Peptides

DOI: 10.1002/ange.201408763

Integrative Chemical Biology Approaches for Identification and Characterization of "Erasers" for Fatty-Acid-Acylated Lysine Residues within Proteins**

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Abstract: *Acylation of proteins with fatty acids is important for the regulation of membrane association, trafficking, subcellular localization, and activity of many cellular proteins. While significant progress has been made in our understanding of the two major forms of protein acylation with fatty acids, N-myristoylation and S-palmitoylation, studies of the acylation of lysine residues, within proteins, with fatty acids have lagged behind. Demonstrated here is the use of integrative chemical biology approaches to examine human sirtuins as de-fatty-acid acylases in vitro and in cells. Photo-crosslinking chemistry is used to investigate enzymes which recognize fatty-acid acylated lysine. Human Sirt2 was identified as a robust lysine de-fatty-acid acylase in vitro. The results also show that Sirt2 can regulate the acylation of lysine residues, of proteins, with fatty acids within cells.*

Acylation of proteins with fatty acids regulates many fundamental cellular processes in eukaryotes such as signal transduction and membrane trafficking.[1] While diverse modifications by acylation, with fatty acids, of different residues of proteins can occur, our current understanding of this type of post-translational modification (PTM) is mainly based on the studies of two well-known acylations: myristoylation of *N*-terminal glycine residues (*N*-myristoylation)[2] and palmitoylation of cysteine residues (*S*-palmitoylation).[3] In contrast, acylation of lysine residues with fatty acids, although identified more than two decades ago,[4] has been little studied. The regulatory mechanisms and cellular functions of the acylation of lysine remains poorly understood, in large part because of a lack of knowledge of cellular enzymes which catalyze the addition (i.e., "writers") or removal (i.e., "erasers") of this PTM.

Recently, Lin and co-workers found that sirtuin 6 (Sirt6), one of seven members of mammalian nicotinamide adenine dinucleotide(NAD) dependent lysine deacetylases,[5] can catalyze the removal of fatty-acid acyl groups from lysine residues of tumor necrosis factor-α (TNF-α), a process through which the secretion of TNF-α can be regulated.[6] A later study by Denu and co-workers revealed that several other mammalian sirtuins also have the ability to catalyze the hydrolysis of a fatty-acid-acylated lysine on a model peptide substrate.[7] However, because of the lack of further characterizations, our understanding of their de-fatty-acid acylase activity and substrate selectivity toward acetylated versus fatty-acid-acylated lysine is still incomplete. More importantly, since the de-fatty-acid acylase activities of these sirtuins were only tested using a peptide-based in vitro assay, it is essential to determine whether they can regulate the acylation of lysine by fatty acids in vivo, and if so, what their endogenous fatty-acid-acylated protein substrates are.

To address these questions, we first sought to examine the ability of human sirtuins to recognize the acylation of lysine with fatty acids. We have previously developed an approach, based on photo-crosslinking chemistry, to study protein–protein interactions mediated by PTMs, such as lysine methylation and threonine phosphorylation.[8] We reasoned that this method would also be useful to investigate the recognition process of lysine acylation by sirtuins, which involves transient interactions between the PTM and the enzymes. To this end, we designed and synthesized a chemical probe (Probe **1**; Figure 1) based on a Lys9-myristoylated histone H3 peptide (H3K9Myr), in which Thr6 was replaced by a diazirine-containing photoreactive amino acid (photo-Leu) to capture enzymes which recognize the myristoyl modification by converting transient protein–protein interactions into irreversible covalent linkages. The probe also has a terminal-alkyne-containing amino acid (propargylglycine) at the peptide *C*-terminus to enable bioorthorgonal conjugation of fluorescence tags for the detection of captured proteins (Figure 1 a,b).

We first tested if Probe **1** could covalently label Sirt6, which is known to catalyze the removal of the myristoyl group from the H3K9Myr peptide in vitro.[6] The recombinant Sirt6 was incubated with Probe **1** and a K9-unmodified control probe (Probe **C**). After irradiation by UV light for 20 minutes, the probe-labeled Sirt6 was then conjugated with rhodamine azide (Rho-N_3) by copper(I)-catalyzed azide–alkyne cycloaddition (click chemistry). The resulting reaction mixtures were resolved by SDS-PAGE and analyzed by in-gel fluorescence scanning. As expected, Sirt6 was selectively labeled by the myristoylated Probe **1** but not by the unmodified Probe **C** (Figure 1 c), thus suggesting that Probe **1** can be used to examine the interactions between N-myristoylated lysine and its erasers. We then used Probe **1** to

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[**] X.D.L. acknowledges support from the Hong Kong Research Grants Council, Early Career Scheme (HKU 709813P), and the University of Hong Kong (Seed Funding Programme for Basic Research 201211159017).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201408763.

