

Activity-Based Probes That Target Functional Subclasses of Phospholipases in Proteomes

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Phospholipases are a large and diverse set of enzymes that metabolize the phospholipid components of cell membranes^{1,2} and participate in key lipid-signaling pathways, such as those that generate eicosanoids³ and endocannabinoids.⁴ Although many phospholipases have been molecularly characterized, others remain poorly defined as unenriched activities in crude cell or tissue extracts. Phospholipases also share considerable sequence homology with other hydrolytic enzymes that accept distinct substrates, such as neutral lipids.² The identification of novel phospholipases would thus benefit from chemical probes that selectively target these enzymes on the basis of their distinct substrate specificities and catalytic properties. Here we present the synthesis and characterization of a set of activity-based protein profiling (ABPP⁵) probes that contain key recognition and reactivity elements for targeting functional subclasses of phospholipases. We show that the labeling profiles of these probes accurately report on the substrate specificity of both characterized and uncharacterized phospholipases in diverse proteomic backgrounds.

A substantial number of phospholipases belong to the serine hydrolase (SH) superfamily of enzymes, imparting upon them susceptibility to proteomic profiling using the fluorophosphonate (FP) class of ABPP probes. Original FP probes^{6,7} and variants thereof⁸ have rather general structures, which grant them broad reactivity across the SH class but does not inform on the possible substrate preferences of individual SH enzymes. To target serine phospholipases, we designed and synthesized phosphatidylcholine (PC) probes containing reactive FP elements at the *sn*-1 and *sn*-2 positions of a PC scaffold (**1** and **2**, respectively; Figure 1A and Supporting Figure 1) connected to an alkyne for click-chemistry conjugation to azide-reporter tags^{9,10} (Figure 1B). We hypothesized that these probes would prove more selective for reacting with phospholipases (over other SHs) than a general FP-alkyne probe (**3**; Figure 1A and Supporting Figure 2), as they resemble the natural substrates of these enzymes. Probes **1** and **2** might further distinguish between phospholipase A₁ (PLA₁) and A₂ (PLA₂) activities, thereby providing additional information on the substrate specificity of targeted enzymes.

For initial characterization of probes **1** and **2**, we assessed their reactivity with the mammalian serine phospholipase DDHD1, which shows selective PLA₁ (but not PLA₂) activity.¹¹ We also assessed the probe reactivity of DDHD2, an enzyme homologous to DDHD1 with less well characterized phospholipase activity. HEK293T cells were transfected with cDNAs encoding FLAG-tagged DDHD1 and DDHD2 enzymes. DDHD1 and DDHD2 were affinity-purified with an anti-FLAG antibody to afford enriched enzyme samples (Supporting Figure 3), and these enzymes were then treated with probe **1**, **2**, or **3** (10 μM) at 37 °C for 1 h. Labeled enzymes were subjected to click chemistry with rhodamine-N₃ (Rh-N₃) and analyzed by SDS-PAGE and in-gel fluorescence scanning (Figure 2). Both enzymes readily reacted with FP-alkyne **3**, as expected for serine

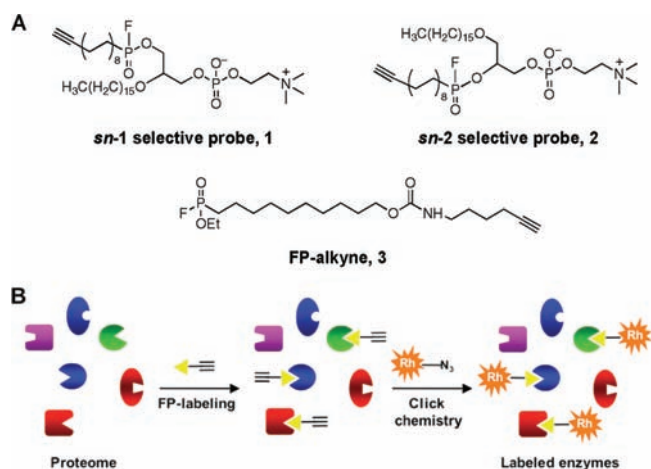


Figure 1. ABPP strategy for serine phospholipases. (A) FP probes that target functional subclasses of phospholipases. (B) Detection of probe-labeled enzymes by click chemistry. Rh = rhodamine.

