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Chemical Reporters for Exploring Protein Acylation

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Abstract

Proteins are acylated by a variety of metabolites that regulates many important cellular pathways in all kingdoms of life. Acyl groups in cells can vary in structure from the smallest unit, acetate, to modified long chain fatty acids, all of which can be activated and covalently attached to diverse amino acid side chains and consequently modulate protein function. For example, acetylation of Lys residues can alter the charge state of proteins and generate new recognition elements for protein–protein interactions. Alternatively, long chain fatty-acylation targets proteins to membranes and enables spatial control of cell signalling. To facilitate the analysis of protein acylation in biology, acyl analogues bearing alkyne or azide tags have been developed that enable fluorescent imaging and proteomic profiling of modified proteins using bioorthogonal ligation methods. Herein, we summarize the currently available acylation chemical reporters and highlight their utility to discover and quantify the roles of protein acylation in biology.

Keywords

protein acylation; chemical reporters; chemical proteomics; acetylation; fatty-acylation; post-translational modification

Introduction

Protein acylation, the post-translational and covalent attachment of acyl groups to proteins, is a widespread protein modification (Fig 1). The reaction is generally catalyzed by acyl-specific transferases, and typically involves the transfer of an acyl group from acyl-Coenzyme A (acyl-CoA) to specific amino acids and positions of substrate proteins. Protein acylation is conserved in all kingdoms of life and is a key regulator of numerous cellular pathways. Acylation can be classified according to the modified amino acids and the length/structure of acyl groups (Fig 1). Protein acylation can have various functions in cells; small acyl group, such as formyl and acetyl, work as recognition elements for protein–protein interactions while long chains of fatty acids, such as palmitate, can target proteins to membranes and affect signal transduction. Acylation typically occurs on amino acid residues (Lys, Cys, Ser/Thr) bearing nucleophilic side chains ($-\text{NH}_2$, $-\text{SH}$, $-\text{OH}$) and therefore referred as N-acylation (amide linkage), S-acylation (thioester linkage) or O-acylation (ester linkage), respectively. There are a few exceptions where acylation occurs at the protein N-

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terminus, such as N-terminal acetylation, *N*-myristoylation on Gly or *N*-palmitoylation on Cys residues.

Over the past few years, several chemical reporters of protein acylation have been developed for fluorescent detection and proteomic profiling of modified proteins using bioorthogonal ligation methods. This review describes the currently available acylation chemical reporters and highlights their utility to discover and quantify the roles of protein acylation in biology. We will first summarize the tools available to study N-acylation, including ϵ -N-Lys acylation (Fig 1A) and N-terminal acetylation (Fig 1B), N-fatty-acylation (*N*-myristoylation and *N*-palmitoylation) (Fig 1C), then the tools available to study S-fatty-acylation (S-palmitoylation) (Fig 1D) and O-acylation, including Ser/Thr acylation (Fig 1E) and Ser/Thr-palmitoylation (Fig 1F).

Chemical reporter strategy to detect protein acylation

Before the invention of bioorthogonal reactions, several methods were employed to study protein acylation. The universal method involved metabolic labelling with radioactive [^3H], [^{14}C] or [^{125}I] acyl derivatives and detection by fluorography [1–3]. These assays suffered from the inherent hazards associated with radioactivity, the high cost of reagents, as well as low sensitivity and long exposure time, from days to months. Alternatively, post-translational modifications can be directly analysed by mass spectrometry [4]. The method has proved useful to study protein acetylation or other small acyl group modifications, but efforts to detect other long-chain fatty acid modifications have been more challenging due to potentially labile linkages and hydrophobic properties of modified peptides [5,6]. The analysis of complex mixtures is even more challenging and often requires fractionation of the sample or purification of the protein of interest. Other methods are available to study specific protein acylation, such as anti-acetyl-Lys antibodies which can be used to detect as well as enrich acetylated proteins [7], but antibodies for lipid modifications have been difficult to generate. The unique chemical reactivity of fatty acid modifications can also be exploited. For example, acyl-biotin exchange (ABE) and acyl-resin-assisted capture (acyl-RAC) have been developed to enrich and analyse S-acylated proteins due to their sensitivity towards hydroxylamine cleavage [8].

