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Chemical Probes for the Rapid Detection of Fatty-Acylated Proteins in Mammalian Cells

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The attachment of saturated fatty acids onto proteins modulates their interactions with membranes and other proteins, which ultimately regulates their signaling properties, subcellular localization, trafficking, and enzymatic activity.[1] In eukaryotes, the two major forms of protein fatty acylation are N-myristoylation and S-palmitoylation (Figure 1A), which are characterized by the addition of myristic acid to the N-terminal glycine of nascent polypeptides or the modification of cysteine residues with palmitic acid, respectively. These two forms of fatty acylation can occur separately on proteins or in concert to regulate protein function and have been shown to play key roles in many biological processes, such as T cell activation, apoptosis, tumorogenesis, neuronal signaling, cellular growth, and differentiation.[1]

The study of protein fatty acylation has been challenging due to limited methods for biochemical analysis.[2] Radiolabeled fatty acids have been utilized to visualize protein lipidation but suffer from low specific activity and are cumbersome to handle.[2] Alternatively, mass spectrometry has also been used to detect fatty acylation of purified proteins.[2] The development of non-radioactive methods to analyze protein fatty acylation in cell lysates or even in vivo would facilitate the analysis of protein fatty acylation. For example, the acyl−biotinyl exchange protocol provides a non-radioactive method for the visualization of protein S-acylation[3] and has enabled the global analysis of protein S-palmitoylation in yeast cell lysates.[4] Nonetheless, this method still requires metabolic labeling with radioactive fatty acids to confirm protein fatty acylation in living cells.[4] Here we describe the development of ω-azido-fatty acids as non-radioactive probes for the rapid detection of protein fatty acylation in mammalian cells (Figure 1B).

To explore whether ω-azido-fatty acids could serve as chemical probes for protein fatty acylation, we synthesized a series of ω-azido-fatty acids **1**−**4** of varying chain length, which contain 12, 14, 15, and 16 carbons, respectively (Figure 1B and Supporting Information Scheme 1). Mammalian cells (RAW264.7 macrophages) were incubated with the panel of ω-azido-fatty acids **1**−**4** and harvested after 6 h. Cell lysates were prepared and subjected to bioorthogonal labeling with phosphine−biotin via the Staudinger ligation[5] (Figure 1B). Biotinylated proteins were then separated by gel electrophoresis and analyzed by streptavidin blotting (Figure 2A). Treatment of mammalian cells with these chemical probes afforded selective protein labeling that was dependent on the chain length of the ω-azido-fatty acids **1**−**4** (Figure 2A). In the absence of any ω-azido-fatty acid, only endogenously biotinylated proteins are detected by streptavidin blotting, demonstrating the specificity of our bioorthogonal labeling conditions (Figure 2A). The 12-carbon ω-azido-fatty acid **1** yields the most robust profile of protein labeling

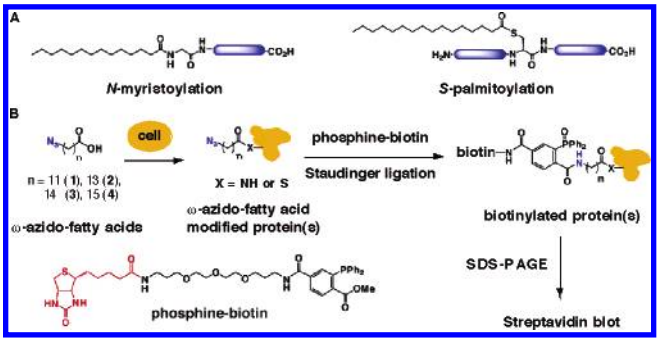

Figure 1. (A) Two major forms of protein fatty acylation in mammalian cells. (B) Strategy for the rapid detection of protein fatty acylation in mammalian cells with ω-azido-fatty acids (**1**−**4**) followed by bioorthogonal labeling with phosphine−biotin via Staudinger ligation. The chain length of the ω-azido-fatty acids (**1**−**4**) corresponds to 12, 14, 15, and 16 carbons, respectively. The chemical synthesis of ω-azido-fatty acids is described in the Supporting Information Scheme 1.

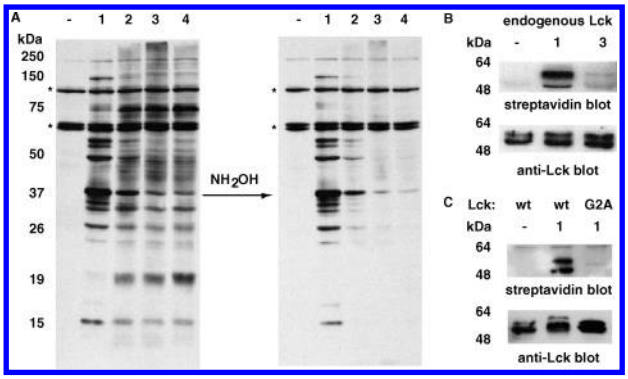

Figure 2. Visualization of ω-azido-fatty acid protein labeling in mammalian cell lysates. (A) Analysis of ω-azido-fatty acid protein labeling in RAW264.7 cell lysates. − or + hydroxylamine (NH_2OH) treatment. Cells were incubated with various ω-azido-fatty acids (**1**, 20 μM), (**2**−**4**, 100 μM), or DMSO control (−) for 6 h and harvested; ∗ marks endogenously biotinylated proteins. (B) The ω-azido-fatty acid labeling of endogenous Lck immunoprecipitated from Jurkat cells. (C) The ω-azido-fatty acid labeling of wild-type Lck and G2A Lck mutant overexpressed in HEK 293T cells.