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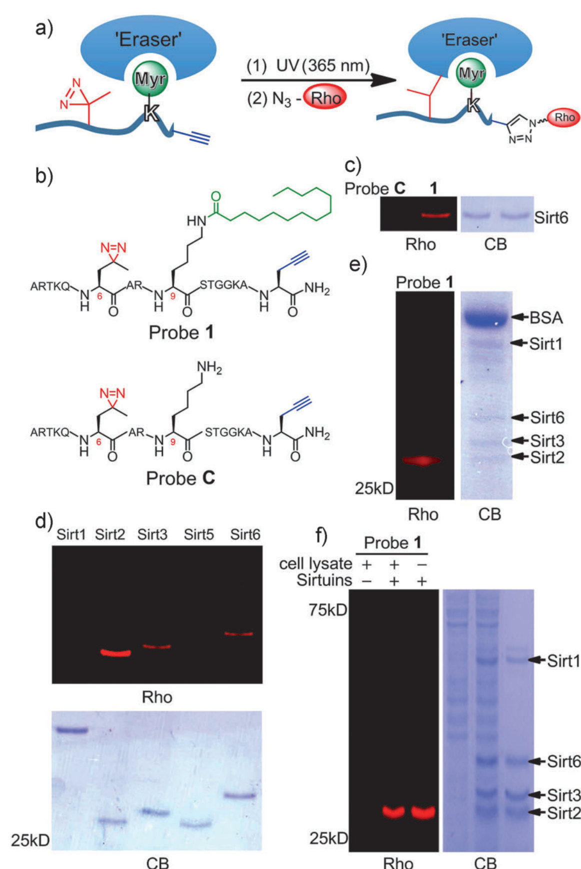
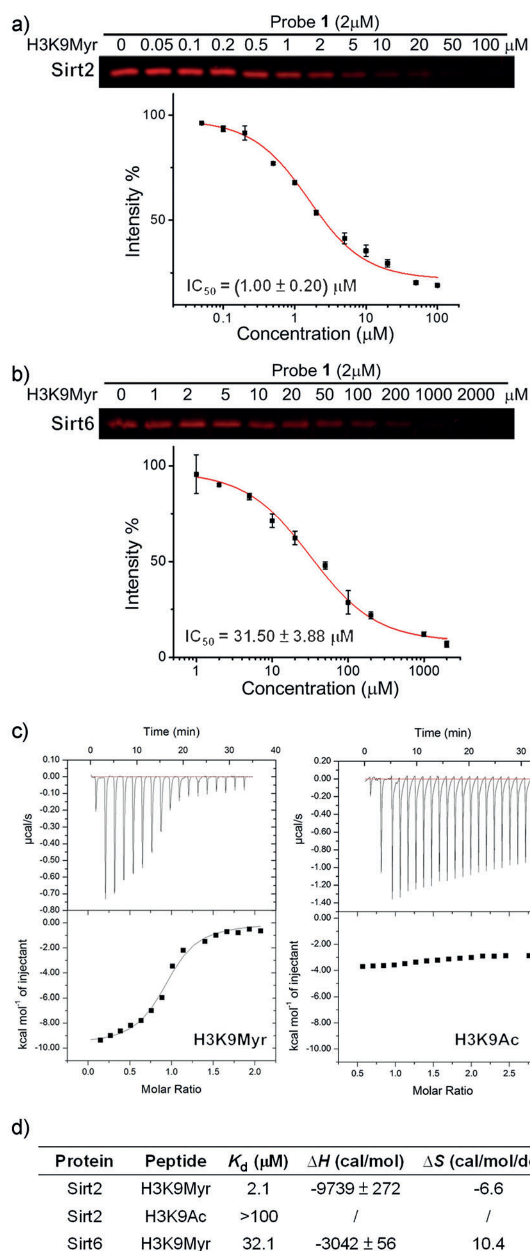


Figure 1. a) Approach for capturing and labeling erasers of protein lysine myristoylation based on photo-crosslinking. b) Chemical structures of the Probes **1** and **C**. c) Sirt6, a known lysine de-fatty-acid acylase, was selectively labeled by Probe **1** but not Probe **C**. d) Labeling of the indicated sirtuins (20 $\mu g\,mL^{-1}$) by Probe **1** (2 μM). e) Probe **1** (1 μM) selectively labeled Sirt2 from a mixture of the indicated sirtuins (1 μM) and BSA (10 μM). f) Probe **1** (1 μM) selectively labeled Sirt2 from whole cell lysate (1.5 $mg\,mL^{-1}$) containing the indicated recombinant sirtuins (1 μM). The proteins were incubated with either Probe **1** or Probe **C**. After UV irradiation ($\lambda = 365$ nm) for 20 min, the labeled proteins were conjugated to rhodamine-azide, resolved by SDS-PAGE, and detected by in-gel fluorescence scanning. The Coomassie blue staining was used as loading controls. Rho = rhodamine fluorescence, CB = Coomassie blue.



