A membrane-associated, fluorogenic reporter for mammalian phospholipase C isozymes

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A diverse group of cell-surface receptors, including many G protein-coupled receptors and receptor tyrosine kinases, activate phospholipase C (PLC) isozymes to hydrolyze phosphatidylinositol 4,5-bisphosphate into the second messengers diacylglycerol and 1,4,5-inositol trisphosphate. Consequently, PLCs control various cellular processes, and their aberrant regulation contributes to many diseases, including cancer, atherosclerosis, and rheumatoid arthritis. Despite the widespread importance of PLCs in human biology and disease, it has been impossible to directly monitor the real-time activation of these enzymes at membranes. To overcome this limitation, here we describe XY-69, a fluorogenic reporter that preferentially partitions into membranes and provides a selective tool for measuring the real-time activity of PLCs as either purified enzymes or in cellular lysates. Indeed, XY-69 faithfully reported the membrane-dependent activation of PLC-β3 by Gαq. Therefore, XY-69 can replace radioactive phosphatidylinositol 4,5-bisphosphate used in conventional PLC assays and will enable high-throughput screens to identify both orthosteric and allosteric PLC inhibitors. In the future, cell-permeable variants of XY-69 represent promising candidates for reporting the activation of PLCs in live cells with high spatiotemporal resolution.

Many extracellular stimuli, including various hormones, growth factors, and neurotransmitters initiate signaling cascades that result in the activation of phospholipase C (PLC) isozymes (1–3). Active PLCs catalyze the conversion of the membrane-bound phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2)3 into two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, which mobilize intracellular stores of Ca2+ and activate protein kinase C isozymes, respectively. Furthermore, depletion of PIP2 also alters the membrane association and/or activity of many proteins that harbor phosphoinositide-binding domains. Consequently, PLCs are key signaling proteins that regulate diverse cellular functions including proliferation, differentiation, migration, and nerve conductance (4). Conversely, aberrant regulation of PLCs contributes to various diseases including cancer (5–7), atherosclerosis (8), and rheumatoid arthritis (9).

The human genome encodes 13 PLCs grouped into six subfamilies (β, δ, γ, ε, ζ, and η). Most PLCs are autoinhibited by an X–Y linker that separates the X- and Y-boxes of the catalytic core (10) and mechanisms that release this autoinhibition are diverse and specific to the various subfamilies. For example, the PLC-β isozymes are directly activated by Gα and Gβγ subunits of heterotrimeric G proteins (11, 12), whereas PLC-γ isozymes are activated upon phosphorylation by tyrosine kinases (13). Similarly, Rac1 activates certain PLC-β isozymes (14) as well as PLC-γ2 (14, 15), but Rac1 binds to distinct regions of the two PLC subgroups. Therefore, to understand how PLCs are regulated to control normal cellular processes and physiology as well as disease states, it is crucial to have convenient assays of PLC activity that differentiate modes of regulations and that can be used in cells.

The canonical assay of the lipase activity of purified PLC isozymes uses radiolabeled PIP2 as substrate. The enzymatic product, IP3, is then quantified by scintillation counting. However, this assay does not allow for continuous monitoring of PLC activity, and handling of radioactive materials requires special training and facilities. Likewise, cellular PLC activity is typically measured from the production of radiolabeled inositol phosphates after biosynthetic incorporation of radioactivity into phosphoinositide pools (16). These assays are subject to cell-dependent differences in the steady-state metabolism of phosphoinositides and variable expression of PLCs. Alternatively, cell-permeable dyes that increase in fluorescence upon binding Ca2+ are also routinely used to monitor PLC activity (17). However, these dyes do not directly measure PLC activity and often generate confounding data due to diverse factors known to affect intracellular Ca2+ concentrations. Similarly, PLC-α1, PLC-β1, and PLC-β3, phosphorylase A; P3K, phosphoinositide 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; PE, phosphatidylethanolamine.
the intracellular movement of fluorescently-tagged domains that bind specific lipid components, e.g. the GFP-tagged PH domain of PLC-δ1, which specifically interacts with PIP2, are also commonly used to monitor the activation of PLCs in live cells (18–20). However, like dyes that are indicators of Ca2+ effects of concentration of free Ca2+ on hydrolysis of XY-69. Hydrolysis of XY-69 (5 μM) by PLC-δ1 (0.023 μM) was measured in the presence of the indicated concentrations of free calcium. The reaction progression was monitored by fluorescence (λex/λem = 485/520 nm). Plots were representatives of three independent experiments. Each experiment was run in triplicates with error bars as standard deviations.

