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Emerging roles for protein S-palmitoylation in immunity from chemical proteomics

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Abstract

The activation of innate and adaptive immune signaling pathways and effector functions often occur at cellular membranes and are regulated by complex mechanisms. Here we review the growing number of proteins known to be regulated by S-palmitoylation in immune cells emerging from recent advances in chemical proteomics. These chemical proteomic studies have highlighted the roles of this dynamic lipid modification in regulating the specificity and strength of immune responses in different lymphocyte populations.

Introduction

S-Palmitoylation is a reversible lipid post-translational modification that occurs on proteins in diverse cell types including lymphocytes that are crucial for the innate and adaptive immune responses. This covalent attachment of the 16-carbon palmitic acid to specific cysteine side chains regulates protein trafficking to membrane compartments in numerous biological processes [1,2]. For example, reversible S-palmitoylation of the small GTPases H- and N-Ras controls their plasma membrane versus Golgi localization and subsequent interaction with signaling partners that regulate cell growth and differentiation [3,4] (Figure 1). This cyclical control of S-palmitoylation is enzymatically mediated by a family of 23 mammalian DHHC motif-containing protein acyl transferases (DHHC-PATs) that catalyze S-palmitoylation [5–7], while the mechanisms and enzymes responsible for depalmitoylation remain enigmatic (See Martin BR review in this Issue). S-palmitoylation is known to recruit multiple key signaling molecules to the immunological synapse in T cells [8] and S-palmitoylated proteins in other lymphocyte populations are emerging with the advent of new technologies (Table 1). Herein, we describe the technical advances that have enabled improved detection and chemical proteomic analysis of S-palmitoylated proteins, and review the emerging roles of protein S-palmitoylation in the immune system that are being revealed through application of these chemical approaches.

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Chemical Technologies for Studying S-Palmitoylation

S-Palmitoylation of the Vesicular Stomatitis Virus Glycoprotein (VSV-G) was first described in 1979 when it was discovered that purified VSV-G contained a lipid species that could not be removed by organic extraction or protein denaturation and digestion [9]. This lipid was identified as palmitic acid and its incorporation into VSV-G was confirmed using radiolabeled ^3H -palmitate [9]. Removal of the lipid with the thioester bond cleaving agent, hydroxylamine, suggested that the lipid modification occurred on a cysteine residue [9]. Demonstrating the incorporation of radiolabeled palmitate into candidate proteins at specific cysteine residues remained a gold standard for defining S-palmitoylation for more than two decades. However, this notoriously cumbersome and insensitive radioactivity-based method has limited the study of S-palmitoylation. Furthermore, unlike other lipid modifications, there is no unique consensus motif for S-palmitoylation and accurate prediction of S-palmitoylation sites on proteins remains challenging.

Two complementary chemical approaches have circumvented these limitations and greatly facilitated the investigation of S-palmitoylation [10]. For example, metabolic labeling of cells with alkynyl palmitate (alk-16) also referred to as 17-octadecynoic acid (ODYA) followed by bioorthogonal chemical ligation of labeled cellular proteins with azide-bearing fluorophores allows robust detection of protein S-palmitoylation and mapping of S-palmitoylation sites [11,12] (Figure 2). Compared to days or weeks for autoradiography, improved fluorescent detection of S-palmitoylated proteins using alk-16/ODYA takes minutes. In addition, bioorthogonal ligation of alk-16/ODYA labeled proteins with affinity tags such as azide-functionalized biotin enables enrichment and identification of S-palmitoylated proteins by mass spectrometry-based proteomics [12–14]. A complementary technique known as acyl-biotin exchange (ABE) has also been employed in recent years for the large-scale characterization of S-palmitoylated proteins [15] (Figure 2). This method first requires capping of free cysteines with thiol-reactive reagents such as N-ethyl maleimide. Palmitic acid is subsequently removed from modified cysteines using hydroxylamine, which cleaves thioester bonds, leaving the previously S-palmitoylated cysteines available for reaction with a thiol-reactive biotin moiety for affinity enrichment followed by identification. In fact, the analyses of S-palmitoyl-proteomes using the ABE method strongly suggest that the scope and functional contribution of this modification in eukaryotes has been largely underestimated [16,17]. For these methods, selectively cleavable reagents and/or high-content proteomics methods were particularly useful for profiling S-palmitoylomes [12–14,18]. Both the bioorthogonal labeling and ABE protocols have been successfully used in yeast and mammalian systems, revealing numerous new S-palmitoylated proteins that were not predicted by existing algorithms [19], including immunity-associated proteins that are the focus of this review.

