian and Schroit, 2003). During the process of apoptosis, flippase activity has been shown to be inhibited, although flippase inhibition alone is not sufficient for PS externalization (Bevers and Williamson, 2010). A putative protein known as a calcium-dependent scramblase, which transports lipids bi-directionally and nonspecifically, must be activated (Bevers and Williamson, 2010). Following scramblase activation, PS and phosphatidylethanolamine (PE), which are initially located on the cytoplasmic side, are exposed to the cell surface, while phosphatidylcholine (PC) and sphingomyelin (SM), which are initially located on the extracellular side, are internalized. Once PS is exposed to the cell surface, it functions as an "eat-me-signal," allowing apoptotic cells to be recognized and engulfed by phagocytes (Ravichandran and Lorenz, 2007; Nagata *et al*., 2010). The phospholipid scramblase also regulates PS exposure in activated platelets, to provide a scaffold for coagulation factors, whose activation is required to control bleeding (Zwaal *et al*., 1992). Although phospholipid scrambling resulting in PS exposure is important in many physiological situations, the molecular mechanism mediating this process was completely unknown until recently. Over the past few years, studies have begun to clarify the details involved in membrane lipid scrambling. In particular, we recently showed that whether apoptotic cells and activated platelets utilize the different protein(s) in promoting PS exposure.

An eight transmembrane domain-containing protein, TMEM16F, was identified as an essential component involved in regulating calcium-dependent phospholipid scrambling (Suzuki *et al*., 2010). TMEM16F belongs to the 10-member TMEM 16 family. Mutations in *TMEM16F* have been identified in patients with Scott syndrome and are associated with defective calcium activation-induced PS exposure, resulting in impaired thrombin generation and mild bleeding (Suzuki *et al*., 2010; Castoldi *et al*., 2011). Similarly, lymphocytes derived from *TMEM16F* knockout mice are defective in calcium-induced PS exposure (Yang *et al*., 2012; Suzuki *et al*., 2013a); however, these cells exhibit normal PS exposure following apoptotic stimulation (Suzuki *et al*., 2013a), as also observed in lymphocytes derived from a Scott syndrome patient (Williamson *et al*., 2001). These results suggest that calcium- and apoptosis-induced phospholipid scrambling may be differentially regulated. Indeed, a six transmembrane domain-containing protein, Xk-related protein 8 (Xkr8) has been shown to regulate apoptosis-dependent phospholipid scrambling when it is cleaved by caspases (Suzuki *et al*., 2013b). The leukemia cell lines Raji and PLB985 are defective in apoptotic PS exposure (Fadeel *et al*., 1999; Fadok *et al*., 2001) due to a limited expression of *Xkr8* resulting from hypermethylation in the promoter region (Suzuki *et al*., 2013b). The exogenous expression of Xkr8 rescues phospholipid scrambling in these cells, confirming the importance of Xkr8 in this process. CED-8, the only Xkr8 homolog in *C. elegans,* also regulates PS exposure, promoting cell engulfment by phagocytes (Suzuki *et al*., 2013b), and indicating that the CED-8/Xkr8 homologs share an evolutionarily conserved role in regulating phospholipid scrambling. In contrast, lymphocytes from *Xkr8* knockout mice exhibit normal PS exposure upon calcium-ionophore stimulation, indicating that apoptotic- and calcium-induced PS exposure are differentially regulated by Xkr8 and TMEM16F, respectively.

The following sections describe cell-based assays that we have developed for calcium- and apoptosis-induced phospholipid scrambling.

2. Assays for calcium-induced phospholipid scrambling

Early studies showed that the treatment of activated platelets with collagen plus thrombin facilitates PS exposure, leading to the enhanced conversion of prothrombin to thrombin by coagulation factors (Bevers *et al*., 1983). At the same time PS exposure is increased, cell surface sphingomyelin is decreased, suggesting that lipids are transported or scrambled from one side of the membrane to the other. This observation was confirmed by monitoring nitrobenzoxadiazole (NBD)-conjugated lipids in red blood cells and platelets stimulated with calcium (Smeets *et al*., 1994; Williamson *et al*., 1995). Here, we describe PS and PE exposure assays, and NBD-PC and NBD-SM incorporation assays using activated lymphocytes.

