Supporting Information for

Ex Uno Plura: Differential labeling of phospholipid biosynthetic pathways with a single bioorthogonal alcohol

Timothy W. Bumpus, Felice J. Liang, and Jeremy M. Baskin*

Department of Chemistry and Chemical Biology and Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, New York, USA

Contents

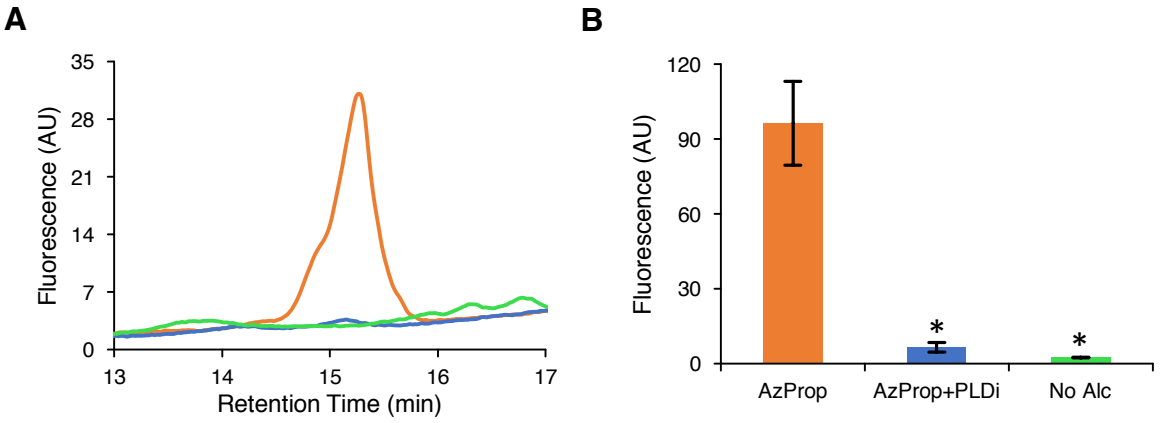

Figure S1. Extended AzProp labeling proceeds via PLD activity and not via the de novo PC biosynthesis pathway. HeLa cells were treated with AzProp (100 μM) or no alcohol for 24 h along with the pan-PLD inhibitor FIPI (PLD_i, 750 nM) or DMSO vehicle. After labeling, cellular lipids were extracted and tagged via SPAAC with BODIPY-BCN (2.5 μM for 16 h) and analyzed by HPLC with fluorescence detection. Shown are (A) representative chromatograms and (B) quantification of fluorescence from those chromatograms (area under the curve). Orange: AzProp; Blue: AzProp + FIPI; Green: no alcohol. For (B), n = 3 and * denotes $p < 0.05$.