(Figure 2A), whereas the longer chain ω-azido-fatty acids **2**−**4** afford similar profiles of protein labeling that are distinct and partially overlapping with ω-azido-fatty acid **1** (Figure 2A). Furthermore, protein labeling with the ω-azido-fatty acids **1**−**3** in living cells was time- and dose-dependent (Supporting Information Figure 1), demonstrating that active cellular metabolism is required for ω-azido-fatty acid incorporation and that polypeptides visualized by this approach are not the result of post-lysis enzymatic activity. The metabolic labeling of mammalian proteins with ω-azido-fatty acids **1**−**4** followed by bioorthogonal reaction with phosphine−biotin affords a very sensitive non-radioactive method to detect

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fatty-acylated proteins, as labeled proteins can be visualized within minutes by streptavidin blotting.

The distinct profiles of protein labeling afforded by ω-azido-fatty acid **1** compared to **2**−**4** suggest that these chemical probes target different sets of fatty-acylated proteins in mammalian cells. To determine whether the ω-azido-fatty acids selectively target N-myristoylated or S-palmitoylated proteins, we subjected ω-azido-fatty acid labeled proteins to hydroxylamine (NH_2OH) treatment, which selectively removes fatty acids attached to proteins via a thioester linkage.[3] Treatment of ω-azido-fatty acid labeled proteins with NH_2OH demonstrated that the polypeptides visualized with ω-azido-fatty acid **1** were resistant to NH_2OH cleavage (Figure 2A). In contrast, proteins visualized with ω-azido-fatty acids **2**−**4** were sensitive to NH_2OH treatment and suggest that the majority of ω-azido-fatty acids **2**−**4** are attached to proteins through a thioester bond (Figure 2A). Proteins labeled with the 14-carbon ω-azido-fatty acid **2** were differentially sensitive to NH_2OH, which suggests that compound **2** may serve as a substrate analogue for both myristic and palmitic acid (Figure 2A). Co-incubation with myristic acid selectively decreased protein labeling with ω-azido-fatty acid **1** in a dose-dependent manner, whereas lauric acid and palmitic acid had no effect at the same concentration (Supporting Information Figure 2A and B). Alternatively, protein labeling with ω-azido-fatty acid **3** was selectively reduced by co-incubation with longer chain fatty acids, such as palmitic acid or stearic acid and not with lauric or myristic acid (Supporting Information Figure 2C). Furthermore, proteins labeled with ω-azido-fatty acid **1** were sensitive to co-incubation with cycloheximide, whereas proteins targeted by ω-azido-fatty acid **3** were not (Supporting Information Figure 2D), consistent with the co-translational addition of myristic acid to proteins by *N*-myristoyltransferases[6] and the post-translational modification of palmitoylated proteins by *S*-palmitoyltransferases.[7] Collectively, these results suggest that the ω-azido-fatty acid **1** selectively targets N-myristoylated proteins, whereas the longer chain ω-azido-fatty acids **2**−**4** primarily label S-acylated proteins.

To further establish fatty-acylated protein labeling with ω-azido-fatty acids, we analyzed a well-characterized fatty-acylated protein, Lck, a Src-family protein kinase that is essential for T cell activation.[8] Human Jurkat T cells were metabolically labeled with ω-azido-fatty acid **1** or **3**, cell lysates were prepared, labeled with phosphine−biotin, immunoprecipitated for endogenous Lck with polyclonal anti-Lck sera,[9] and analyzed for fatty acylation by streptavidin blotting (Figure 2B). Lck immunoprecipitated from Jurkat cell lysates was efficiently labeled with the myristic acid analogue **1** as well as by the palmitic acid analogue **3**, albeit at much lower levels (Figure 2B). The differences observed between Lck labeling with compounds **1** and **3** are consistent with N-myristoylation being a constitutive modification, whereas S-palmitoylation is often dynamic and substoichiometric. These results are similar to those observed using radiolabeled fatty acids.[10] To unequivocally demonstrate that the myristic acid analogue **1** labels the N-terminal glycine residue of Lck and not the side chain of lysine residues elsewhere in the protein, we analyzed wild-type Lck and an N-terminal glycine to alanine Lck mutant (G2A), which blocks N-myristoylation of proteins.[11] Human embryonic kidney (HEK) 293T cells were transfected with cDNA constructs encoding wild-type Lck or the G2A mutant Lck, labeled with the myristic acid analogue **1** and analyzed for Lck N-myristoylation by streptavidin blotting after immunoprecipitation (Figure 2C). The myristic acid analogue **1** only labeled wild-type Lck and not the G2A mutant, demonstrating that compound **1** was attached specifically to the N-terminal glycine of Lck. Collectively, these experiments establish that ω-azido-fatty acids can be efficiently metabolized by mammalian cells and selectively installed on sites of fatty acylation on proteins, depending on the chain length of the ω-azido-fatty acids.

New tools are needed to further our understanding of protein fatty acylation. Here we demonstrate that ω-azido-fatty acids **1** and **3** can be efficiently metabolized by mammalian cells and serve as selective probes to rapidly visualize N-myristoylation and S-acylation, respectively. In addition to the more sensitive detection of fatty-acylated proteins with these chemical probes, the ability to biotinylate fatty-acylated proteins provides an opportunity for enrichment and proteomic analysis of lipidated proteins. These studies are currently underway and will be reported in due course. The development ω-azido-fatty acids adds to the toolbox of chemical reporters to monitor protein glycosylation[12] and farnesylation.[13]

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Supporting Information Available: Procedures for chemical synthesis, metabolic labeling, and streptavidin blots. This material is available free of charge via the Internet at http://pubs.acs.org.

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