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Bifunctional Fatty Acid Chemical Reporter for Analyzing S-palmitoylated Membrane Protein-Protein Interactions in Mammalian Cells

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Abstract

Studying the functions of S-palmitoylated proteins in cells can be challenging due to the membrane targeting property and dynamic nature of protein S-palmitoylation. New strategies are therefore needed to specifically capture S-palmitoylated protein complexes in cellular membranes for dissecting their functions *in vivo*. Here we present a bifunctional fatty acid chemical reporter, x-alk-16, which contains an alkyne and a diazirine, for metabolic labeling of S-palmitoylated proteins and photocrosslinking of their involved protein complexes in mammalian cells. We demonstrate that x-alk-16 can be metabolically incorporated into known S-palmitoylated proteins such as H-Ras and IFITM3, a potent antiviral protein, and induce covalent crosslinking of IFITM3 oligomerization as well as its specific interactions with other membrane proteins upon in-cell photoactivation. Moreover, integration of x-alk-16-induced photocrosslinking with label-free quantitative proteomics allows identification of new IFITM3 interacting proteins.

The discovery of many S-palmitoylated proteins by recent proteomic studies suggests broader roles of protein S-palmitoylation in regulating eukaryotic biology than previously appreciated.¹ As a reversible and dynamic posttranslational modification, S-palmitoylation controls the localization and protein-protein interactions of many membrane proteins.² Distinguishing the interacting partners of S-palmitoylation proteins from those of their unmodified forms is crucial for understanding the functions of this lipid modification in a variety of biological pathways. The analysis of S-palmitoylated membrane protein-protein interactions in cells, however, can be challenging compared to soluble proteins.³ Membrane protein complexes may only be maintained under a native and unique lipid environment, which is often destroyed during cell lysis and difficult to reconstitute *in vitro*. Moreover, the hydrophobic and amphiphilic nature of membrane proteins makes classical techniques such as co-immunoprecipitation, two-hybrid systems and native gel electrophoresis more difficult to implement compared to soluble proteins.^{3a} These technical challenges are further

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ASSOCIATED CONTENT

Supporting Information

Synthesis of x-alk-16, experimental methods, and supplementary figures are available free of charge via the Internet at <http://pubs.acs.org>.

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exacerbated by the dynamic nature of S-palmitoylation. New tools are therefore needed to characterize S-palmitoylated membrane protein complexes in living cells for functional studies.

To facilitate the characterization of protein-protein interactions in cells, photocrosslinking methods have provided powerful approaches by generating covalent bonds between protein interaction partners in response to light.⁴ Owing to the newly introduced covalent bonds, weak and transient protein interactions can be trapped as covalent complexes for biochemical analysis even with harsh cell lysis and stringent protein purification conditions. Notably, photocrosslinking can be conducted in live and intact cells to stabilize and capture native membrane protein-protein interactions. For instance, a variety of photocrosslinking methods have been utilized to characterize protein-protein interactions⁵, glycan-protein interactions^{5b} as well as lipid-protein interactions⁶. Herein, we show that a bifunctional fatty acid chemical reporter, x-alk-16, which contains a clickable alkyne and a photoactivatable diazirine, can be metabolically incorporated into S-palmitoylated proteins and allows photocrosslinking of their interacting partners in mammalian cells (Figure 1).

To photocrosslink S-palmitoylated protein complexes, we synthesized a bifunctionalized fatty acid chemical reporter with a terminal alkyne and an internal diazirine, x-alk-16 (Figure 1 and Supporting Information Scheme S1). We envisioned that insertion of an internal diazirine into a previously described chemical reporter for S-palmitoylation, alk-16,⁷ would not significantly change the properties of the long-chain fatty acid, so the resulting palmitic acid analog could still be utilized by native enzymes for S-palmitoylation in mammalian cells. Once incorporated into S-palmitoylated proteins, the diazirine group could facilitate covalent crosslinking of x-alk-16-modified proteins with their interacting proteins upon photoactivation (Figure 1).

We first tested whether x-alk-16 could serve as a chemical reporter for S-palmitoylation in mammalian cells. Human embryonic kidney (HEK) 293T cells were incubated with 50 μ M alk-16 or x-alk-16 for 6 h and harvested. Cell lysates were prepared, reacted with azido-rhodamine (az-rho) and analyzed by in-gel fluorescence (Figure 2a). Like alk-16, incubation of cells with x-alk-16 afforded robust protein labeling relative to DMSO control (Figure 2a). In addition, protein labeling with x-alk-16 in living cells was dose- and time-dependent (Supporting Information Figure S1), indicating that active cellular metabolism is required for its incorporation. Comparative proteomic analysis of alk-16 and x-alk-16 labeled proteins after click reaction with azido-biotin and streptavidin enrichment indicated over 70% overlap of labeling between these two fatty acid chemical reporters, including many known S-palmitoylated proteins (Supporting Information Figure S2 and Table S1).