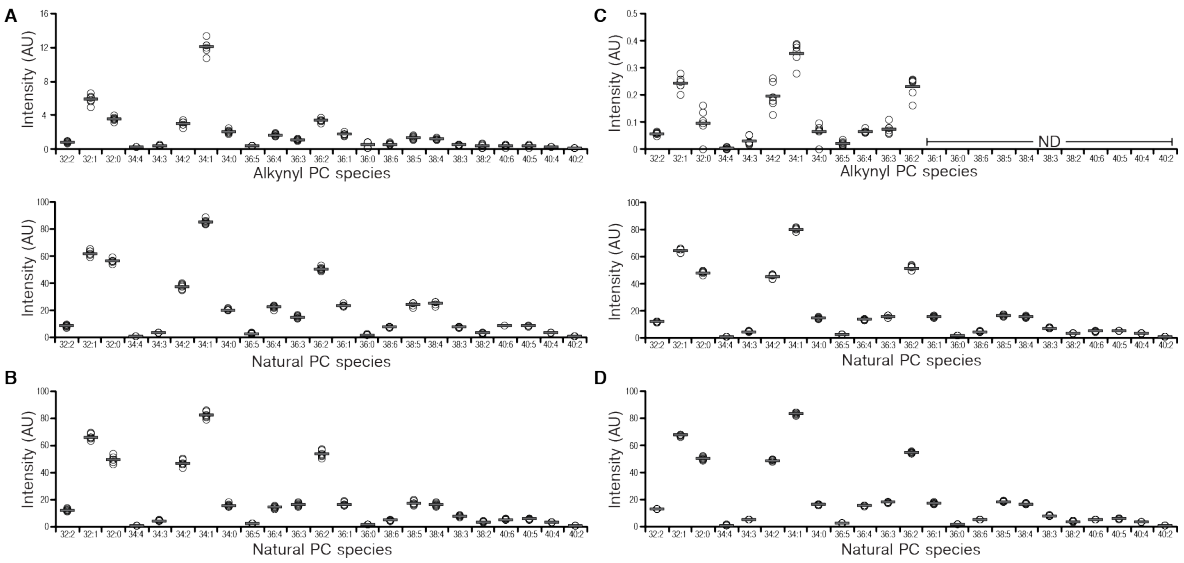

Figure S2. Lipidomics analysis of alkynyl and natural phosphatidylcholine (PC) species detected following ProCho labeling. HeLa cells were treated with (A) ProCho (100 μM) for 24 h, (B) no alcohol for 24 h, (C) ProCho (100 μM) and PMA (100 nM) for 20 min, or (D) no alcohol and PMA (100 nM) for 20 min. After labeling, the cells were lysed and lipid extracts were tagged via CuAAC with 6-azido-1-hexanol, followed by analysis by LC-ESI-MS. We note that this derivatization step increases the mass and modifies the polarity of the alkyne-labeled lipids, which facilitates their identification by LC-ESI-MS. Shown is quantification (area under extracted ion chromatogram curve) of alkynyl or natural PC species as indicated, denoted by total number of carbons in fatty acyl/ether tail:number of double bonds in tail. The intensity values on the y-axis represent relative ion abundances for the natural and alkynyl PC species shown within this figure. Given the similarity of all of these molecular species, the relative ion abundances are a good approximation for the relative amount of each lipid species. Hollow circles indicate value for each individual biological replicate (n = 6), and horizontal bar indicates mean. N.D., not detected.

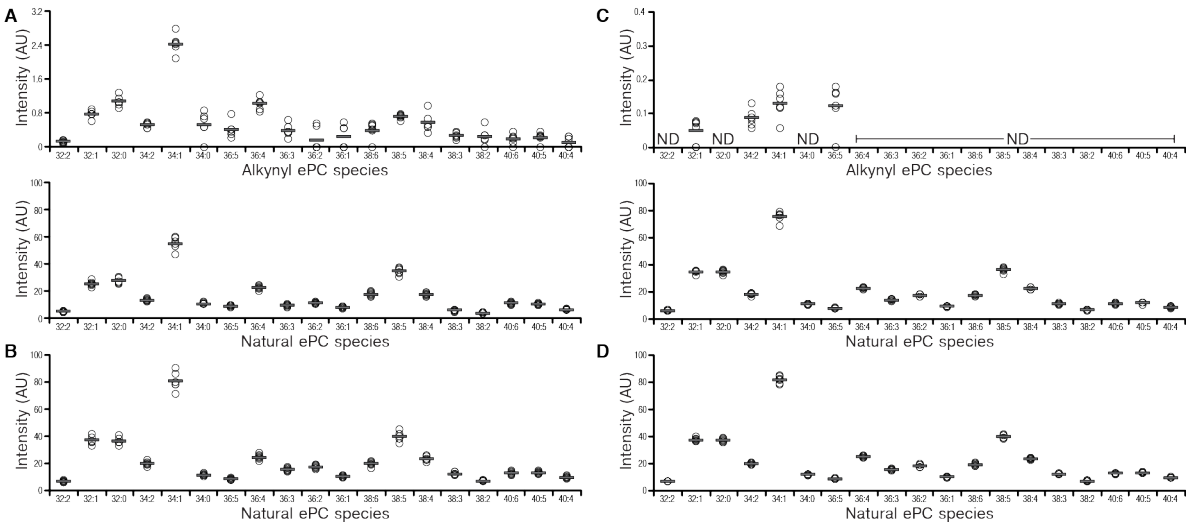

Figure S3. Lipidomics analysis of alkynyl and natural ether phosphatidylcholine (ePC) species detected following ProCho labeling. HeLa cells were treated with (A) ProCho (100 μM) for 24 h, (B) no alcohol for 24 h, (C) ProCho (100 μM) and PMA (100 nM) for 20 min, or (D) no alcohol and PMA (100 nM) for 20 min. After labeling, the cells were lysed and lipid extracts were tagged via CuAAC with 6-azido-1-hexanol, followed by analysis by LC-ESI-MS. We note that this derivatization step increases the mass and modifies the polarity of the alkyne-labeled lipids, which facilitates their identification by LC-ESI-MS. Shown is quantification (area under extracted ion chromatogram curve) of alkynyl or natural ePC species as indicated, denoted by total number of carbons in fatty acyl/ether tail:number of double bonds in tail. The intensity values on the y-axis represent relative ion abundances for the natural and alkynyl ePC species shown within this figure. Given the similarity of all of these molecular species, the relative ion abundances are a good approximation for the relative amount of each lipid species. Hollow circles indicate value for each individual biological replicate (n = 6), and horizontal bar indicates mean. N.D., not detected.

