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Photoaffinity labeling approaches to elucidate lipid–protein interactions

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Abstract

Lipid–protein interactions serve as the basis for many of the diverse roles of lipids. However, these noncovalent binding events are often weak, transient, or dependent upon environmental cues. Photoaffinity labeling can preserve these interactions under native conditions, enabling their biochemical profiling. Typically, photoaffinity labeling probes contain a diazirine photocrosslinker and a click chemistry handle for enrichment and downstream analysis. In this review, we summarize recent advances in the understanding the mechanisms of diazirine photocrosslinking, and we provide an overview of recent applications of photoaffinity labeling to reveal the interactions of diverse types of lipids with specific members of the proteome.

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Keywords

Photoaffinity labeling, Photocrosslinking, Lipid, Chemoproteomics, Diazirine.

Abbreviations

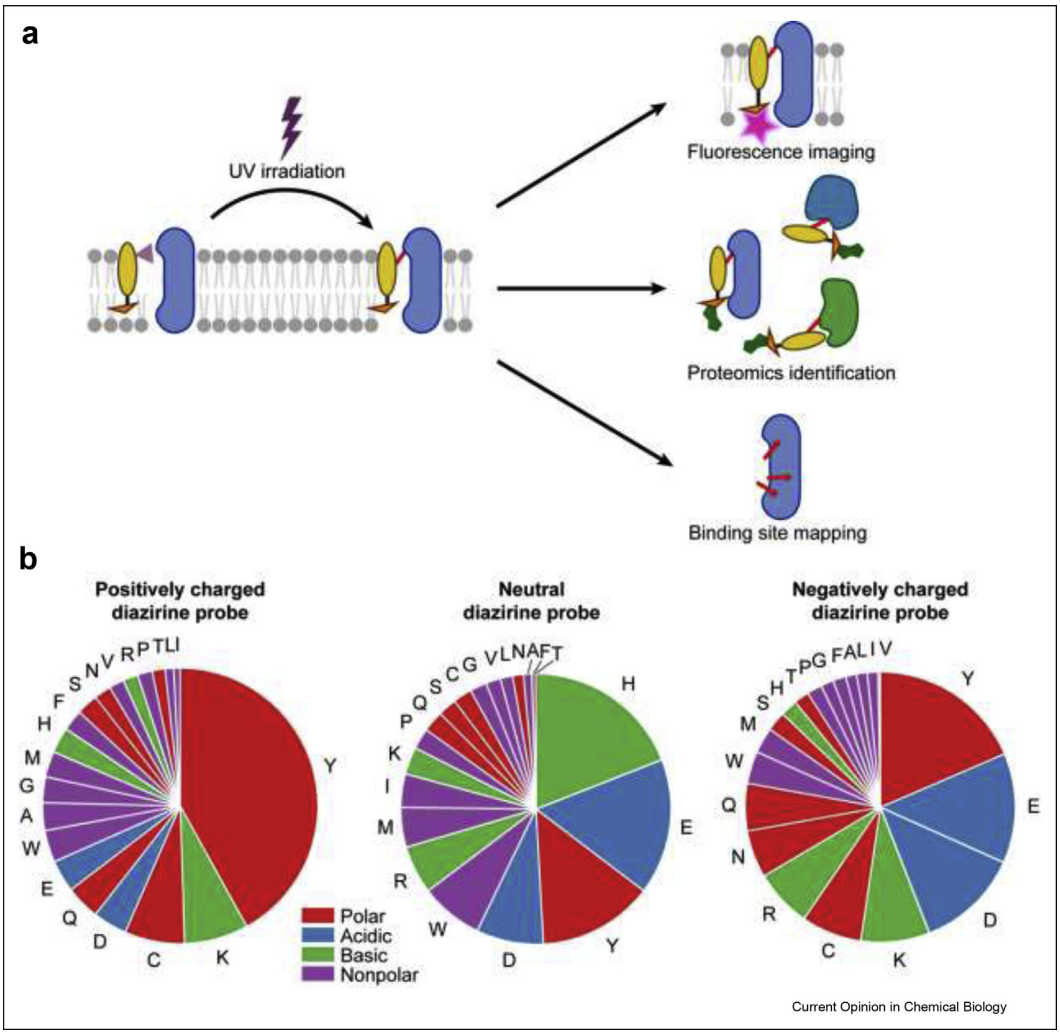
NMT, N-myristoyltransferase; 20(*S*)–OHC, 20(*S*)-hydroxycholesterol; PAL, photoaffinity labeling; PC, phosphatidylcholine; PEth, phosphatidylethanol; PI, phosphatidylinositol; PIP, phosphoinositide; PLD, phospholipase D; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPD, trifluoromethylphenyl diazirine; UV, ultraviolet.

