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Proteomic analysis of fatty-acylated proteins

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Protein fatty-acylation in eukaryotes has been associated with many fundamental biological processes. However, the diversity, abundance and regulatory mechanisms of protein fatty-acylation *in vivo* remain to be explored. Herein, we review the proteomic analysis of fatty-acylated proteins, with a focus on *N*-myristoylation and *S*-palmitoylation. We then highlight major challenges and emerging methods for direct site identification, quantitation, and lipid structure characterization to understand the functions and regulatory mechanisms of fatty-acylated proteins in physiology and disease.

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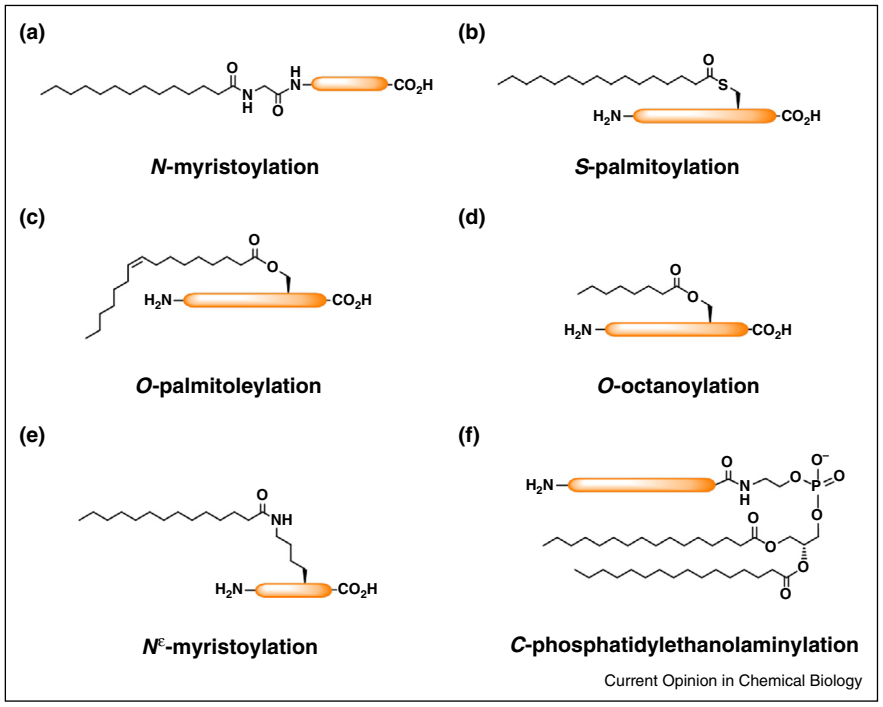
Introduction

Protein fatty-acylation describes the covalent attachment of diverse fatty acids onto a variety of amino acid residues on proteins, including *N*-myristoylation, *S*-palmitoylation (or called *S*-fatty-acylation), N^{ε}-fatty-acylation, *O*-fatty-acylation (e.g., *O*-palmitoleylation and *O*-octanoylation), glycosylphosphatidylinositol (GPI)-anchor modification, and *C*-phosphatidylethanolaminylation (Figure 1) [1]. *N*-myristoylation and *S*-palmitoylation are the two most prominent forms of protein fatty-acylation (Figure 1a,b), comprising the addition of myristate, a 14-carbon saturated fatty acid, and palmitate, a 16-carbon saturated fatty acid, onto *N*-terminal glycines and cysteines of proteins, respectively. Serine and threonine residues can also be modified by different fatty acids, such as *O*-palmitoleylation and *O*-octanoylation (Figure 1c,d). In addition, fatty-acylation also occurs on the epsilon-NH_2 groups of lysine side chains (Figure 1e). *C*-Phosphatidylethanolaminylation is a less prevalent form of fatty-acylation, which involves conjugation of phosphatidylethanolamine to the *C*-terminal glycine residue of LC3/Atg8, a key protein in autophagy (Figure 1f). Fatty acids can also be attached to the *C*-termini of proteins post-translationally through glycosylphosphatidylinositol (GPI) anchors.

Fatty-acylation can not only target proteins to specific membrane compartments, but also broadly influence protein–protein interactions and protein activity [2]. As a result, protein fatty-acylation is now well-recognized to regulate a variety of biological processes in eukaryotes, such as cell division and differentiation, synaptic transmission, immunity, and more [2].