2.1. Cells, reagents and buffers

2.1.1. Cells

1. Ba/F3 cells (Palacios and Steinmetz, 1985) are maintained in RPMI1640 containing 10% fetal calf serum (FCS), 45 units/ml mouse IL-3, and 50 μM β-mercaptoethanol.

2. PLB985 cells (Tucker *et al*., 1987) are maintained in RPMI1640 containing 10% FCS and 50 μM β-mercaptoethanol.
3. Immortalized fetal thymocytes (IFETs) (Imao and Nagata, 2013; Suzuki *et al*., 2013a) are cultured in DMEM containing10% FCS, 1 x non-essential amino acids (Gibco), 10 mM Hepes-NaOH buffer (pH 7.4), 50 μM β-mercaptoethanol, and GlutaMax (Gibco).

2.1.2. Reagents

1. PS exposure: AnnexinV-Cy5 (Biovision), Milk fat globule EGF factor 8 (MFG-E8)-FITC (Hematologic Technologies), Propidium Iodide (PI) (Sigma), Ca^{2+} ionophore A23187 (Sigma).
2. PE exposure: Biotin-labeled Ro09-0198 (Dr. Umeda's laboratory, Kyoto University), Streptavidin-APC (BD Biosciences).
3. Lipid incorporation: 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC) (Avanti), N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sphingosine-1-phosphocholine (NBD-SM) (Avanti). NBD-lipids in chloroform were dried with nitrogen gas to avoid oxidization and resuspended in DMSO to a final concentration of 1 mM. NBD-lipids in DMSO can be stored at -20 °C for at least one month.

2.1.3 Buffers

1. Annexin buffer: 2.5 mM $CaCl_2$, 140 mM NaCl, 10 mM Hepes-NaOH (pH 7.4).
2. Lipid incorporation buffer: Hank's Balanced Salt Solution (HBSS) (Gibco), 1 mM $CaCl_2$.
3. Lipid extraction buffer: HBSS, 1 mM $CaCl_2$, 5 mg/ml Fatty acid-free BSA (Sigma), 500 nM Sytox blue (Molecular Probes).

2.2. Protocol for the phospholipid exposure assay

PS exposure can be evaluated using the PS-binding proteins AnnexinV (Koopman *et al*., 1994) or MFG-E8 (Hanayama *et al*., 2002). PE exposure can be detected using the PE-binding peptide RO09-0198 (Emoto *et al*., 1997). In both cases, necrotic cells or membrane-broken cells can be identified by staining with PI or Sytox Blue.

*2.2.1. Phosphatidylserin*e *exposure*

1. Transfer 2 $\times 10^6$ cells to a 15-ml tube.
2. Centrifuge (300 × g, 2 min, room temperature) and wash cells with 1 ml PBS.
3. Centrifuge and wash cells with 1 ml Annexin buffer.
4. Centrifuge and resuspend cells in 2 ml Annexin buffer.

5. Transfer 1 ml of the cell suspension to a 1.5-ml tube.
6. Add 1 μl AnnexinV-Cy5 (1:1000) and 10 μl of 500 μg/ml PI (final concentration: 5 μg/ml).
   *AnnexinV can be replaced with MFG-E8-FITC (final concentration: 83 ng/ml).
7. Incubate in a heat block at 20 ^{o}C for 3 min.
8. Transfer 500 μl of the stained cells to a FACS tube with a cap filter.
9. Stimulate cells with 5 μl of 300 μM A23187 (final concentration: 3 μM).
   *The A23187 concentration should be optimized for different cell types by titrating from 0.5 to 10 μM. The optimal A23187 concentration was found to be 0.5 μM for Ba/F3 pro-B cells, 10 μM for PLB985 myeloid progenitor cells, and 3 μM for IFETs.
10. Analyze stained and activated cells by FACSAria at 20 ^{o}C.

2.2.2. Phosphatidylethanolamine exposure

At step 6 in the PS exposure assay, cells are resuspended in Annexin buffer with RO09-0198-biotin, Streptavidin-APC (1 μg/ml), and 5 μg/ml PI (the RO09-0198-biotin and Streptavidin-APC are mixed in Annexin buffer prior to use). The RO09-0198 concentration should be optimized.