To further confirm that x-alk-16 is able to specifically label S-palmitoylated proteins, we investigated two well-characterized S-palmitoylated proteins, H-Ras and IFITM3.⁸ HEK293T cells were transfected with plasmids encoding HA-tagged wild-type proteins or palmitoylation-deficient mutants, and labeled with alk-16 or x-alk-16. The proteins were then immunoprecipitated, reacted with az-rho and analyzed by in-gel fluorescence. Wild-type HA-tagged H-Ras and IFITM3 were efficiently labeled with both alk-16 and x-alk-16, whereas S-palmitoylation-deficient mutants of HA-H-Ras (C181, 184S) and HA-IFITM3

(C71, 72, 105A) showed no fluorescence labeling with either reporter (Figure 2b), indicating that x-alk-16, like alk-16, is specifically incorporated into key S-palmitoylation sites of both H-Ras and IFITM3.

We then examined whether the incorporation of x-alk-16 could induce crosslinking of S-palmitoylated protein complexes in cells. For these studies, we initially focused on IFITM3 photocrosslinking with its interacting proteins as part of ongoing efforts in our lab to characterize the antiviral mechanisms of this protein.⁸ HEK293T cells were thus transfected with HA-IFITM3, labeled with fatty acid reporters, and then irradiated with UV light at 365 nm. Cell lysates were prepared and analyzed by western blot. Apparent higher molecular weight complexes were observed for HA-IFITM3 only in the x-alk-16 labeled and UV-irradiated sample (Figure 3a). By contrast, no crosslinking occurred for HA-IFITM3 in DMSO, alk-16 labeled samples or in the x-alk-16 labeled non-irradiated sample (Figure 3a). Photocrosslinking conditions, including x-alk-16 dose, labeling time, and irradiation time, were then optimized for HA-IFITM3 (Supporting Information Figure S3). Cells were generally incubated with 50 μ M of x-alk-16 for 2 h, and then UV irradiated for 5 min to minimize the cytotoxic effects of UV light, which is sufficient to achieve nearly maximal photocrosslinking. The cell lysates were also reacted with azido-biotin and enriched with streptavidin beads before elution for western blotting analysis. Crosslinking complexes of HA-IFITM3 were only detected in x-alk-16 labeled and UV-irradiated sample after clicked with azido-biotin (Supporting Information Figure S4). Together, these results demonstrate that the crosslinking is dependent on UV irradiation and mediated by x-alk-16.

Photocrosslinking induced by x-alk-16 was not limited to overexpressed IFITM3. Other HA-tagged S-palmitoylated membrane proteins (CD9, and CD81) were also photocrosslinked to form higher molecular weight complexes when cells were labeled with x-alk-16 and irradiated with UV (Supporting Information Figure S5a). In addition, x-alk-16-induced photocrosslinking was observed for endogenously expressed S-palmitoylated proteins (IFITM3, CAV1, and CANX) (Supporting Information Figure S5b). By contrast, under same conditions no photocrosslinking occurred for non-palmitoylated proteins, such as p53 and green fluorescent protein (GFP) (Supporting Information Figure S6). It is noteworthy that overexpressed H-Ras was not photocrosslinked so obviously as those shown above (Supporting Information Figure S5a). We reasoned that the low steady-state palmitoylation level of H-Ras⁹ may affect its photocrosslinking efficiency, and that the abundance of H-Ras photocrosslinking complexes might be too low for detection with western blotting.

Next we focused on the characterization of x-alk-16-induced photocrosslinking of IFITM3. We performed additional experiments with IFITM3 S-palmitoylation-deficient mutant HA-IFITM3-Palm⁻. Photocrosslinked bands with higher molecular weights were also observed for HA-IFITM3-Palm⁻, however, much weaker than those observed for wild-type protein (Figure 3a). To eliminate the interference from IFITM3 lysine ubiquitination (marked by asterisks) previously reported by our laboratory^{8b}, we also examined x-alk-16 induced photocrosslinking on ubiquitination-deficient mutants of IFITM3, HA-IFITM3-Ub⁻ and HA-IFITM3-Ub⁻-Palm⁻. UV- and x-alk-16-dependent molecular weight shifts on these mutants are more evident (Figure 3a). Interestingly, HA-IFITM3-Ub⁻-Palm⁻ still exhibited

a detectable, albeit much weaker, amount of UV- and x-alk-16-dependent crosslinking in the ~30 kDa region (Figure 3a), probably resulted from photocrosslinking with x-alk-16-modified endogenous proteins or phospholipid-mediated photocrosslinking of membrane proteins, as diazirine-containing fatty acid analogs have been shown to be metabolized into cellular phospholipids.^{6a,6f}