Historically, protein fatty-acylation was difficult to study largely due to lack of specific antibodies and limited detection methods. To overcome these limitations, selective chemical labeling methods have been developed to tag specific forms of protein fatty-acylation [1,3]. For example, fatty acid chemical reporters have provided an efficient approach for non-radioactive detection and large-scale analysis of fatty-acylated proteins when combined with bioorthogonal reactions and mass spectrometry-based proteomics (Figure 2a). Fatty acid chemical reporters contain unique chemical functionality (e.g., alkyne or azide) and can be metabolically incorporated into fatty-acylated proteins [4]. The alkyne or azide tag introduced into fatty-acylated proteins allows bioorthogonal reactions with either fluorophores for rapid and sensitive visualization of fatty-acylated proteins by in-gel fluorescence imaging or affinity tags (e.g., biotin) for selective enrichment and large-scale proteomic identification (Figure 2a). This chemical reporter strategy has been widely employed for the global analysis of *N*-myristoylated and *S*-palmitoylated proteins, and can in principle be used for other fatty-acylated proteins [5•]. Alternatively, *S*-fatty-acylated proteins can also be selectively labeled and enriched by the acyl-biotin exchange (ABE) protocol, which exploits the hydroxylamine (NH_2OH) sensitivity of thioester bonds in *S*-palmitoylated proteins (Figure 2b) [6,7]. ABE encompasses reduction of disulfide bonds, capping of free cysteines with *N*-ethyl maleimide (NEM), cleavage of thioester bonds with NH_2OH, capture of newly liberated cysteines with HPDP–biotin, streptavidin pull-down, and elution of *S*-acylated proteins for western blotting or proteomic analysis. An improved version of ABE that requires fewer steps, that is, acyl resin-assisted capture (acyl-RAC), has recently been developed [8]. Following capping and NH_2OH treatment, newly liberated cysteines are captured on thiosepharose resin *via* the formation of

Figure 1



Fatty-acylated proteins in eukaryotes. **(a)** *N*-myristoylation. **(b)** *S*-palmitoylation. **(c)** *O*-palmitoleylation. **(d)** *O*-octanoylation. **(e)** N^{ε}-fatty-acylation. **(f)** *C*-phosphatidylethanolaminylation.

disulfide bonds, which can subsequently be reduced to elute captured proteins.

The selective chemical methods have allowed the large-scale analysis of fatty-acylated proteins, especially *N*-myristoylated and *S*-palmitoylated proteins, in different cell-types and animals. Notably, more than 300 fatty-acylated proteins have been identified to date, suggesting broader roles of fatty-acylation in regulating eukaryotic biology than previously appreciated [2]. In this review, we summarize the large-scale *N*-myristoylome and *S*-palmitoylome profiling studies, with a focus on those appeared in the past two years, and discuss the current status and unmet challenges for proteome-wide analysis of other fatty-acylated proteins. We then highlight emerging chemical biology and proteomic methods for higher resolution analysis of fatty-acylated proteins and close with an outlook on future developments needed in the fatty-acylation field.

N-myristoylation profiling

Protein *N*-myristoylation is catalyzed by *N*-myristoyltransferases (NMTs) that use myristoyl-CoA and typically modify *N*-terminal glycine residues of proteins co-translationally [9]. Alternatively, post-translational *N*-myristoylation can occur during apoptosis following caspase cleavage of proteins to expose *N*-terminal glycine residues. *N*-myristoylation can control protein subcellular localization and activity by promoting protein–membrane and protein–protein interactions and is involved in a wide variety of cellular processes, ranging from T cell activation, programmed cell death, and microbial infections [9].

Myristic acid analogs functionalized at the ω-position with an alkyne or azide group, such as alk-11, alk-12, az-11, and az-12, have been developed as chemical reporters to study *N*-myristoylated proteins [10–12], which have become the method of choice for large-scale proteomic analysis of *N*-myristoylation. Comparative studies have previously shown that alk-12, in combination with azide-tagged fluorophores or biotin, gives minimal background labeling, and that alk-12 preferentially labels *N*-myristoylated proteins compared to longer chain fatty acid reporters [13,14]. However, due to highly promiscuous fatty-acylation machinery and fatty acid metabolism, alk-12 has also been shown to label other types of fatty-acylated proteins [14], including N^{ε}-myristoylated proteins [15•,16], *S*-palmitoylated proteins, and GPI-anchor modified proteins [14,17••], which can complicate *N*-myristoylome profiling studies. To differentiate *N*-myristoylated proteins from other fatty-acylated proteins in proteome-wide studies using alk-12 labeling, several strategies have been applied. The first strategy involves combination of potent NMT inhibitors with alk-12 labeling and quantitative proteomic analysis to quantify the relative abundance of alk-12-labeled proteins in