Analytical Biochemistry xxx (2015) 1–7

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Click chemistry armed enzyme-linked immunosorbent assay to measure palmitoylation by hedgehog acyltransferase

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ARTICLE INFO

Article history:
Received 26 June 2015
Received in revised form
20 August 2015
Accepted 21 August 2015
Available online xxx

Keywords:
Hedgehog acyltransferase
Protein palmitoylation
Click chemistry
MBOAT

ABSTRACT

Hedgehog signaling is critical for correct embryogenesis and tissue development. However, on maturation, signaling is also found to be aberrantly activated in many cancers. Palmitoylation of the secreted signaling protein sonic hedgehog (Shh) by the enzyme hedgehog acyltransferase (Hhat) is required for functional signaling. To quantify this important posttranslational modification, many in vitro Shh palmitoylation assays employ radiolabeled fatty acids, which have limitations in terms of cost and safety. Here we present a click chemistry armed enzyme-linked immunosorbent assay (click–ELISA) for assessment of Hhat activity through acylation of biotinylated Shh peptide with an alkyne-tagged palmitoyl-CoA (coenzyme A) analogue. Click chemistry functionalization of the alkyne tag with azido-FLAG peptide allows analysis through an ELISA protocol and colorimetric readout. This assay format identified the detergent *n*-dodecyl β-D-maltopyranoside as an improved solubilizing agent for Hhat activity. Quantification of the potency of RU-SKI small molecule Hhat inhibitors by click–ELISA indicated IC_{50} values in the low- or sub-micromolar range. A stopped assay format was also employed that allows measurement of Hhat kinetic parameters where saturating substrate concentrations exceed the binding capacity of the streptavidin-coated plate. Therefore, click–ELISA represents a nonradioactive method for assessing protein palmitoylation in vitro that is readily expandable to other classes of protein lipidation.

Posttranslational modification (PTM) of proteins through S-acylation of cysteine residues with predominantly C16:0 fatty acids (termed *palmitoylation*) is one of the most common forms of PTM. The palmitoyl acyltransferase family of proteins is associated with a range of diseases, including neurological disorders and cancer [1]. Classically, protein lipidation has been studied through the application of radiolabeled lipids to facilitate detection [2]. However, such methods are limited in their utility due to requirements for lengthy detection times, the hazards of using radioactive materials, and the associated high costs of materials and disposal. With the advent of the click chemistry era, the study of protein lipidation has advanced significantly. Here, fatty acids labeled with azide or alkyne bioorthogonal reporters undergo copper(I)-catalyzed (3 + 2) cycloaddition for the attachment of reporter moieties. Such reporters have allowed detailed analysis of lipidation through a range of techniques, including Western blotting and in-gel

Abbreviations: PTM, posttranslational modification; HTS, high-throughput screen; GOAT, ghrelin-*O*-acyltransferase; cat–ELCCA, catalytic assay using an enzyme-linked click chemistry assay; CoA, coenzyme A; HRP, horseradish peroxidase; Hhat, hedgehog acyltransferase; MBOAT, membrane bound *O*-acyltransferase; Shh, sonic hedgehog; Hh, hedgehog; click–ELISA, click chemistry armed enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; HEK293a, human embryonic kidney 293a; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT, room temperature; PBS, phosphate-buffered saline; YnC_{15}, heptadec-16-ynoic acid; BSA, bovine serum albumin; TCEP, tris(2-carboxyethyl) phosphine; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; OTG, *n*-octyl β-D-glucopyranoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DDM, *n*-dodecyl β-D-maltopyranoside.

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http://dx.doi.org/10.1016/j.ab.2015.08.025
0003-2697/

fluorescence [3]. One of the most powerful applications of click chemistry to in vivo studies is through affinity enrichment and proteomic analysis. This approach has identified changes in the N-myristoylated proteome during the cell cycle and vertebrate development [4,5] and has been applied to validate the lipid transferase *N*-myristoyl transferase as an antimalarial drug target in *Plasmodium falciparum* [6].

During recent years, click chemistry has also been applied to in vitro studies of lipidation to afford a nonradioactive high-throughput screen (HTS) format [7]. The enzyme ghrelin-*O*-acyltransferase (GOAT), which is responsible for the octanoylation of the growth hormone releasing peptide ghrelin, was assessed through a catalytic assay using an enzyme-linked click chemistry assay (cat–ELCCA). A biotinylated substrate ghrelin peptide is bound to a streptavidin-coated plate and incubated with GOAT-containing membrane fractions and alkynyl-tagged *n*-octanoyl-CoA (coenzyme A). The resulting alkynylated peptide is then subjected to labeling via click chemistry with azido-HRP (horseradish peroxidase) to facilitate product detection through fluorogenic deacetylation of Amplex Red in the presence of hydrogen peroxide catalyzed by HRP [7]. This methodology allowed the assessment of GOAT activity and measurement of kinetic parameters along with screening to identify small molecule inhibitors [8].