Protein	Peptide	K_d (μM)	ΔH (cal/mol)	ΔS (cal/mol/deg)
Sirt2	H3K9Myr	2.1	-9739 ± 272	-6.6
Sirt2	H3K9Ac	>100	/	/
Sirt6	H3K9Myr	32.1	-3042 ± 56	10.4

Figure 2. The H3K9Myr peptide inhibited the Probe **1** (2 μM) labeling of a) Sirt2 (20 $\mu g\,mL^{-1}$) and b) Sirt6 (20 $\mu g\,mL^{-1}$). Data are averages $\pm$ s.e. ($n = 3$). c) Isothermal titration calorimetry (ITC) measurement for the binding affinity of Sirt2 toward the H3K9Myr (left) and H3K9Ac (right) peptide. d) Summary of dissociation constants (K_d), enthalpy changes (ΔH), and entropy changes (ΔS) of Sirt2 and Sirt6 for the histone peptides.

investigate the ability of other sirtuins to recognize lysine myristoylation. In addition to Sirt6, Sirt2 and Sirt3 were also labeled by Probe **1** (Figure 1 d), thus indicating they are likely to recognize lysine myristoylation.

Notably, upon photoaffinity labeling by Probe **1**, Sirt2 showed a much stronger signal than Sirt6 and Sirt3. This result prompted us to further compare the relative ability of these sirtuins to recognize and bind myristoyl lysine. We then mixed Sirt1, Sirt2, Sirt3, and Sirt6, at the same concentration, together with a tenfold excess of BSA and total cell lysates of human HeLa S3 cells. We found that Sirt2 was preferentially labeled by Probe **1** from these complex protein mixtures (Figure 1 e,f), thus suggesting that among the tested sirtuins, Sirt2 has the strongest interaction with H3K9Myr. Consistent with this result, the labeling of Sirt2 competed with a native H3K9Myr peptide with an $IC_{50} = 1.0$ μM, while a much higher IC_{50} value (31.5 μM) was determined for the competition of Sirt6 labeling by using the same peptide (Figure 2 a,b). In addition, we found that the labeling of Sirt2 by Probe **1** can also compete with the H3 peptides having a longer (i.e., palmitoyl) or shorter (i.e., lauroyl) acyl group at Lys9, as well as a histone H4 peptide with myristoyl at Lys16 (see Figure S1 in the Supporting Information), thus suggesting that Sirt2 may preferentially recognize a broad scope of long-chain fatty-acid-acylated proteins. Finally, the direct measurement of binding affinity using isothermal titration calorimetry (ITC) revealed that Sirt2 ($K_d = 2.1$ μM) bound the H3K9Myr

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peptide much tighter than Sirt6 ($K_d = 32.1$ μM; Figure 2 c,d; see Figure S2). Interestingly, the interaction of Sirt2 with this H3K9Myr peptide was also much stronger than its interaction with a K9-acetylated histone H3 (H3K9Ac) peptide ($K_d >$ 100 μM; Figure 2 c,d), a known in vitro substrate of Sirt2.[9] Taken together, Sirt2, compared to other sirtuin family members, demonstrated the better ability to recognize and interact with a peptide substrate containing a fatty-acid-acylated lysine. Therefore, we focused on this enzyme for further studies.

We next examined the de-fatty-acid acylase activity of Sirt2. High-performance liquid chromatography (HPLC), in combination with mass spectrometry and ultraviolet spectroscopy, was used to monitor the hydrolysis of the H3K9Myr peptides. Like Sirt6, Sirt2 efficiently catalyzed the removal of the myristoyl group from the peptide (Figure 3 a,b). To quantitatively compare the demyristoylase activity of Sirt2 and Sirt6, we carried out kinetic studies on these enzymes. The steady-state kinetics data (Figure 3 c) suggested that the