T cells

T lymphocytes play a primary role in cell-mediated immunity and exist as several distinct subtypes broadly grouped as cytokine-secreting CD4⁺ T cells or cytotoxic CD8⁺ T cells [20]. Engagement of the T cell receptor (TCR) by peptide/major histocompatibility complexes (MHC) on antigen presenting cells induces the activation and proliferation of T

cells [21]. In this context, analysis of key proteins in T cell signaling has revealed an important role for *S*-palmitoylation in immunity [8]. For example, *S*-palmitoylation of signaling molecules such as Src family signaling kinases LCK [22,23] and FYN [24], transmembrane adaptor LAT (linker for activation of T cells) [25], and co-receptors CD4 [26] and CD8 [27] is required for effective TCR signaling [8]. Disruption of *S*-palmitoylation results in loss of membrane raft association by these proteins, concomitant with abrogation of TCR signaling [25,27–31]. Substitution of palmitate on LCK with a more hydrophilic oxygen-substituted palmitate analog that disrupts lipid packing also reduces raft association and weakens its signaling activity [32]. Although artificial targeting of chimeric LCK and LAT constructs to the plasma membrane without raft association restored their function in TCR signaling, their signaling properties are altered [33,34]. In addition, kinetics of palmitate turnover on LCK, which is accelerated upon T cell activation [35], is consistent with its cytosol-membrane exchange kinetics (~50 s) [36]. Together, these observations strongly indicate an important role for *S*-palmitoylation-mediated targeting to membrane microdomains in the modulation of TCR signaling.

Given the extensive characterization of *S*-palmitoylated proteins in T cells with ³H-palmitate, this cell type provided an excellent testing ground for the large-scale identification of *S*-palmitoylated proteins using the alk-16/ODYA palmitate reporter. Martin and Cravatt profiled alk-16/ODYA-labeled proteins from membrane fractions of Jurkat T cells, a human CD4⁺ T cell lymphoma line [12]. Validating the utility of the bioorthogonal labeling approach, T cell proteins known to be *S*-palmitoylated such as FYN, LCK, LAT, and CD4 were identified among more than 300 additional proteins, many of which, such as Ras isoforms, were also known to be *S*-palmitoylated in other cell types [12]. Also included in this list were novel *S*-palmitoylated proteins, such as the uncharacterized FAM108B1 serine hydrolase that mislocalizes when its cysteine-rich *S*-palmitoylated domain is deleted [12]. A complementary proteomic study of total cell lysates from alk-16/ODYA-labeled Jurkat T cells from our laboratory revealed a partially overlapping list of over 200 known and candidate *S*-palmitoylated proteins. The latter included histone H3 variants, which were found to be *S*-palmitoylated on cysteine 110 suggesting a novel potential role for this modification in histone H3 function [14]. The integration of stable isotope labeling by amino acids in T cell cultures with pulse-chase alk-16/ODYA proteomic studies has revealed distinct subsets of signaling proteins that are dynamically *S*-palmitoylated [37], suggesting that not all fatty-acylated proteins are subjected to temporal regulation like the Ras GTPases. These *S*-palmitoyl-proteomic data in T cells serve as a rich resource for further functional studies and paves the way for evaluating the role of *S*-palmitoylation in other cell types.

Dendritic cells

A defining feature of dendritic cells is their potent ability to present antigenic peptides on MHC class I and II molecules to T cells [38]. The site of engagement of MHC molecules on antigen presenting cells with the TCR is known as the immunological synapse [39]. While it is clear that *S*-palmitoylation controls recruitment of many T cell signaling molecules to the synapse membrane, the role of *S*-palmitoylation in forming and maintaining the antigen presenting cell side of the synapse is less appreciated. Even so, some MHC molecules themselves and other antigen presentation-associated proteins, such as the MHC-associated

invariant chain and tetraspanins, have been shown to be *S*-palmitoylated [40–42], lending credence to the hypothesis that *S*-palmitoylation is important at both sides of this cell-to-cell signaling interface. Furthermore, *S*-palmitoylation has been reported for several cell surface receptors responsible for antigen uptake or dendritic cell activation during virus infections such as CD36 [43,44] and the interferon α/β receptor [45–47], respectively.