2.3. Protocol for the phospholipid incorporation assay

Lipid incorporation can be observed using fluorescence-conjugated lipids (NBD-lipids) (Mclntyre and Sleight, 1991), and internalized lipids can be measured by the specific removal of outer layer lipids using fatty acid-free BSA (Haest *et al*., 1981). The ATP-dependent aminophospholipid translocases, or flippases, have the ability to incorporate PS and PE, but not PC (Colleau *et al*., 1991). While, scramblases non-specifically incorporate phospholipids (Williamson *et al*., 1992). Thus, NBD-PC is more suitable than NBD-PS to assay the scramblase. Necrotic cells or membrane-broken cells can be detected by staining with Sytox blue. It should be noted that A23187 itself possesses a similar, but weak, fluorescence spectrum to Sytox blue.

2.3.1. Nitrobenzoxadiazole-labeled phospholipid incorporation

1. Transfer 4 $\times 10^{6}$ cells to a 15-ml tube.
2. Centrifuge (300 × g, 2 min, room temperature) and wash cells with 1 ml HBSS.
3. Centrifuge and wash cells with 1 ml HBSS containing 1 mM $CaCl_2$ (HBSS/$CaCl_2$).
4. Centrifuge and resuspend cells in 2 ml cold HBSS/$CaCl_2$.
5. Transfer 600 μl of the washed cells to two 1.5-ml tubes (one for DMSO and one for A23187).
6. Incubate in an aluminum block on ice for 7 min.
7. Add 600 μl of 200 nM NBD-PC dissolved in HBSS/ $CaCl_2$ to the cell suspension (NBD-PC final concentration: 100 nM).

*The NBD-PC concentration should be optimized for different cell types by titrating from 0.1 to 1 μM.

8. Place the cell suspension on ice for 3 min.
9. As a sample for time 0, mix 200 μl of the cell suspension with 200 μl of the chilled lipid extraction buffer, and keep on ice.
10. To the remaining 1 ml cell suspension kept at step 8, add 5 μl DMSO or 5 μl 50 μM A23187 (to a final concentration of 250 nM) to 1 ml cell suspension. *The A23187 concentration should be optimized for different cell types by titrating from 0.25 to 1 μM.
11. Incubate in a heat block at 15 ^{o}C.

    *The incubation temperature can be increased up to 25 ^{o}C, but the cells may lyse at higher temperatures, depending on the cell type and the labeled lipid used. At lower temperature (15-25 ^{o}C), scramblase-independent incorporation (such as endocytosis) may be prevented.
12. Collect 150-μl samples at 1, 2, 4, 6, and 8 min and add to 150 μl of the chilled lipid extraction buffer or the same buffer without BSA, and keep on ice.
13. Analyze the Sytox blue-negative population of the applied cells for the incorporated NBD-PC by flow cytometry. The BSA-non-extractable NBD-PC signal represents incorporated lipids that have been internalized. The BSA-non-treated NBD-PC signal represents total lipids, including those attached to the cell surface and those that have been internalized.

3. Assays for apoptosis-induced phospholipid scrambling

PS exposed on the cell surface was first suggested to be an “eat-me-signal” by studies in which red blood cells were labeled with the fluorescence-conjugated PS (NBD-PS); unincorporated NBD-PS remaining on the surface of the red blood cells was found to facilitate their uptake by macrophages (Tanaka and Shroit, 1983). Approximately 10 years later, apoptotic lymphocytes exposing PS were shown to be engulfed by phagocytes in a PS-dependent manner (Fadok *et al*., 1992). When PS is exposed to the cell surface, PC or SM, located on the outer surface of the membrane become internalized, suggesting the existence of proteins that transport lipids bi-directionally and nonspecifically (Williamson *et al*., 2001). Here, we describe PS and PE exposure assays and NBD-PC and NBD-SM incorporation assays using apoptotic leukocytes.

3.1. Cells, reagents, and buffer

3.1.1. Cells

1. WR19L cells (ATCC, #TIB-52) expressing mouse Fas (Ogasawara *et al*., 1993) are maintained in RPMI1640 containing 10% FCS and 50 μM β-mercaptoethanol.
2. Raji cells (ATCC, #CCL-86) are maintained in RPMI1640 containing 10% FCS and 50 μM β-mercaptoethanol.

3. Culture condition of PLB985 cells is described in 2.1.1.

3.1.2. Reagents

Most of the reagents and buffers used here were described in section 2.1.2. The reagents used specifically in this section include: Fas ligand and staurosporine (Kyowa Hakko) to induce apoptosis.