Figure 1. Design and validation of XY-69. A, the fluorescein derivative (green) of XY-69 is efficiently quenched by close proximity to a DABCYL moiety (red) attached to the 6-position of the inositol. Cleavage of XY-69 by PLCs dissociates the fluorophore/quencher pair so that the fluorescein portion becomes highly fluorescent (λex/em = 485/520 nm). The long alkyl chain (blue) serves to increase the affinity of XY-69 for membranes. B, excitation and emission spectra of XY-69 after hydrolysis by wildtype (WT) PLC-δ1 and catalytically inactive PLC-δ1 (E341A). C, effects of concentration of free Ca2+ on hydrolysis of XY-69. Hydrolysis of XY-69 (5 μM) by PLC-δ1 (0.023 μM) was measured in the presence of the indicated concentrations of free calcium. The reaction progression was monitored by fluorescence (λex/λem = 485/520 nm). Plots were representatives of three independent experiments. Each experiment was run in triplicates with error bars as standard deviations.

Results and discussion

Design and synthesis of XY-69

We developed XY-69 to robustly monitor PLC activity at membranes (Fig. 1A). The design of XY-69 relies on the fact that the 6-hydroxyl position of PIP2 remains solvent exposed within the active site of PLCs, such that modifications at this site minimally compromise the capacity of PIP2 derivatives to function as PLC substrates (25). Therefore, to create XY-69, fluorescein was introduced at the sn-1 position of PIP2, whereas 4-(dimethylaminoazo)benzene 4-carboxylic acid (DABCYL) was introduced at the 6-hydroxyl position. This arrangement is expected to effectively quench the intrinsic fluorescence of fluorescein by the DABCYL moiety. However, once XY-69 is cleaved by PLCs, the fluorophore/quencher pair will be separated resulting in a dramatic increase in the quantum yield of fluorescein. XY-69 was also designed to retain a long alkyl chain (C15H31) at the sn-2 position to favor the partitioning of XY-69 into lipid membranes. Therefore, much like diacylglycerol, the fluorescent product of XY-69 hydrolysis is expected to remain in lipid membranes.

The synthesis of XY-69 (Fig. 2) started with the inositol intermediate 3 that we previously developed (25). Olefin metathesis of 3 with the terminal alkene N-tert-butoxycarbonyl-2-allyloxyethanolamine 4 in the presence of Hoveyda-Grubbs catalyst followed by hydrogenation produced 5. The 4- and 5-hydroxyl groups in 5 were phosphorylated through reactions with dibenzyl diisopropylphosphoramidite followed by oxidation with meta-chloroperoxybenzoic acid (mCPBA). The resulting phosphate ester was treated with tetrabutylammonium fluoride (TBAF) to remove the tert-butyldiphenylsilyl (TBDPS) protective group. Phosphorylation of 6 with compound 7, which was prepared according to literature protocols.
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XY-69 was initially incubated with purified PLC-δ1 at 37 °C for 1 h and the fluorescence excitation and emission spectra of the reaction mixture were recorded (Fig. 1B). Excitation and emission maxima were 490 and 518 nm, respectively. These maxima are similar to the equivalent numbers for isolated fluorescein (490 and 525 nm). Liquid chromatography-mass spectrometry (LC-MS) analysis of the assay mixture was also carried out and confirmed the formation of the expected product 1 (Fig. S1). In subsequent experiments, cleavage of XY-69 was detected at 520 nm after excitation at 485 nm.

PLCs require a Ca²⁺ cofactor to stabilize the transition state during the hydrolysis of PIP₂ (27, 28). We thus investigated the effect of Ca²⁺ concentration on XY-69 hydrolysis (Fig. 1C). As expected, increasing the concentration of free Ca²⁺ led to increased rates of hydrolysis of XY-69 by PLC-δ1 and this behavior mirrored earlier work with WH-15 (24). Conversely, without free Ca²⁺, there was essentially no hydrolysis of XY-69. Consequently, amounts of free Ca²⁺ were tightly controlled throughout this work to ensure efficient enzymatic reactions. To demonstrate that XY-69 can be used to monitor PLC activity, the real-time fluorescence of detergent-solubilized XY-69 incubated with purified PLC-δ1 was recorded (Fig. 3A). Purified PLC-δ1 harboring a single mutation (E341A) within its active site has undetectable lipase activity (29) and was used in a parallel reaction. XY-69 generated approximately a 20-fold increase in fluorescence with PLC-δ1 relative to an identically treated sample containing PLC-δ1 (E341A). These results strongly suggest that the fluorescence increase arises from the enzymatic action of PLC-δ1 on XY-69.