In an initial attempt at profiling the *S*-palmitoyl-proteome of dendritic cells, our group identified 157 alk-16/ODYA-labeled proteins in the DC2.4 cell line [13]. A majority of the identified proteins were previously reported in other cell types, and included calnexin, transferrin receptor, mannose-6-phosphate receptor, N-Ras, G protein subunits, and the tetraspanin CD9. Additionally, several novel candidate *S*-palmitoylated proteins involved in immune responses were identified. In particular, *S*-palmitoylation of the interferon-induced transmembrane protein 3 (IFITM3) and that of other family members, IFITM1 and IFITM2, was confirmed [13]. Upon mapping the sites of IFITM3 to three membrane-proximal cysteines, it was discovered that *S*-palmitoylation controls IFITM3 to clustering in endoplasmic reticulum and endolysosomal membrane compartments, and is required for the full activity of IFITM3 in inhibiting influenza virus infection [13,48]. These studies nicely complemented a genome-wide RNA interference screen that also identified IFITM3 as an influenza virus antagonist [49], and IFITM3 loss-of-function studies in mice and humans [50,51]. While the precise mechanism of action for IFITM3 antiviral activity is still under investigation, additional studies have suggested that IFITM3 alters the endolysosomal membrane compartment through which the influenza virus normally trafficks [48,52,53], and is likely not a transmembrane protein, but rather an intramembrane protein whose complete targeting and association with membranes requires *S*-palmitoylation [48]. These early chemical proteomic studies have uncovered new *S*-palmitoylated proteins in dendritic cells and highlight an unappreciated role for *S*-palmitoylation in host defense against viral pathogens.

Macrophages

Macrophages serve as phagocytes in the immune system engulfing pathogens and cellular debris for digestion in lysosomal compartments [54]. Membrane remodeling involved in phagocytosis and other immunomodulatory functions of macrophages such as cytokine secretion are likely to involve *S*-palmitoylated proteins. Support for this hypothesis is provided by studies of syntaxin-7, an *S*-palmitoylated protein involved in macrophage phagocytosis [55,56], and SNAP-23, an *S*-palmitoylated membrane fusion protein involved in tumor necrosis factor α secretion [57,58]. Merrick and colleagues recently employed the ABE method to profile *S*-palmitoylated proteins in the murine RAW264.7 macrophage line [18]. This study proved fruitful in identifying 98 candidate proteins, 76% of which were previously suggested to be *S*-palmitoylated by other studies in mammalian cells including syntaxin-7 and SNAP-23. This significant overlap with the literature validated the methodology and suggested that the 24 novel candidates identified in this study are likely to be *S*-palmitoylated. These results also supported a role for *S*-palmitoylation in targeting proteins to lipid rafts as 48% of the identified proteins were also seen in a separate proteomic analysis of macrophage detergent-resistant membranes [18,59]. Among the novel candidate proteins without previous report of *S*-palmitoylation is LAT2, a non-T cell-

specific homologue of LAT that participates in signaling cascades originating at the plasma membrane [60], though the *S*-palmitoylation of this molecule has yet to be followed up in terms of function. Phospholipid scramblase 3 (PLSCR3) was also identified in this study and its sites of *S*-palmitoylation were mapped to a cluster of five cysteines. An *S*-palmitoylation-deficient PLSCR3 mutant was mislocalized from the mitochondria to the nucleus, providing the first example of protein targeting to the mitochondria by *S*-palmitoylation [18]. Given the crucial role of the mitochondria in initiating programmed cell death, the authors examined the role of PLSCR3 in apoptosis. Interestingly, overexpression of PLSCR3 sensitized macrophages to etoposide-induced apoptosis, and this was largely dependent on PLSCR3 *S*-palmitoylation and mitochondrial localization [18]. Thus, *S*-palmitoylation plays a role in the pro-apoptotic function of PLSCR3 and our understanding of the role of *S*-palmitoylation in macrophage biology continues to expand.

B cells

The primary function of B cells is to recognize antigen through the B cell receptor (BCR) and subsequently produce and secrete antigen-specific antibodies [61]. These cells mediate the humoral immune response important for the opsonization and neutralization of pathogens [62]. B cells have long been recognized as a model system for studying the importance of tetraspanin-rich membrane microdomains characterized by the presence of numerous *S*-palmitoylated tetraspanin proteins that provide a structural framework for receptors to transmit signals to intracellular molecules [63]. In fact, *S*-palmitoylation of the tetraspanin CD81 is necessary for the stabilization of the CD19/CD21/CD81/BCR complex that leads to enhanced BCR signaling upon antigen binding [64]. Again, the example set by *S*-palmitoylation studies of tetraspanins and other B cell proteins using classical techniques primed an interest in large-scale analysis of B cell *S*-palmitoylation. Thus, Ivaldi and coworkers performed ABE and proteomics to identify *S*-palmitoylated proteins in a human B lymphoid cell line [65]. Of 95 putative *S*-palmitoylated proteins, 46 were distinct MHC isoforms and 39 were suggested by other proteomic studies or were previously confirmed on a candidate basis. These 39 proteins included tetraspanins CD81, CD9, CD63, and CD82, as well as IFITM1, N-Ras, and LCK. Of the 10 newly identified molecules, the immunoregulatory, B cell-specific CD20 molecule and CD23, which is important for B cell development, were validated by demonstrating alk-16/ODYA incorporation on specific cysteine residues [65]. Both of these molecules are important drug targets in human B cell disorders, such as lymphoma, underscoring the importance of defining the cell biology governing the action of these proteins [66,67]. Thus, the success of using these new chemical tools, both ABE and alk-16/ODYA, in B lymphocytes presents an exciting opportunity to continue to learn more about the control of B cell function and differentiation by *S*-palmitoylation.