Over the past decade, the considerable advances in bioorthogonal reactions have permitted the development of a chemical reporter strategy to study protein acylation (Fig 2) [9]. The strategy is based on the introduction of a small chemical tag to the acyl modification of interest. The tag is usually small (alkyne or azide reporter) as it needs to be accommodated in the acyltransferases. Ideally, alkyne- or azide-tagged acyl reporters should mimic the chain length of the natural acyl substrate and be transferred with similar efficiency to the proteins (Table 1). Acylation reporters can be employed to metabolically label proteins in cells (acetate or acid reporter) and *in vitro* (acyl-CoA reporter) (Fig 2A,B). The alkyne or azide tag enables the bioorthogonal reaction with fluorophores for rapid and sensitive visualization of tagged proteins by in-gel fluorescence or affinity tags (biotin) for selective enrichment of tagged proteins for western blot analysis and mass-spectrometry-based proteomics (Fig 2C). Alkyne and azide-tagged reporters have been developed for the detection of protein short chain acylation [7,10], *N*-myristoylation [8,11,12] and S-

palmitoylation in eukaryotes [8,11–13] as well as fatty-acylated proteins in bacteria [14] (Table 1). Azido-fatty acids were initially developed and are compatible with Cu^I-catalyzed azide-alkyne cycloaddition (CuAAC), Staudinger ligation, strain-promoted alkyne-azide cycloaddition (SPAAC) (Fig 2A), but alkyne-tagged reporters have been used in most studies as they yield the most specific and efficient two-step labelling with CuAAC (Fig 2B) [15]. Recently, quantitative proteomics methods such as SILAC (stable isotope labelling by amino acids in cell culture) [16], have also been used in combination with acylation chemical reporters to quantify protein acylation. This technique allows the evaluation of signal/background ratios to identify acylated proteins with high confidence as well as the comparison of the levels of protein acylation in various biological samples. The improved detection and proteomic analysis of acylated proteins have enabled the discovery of novel modified proteins, characterization of endogenous regulatory mechanisms and specificity of small molecule inhibitors targeted at acylation enzymes.

ε-N-Lys acylation

Acylation of Lys side chains is a highly conserved protein post-translational modification that plays key roles in the regulation of protein function in a variety of biological pathways, most notably in the epigenetic control of gene expression [17]. The most prominent type of Lys acylation is acetylation, the attachment of a C2-acid to ε-amine side-chain (Fig 1A). This modification has been widely studied for histone modifications and its role in gene regulation. Histones are acylated by Lys acetyltransferases (KAT) to activate transcription and deacetylated by Lys deacetylases (KDACs) to repress transcription. In addition, proteins are often modified by other types of acylation, such as formylation, propionylation, butyrylation, crotonylation, malonylation, succinylation, myristoylation. In recent years, there has been increasing evidence that non-enzymatic acylation can lead to protein modification [18] and it has been suggested that sirtuins are key deacylases of these modifications. To address the substrate specificity of specific acetyltransferases and deacetylases, alkyne analogues of Lys acetylation have been developed [19] (Table 1). In-gel fluorescence and proteomics analysis showed that 4-pentynoyl-CoA and 4-pentynoate label known and putative Lys-acetylated proteins *in vitro* and in cells respectively. *In vitro* studies with 4-pentynoyl-CoA and purified Lys acetyltransferase p300 in cell lysate allowed the identification of the acetyltransferase-specific substrates [20]. Mass spectrometry analysis of proteins labelled with 4-pentynoyl-CoA or 4-pentynoate confirmed that these chemical reporters were incorporated onto Lys residues. Nevertheless, these probes could also be employed to study N-terminal acetylation and Ser/Thr acetylation. Recently, an alkyne-tagged chemical reporter of malonylation, malAM-yne, has been reported [10] (Table 1). 2-Propargyl-malonate (mal-yne) was masked by acetoxymethyl (AM) protecting group, later hydrolysed in cells by lipases, in order to enhance cell-permeability. The probe was combined with chemical proteomics to identify some known malonylated proteins as well as more than 300 putative substrates of malonylation.