To further test whether XY-69 functions as a substrate for other PLC isoforms, the reporter was incubated with either purified PLC-β3 or -γ1 in reactions analogous to that described for PLC-δ1. As shown in Fig. 3A, XY-69 is hydrolyzed with similar kinetics by each of the PLC isozymes. The catalytic domains of PLC isozymes are highly conserved (30) and consequently, XY-69 is likely a general PLC substrate.

We also tested whether other lipid-metabolizing enzymes that utilize PIP₂ as a substrate could generate fluorescent signals from XY-69. For these tests, we also produced XY-23 (Fig. 3B), which contains a fluorescein moiety but not the DABCYL quencher of XY-69. Consequently, XY-23 is intrinsically fluorescent and enzymatic transformations of XY-23 should invariably generate new fluorescent products. In this respect, XY-23 is highly similar to PIP₂ and is expected to be a substrate for isozymes of phospholipase D (PLD), phospholipase A (PLA), and phosphoinositide 3-kinase (PI3K), as well as PLCs (31). Both XY-23 and XY-69 were subjected to enzymatic reactions with PLCs (-δ1, -γ1, and -β3), PLD1, PLA2, and PI3Kα. The reaction mixtures were then separated by thin layer chromatography (TLC) and visualized by fluorescence. As shown in Fig. 3C, for XY-23, each lipid-metabolizing enzyme produced a new fluorescent product. In contrast, only the PLCs were capable of hydrolyzing XY-69, attesting to the PLC-selective nature of this fluorogenic reporter.
Membrane association and Gαq activation of XY-69

PHY2 is a membrane-associated phospholipid and PLCs catalyze the hydrolysis of PHY2 at the lipid–aqueous interface. Therefore, XY-69 was assessed for capacity to incorporate into membranes used to assay PLC activity. We originally attempted to use a variety of polybasic carriers including histones (32) and polyamines (32) and liposomes (33) to deliver XY-69 or PHY2 into cells with the expectation that these PHY2 analogs would partition into cellular membranes. However, results were inconsistent with low efficiencies of delivery and high sequestration into endosomal compartments. Instead, we used an in vitro membrane association assay originally developed to measure protein–lipid interactions. In this format, liposomes incubated with either XY-69 or WH-15 were recovered after centrifugation in a sucrose gradient (Fig. 5A). Quantification of the top (lipid) and bottom sucrose layers (Fig. 5B) indicated that ~90% of XY-69 incorporated into the lipid fraction. In contrast, WH-15 preferentially (~60%) partitioned into the aqueous fraction. Therefore, XY-69 efficiently partitions into lipid vesicles that mimic cellular membranes.

XY-69 was subsequently reconstituted into lipid vesicles and used to monitor the phospholipase activities of purified PLCs (Fig. 6A). Under these conditions, XY-69 was hydrolyzed with similar kinetics by PLC-δ1, -β3, and -γ1. In contrast, catalytically inactive PLC-δ1 (E341A) was unable to hydrolyze XY-69.

We previously showed that the soluble, fluorogenic substrate WH-15 cannot be used to monitor the activation of PLC-β
isozymes by membrane-resident Gaq (24). In contrast, XY-69 reconstituted into lipid vesicles with Gaq readily reports this activation (Fig. 6B). Gaq increased the initial rate of XY-69 hydrolysis by PLC-β3 ~12-fold, consistent with measurements when [3H]PIP2 was used as the substrate (Fig. 6C). For comparison, PLC-β3 (PH-C2), which is not responsive to Gaq in conventional assays (24), is also unresponsive to Gaq in this assay.

Finally, to demonstrate that XY-69 has the capacity to report activity of endogenous PLCs, HEK293 cells transfected with plasmid encoding Gaq (Q209L) or the parent vector were lysed 48 h after transfection with assay buffer containing XY-69. As expected, cells transfected with the parent vector showed essentially no increase in fluorescence associated with the hydrolysis of XY-69 (Fig. 7, A and B). In contrast, cells transfected with Gaq (Q209L) showed an approximate 10-fold increase in fluorescence related to the robust hydrolysis of XY-69 (Fig. 7, A and B). These results are consistent with the equivalent comparison using conventional labeling of cells with [3H]inositol followed by radioactivity counting of [3H]inositol phosphates produced by PLCs (Fig. 7C). Taken together, these results demonstrate that XY-69 successfully mimics PIP2 to report the regulation of PLCs operating at membranes. Consequently, XY-69 is able to replace [3H]PIP2 used in conventional assays used to measure the phospholipase activity of PLCs.