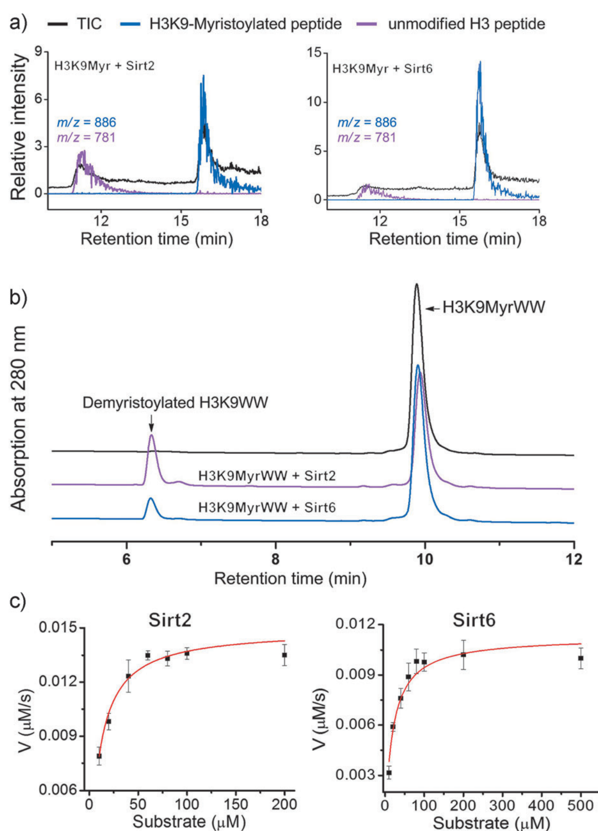

Figure 3. a) LC-MS traces and b) HPLC-UV traces showing de-myristoylation of histone H3K9-myristoylated peptide ARTKQTARK(Myr)STGGKA and KQTARK(Myr)STGGWW, respectively, catalyzed by Sirt2 and Sirt6. The enzyme (1 μM) was incubated with the myristoylated peptide (200 μM) for 60 min in the presence of 1 mM NAD, and then analyzed. In (a), the black traces show total ion intensity for all ion species with m/z values from 300 to 2000 (i.e., total ion counts, TIC); purple traces show ion intensity (5× magnified) for the masses of demyristoylated (unmodified) H3 peptides; and blue traces show ion intensity (5× magnified) for the masses of K9-myristoylated H3 peptide. c) Michaelis–Menten curves show the catalytic efficiencies of Sirt2 and Sirt6 toward the H3K9Myr peptide. Data are average ± s.e., $n = 3$.

catalytic efficiency of Sirt2 ($k_{cat}/K_m = 1700\ s^{-1}M^{-1}$) for the hydrolysis of the H3K9Myr peptide was around 2.8-fold higher than that of Sirt6 ($k_{cat}/K_m = 608\ s^{-1}M^{-1}$). Interestingly, the demyristoylation activity of Sirt2 is close to its deacetylation activity toward an H3K9Ac peptide ($k_{cat}/K_m = 3160\ s^{-1}M^{-1}$; see Figure S5). The K_m value of Sirt2 for the myristoyl peptide (8.6 μM) was much lower than that for the acetylated peptide (66.7 μM), thus agreeing well with the determined binding affinities of the enzyme toward these two peptides (Table 1). These enzymology data demonstrated that Sirt2 can function as a lysine de-fatty-acid acylase in vitro.

Table 1: Kinetic parameters of Sirt2 and Sirt6 on the acetyl and myristoyl H3K9 peptides.

Enzyme	Substrate	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$s^{-1}\ M^{-1}$]
Sirt2	H3K9Myr	$1.47 \pm 0.03 \times 10^{-2}$	8.62 ± 1.08	1.70×10^3
	H3K9Ac	0.211 ± 0.008	66.7 ± 9.12	3.16×10^3
Sirt6	H3K9Myr	$1.12 \pm 0.04 \times 10^{-2}$	18.4 ± 3.16	6.08×10^2