Materials and Methods

General materials and methods

All chemical reagents were analytical grade, obtained from commercial suppliers, and used without further purification, unless otherwise indicated. Where indicated, solvents were degassed by sparging with argon for 10 min. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Echelon Biosciences, 5-fluoro-2-indolyl deschlorohalopemide (FIPI) was purchased from Cayman Chemical; phorbol 12-myristate 13-acetate (PMA) was purchased from Santa Cruz Biotechnology; *Arachis hypogaea* PLD, Azide-fluor 488, and Azide-fluor 545 were purchased from Sigma-Aldrich. HPLC analysis was performed on a Shimadzu LC-20AR HPLC equipped with an SPD-20AV UV/Vis detector, an RF-20A fluorescence detector, and a Phenomenex Luna silica 3 μm 100 Å 25 cm x 4.6 mm column. LCMS analysis was performed on an Agilent 6230 electrospray ionization–time-of-flight (ESI–TOF) MS coupled to an Agilent 1260 HPLC equipped with a Phenomenex Luna silica 3 μm 100 Å 5 cm x 2.0 mm column. Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), and 0.05% trypsin-EDTA were purchased from Corning. Fetal bovine serum (FBS) was purchased from Thermo Fisher. Imaging was performed on a Zeiss LSM 800 confocal laser scanning microscope equipped with 20X 0.8 NA and 40X 1.4 NA Plan Apochromat objectives, 405, 488, 561, and 640 nm solid-state lasers, and two GaAsP PMT detectors. Image analysis was performed using the Zeiss Zen Blue 2.3 and FIJI software packages. All micrographs shown are maximum-intensity z-projections. Images shown in Figures 2–4 are representative of at least n = 15 different cells imaged in at least two independent experiments.

Synthetic procedures

3-azido-1-propanol[1], BODIPY-BCN[2], and ProCho[3] were prepared according to literature procedures. 6-azido-1-hexanol[4] was prepared using a method analogous to that described in ref. 1.

Cell culture

HeLa cells were maintained in a 5% CO_2, water-saturated atmosphere at 37 °C and grown in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Cell densities were maintained between 10^5 and $1.6x10^6$ cells/mL.

Generation of phosphatidyl propargylcholine by an *in vitro* PLD reaction

A 1.5 mL conical tube was charged with 10 μL of an 8 mg/mL chloroform solution of DOPC. The chloroform was removed under a stream of air. The DOPC was then dissolved in 1.5 μL of 50 mM SDS, 3 μL of 1 M sodium acetate (pH 5.6) and 19.5 μL of 118.5 mM ProCho in water. Subsequently, 3 μL of 500 mM calcium chloride and 2 μL of deionized water were added and the solution was vortexed. Finally, 1 μL of a freshly made solution of *Arachis hypogaea* PLD (2 U/μL) was added, and the reaction was placed at 30 °C for 90 min. The reaction was then diluted with 70 μL of PBS, 250 μL of methanol, 250 μL of chloroform, and 125 μL of 20 mM acetic acid. This solution was vortexed for 1 min and then centrifuged for 2 min at 16,000 x g. The organic layer was then removed and saved, an additional 250 μL of chloroform was added to the remaining aqueous layer, and the sample was vortexed and centrifuged as before. The two organic layers were then combined and dried under a stream of N_2.