In summary, we have developed a membrane-bound fluorogenic reporter, XY-69, that monitors the lipase activity of PLC isozymes in real-time with high sensitivity. The reporter works with both detergent micelles and lipid vesicles and can be used with either purified PLCs or cellular lysates. Furthermore, XY-69 captures the activation of PLC activity through intrinsic regulation or activation of Gaq, which requires the presence of membranes. Consequently, XY-69 is suitable to replace radiolabeled PIP2 that is used in the canonical enzymatic assay of PLC activity, with the advantages of continuous monitoring, high-throughput and avoidance of using radioactive materials. These features enable XY-69 to be used for development of isozyme-selective inhibitors of PLCs. Given the general significance of PLC isozymes, isozyme-selective inhibitors will likely find applications as novel chemical probes or therapeutics for PLC-associated diseases. Finally, other derivatives of phosphoinositides such as PIP4 and PI3P have been delivered into cells through caging negatively charged phosphates to produce the corresponding charge-neutral esters (34, 35). Accordingly, when a suitable delivery method is available, XY-69 could be used to monitor PLC activity in live cells with high spatiotemporal resolution.

**Experimental procedures**

**PLC assay in detergent micelles**

In a PerkinElmer ProxiPlate™-384 Plus F black plate, XY-69 was added to a final concentration of 5 μM in assay buffer containing HEPES (50 mM, pH 7.4), KCl (70 mM), CaCl2 (3 mM), EGTA (3 mM), DTT (2 mM), cholate (0.5%), and fatty acid-free BSA (0.2 mg/ml). The free Ca2+ concentration in this buffer is calculated as 18.9 μM according to the Ca-EGTA Calculator v1.3 program using constants from Theo Schoenmakers’ chelator (36). The PLC-81 (25 ng), PLC-81 (E341A) (25 ng), PLC-γ1 (100 pg), or PLC-β3 (50 ng) enzymes in the same buffer were then added to initiate enzymatic reaction at 37 °C. The final volume of the assay was 10 μl. The progression of the assay was monitored continuously by fluorescence intensity of the reaction mixture on a PerkinElmer Wallac EnVision 2103 multilabel reader with an excitation wavelength of 485 nm (bandwidth of 10 nm) and an emission wavelength of 520 nm (bandwidth of 10 nm).

When cell lysates were used in this assay, HEK293 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) containing fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C. Cells were plated in a 12-well dish at a density of 60,000 cells/well in DMEM and 24 h after plating were transfected with the indicated DNA at 300 ng/well using Continuum (Gemini BioSciences). Forty-eight hours post-transfection, cells were washed with PBS, and lysed with RIPA buffer containing NaCl (150 mM), Nonidet P-40 (1%), SDS (0.1%), Tris-HCl (50 mM, pH 8), and sodium deoxycholate (12 mM). Western blotting was performed to confirm the expression of PLC-β3 using anti-PLC β3 (Santa Cruz Biotechnology). Lysates were normalized for total protein concentration prior to use in the reporter assay as described above.
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PLC assay in lipid vesicles

Lipid vesicles containing liver phosphatidyethanolamine (PE, 330 μM), brain PIP<sub>2</sub> (30 μM), and XY-69 (7.5 μM) were generated by mixing the lipids, drying the mixture under a stream of nitrogen, and re-suspending the dried lipid mixture in HEPES (20 mM, pH 7.4) using a probe sonicator. In parallel, PLC isoforms were diluted to the desired concentration with a buffer containing HEPES (20 mM, pH 7.4), NaCl (50 mM), dithiothreitol (DTT, 2 mM), and fatty acid-free BSA (1 mg/ml). To a PerkinElmer ProxiPlate<sup>TM</sup> 384 Plus F black plate was added 6× buffer (2 μl) containing HEPES (80 mM, pH 7.4), KCl (420 mM), DTT (4 mM), CaCl<sub>2</sub> (3 mM), NaF (20 mM), and 0.3 mg/ml of fatty acid-free BSA to final concentrations of 10 and 20 nM, respectively. G<sub>α<sub>q</sub></sub> was then diluted with the same 2× assay buffer to the final concentration of 480 nM. To a PerkinElmer ProxiPlate<sup>TM</sup> 384 Plus F black plate was added 2× assay buffer containing HEPES (20 mM, pH 7.4), KCl (40 mM), NaCl (30 mM), EGTA (6 mM), AlCl<sub>3</sub> (60 mM), MgCl<sub>2</sub> (5 mM), DTT (4 mM), CaCl<sub>2</sub> (3 mM), NaF (20 mM), and 0.3 mg/ml of fatty acid-free BSA to final concentrations of 10 and 20 nM, respectively. G<sub>α<sub>q</sub></sub> was then diluted with the same 2× assay buffer to the final concentration of 480 nM. To a PerkinElmer ProxiPlate<sup>TM</sup> 384 Plus F black plate was added 2× assay buffer and 4 μl of G<sub>α<sub>q</sub></sub> solution, followed by the addition of 6 μl of lipids to initiate the reaction. The assay was incubated at room temperature and fluorescence was recorded as described above.