To identify specific substrates of the three major families of lysine acyltransferases (KATs), GCN5/PCAF, MYST and p300/CBP families, modified acylation chemical reporters were developed for engineered KATs [21]. Several residues in the active site of the KAT of interest were mutated to accommodate larger size acyl groups. Azide and alkyne-tagged

acyl-CoA analogues of different chain lengths were prepared and their ability to be transferred to N-terminal 20 amino acid H3 peptide or to proteins in cell lysate by wild-type or engineered KAT enzyme was tested *in vitro*. 5-Hexynoyl-CoA gave high selectivity for the engineered GCN5 (T612G/F622A and T612G/L531) and MYST family enzyme (MOF I317A and I317A/L531A) and 3-azido-propanoyl CoA was also a suitable cofactor for MOF I317A. The KAT p300 could accommodate 4-pentynoyl CoA as previously described [19] but also 3-azido-propanoyl-CoA. While these studies have only been carried out *in vitro*, cell-permeable probes in combination with engineered KATs could be employed to study specific substrates in cells.

ϵ -N-Lys fatty-acylation

Lys side-chain fatty acylation was discovered more than 20 years ago when TNF- α and Interleukin-1- α were shown to be modified by myristate using mass spectrometry [22,23]. This Lys modification has received more interest recently as chemical reporter of fatty-acylation, alk-14, and *in vitro* biochemical assays revealed that Sirt6, a sirtuin-family deacylase, can remove long-chain fatty-acids, such as myristate, on Lys side-chains [24]. This process was shown to be critical for the secretion of the proinflammatory cytokine TNF- α in mammalian cells. Subsequently, *in vitro* studies have revealed the specificity of the seven sirtuin enzymes for deacylating Lys modified with a wide range of fatty acids [25]. More recently, a photocrosslinker chemical probe based on a Lys-myristoylated histone H3 peptide was developed to assess the ability of five human sirtuins to recognize fatty-acylation of Lys side chains *in vitro* [26]. Interestingly, Sirt2 was more readily labelled by the probe, indicating that Sirt2 may have a higher deacylase activity than Sirt6 and *in vitro* biochemical assays were performed to confirm this difference [26]. Subsequently, comparison of alk-14 labelling in HeLa cells subjected to a knockdown or chemical inhibition of Sirt2 revealed an increase of labelling of several proteins by in-gel fluorescence when Sirt2 activity was decreased. While alk-14 labels myristoylated proteins and fatty-acylated Lys, an increase of labelling of several proteins in the sample missing Sirt2 activity revealed that acylation of Lys for these proteins was regulated by Sirt2 in cells. The large-scale proteomic analysis of Lys side-chain acylation using the chemical reporter of fatty-acylation combined with a knockdown or inhibition of specific deacylase enzymes should shed some light on the extent of this protein modification as well as its function and regulation.

N-myristoylation

Protein N-myristoylation describes the covalent modification of N-terminal Gly residues with a C-14 fatty-acid (myristate) [27]. This fatty acid is transferred from myristoyl-CoA to substrate proteins by N-myristoyl transferase (NMT). In humans and high organisms, NMT is encoded by two genes (*nmt1* and *nmt2*) while in lower eukaryotes, only one gene has been characterised. N-myristoylation increases proteins' hydrophobicity and thus membrane association. It has been associated with the development and progression of many diseases, such as microbial and viral infections, cancer or more recently to the Fragile X syndrome [27,28]. While this modification mostly occurs co-translationally, a few examples of post-translationally modified proteins, such as the pro-apoptotic protein BID, have been reported.

Post-translational myristoylation is typically preceded by caspase cleavage of the protein in the context of apoptosis, which yields proteins with *de novo* N-terminal Gly residues [27].