Fas Ligand

The leucine-zipper tagged human Fas ligand (Shiraishi et al., 2004) was prepared by introducing its expression vector into COS-7 cells. In brief, one hundred fifty μg of the expression vector was mixed with $3x10^7$ COS-7 cells in 3 ml of kPBS (30.8 mM NaCl, 121 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4) and subjected to electroporation using a Gene-pulser (960 μF, 0.23 kV) (Bio-Rad). After electroporation, the cells in ten 10-cm plates were cultured for 3 days in DMEM containing 1% FCS, and the culture supernatant was collected. The cells were further cultured for two days with fresh medium, and the conditioned medium was combined with the medium collected at day 3 (a total of 150 ml). A 225 ml of saturated ammonium sulfate was added to the conditioned medium at 4 ^oC to obtain 60% saturation of ammonium sulfate, and the proteins were precipitated by centrifugation at 15,000 g for 30 min. The precipitated proteins were dissolved in 4 ml of PBS, dialyzed against PBS, filtered through a 0.22 μm filter, and stored at -80 ^oC until use. The biological activity of Fas ligand was determined with WR19L cells expressing mouse Fas as described (Tanaka *et al.*, 1997), and one unit is defined as the dilution that gives a half-maximum response.

3.2. Protocol for the phospholipid exposure assay

Like the Ca^{2+}-induced PS exposure, PS exposed during apoptosis can be detected using the PS-binding proteins Annexin V (Koopman *et al.*, 1994) or MFG-E8 (Hanayama *et al.*, 2002), while PE exposure is detected with RO09-0198 (Emoto *et al.*, 1997). Apoptotic cells later undergo secondary necrosis, and these cells are stained by AnnexinV and PI (or Sytox blue).

3.2.1. Phosphatidylserine exposure

1. Transfer 5×10^5 cells in 500 μl medium to a 24-well plate.
2. Stimulate cells with adequate apoptotic stimuli.
   WR19L cells expressing mouse Fas are stimulated with 10 units/ml Fas ligand for 60 min.
   Raji cells are stimulated with 400 units/ml Fas ligand for 2 h.
   PLB985 cells suspended in PBS are exposed to 2000 J/m^2 UV irradiation and cultured in medium for 2 to 3 h, or treated with 10 μM staurosporine for 2 to 4 h.
3. Transfer 230 μl of the cell suspension to a 96-well round-bottom plate.
4. Centrifuge (300 g, 2 min, 4 ^oC) and wash cells with 200 μl PBS.

5. Centrifuge and wash cells with 200 μl Annexin buffer.
6. Centrifuge and resuspend cells in 100 μl Annexin buffer containing AnnexinV-Cy5 (1:1000).
7. Incubate cells on ice for 15 min.
8. Add 100 μl Annexin buffer containing 10 μg/ml PI.
9. Incubate cells on ice for 2 min.
10. Analyze stained cells with flow cytometry.

3.2.2. Phosphatidylethanolamine exposure

The cells are treated with apoptotic stimuli as described above (steps 1 to 5).

6. Centrifuge and resuspend cells in 100 μl Annexin buffer with biotin-RO09-0198.
7. Incubate cells on ice for 15 min.
8. Add 100 μl Annexin buffer, centrifuge, and wash cells with 200 μl Annexin buffer.
9. Centrifuge and resuspend cells in 100 μl Annexin buffer with Streptavidin-APC (1 μg/ml).
10. Incubate cells on ice for 15 min.
11. Add 100 μl Annexin buffer, centrifuge, and wash cells with 200 μl Annexin buffer.
12. Centrifuge and resuspend cells in 200 μl Annexin buffer containing 5 μg/ml PI.
13. Analyze stained cells with flow cytometry.

3.3. Protocol for the phospholipid incorporation assay

Like the Ca^{2+}-induced scrambling of phospholipids, the scrambling of phospholipids during apoptosis can be monitored using NBD-PC, and internalized lipids can be distinguished from those bound to the cells by the specific removal of outer layer lipids using fatty acid-free BSA (Haest *et al*., 1981). The apoptotic PS exposure is caspase-dependent (Martin *et al*., 1996), and when the cells undergo the secondary necrosis, phospholipid scrambling may occur at the membrane-broken sites in a scramblase-independent fashion. Thus, timing to assay the scramblase activity in apoptotic cells is critical.

3.3.1. Nitrobenzoxadiazole-labeled phospholipid incorporation

Cells were stimulated with apoptotic stimuli as described above (3.2.1).