Inspired by the success of the cat–ELCCA approach, we sought to develop a method to measure activity and kinetics of the enzyme hedgehog acyltransferase (Hhat). Hhat is a multipass transmembrane protein [9,10] and, like GOAT and porcupine, is a member of the membrane bound *O*-acyltransferase (MBOAT) family of enzymes. MBOATs show similar topology in regions surrounding conserved residues that are required for catalysis [11]. Hhat is responsible for the palmitoylation of sonic hedgehog (Shh), a secreted morphogen that is involved in neurogenesis during embryonic development and is aberrantly activated in mature tissues leading to carcinogenesis [12,13]. Shh is palmitoylated via an amide linkage on the N-terminal cysteine following signal peptide cleavage, most likely by initial palmitoylation on the side chain of the cysteine residue, with the palmitate group subsequently undergoing an S–N acyl shift to the N terminus [14]. Palmitoylation of Shh has been shown to be essential for Shh signaling, thereby making Hhat an attractive target for therapeutic intervention and as a tool to investigate the hedgehog (Hh) pathway [15]. Indeed, a class of 5-acyl-6,7-dihydrothieno[3,2-*c*] pyridines was recently identified as inhibitors of Hhat using highly ionizing ^{125}I-labeled palmitoyl-CoA in a scintillation proximity assay HTS [16]. However, contrary to the proposed therapeutic benefit of Hh pathway inhibition, several recent publications have demonstrated that formation of an Hh signaling promoted stromal matrix around tumors actually results in restriction of tumor growth [17–19]. This dramatic contrast in the prognosis of Hh inhibition highlights the need for better understanding of the Hh pathway, requiring both improved assays and chemical tools. To date, many studies of Hhat activity have employed radiolabeled palmitate [14,16,20–23], which may impede both the analysis and development of existing inhibitors and the identification of new alternative series with improved properties. To this end, we employed a click chemistry armed enzyme-linked immunosorbent assay (click–ELISA) format to study Hhat activity, kinetic parameters, and assessment of Hhat inhibitors.

Materials and methods

Plasmid construction and cell culture

Human Hhat cDNA (accession number BC117130) base pairs 4 to 1479 was amplified by polymerase chain reaction (PCR) (forward primer: AGGAGATATACCATGCTGCCCCGATGGGAACTGG; reverse primer: CAGAACTTCCAGTTTGTCCGTGGCGTAGGTCTGGGC) and Q4 inserted by ligation independent In-Fusion cloning (Takara Bio/Clontech, France) [24] into the expression vector, pOPINEneo-3C-FLAG. This vector was produced by incorporating a C-terminal 3C protease cleavage site, followed by a C-terminal FLAG and 8 × histidine epitope into the plasmid, ptriex2neo (Novagen). The human embryonic kidney 293a (HEK293a) line was transfected with pOPINEneo-Hhat-3C-FLAG-His$_8$ using TurboFect (Thermo Scientific) according to the manufacturer's instructions. Single colonies were ring cloned and selected for stable transfection through culturing in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 9% fetal bovine serum (Invitrogen) and 500 μg/ml G418 (Sigma) for at least five passages. Resistant cells were assessed by anti-polyhistidine immunoblotting, and the highest expressing cell line was selected for further protein expression. Cells were passaged as described previously [15].

Immunoblotting

Protein samples were supplemented with reducing NuPAGE Sample Buffer (Fisher), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 15% gel, and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore). Membranes were blocked at room temperature (RT) for 1 h in phosphate-buffered saline (PBS) with 5% skimmed milk and then incubated with α-polyhistidine-HRP monoclonal antibody (1:3000, R&D Systems) for 16 h at 4 °C. Bound immunocomplexes were detected with ECL Plus (Pierce) and visualized on an Ettan DIGE Imager (GE Healthcare). For normalization of Hhat concentration, band intensities were quantified using ImageQuant software (GE Healthcare).

Protein expression and solubilization

Buffers and conditions for Hhat expression and activity were taken from existing literature protocols [14] with the following modifications: HEK293a cells stably transfected with pOPINE-Hhat-3C-FLAG-His$_8$ were grown to 90% confluence in 2 × T175 flasks, harvested via trypsinization, washed with 10 ml of 1 × PBS, and stored at −80 °C. Pellets were lysed as described in the literature [14]. Unlysed cells were removed by centrifugation at 1000 rcf for 10 min at 4 °C, and the resulting supernatant was separated into soluble (S100) and membrane (P100) fractions through centrifugation at 100,000 rcf for 1 h at 4 °C. The P100 fraction was resuspended in 8 ml of solubilization buffer (20 mM Hepes [pH 7.3], 350 mM NaCl, and 5% glycerol) supplemented with 1% (w/v) detergent and incubated for 60 min on ice, followed by centrifugation at 100,000 rcf for 1 h at 4 °C to afford a solubilized membrane fraction [P100(sol)] and nonsolubilized material [P100(n/sol)], which were stored at −80 °C.

Peptide and inhibitor synthesis

Heptadec-16-ynoic acid (YnC$_{15}$) was synthesized according to existing literature protocols [25,26] and coupled to CoA using 1,1′-carbonyl-diimidazole activation. 4-Azidobutyric acid was prepared in two steps from ethyl 4-bromobutyrate. Residues 1 to 11 of the mature Shh protein (CGPGRGFGKRK) were used as the substrate for Hhat-catalyzed reactions, and Shh(1–11)-PEG$_3$-biotin, YnC$_{15}$-Shh(1–11)-PEG$_3$-biotin, and azido-FLAG (DYKDDDDK) peptides were synthesized using standard solid phase peptide synthesis protocols; full experimental information and characterization can be found in the online supplementary material. Synthesis of RU-SKI inhibitors was performed following our previously described

Please cite this article in press as: T. Lanyon-Hogg, et al., Click chemistry armed enzyme-linked immunosorbent assay to measure palmitoylation by hedgehog acyltransferase, Analytical Biochemistry (2015), http://dx.doi.org/10.1016/j.ab.2015.08.025