Alkyne and azide-analogues of myristic acid have been developed to study *N*-myristoylation (Table 1) [8,11,12], but alk-12 is the most widely used reporter [29]. While this chemical reporter labels *N*-myristoylated proteins, it was shown to label other types of lipid-modified proteins, such as *S*-palmitoylated proteins [30] and GPI-anchored proteins [31]. To distinguish *N*-myristoylation from these other forms of fatty-acylation cell lysates were treated with hydroxylamine to hydrolyse thioester and ester bonds found in *S*-palmitoylated and GPI-anchored substrates, which facilitated selective detection and profiling of *N*-myristoylated proteins [31]. Recently, a combination of potent NMT inhibitors, alk-12 and chemical proteomic were employed to identify known and novel *N*-myristoylated proteins in malaria parasites [31]. Alk-12 was combined with in-gel fluorescence to confirm that the NMT inhibitors were acting on-target in cells, as seen by a decrease of labelling by in-gel fluorescence when the concentration of inhibitor was increased, which helps validate NMT as an antimalarial drug target. The strategy was further refined and applied to study co- and post-translational *N*-myristoylation in cancer cells [32]. A concentration range of the inhibitor, combined with alk-12 labelling and quantitative proteomics, allowed the identification of >100 targets of myristoylation in a native context in mammalian cells, including more than 30 post-translational *N*-myristoylated substrates. Additionally, new reagents allowing the direct identification of the site of *N*-myristoylation by mass spectrometry were described. Furthermore, the application of alk-12 in the context of bacterial infection has revealed the first example of hydrolysis of the myristoyl moiety at the N-terminal Gly of host proteins, by a secreted bacterial effector, IpaJ [33]. This cleavage is irreversible since the N-terminal glycine is also cleaved from the host protein. This unique proteolytic activity was shown to contribute to the virulence of bacterial pathogen *Shigella flexneri*.

***N*-palmitoylation**

Palmitoylation has also been shown to occur at N-terminal Cys residues of several proteins, via an amide linkage. Reported examples to date include the Gα_s subunit and secreted signalling proteins, the Hedgehog proteins and Spitz [34]. Although significantly less studied, this modification has recently been shown to be essential to Sonic Hedgehog signalling, using alk-15 in human carcinoma cells [35]. Abnormal Sonic Hedgehog signalling has been implicated in the proliferation of numerous cancer cell lines. Interestingly, a decrease of Sonic hedgehog palmitoylation, catalysed by Hedgehog acyltransferase, was shown to decrease Sonic hedgehog signalling, proliferation and invasiveness of human carcinoma cells.

***S*-palmitoylation**

S-Palmitoylation is the reversible attachment of a 16 carbon-long fatty acid chain to Cys residues of a protein via a thioester linkage. *S*-Palmitoylation is catalysed by palmitoyltransferases, commonly referred to as DHHC-enzymes, due to their conserved Asp-His-His-Cys catalytic motif [36]. *S*-Palmitoylation is a dynamic and regulated process

[37] that can be reversed by the action of thioesterases. S-palmitoylation increases the protein affinity for membranes and facilitates their correct subcellular localization [37]. While several chemical reporters of S-palmitoylation have been reported (Table 1), alk-16 is the optimal reporter as shorter chains can also label *N*-myristoylated proteins [15].

There are several recent examples of the application of alk-16, in identifying new S-palmitoylated proteins by chemical proteomics, in a wide-range of organisms. These studies have greatly expanded the number of fatty-acylated proteins to several hundreds and revealed new roles for S-palmitoylation [30,38,39]. S-Palmitoylome profiling in DC2.4 immune cells revealed that the Interferon-induced transmembrane protein 3 (IFITM3) [38] and Toll-like receptor 2 (TLR2) [40], are S-palmitoylated and further studies have highlighted the role of these modifications in immunity. Notably, S-palmitoylation of IFITM3 is crucial for host resistance to influenza virus infection [38] while S-palmitoylation of TLR2 plays a critical role in the inflammatory response to microbial ligands [40].

Palmitic acid chemical reporter labelling has also been employed to study protein S-palmitoylation dynamics in cells. In fission yeast, alk-16 labelling revealed an increase of S-palmitoylation during meiosis compared to vegetative growth [41]. This alteration was shown to be dependent on the DHHC-palmitoyltransferase Erf2 as no increase of palmitoylation was observed in Erf2-deficient cells. To characterize proteins which are fatty-acylated by Erf2 during meiosis, alk-16 labeling was combined with comparative proteomics. A small GTPase Rho3 was shown to be preferentially S-palmitoylated in meiotic cells and further experiments revealed that levels of Rho3 S-palmitoylation were tuned by precise levels of Erf2, which contributes to the regulation of meiosis in fission yeast.