**Measurement of activity of endogenous PLCs**

HEK293 cells were plated in 6-well tissue culture plates at the density of 150,000 cells/well in DMEM supplemented with 10% fetal bovine serum and incubated overnight in a humidified, 37 °C, 5% CO<sub>2</sub> incubator. Cells were transfected with 300 ng of pCHA-lic control vector or 30 ng of pCHA GNAQ Q209L DNA plus 270 ng of pcHA-lic control using Continuum transfection reagent (Gemini Bio Products). After 48 h, the medium was removed and replaced with 400 μl of inositol-free DMEM. The cells were cultured for another 24 h and then washed with ice-cold PBS before being collected with PBS. The suspension was briefly spun to pellet, the supernatant was removed, and the resulting pellets were snap-frozen with liquid N<sub>2</sub> and stored at −80 °C for subsequent uses.

The cell pellets from two wells were combined in a microcentrifuge tube on ice by resuspending the cells with 80 μl of lipid vesicles containing liver PE (500 μM), brain PIP<sub>2</sub> (50 μM), and XY-69 (10 μM) in HEPES (20 mM, pH 7.4) and 80 μl of 2× assay buffer containing HEPES (20 mM, pH 7.4), KCl (40 mM), NaCl (30 mM), MgCl<sub>2</sub> (5 mM), AlCl<sub>3</sub> (0.06 mM), NaF (20 mM), DTT (4 mM), EGTA (6 mM), CaCl<sub>2</sub> (3 mM), and 0.3 mg/ml of fatty acid-free BSA. The cells were lysed by passing through a syringe with a 27-gauge hypodermic needle 10 times. The resulting mixture was then added, followed by addition of PLC protein (2 μl) at concentrations of 11.4, 18.9, 67.3, and 66.6 nM for PLC-δ1, -δ1 (E341A), -γ1, and -β3, respectively, to initiate the assay. The assay was incubated at 37 °C and fluorescence was recorded as described above.

**Figure 6. XY-69 captures the activation of PLC activity through both intrinsic and external regulations.** A, indicated PLCs, including δ1 (11.4 nM), δ1 (E341A) (18.9 nM), γ1 (67.3 nM), and β3 (66.6 nM), were added to XY-69 (5 μM) incorporated into lipid vesicles and fluorescence monitored (λ<sub>ex</sub> = 485/520 nm). Plots were representatives of three independent experiments. Each experiment was run in triplicates with error bars as standard deviations. B, activation of PLC-β3 by G<sub>α<sub>q</sub></sub> incorporated into lipid vesicles containing XY-69. As indicated, half the vesicles were incubated with purified G<sub>α<sub>q</sub></sub> (160 nM) activated with aluminum fluoride before addition of either wildtype PLC-β3 (1.7 nM) or the equivalent amount of a form (PH-C2) that does not respond to G<sub>α<sub>q</sub></sub>. Samples were subsequently monitored by fluorescence. Plots were representatives of three independent experiments. Each experiment was run in triplicates with error bars as standard deviations. C, hydrolysis of [<sup>3</sup>H]PIP<sub>2</sub> by PLC-β3. Hydrolysis of [<sup>3</sup>H]PIP<sub>2</sub> was measured with reconstituted phospholipid vesicles for purified PLC-β3 (1.7 nM) or PLC-β3 (1.7 nM) + G<sub>α<sub>q</sub></sub> (160 nM). [<sup>3</sup>H]Inositol phosphates were then isolated and quantified. Data are represented as three independent experiments. Each data point is an average of two replicates in one independent experiment.
was then incubated at 37 °C. At an indicated time point, 30 μl of mixture was taken out and centrifuged for 40 s, and 10 μl of supernatant was transferred to a PerkinElmer ProxiPlate™ 384 Plus F black plate for fluorescence measurement as described above. The activity of endogenous PLCs was calculated from the fluorescence increase as the slope. For comparison, the endogenous PLC activity was also measured by labeling cells with 1 μCi of myo-[3H]inositol (PerkinElmer) and quantifying [3H]inositol phosphates as previously described.