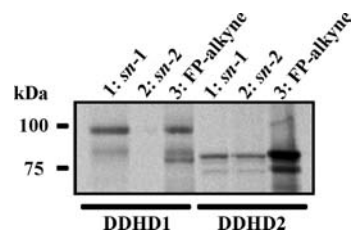


Figure 2. DDHD1 and DDHD2 labeling with FP probes. **1**–**3** (10 μM) were incubated with DDHD1 and DDHD2 for 1 h at 37 °C and reacted with Rh-N₃ using click chemistry, after which the reactions were visualized by SDS-PAGE and in-gel fluorescence scanning (fluorescent gel shown in grayscale).

phospholipases. Notably, DDHD1 showed much greater reactivity with **1** than with **2**, consistent with the reported PLA₁-selective catalytic activity of this enzyme.¹¹ In contrast, DDHD2 displayed similar reactivities with the two probes **1** and **2**. To quantify the relative reactivity of **1** and **2** with DDHD lipases, we treated DDHD1 and DDHD2 with the probes at concentrations ranging from 3 nM to 50 μM and quantified the labeling signals. Probe **1** potently reacted with DDHD1, showing half-maximal labeling at ~300 nM, while probe **2** exhibited much weaker labeling (partial labeling at 20–50 μM; see Supporting Figure 4). The two probes reacted similarly with DDHD2, showing half-maximal labeling at low-micromolar concentrations (1–3 μM). These data demonstrate that probes **1** and **2** accurately report on the PLA₁-selective catalytic activity of DDHD1 and further suggest that DDHD2, which displays similar reactivities with the two probes, might exhibit both PLA₁ and PLA₂ activity.¹²

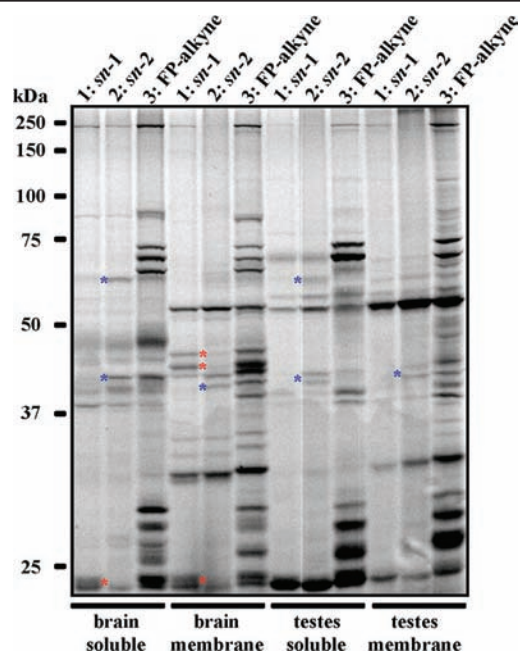


Figure 3. ABPP of mouse tissue proteomes with probes 1–3. Soluble and membrane proteomes (2 mg/mL) were incubated with 1 or 2 (10 μ M) or 3 (2 μ M) for 1 h at 37 $^{\circ}$ C. Probe-labeled enzymes were reacted with Rh–N₃ using click chemistry, resolved by SDS-PAGE, and detected by in-gel fluorescence scanning. Red and blue asterisks mark proteins selectively labeled with the *sn*-1 and *sn*-2 probes, respectively.

We next compared the labeling profiles of probes 1–3 in mouse tissue proteomes (Figure 3). Several proteins showed selective reactivity with either 1 (red asterisks) or 2 (blue asterisks). Interestingly, a subset of 2-labeled proteins detected in the soluble testis proteome did not react with either 1 or 3, suggesting that they may be PLA₂-type enzymes that preferentially react with substrate-like ABPP probes.