Dual labelling with alk-16 and a protein synthesis reporter provides a robust approach to quantify the turnover of S-palmitoylation on native proteins. The method was applied in Jurkat T cells and demonstrated an increase rate of depalmitoylation of signalling kinases such as Lck upon T-cell activation [42]. Furthermore, treatment with a non-specific lipase inhibitor reduced the rate of palmitoylation turnover, implicating serine hydrolases in the depalmitoylation of proteins in T cells. Subsequently, the large-scale analysis of S-palmitoylation dynamics using pulse-chase with alk-16, a serine lipase-selective inhibitor and quantitative chemical proteomics revealed that many proteins are stably fatty-acylated, but other signalling proteins, notably oncogenes undergo rapid S-acylation cycles [43]. The study confirmed that protein depalmitoylation by thioesterases are an important regulatory mechanism for proteins with a fast palmitoylation turnover.

Beyond eukaryotes, fatty acid chemical reporters labelling is providing unique insight in other organisms. For example, fatty acid chemical reporters have revealed non-canonical forms of protein lipidation in *E. coli* [14] as well as fatty-acylation of secreted bacterial effectors from *S. typhimurium* [44]. Moreover, alk-16 was used in combination with SILAC to identify several hundreds of S-palmitoylated proteins in *Plasmodium falciparum* [45]. The method was compared with the proteomics identification of S-palmitoylated proteins by the acyl-biotin exchange protocol [46]. Both methods were shown to give overlapping dataset of

S-palmitoylated proteins but the study suggested that the methods are best used in combination for validation of protein substrates.

O-palmitoleoylation

Secreted lipid-modified glycoproteins Wnt proteins, such as Wnt3a or *Drosophila* Wingless (wnt-1) are also readily labelled by chemical reporters of fatty-acylation. These proteins are of particular interest as they have been suggested to be dually fatty-acylated. For example, Wnt-3a is S-palmitoylated on a conserved Cys residue (Cys77) and modified with a monounsaturated palmitoleic acid moiety linked via an ester bond (O-palmitoleoylation) by Porcupine on a conserved serine (Ser209) [47]. These fatty-acylations have been suggested to be essential for protein trafficking and the function of the Wnt proteins. Az-15 metabolic labelling of overexpressed Wnt proteins in *Drosophila* showed that S-palmitoylation is required for secretion of some, but not all Wnt proteins [48]. A recent study demonstrated that alk-16 can label both Cys77 and Ser209, and that alk-16 labelling of only Ser209 increases with the overexpression of Porcupine [49], suggesting Cys77 is not fatty-acylated by Porcupine.

Imaging of fatty-acylated proteins

Alkynyl-fatty acid labelling has also been used to visualize S-palmitoylated proteins by fluorescence microscopy after bioorthogonal labelling with azide-reagents [15,50]. The two-step labelling has been limited to fixed cells, but recent developments in vibrational spectroscopy has enabled *in vivo* imaging of alkynyl-fatty acids using stimulated raman-scattering (SRS) spectroscopy [51]. Notably, the SRS spectroscopy allowed the sensitive visualization of high levels of alk-16 in THP-1 macrophages and *C. elegans* [51]. Alk-16 was shown to localize into lipid droplets, probably in the form of triglycerides, but the method could also be applied to track S-palmitoylated proteins in live cells. However, the two methods described above only allow the visualization of the whole palmitoylated proteome and cannot image specific fatty-acylated proteins. To overcome this problem, a new method to image specific fatty-acylated proteins in single cells based on the use of alk-16 and *in situ* proximity ligation has been developed to track S-palmitoylated Wnt-3a in cells [49]. A similar approach was then used to detect Hedgehog, Tubulin, Ras [52], suggesting that the method could be applied to a variety of fatty-acylated proteins and could specifically reveal intracellular localization of S-palmitoylated proteins.