TLC analysis of assay mixtures

XY-69 or XY-23 was added to various assay buffers (30 μl) to reach a final concentration of 67 μM. The components for assay buffers included: 1) HEPES (50 mM, pH 7.4), KCl (70 mM), CaCl2 (3 mM), EGTA (3 mM), DTT (2 mM), cholate (0.5%), and fatty acid-free BSA (0.2 mg/ml) for PLCs; 2) Tris-HCl (12.5 mM, pH 8.0), SDS (6.2 mM), and EtOH (0.1%) for PLD1; 3) Tris-HCl (50 mM, pH 7.5), KCl (150 mM), and CaCl2 (10 mM) for PLA2; and 4) MOPS (50 mM, pH 6.5), NaCl (100 mM), sodium cholate (0.5 mM), DTT (1 mM), MgCl2 (10 mM), and ATP (2 mM) for PI3Kα. The enzymes PLC-δ1 (25 ng), PLC-δ1 (E341A) (25 ng), PLC-γ1 (100 pg), PLC-β3 (50 ng), PLD1 (40 units), PLA2 (2 units), or PI3Kα (15 ng) were then added to the initial enzymatic reactions. The assay mixtures were incubated at 37 °C for 1 h, and samples (1 μl) were taken out and spotted on TLC plates (Merck, Silica Gel-60). The solvents used for TLC were CHCl3:MeOH:H2O (100:20:1) for reactions with PLCs and PLD and CHCl3:acetone:MeOH:HOAc:H2O (80:30:26:24:14) for PLA2 and PI3Kα, respectively. The products were detected by fluorescence with a Typhoon 9400 Variable Mode Imager (λex/em = 488/520 nm).

Membrane association assay

The liposome mixture was prepared from stock solutions of lipids in CHCl3 for a final composition of 45% phosphatidylcholine, 25% PE, 15% cholesterol, 10% phosphatidylinositol, and 5% phosphatidylserine. The solvent was blown off under a stream of nitrogen followed by drying under vacuum for at least 1 h. The lipid film was then suspended in buffer composed of MOPS (50 mM, pH 6.7), NaCl (100 mM), DTT (1 mM), and MgCl2 (10 mM) to a concentration of 2 mM. Liposomes were extruded through a 0.03-μm pore size polycarbonate filter membrane at least 11 times back and forth. XY-69 (10 μM) or WH-15 (10 μM) and liposomes (1 mM) were incubated in buffer at room temperature for 5 min in a total volume of 150 μl. The suspension was adjusted to 30% sucrose by the addition of 100 μl of 75% (w/v) sucrose in buffer followed by mixing. Buffer (200 μl) containing 25% (w/v) sucrose was then overlaid on the high-sucrose suspension followed by 50 μl of buffer containing no sucrose. The sample was centrifuged at 55,000 rpm in a Beckman swinging-bucket rotor (TLS 55) for 1 h at 4 °C. The bottom 350 μl and top 150 μl were manually collected using a syringe and adjusted to the same volume (350 μl). Each fraction was then diluted with 1× PLC buffer containing HEPES (50 mM, pH 7.4), KCl (70 mM), CaCl2 (3 mM), EGTA (3 mM), DTT (2 mM), and cholate (0.5%). Fractions containing XY-69 were diluted 1:50, whereas WH-15 fractions were diluted 1:3. PLC-δ1 was diluted to 10 ng/μl in 1× PLC buffer with 1 mg/ml of fatty acid-free BSA. In a PerkinElmer ProxiPlate™-384 Plus F black plate, 5 μl of the above reporter solution was mixed with 5 μl of PLC-δ1 or PLC dilution buffer. After incubation at room temperature for 6 h, fluorescence intensity was measured on a PerkinElmer Wallac EnVision 2103 multilabel reader as described before. The fluorescence intensity of the samples treated with PLC were subtracted with that of the samples treated with PLC dilution buffer. The fluorescence intensity differences were used to calculate the amount of XY-69 or WH-15, which was then used to calculate the reporter distribution.

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