1. Transfer 2×10^6 cells to a 15-ml tube.
2. Centrifuge (300 × g, 2 min, room temperature) and wash cells with 1 ml HBSS.
3. Centrifuge and wash cells with 1 ml HBSS/$CaCl_2$.
4. Centrifuge and resuspend cells in 1 ml cold HBSS/$CaCl_2$.
5. Transfer 600 μl of the cell suspension to a 1.5-ml tube.
6. Incubate in an aluminum block on ice for 7 min.
7. Add 600 μl of 200 nM NBD-lipids in HBSS/ $CaCl_2$ to the cell suspension (NBD-lipids final

concentration: 100 nM). *NBD-lipid concentration should be optimized for different cell types by titrating from 0.1 to 1 μM.

8. Place the cell suspension on ice for 3 min.
9. As a sample for time 0, mix 200 μl of the cell suspension with 200 μl of the chilled lipid extraction buffer, and keep on ice.
10. Place the remaining 1 ml cell suspension at step 8 onto a heat block, and incubate at 20 ^{o}C.
11. Take 150-μl aliquots at 1, 2, 4, 6, and 8 min and mix with 150 μl of lipid extraction buffer or the same buffer without BSA, and keep on ice.
12. Analyze the Sytox blue-negative population for the incorporation of fluorescent lipids by flow cytometry.

Figure legend

Figure 1. Ca^{2+}-induced scrambling of phospholipids promoted by TMEM16F.

(A) Ca^{2+} ionophore-induced PS exposure. The wild-type and TMEM16F$^{-/-}$ IFETs were treated at 20°C with 3.0 μM A23187, and Annexin V-binding to the cells was monitored by flow cytometry for 10 min. (B) Ca^{2+} ionophore-induced lipid internalization. The wild-type and TMEM16F$^{-/-}$ IFETs were treated at 15°C with 250 nM A23187 in the presence of NBD-PC. Using aliquots of the reaction mixture, the BSA-non extractable level of NBD-PC in the Sytox Blue-negative population was determined at the indicated time by flow cytometry and shown in mean fluorescence intensity.

Figure 2 Apoptosis-induced scrambling of phospholipids promoted by Xkr8.

(A and B) Apoptotic exposure of PS and PE. PLB-985 and PLB-985 transformants expressing Xkr8 were treated with staurosporine (STS), stained with AnnexinV-Cy5 (A) or biotin-RO peptide/APC-streptavidin (B), and PI, and analyzed by flow cytometry. (C and D) Scrambling of PC and SM in apoptotic cells. PLB-985 and PLB-985-transformants expressingXkr8 were treated with STS and incubated with NBD-PC (C) or NBD-SM (D), and the incorporated lipids were analyzed by flow cytometry. The fluorescence intensity in the SytoxBlue-negative fraction is shown in arbitrary units.

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Figure 1

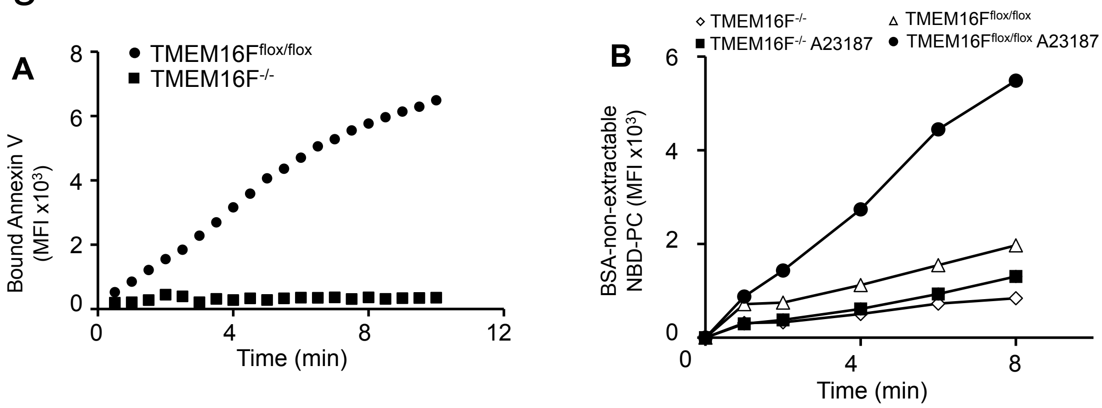

Figure 2