Perspectives

Over the past decade, chemical reporters have significantly enhanced the detection of protein acylation. Notably, numerous studies combining the chemical reporters of protein acylation with proteomics have revealed the acylated proteome in various organisms and cell lines, particularly for S-palmitoylation and *N*-myristoylation. The important next goal would be to identify the protein acylation sites by chemical proteomics. Recent advances have allowed to detect the sites of S-palmitoylation, using expensive commercially available ICAT reagents [53] or a modified acyl-biotin exchange (ABE) protocol [54], as well as the modification sites for *N*-myristoylation [31,32]. Nevertheless, new methods are still needed for the unbiased identification of acylation modification sites. While chemical reporters are

now available to study a wide range of protein acylation, the modification of proteins with cellular long chain fatty acid or unsaturated fatty acid has been significantly less studied. Long chain fatty acids can be metabolized in cells through chain elongation or shortage, β -oxidation, or incorporation into phospholipids [55]. To address the metabolism of fatty acid reporters, HDYOA (15-hexadecynyloxyacetic acid) (Table 1) was developed to limit β -oxidation and improve the efficiency of chemical reporter labelling *in vivo* [56]. Moreover, stearate (C18) and oleic acid (C18:1) were also reported to modify Cys [57] and Lys residues [25]. Chemical reporters of these modified fatty acids could be developed to understand the scope and function of these protein modifications. Combining CRISPR/Cas9 technology gene editing methods with chemical reporters of fatty-acylation and quantitative proteomics should also allow the identification of key targets of acylation associated enzymes. These methods should provide a better understanding of numerous metabolic pathways in a variety of model organisms and also help characterize protein acylation on human diseases.

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Abbreviations

ABE	acyl-biotin exchange
CoA	Coenzyme A
CuAAC	Cu ^I -catalyzed azide-alkyne cycloaddition
ICAT	isotope-coded affinity tag
HDYOA	15-hexadecynyloxyacetic acid
IFITM3	Interferon-induced transmembrane protein 3
KAT	lysine acyltransferase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NMT	<i>N</i> -myristoyltransferase
17-ODYA	17-octadecynoic acid
RAC	resin-assisted capture
Shh	Sonic hedgehog
SILAC	stable isotope labelling by amino acids in cell culture
SPAAC	strain-promoted alkyne-azide cycloaddition
TLR2	Toll-like receptor 2
Wnt	Wingless proteins

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Figure 1. Different types of protein acylation. A) Lys acylation. B) N-terminal acetylation. C) *N*-myristoylation and *N*-palmitoylation. D) *S*-palmitoylation. E) Ser/Thr acylation. E) Ser/Thr-palmitoylation.

A) Azide-tagged chemical reporters **B) Alkyne-tagged chemical reporters** **C) Affinity enrichment and quantitative proteomics** **Figure 2.**

Chemical reporter strategy for analyzing protein acylation. A) Azide-tagged acylation chemical reporters and fluorescence detection. B) Alkyne-tagged acylation chemical reporter and fluorescence detection. C) Affinity enrichment of alkyne-tagged proteins and mass spectrometry-based analysis for comparative proteomics. Quantitative comparison between biological samples (State A and State B) can be achieved by the incorporation of an isotopic (SILAC, dimethyl labelling) or isobaric tag (iTRAQ) or by label-free quantification.

Table 1

Acylation chemical reporters, *in vivo* (acetate analogues) and *in vitro* (acyl-CoA analogues).

Acylation	Probe structure	Probe name
Acetylation, propionylation, butyrylation		4-pentynoate
$n = 1; 2$	$n = 2$; 4-pentynoyl-CoA $n = 3$; 5-hexynoyl-CoA
	3-azidopropanoyl CoA
Malonylation		malAM-yne mal-yne
	$n = 10$; az-11 $n = 11$; az-12	
N-myristoylation	$n = 10$; alk-11 $n = 11$; alk-12	az-11-CoA
	alk-12-CoA
	az-15
S-palmitoylation and N-palmitoylation	$n = 13$; alk-14 $n = 14$; alk-15 $n = 15$; alk-16, 17-ODYA	az-14-CoA
	HDYOA