Finally, we sought to determine whether Sirt2 can regulate the acylation of lysine by fatty acids in cells. To test this, we perturbed the enzymatic activity of Sirt2 in HeLa cells by siRNA-induced Sirt2 knockdown and a specific small-molecule inhibitor of Sirt2 (AGK2),[10] respectively, and monitored the changes in the levels of fatty-acid-acylated proteins in the cells using a previously developed chemical reporter (alk-14).[11] The HeLa cells were cultured in the presence of alk-14, which potentially labels proteins with fatty-acid-acylated Cys, Lys, and *N*-terminal Gly residues. After harvesting the cells, the cytosolic and nuclear lysates were prepared and subjected to an azide–alkyne click chemistry to conjugate the alk-14-labeled proteins with a rhodamine dye. The labeled proteins were then treated with hydroxylamine (NH_2OH) to uncouple the proteinshaving fatty-acid-acylated Cys residues. The remaining proteins with fatty-acid acylated Lys and *N*-terminal Gly residues were finally resolved by SDS-PAGE and visualized by in-gel fluorescent scanning (Figure 4 a). As shown in Figure 4 b and Figure S6, the fluorescence signals of many bands corresponding to proteins labeled by alk-14, from the cytosolic lysates, were significantly increased by both Sirt2 knockdown and inhibition. Given that Sirt2 cannot catalyze the hydrolysis of the acyalated *N*-terminal Gly residues (see Figure S3), it is likely that the levels of aclyated lysine for these cytosolic proteins were regulated by Sirt2 in the cells. For the nuclear lysates, we focused on examining whether acylation of lysine by fatty acids occurs on histones, which are known to carry diverse PTMs. Indeed, we found that the labeling of core histones by alk-14 was enhanced in the cells treated with Sirt2 siRNA or its inhibitor (Figure 4 b), thus suggesting that Sirt2 could regulate the acylation of lysine, by fatty acids, on histones.

In summary, we have used integrative chemical biology approaches to demonstrate that human Sirt2 catalyzes the hydrolysis of fatty-acid-acylated lysine residues in vitro and within cells. This finding opens new opportunities to investigate the biological significance of acylation of lysines by fatty acids within proteins, process which has been under-

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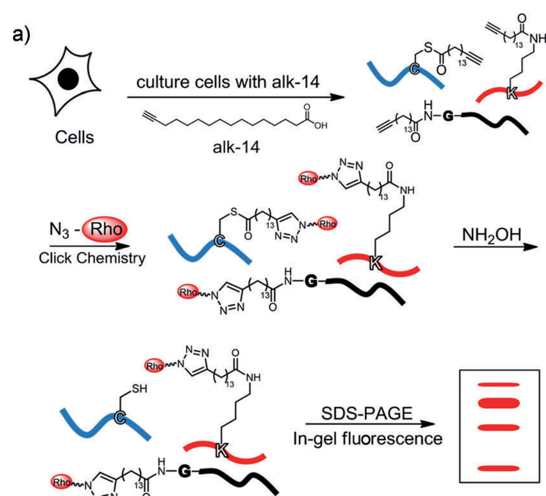

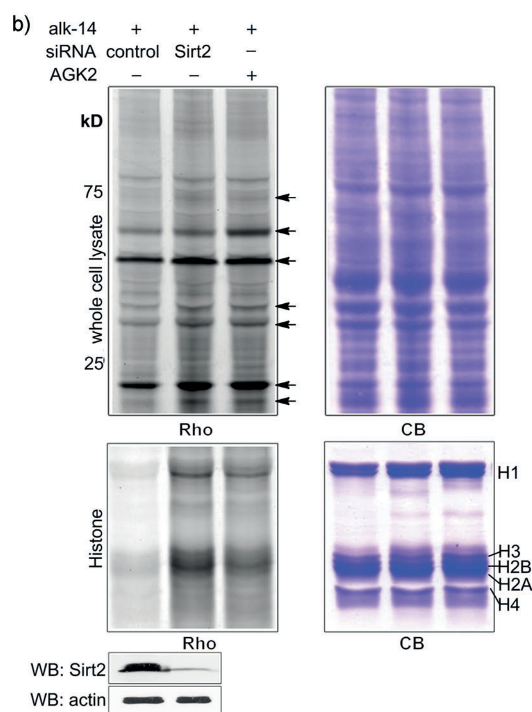

Figure 4. a) Detection of fatty-acid-acylated proteins using chemical reporter alk-14. b) Level of cytosolic proteins acylated by fatty acids (upper panel) and core histones (lower panel) extracted from HeLa cells was regulated by Sirt2. The arrows highlight the cytosolic proteins whose levels of fatty-acid-acylated residues were increased after the cells were treated with Sirt2 siRNA and AGK2, a selective Sirt2 inhibitor. WB = Western blot.

recognized for more than two decades. The photo-crosslinking strategy used in this study should also be applicable for examining interactions between other PTMs and their erasers. In contrast, the identification of Sirt2 as a robust de-fatty-acid acylase may also help to unravel unknown cellular mechanisms controlled by this enzyme, which has been considered solely as a deacetylase until now. Comprehensive profiling of cellular fatty-acid-acylated substrates of Sirt2, using a chemical reporter for acylation by fatty acids in combination with quantitative proteomics methods, is an important next step and will be reported in due course.

Received: September 3, 2014
Revised: October 20, 2014
Published online: December 4, 2014

Keywords: click chemistry · fatty acids · peptides · photochemistry · protein modifications

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