Samples were subjected to CuAAC labeling based on a modified version of the method described by Thiele et al.[5] Briefly, to the lipid residue in a 1.5 mL Eppendorf tube was added 7 μL of degassed chloroform followed by 30 μL of a reaction master mix (2.95 μL of 11.72 mM Az488 in DMSO, 78 μL of 10 mM [acetonitrile]$_4$CuBF$_4$ in degassed methanol, and 312 μL of degassed ethanol). The tube was briefly flushed with argon and placed in a 42 °C water bath, such that the top half of the tube was not submerged. After 5 h, the reactions were diluted with 113 μL of a chloroform:methanol:water (73:23:3) mixture and filtered (0.45 μm) for analysis. Analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (80:19.5:0.5) and solvent B was chloroform: methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a linear gradient from 100% A to 100% B over 10 min, and 10 min of isocratic separation in 100% B.

Detection of cellular PLD activity by HPLC analysis with ProCho

HeLa cells (500,000) were seeded in a 60-mm dish and allowed to grow overnight. Cells were first treated with the indicated PLD inhibitor (FIPI/PLD$_i$, 750 nM; VU0359595/PLD1$_i$, 250 nM; VU0364739/PLD2$_i$, 350 nM) from 1000X DMSO stock solutions or an equivalent volume of DMSO for 30 min in Tyrode's-HEPES buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL glucose, 1 mg/mL bovine serum albumin, 20 mM HEPES, pH 7.4). The buffer then exchanged for Tyrode's-HEPES containing ProCho (100 μM) along with PMA (100 nM) and PLD inhibitor as necessary and incubated for 20 min. The treatment buffer was then aspirated, and the cells were rinsed with 1 mL cold PBS. The cells were then scraped from the dish and subjected to a modified Bligh-Dyer[6] extraction. In brief: 100 μL of cold PBS, 125 μL of cold acetic acid (20 mM in water), and 250 μL of cold methanol were added to the aspirated dish. The cells were

then scraped off the dish and transferred to 1.5 mL conical tubes. To the suspension was added 250 μL of chloroform. The mixture was then vortexed for 1 min and centrifuged at 16,000 x g for 2 min. The organic layer (bottom) was removed and placed in a separate conical tube, and a further 250 μL chloroform was added to the remaining aqueous layer. The vortexing and centrifugation steps were repeated, and the two organic layers were combined and dried under a stream of N_2.

Samples for analysis by HPLC with fluorescence detection were subjected to CuAAC labeling based on a modified version of the method described by Thiele et al.[5] Briefly, to the lipid residue in a 1.5 mL Eppendorf tube was added 7 μL of degassed chloroform followed by 30 μL of a reaction master mix (2.95 μL of 11.72 mM Az488 in DMSO, 78 μL of 10 mM $[acetonitrile]_4CuBF_4$ in degassed methanol, and 312 μL of degassed ethanol). The tube was briefly flushed with argon and placed in a 42 °C water bath, such that the top half of the tube was not submerged. After 5 h, the reactions were diluted with 113 μL of a chloroform:methanol:water (73:23:3) mixture and filtered (0.45 μm) for analysis. Analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (80:19.5:0.5) and solvent B was chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a linear gradient from 100% A to 100% B over 10 min, and 10 min of isocratic separation in 100% B.

Samples for analysis by LCMS were subjected to CuAAC labeling based on a modified version of the method described by Thiele et al.[5] Briefly, to the lipid residue in a 1.5 mL conical tube was added 7 μL of degassed chloroform followed by 30 μL of a reaction master mix (e.g., for 10 reactions, master mix contains 1 mg of 6-azido-1-hexanol (representing a substantial molar excess), 60 μL of 10 mM $[acetonitrile]_4CuBF_4$ in degassed acetonitrile, and 240 μL of degassed

ethanol). The tube was briefly flushed with argon and placed in a 42 °C water bath, such that the top half of the tube was not submerged. After 5 h, the reactions were diluted with 113 μL of a chloroform:methanol:water (73:23:3) mixture and filtered (0.45 μm) for analysis. LCMS analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (85:15:0.5) and solvent B was chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a linear gradient from 100% A to 100% B over 9 min. Phospholipid species were detected using a dual ESI source operating in positive mode, acquiring in extended dynamic range from m/z 100–1700 at one spectrum per second; gas temperature: 325 ºC; drying gas 10 L/min; nebulizer: 20 psig; fragmentor 300 V.