Some important phospholipase activities have to date been characterized only in tissue/cell extracts, and the enzyme(s) responsible for these activities remain unidentified. One such phospholipase activity is the calcium-dependent transacylase (CDTA) enzyme implicated in the biosynthesis of *N*-acylethanolamines (NAEs), including the endocannabinoid anandamide.⁴ CDTA activity has been detected in specific rodent tissues, such as brain,¹³ where it transfers the *sn*-1 acyl chain of PC to phosphatidylethanolamine (PE) to form *N*-acyl-PE (NAPE), which is then converted to NAE by one of multiple pathways.⁴ Despite extensive research efforts,^{13,14} the molecular identification of CDTA has not yet been achieved, likely indicating that it is a very low abundance enzyme. Affinity probes that selectively react with CDTA could prove to be of value for enriching and identifying this enzyme from tissue proteomes. Toward this end, we found that CDTA is inhibited by the *sn*-1 probe 1 with much greater potency than the *sn*-2 probe 2 (IC₅₀ values of 3 and 165 nM, respectively; Figure 4). Future comparative ABPP studies that characterize the protein targets of probes 1 and 2 over a concentration range where they show differential reactivity with CDTA (yellow box, Figure 4) may facilitate the identification of this enzyme.

In conclusion, we have synthesized and characterized ABPP probes that target functional subclasses of serine phospholipases. Placement of an alkyne-conjugated FP reactive group in the *sn*-1

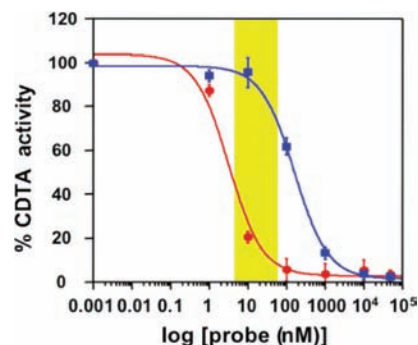


Figure 4. CDTA inhibition with probes 1 and 2. Rat brain cortical membrane proteome (50 μ g) was incubated with 1 or 2 (0.001 nM–50 μ M) in the presence of 2 mM dithiothreitol, 6 mM CaCl₂, and 0.5% NP₄₀ for 1 h at 37 $^{\circ}$ C and assayed for CDTA activity using [1,2-¹⁴C]PC. Formation of NAPE was monitored by thin-layer chromatography and visualized by autoradiography. Curves represent average values \pm standard error (SE) for three experiments (see Supporting Figure 5 for primary autoradiography data). The yellow box marks a concentration range over which probe 1 selectively inhibited CDTA activity.

and *sn*-2 positions of PC provided probes 1 and 2, respectively, whose reactivity profiles accurately reported on the *sn*-1 specificity of both the DDHD1 and CDTA enzymes. Since several additional proteins that selectively reacted with 1 or 2 were identified in tissue proteomes, we anticipate that these probes and variants thereof¹⁵ will facilitate the discovery of new serine phospholipases with *sn*-1 and/or *sn*-2 specificity.

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Supporting Information Available: Synthesis, experimental protocols, and figures showing overexpression of DDHD1 and DDHD2 and substrate assays with CDTA, DDHD1, and DDHD2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- We attempted to characterize the activity of DDHD2 with PC substrates containing *sn*-1 ester/*sn*-2 ether or *sn*-1 ether/*sn*-2 ester linkages (Supporting Figure 6), but unlike DDHD1, DDHD2 did not accept substrates having ether linkages at the *sn*-1/*sn*-2 positions.
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- Phospholipases can also show head-group selectivity, and we therefore envision that variants of probes 1 and 2 in which the choline group is replaced with serine, ethanolamine, or inositol may also prove useful for characterizing additional types of phospholipases.

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