Perspectives

The utility of the newly developed tools for studying *S*-palmitoylation has been demonstrated by the *S*-palmitoyl-proteomics studies reviewed here. Of the novel *S*-palmitoylated proteins that have been identified, only a limited number have been functionally characterized. In T cells the lymphocyte specific CD5 protein and the TCR

delta chain (CD38), whose deficiency leads to severe combined immunodeficiency syndrome, were identified from *S*-palmitoyl-proteomics but not further characterized [12,14,37]. Given the essential nature of CD38 in T cell development [68] and the presence of a conserved cysteine at the cytoplasmic edge of its transmembrane domain, it would be interesting to determine if CD38 *S*-palmitoylation affects T cell function. Likewise, MHC molecules and other known *S*-palmitoylated proteins such as additional small GTPases and tetraspanins were not identified in palmitoyl-proteome studies of DC2.4 cells [13], indicating that improvements in protein recovery and mass spectrometry techniques may yet increase our understanding of *S*-palmitoylation in dendritic cells and other lymphocyte subtypes. Improvements in sensitivity should afford new opportunities to compare *S*-palmitoylation in primary resting and activated lymphocyte populations in various developmental stages. Importantly, the key enzymes (DHHC-PATs and thioesterases) regulating the *S*-palmitoylation of specific proteins in lymphocyte subsets are largely unknown and represent a crucial area for future investigation. As protein *S*-palmitoylation controls interactions in cellular membranes, additional studies of this lipid-modification will continue to be important for understanding the strength and specificity of cell signaling events in innate and adaptive immunity.

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Highlights

The alk-16/ODYA chemical reporter and the acyl-biotin exchange methods for studying protein *S*-palmitoylation are presented.

The role of *S*-palmitoylation in the control of the immune response is highlighted.

Recent *S*-palmitoyl proteome studies performed in T cells, dendritic cells, macrophages, and B cells using new chemical proteomic methods are reviewed.

Figure 1. Dynamic S-palmitoylation of H-Ras

H-Ras is irreversibly prenylated at its C-terminus followed by cycles of S-palmitoylation and depalmitoylation that are mediated by DHHC-PATs and thioesterases, respectively. Prenylation of H-Ras targets the protein to internal organelle membranes while the addition of S-palmitoylation targets H-Ras to the plasma membrane.

Figure 2. Chemical methods for the study of S-palmitoylation

(A) Metabolic labeling of cells with the alkynyl-palmitate reporter, alk-16/ODYA, allows incorporation of reporter onto proteins at sites of S-palmitoylation. Labeled proteins are reacted with azido-modified fluorescent or affinity tags via the copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction commonly referred to as "click chemistry." This method allows robust fluorescent visualization or affinity enrichment and identification of S-palmitoylated proteins. (B) The ABE method requires capping of free cysteines with thiol-reactive reagents such as N-ethylmaleimide (NEM) followed by removal of S-palmitoylation with NH_2OH . Exposed cysteines on previously palmitoylated proteins are then reacted with a thiol-reactive biotin analogue. Streptavidin blotting can be performed to visualize S-palmitoylated proteins. Alternatively, affinity enrichment of the biotin-labeled proteins can be performed for identification of S-palmitoylated proteins.

Table 1
S-palmitoylated proteins in lymphocytes

Examples of S-palmitoylated proteins that are either important for the function of the indicated lymphocyte populations or were discovered to be palmitoylated in a chemical proteomic study on the indicated cell type are shown. Examples include proteins that were classically identified on a candidate basis using H³-palmitate, as well as proteins that have been more recently identified in chemical proteomic screens using ABE and/or alk-16/ODYA.

Cell type	Protein	Method of study	Reference
<i>T cells</i>	FYN	³ H-Palmitate	22
	LCK	³ H-Palmitate	23,24
	LAT	³ H-Palmitate	25
	CD4	³ H-Palmitate	26
	CD8	³ H-Palmitate	27
	FAM108B1	alk-16/ODYA	12
	Histone H3	alk-16/ODYA	14
<i>Dendritic cells</i>	MHC class I	³ H-Palmitate	40
	Invariant chain	³ H-Palmitate	41
	IFITM3	alk-16/ODYA	13
<i>Macrophages</i>	STX7	³ H-Palmitate	56
	SNAP-23	³ H-Palmitate	57
	PLSCR3	ABE and H ³ -Palmitate	18
<i>B cells</i>	CD81	³ H-Palmitate	64
	CD20	ABE and alk-16/ODYA	65
	CD23	ABE and alk-16/ODYA	65