The apparent molecular weights of photocrosslinked complexes of IFITM3 suggest that IFITM3 dimers or oligomers might be captured by x-alk-16 photocrosslinking. To examine higher order IFITM3 complexes in cells and eliminate the interference from photocrosslinking of x-alk-16-modified endogenous proteins, HEK293T cells coexpressing HA- and myc-tagged IFITM3 were labeled with x-alk-16 and UV-irradiated. Immunoprecipitation was then performed using anti-HA-coupled beads and the eluted samples were analyzed for the presence of myc-IFITM3 by western blotting with an anti-myc antibody. Myc-IFITM3 at 15 kDa was detected in all samples coexpressing HA-IFITM3, while not in HA-vector samples, regardless of whether the samples were labeled with x-alk-16 or irradiated with UV, indicating that myc-IFITM3 was co-immunoprecipitated with HA-IFITM₃ (Supporting Information Figure S7a). This result is consistent with a recent report suggesting intermolecular interaction of IFITM₃.¹⁰ A ~30 kDa complex, twice of IFITM₃ molecular weight, appeared only in the sample labeled with x-alk-16 and treated with UV (Figure 3b and Supporting Information Figure S7a). An identical complex was detected in a reciprocal experiment (Supporting Information Figure S7b), in which immunoprecipitation was performed using anti-myc-coupled beads, followed by western blotting using an anti-HA antibody, suggesting that x-alk-16 enables capture of IFITM₃ dimerization upon UV irradiation. Interestingly, much weaker or no photocrosslinked dimer band was observed in samples coexpressing HA-IFITM₃/myc-IFITM₃-Palm or HA-IFITM₃-Palm /myc-IFITM₃-Palm (Figure 3b). Taken together, these results demonstrate that IFITM₃ oligomerization revealed by x-alk-16 photocrosslinking is largely dependent on IFITM3 S-palmitoylation.

To examine whether x-alk-16 can capture the interaction of IFITM3 with its known interacting partners, we focused on vesicle-membrane-protein-associated protein A (VAPA), a protein involved in intracellular cholesterol homeostasis and recently reported to interact with IFITM3¹¹. HEK293T cells coexpressing HA-IFITM3 and myc-VAPA were labeled with x-alk-16, irradiated, and then lysed for immunoprecipitation with anti-HA-coupled beads. The immunoprecipitates were analyzed by western blotting with an anti-myc antibody. As expected, co-immunoprecipitated myc-VAPA at 30 kDa was detected in all samples coexpressing HA-IFITM3 and myc-VAPA, whereas new bands at 45 kDa and 60 kDa appeared only in x-alk-16 photocrosslinking sample (Supporting Information Figure S8a), demonstrating the formation of IFITM3-VAPA and likely dimeric IFITM3-VAPA complexes, respectively. These photocrosslinked complexes were also detected in the reciprocal immunoprecipitation experiment (Supporting Information Figure S8b). More importantly, a VAPA truncation mutant, VAPA¹⁻²²⁷, lacking the key domain for interaction with IFITM3¹¹ did not co-immunoprecipitate and crosslink with IFITM3 (Supporting Information Figure S8a), suggesting protein interaction domains are required for x-alk-16-induced photocrosslinking complex formation. In addition, although myc-IFITM3 co-immunoprecipitated with CAV1-HA and HA-CD9 (Supporting Information Figure S9), two

unrelated S-palmitoylated membrane proteins, neither of them photocrosslinked with myc-IFITM3 to form high molecular weight complexes (Figure S9), further confirming the specificity of x-alk-16-induced photocrosslinking of IFITM3. These results demonstrate that in addition to IFITM3 homotypic interactions, x-alk-16 also enables specific photocrosslinking of IFITM3 with other interacting proteins.