Detection of cellular PC biosynthesis by HPLC

HeLa cells (500,000) were seeded in a 60-mm dish and allowed to grow overnight. Cells were first treated with the indicated PLD inhibitor (FIPI, 750 nM) from 1000X DMSO stock solutions or an equivalent volume of DMSO for 30 min in DMEM. The media then exchanged for media containing the indicated alcohol (ProCho or AzProp (100 μM)) and PLD inhibitor as necessary and incubated for 24 h. The treatment media was then aspirated, and the cells were rinsed with 1 mL cold PBS. For analysis of the ProCho-labeled samples, the cells were subjected to lipid extraction, CuAAC tagging with Az488 (for HPLC) or 6-azido-1-hexanol (for LCMS), and fluorescence-coupled HPLC analysis or LCMS analysis was performed, respectively, as described in the previous section. For samples treated with AzProp, lipid extracts were generated using a modified Bligh-Dyer protocol as above, followed by SPAAC reaction with BODIPY-BCN (2.5 μM in a total reaction volume of 37 μL in a 73:23:3 mixture of chloroform:methanol:water)

overnight at 42 °C. These reactions were then diluted to a total volume of 150 μL by addition of 113 μL of a 73:23:3 mixture of chloroform:methanol:water and filtered (0.45 μm). Analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (95:7:0.5) and solvent B was chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a linear gradient from 100% A to 100% B over 25 min.

Labeling PLD activity with ProCho for imaging

HeLa cells (40,000 cells) were seeded on 12-mm round cover slips (#1.5) in the well of a 12-well dish and allowed to grow overnight. Cells were treated with ProCho (100 μM) and PMA (100 nM) for 20 min in Tyrode-HEPES buffer. The cells were then rinsed three times with PBS and fixed in 4% paraformaldehyde for 20 min. After three rinses with PBS and two with TBS, the cells were subjected to CuAAC labeling as previously described[7], by deposition of the cover slip on top of a 50 μL droplet of CuAAC reaction mixture, which was prepared immediately prior to use and consisted of these reagents added in the following order: 100 mM Tris pH 8.5, 5 μM Az545, 1 mM $CuSO_4$, and 50 mM sodium ascorbate. The cells were rinsed three times with TBS, three times with 500 mM sodium chloride, and three times with PBS. The cover slips were then mounted on glass microscope slides in ProLong Diamond with DAPI and allowed to solidify in the dark overnight at room temperature.

Labeling de novo PC biosynthesis with ProCho for imaging

Samples were prepared based on the protocol previously described by Salic and coworkers[3]. In brief, HeLa cells (40,000 cells) were seeded on 12-mm round cover slips (#1.5) in

the well of a 12-well dish and allowed to grow overnight. Cells were treated with ProCho (100 μM) in DMEM for 24 h with FIPI (750 nM) as indicated. The cells were then rinsed, fixed, labeled via CuAAC, and mounted on slides as described above.

Two-color labeling of PLD activity and PC biosynthesis with AzProp and ProCho for imaging

HeLa cells (40,000 cells) were seeded on 12-mm round cover slips (#1.5) in the well of a 12-well dish and allowed to grow overnight. For two-color labeling of PLD activity (Fig. 4A–B), cells were treated with ProCho (100 μM), AzProp (750 μM), and PMA (100 nM) for 20 min in Tyrode-HEPES buffer. For two-color labeling of PC biosynthesis and PLD activity (Fig. 4C–D), cells were treated with ProCho (100 μM) for 24 h in DMEM. After 24 h, the cells were rinsed three times with PBS and incubated with AzProp (1 mM) in Tyrode-HEPES buffer for 20 min, followed by addition of PMA (100 nM) for an additional 20 min as previously described [8].

In all cases the cells were then rinsed three times with PBS and labeled with BODIPY-BCN (1 μM) for 10 min at 37 °C, after which the cells were rinsed three times with PBS and incubated in Tyrode-HEPES buffer for 10 min. Cells were then fixed in 4% paraformaldehyde for 20 min, rinsed three times with PBS and two times with TBS, and subjected to CuAAC labeling as previously described[7], by deposition of the cover slip on top of a 50 μL droplet of CuAAC reaction mixture, which was prepared immediately prior to use and consisted of these reagents added in the following order: 100 mM Tris pH 8.5, 5 μM Az545, 1 mM $CuSO_4$, and 50 mM sodium ascorbate. The cells were rinsed three times with TBS, three times with 500 mM sodium chloride, and three times with PBS. The cover slips were then mounted on glass microscope slides in ProLong Diamond with DAPI and allowed to solidify in the dark overnight at room temperature.

Safety statement

No unexpected or unusually high safety hazards were encountered.

Supporting Information References

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