Introduction

Lipids play diverse roles in biology. These include forming membranes that facilitate compartmentalization of cells and organelles, serving as energy stores in lipid droplets and adipose tissue, acting as signaling molecules to initiate or propagate cascades that alter cell and tissue behavior, and being used as protein post-translational modifications to change protein localization and activity. In many cases, the interactions of lipids with proteins provide the basis for these biological functions. Therefore, methods to profile lipid–protein interactions have attracted interest in recent years. Whereas certain lipids possess enough polar functionality to enable strong and specific interactions with target proteins, other lipids rely on weaker, transient hydrophobic–hydrophobic interactions and/or indirect effects through modulation of membrane biophysical properties to relay information to protein targets. The transient nature of these interactions and their occasional reliance on environmental cues or a native membrane environment pose challenges to studying these interactions *in vitro*.

Photoaffinity labeling (PAL) addresses these issues by trapping noncovalent, intermolecular interactions such as those in a lipid–protein complex via formation of a covalent bond between the lipid and protein, under native conditions. Typically, a synthetic analog of the lipid that bears both a photocrosslinking group and a click chemistry handle is introduced into cells or cell lysates as a proxy for the lipid of interest (Figure 1a). Ultraviolet (UV) light activates the photocrosslinking moiety, generating a highly reactive species that forms a covalent bond with nearby proteins. This artificially stabilized complex is amenable to biochemical analysis and mass spectrometry-based profiling. A variety of classic photocrosslinking groups can serve this function, such as diazirine (**1,** Figure 2), phenyl azide, aryl ketone, and diazo. Recently, 3-benzoylquinoxalinone [1] **(2,** Figure 2) was reported as a novel photocrosslinking warhead with fluorogenic properties. However, in recent years, and in particular in the intervening time since this topic was last reviewed in this journal [2], researchers have largely converged on the diazirine as the optimal photocrosslinker for profiling lipid–protein interactions, presumably due to its small size, high photocrosslinking activity, and a less phototoxic wavelength (360 nm) required for activation [3]. The compact size of diazirines is of special importance for designing lipid probes, as lipids themselves are rather small, and introducing a bulky photocrosslinking group into a lipid would drastically change its biophysical and biochemical properties, and potentially even intracellular localization.

Figure 1



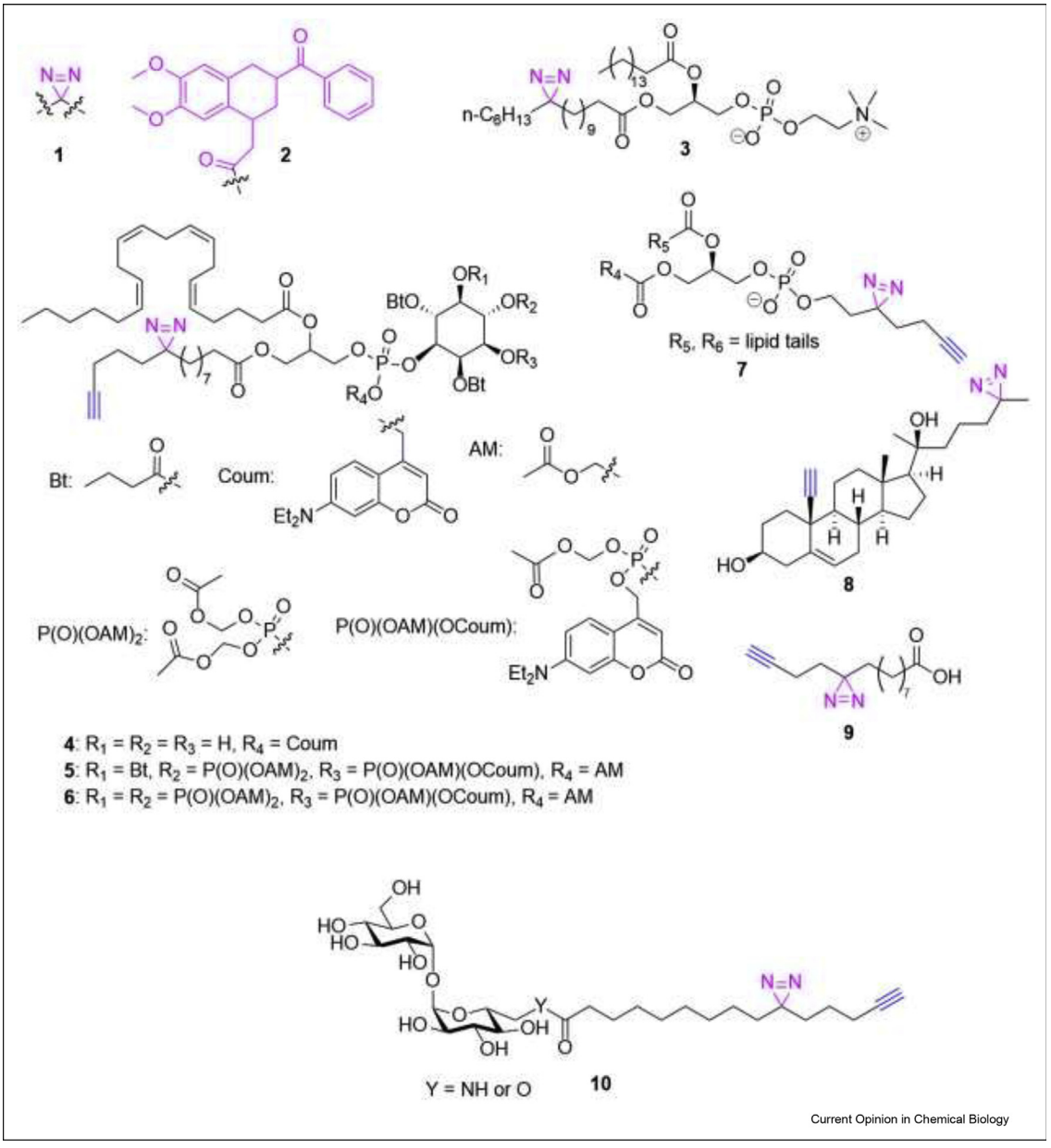
Photoaffinity labeling captures lipid–protein interactions in native conditions. (**a**) General schematic of photoaffinity labeling. A lipid analog (yellow) bearing a photocrosslinker (purple triangle) and click chemistry handle (orange triangle) is introduced into the cell. After UV irradiation, the probe captures nearby protein binders (red bonds), enabling downstream analysis such as fluorescence imaging, protein identification by proteomics, and binding site mapping. (**b**) Labeling frequencies of different amino acid residues by diazirine probes bearing one negative charge, no charge, or one positive charge, based on proteome-wide site mapping. Data from Ziemianowicz et al. [7].