Finally, we sought to identify new IFITM3-interacting proteins using our photocrosslinking strategy coupled with label-free quantitative proteomic analysis. Briefly, HEK 293T cells were transfected with HA-IFITM3, labeled with x-alk-16, and UV-irradiated in photocrosslinking sample group with four biological replicates. In non-photocrosslinking control group, HA-IFITM3-transfected cells were either not labeled with x-alk-16 or not irradiated with UV, each experimental design having three biological replicates. All samples were processed in parallel, including anti-HA immunoprecipitation, in-solution trypsin digestion, and LC-MS/MS analysis (Supporting Information Figure S10a). Proteins were then identified and quantified with the label-free MaxLFQ algorithm in MaxQuant software suite¹², which revealed 12 proteins that were enriched in x-alk-16 photocrosslinking samples versus non-photocrosslinking controls and are therefore IFITM3-interacting candidate proteins (Figure 4a). Notably, 9 of the 12 proteins appear to be membrane-associated proteins. Functional annotation revealed an enrichment of these proteins involved in ER membrane protein quality control and sterol metabolism (Supporting Information Figure S10b and S10c). To confirm the reliability and robustness of our photocrosslinking proteomics dataset, we picked two hits, CANX and BCAP31, for further validation. HA-IFITM3 and myc-tagged candidates were introduced into HEK293T cells which were then subjected to photocrosslinking, immunoprecipitated, and analyzed by western blotting. As shown in Figure 4b, CANX and BCAP31 indeed interact with IFITM3 as revealed by co-immunoprecipitation. More importantly, photocrosslinking complexes of IFITM3-CANX and IFITM3-BCAP31 with high apparent molecular weights were observed only in the photocrosslinking samples. Together, these results demonstrate that our x-alk-16 photocrosslinking strategy coupled with quantitative proteomic analysis allows identification of new IFITM3-interacting proteins.

In summary, we present a bifunctional fatty acid reporter x-alk-16 with a bioorthogonal alkyne detection tag and a photoactivatable diazirine for studying membrane protein interactions of S-palmitoylated proteins. We show that x-alk-16 is readily incorporated into S-palmitoylated proteins by metabolic labeling and enables covalent crosslinking of S-palmitoylated proteins with their interacting partners upon photoactivation in intact mammalian cells. Using this photocrosslinkable fatty acid chemical reporter, we have demonstrated the oligomerization of IFITM3 and its specific interaction with VAPA, both of which are important for the full antiviral activity of IFITM3. Integration of x-alk-16-enabled photocrosslinking with label-free quantitative proteomic studies also revealed new candidate IFITM3 interacting proteins that may uncover key cellular factors important for host resistance to virus infection in the future. This bifunctional lipid chemical reporter and photocrosslinking proteomics should afford new opportunities to characterize S-palmitoylated membrane protein complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Strategy for characterizing S-palmitoylated membrane protein complexes in living cells. Metabolic incorporation of a bifunctional fatty acid chemical reporter, x-alk-16, equipped with an alkyne and a diazirine allows bioorthogonal detection of S-palmitoylated proteins and in-cell photocrosslinking of S-palmitoylated protein complexes of interest (POI) for western blotting and proteomic analysis after immunopurification.

Figure 2.

Analysis of x-alk-16 incorporation into S-palmitoylated proteins in mammalian cells. (A) HEK293T cells were metabolically labeled with 50 μ M alk-16, x-alk-16 or DMSO, and lysed. Cell lysates were reacted with azidorhodamine (az-rho) and analyzed by in-gel fluorescence. (B) Known S-palmitoylated proteins, HA-tagged H-Ras and IFITM3 can be metabolically labeled with x-alk-16 on key Cys residues as judged by in-gel fluorescence detection. Coomassie blue staining in (A) and anti-HA western blots in (B) are included as protein loading controls.

Figure 3.

Photocrosslinking of IFITM3 induced by x-alk-16 incorporation. (A) Detection of x-alk-16-induced IFITM3 photocrosslinking complexes in whole cell lysate (WCL) by anti-HA western blot. IFITM3 ubiquitination bands are marked with asterisks. (B) Validation of x-alk-16-induced photocrosslinking of IFITM3 dimerization (double asterisk) by immunoprecipitation and western blot.

Figure 4.

Proteomic analysis of S-palmitoylated IFITM3-interacting partners captured by x-alk-16 photocrosslinking in mammalian cells. (A) Volcano plot representing results of the label-free quantitative proteomic analysis of IFITM3 pull-downs. The logarithmic ratios of protein intensities in photocrosslinking sample group (PXL) over non-photocrosslinking control group (non-PXL) were plotted against negative logarithmic p -values of the t test performed from multiple replicates. A hyperbolic curve (red dotted line) separates specific IFITM3-crosslinking proteins (red dots) from background (grey dots). (B) Validation of x-alk-16-induced photocrosslinking of IFITM3-CANX and IFITM3-BCAP31 complexes by anti-HA immunoprecipitation and anti-myc western blotting.