In this review, we will highlight advances in the last few years for using PAL for profiling lipid–protein interactions. We will begin by discussing advances in our understanding of diazirine photocrosslinking mechanisms that inform probe design, and then we will discuss recent examples of PAL-based lipid probes for elucidating general and specific lipid–protein interactions across several classes of lipids and in experimental systems ranging from eukaryotic and prokaryotic cells.

New insights into diazirine-based photocrosslinking

Recent efforts have greatly improved our understanding of diazirine probes and their photocrosslinking mechanisms. Upon UV irradiation, diazirines can either extrude a molecule of N_2 to form a highly reactive carbene or rearrange to form a diazo intermediate [4]. Depending on the chemical environment of the diazirine, diazo products can either be highly reactive, and thus contribute significantly to the labeling, in the case of dialkyl diazirines, or be stabilized by nearby groups such as in trifluoromethylphenyl diazirines (TPD), where they persist as stable byproducts. Previously, it was thought that diazirine photocrosslinking went only through carbene intermediates, which have very short half-lives and were thought to be rather non-selective in their labeling site preferences. To revisit this issue, the Fox lab took advantage of a bioorthogonal [3 + 2] cycloaddition between cyclooctynes and diazo compounds, designing a cyclooctyne-based diazo quencher

Figure 2



Chemical structures of probes used for photoaffinity labeling of lipid–protein interactions. Diazirine (**1**) has emerged as the most commonly used photocrosslinker, whereas a newer photocrosslinker (3-benzoylquinoxalinone, **2**) has also been developed [1]. Lipids **3**–**10** are representative PAL probes for studying lipid–protein interactions discussed herein [13,16,17,23,32,40].

to trap diazo intermediates deriving from UV activation of a dialkyl diazirine. Using this probe, they demonstrated that diazo intermediates might contribute to nearly half of the photocrosslinking products of dialkyl diazirines under certain circumstances [5].

Under aqueous conditions, diazo compounds formed from dialkyl diazirines rapidly protonate to diazonium compounds, which react in esterification reactions with carboxylic acid groups on amino acid side chains, reminiscent of the classic synthetic transformation of diazomethane with carboxylic acids to form methyl esters. The Woo and Schriemer labs separately showed that this diazo-based mechanism has real-life consequences on the labeling preferences of amino acid side chains (Figure 1b) [6,7]. Alkyl diazirines strongly prefer amino acids with a heteroatom on the side chain, whereas trifluoromethylphenyl diazirines (TPD), which form reactive carbenes but stable diazo compounds, have a reduced preference for certain amino acid

residues, including acidic residues. These differences in labeling preferences can translate to different protein identification. For example, varying the functional groups around the diazirine in a probe of the natural product staurosporine resulted in substantial differences in the identities of enriched proteins [8]. The divergence in labeled proteome is likely due to both differences in residue preferences, as well as the different molecular shape and electronic structure. However, despite this variability, strong binding targets of staurosporine were identified using all three probes, highlighting the importance of careful probe design and downstream validation of results from mass spectrometry experiments.

These studies also identified that diazirine compounds tend to prefer membrane proteins, presumably due to the higher pK_a of acidic residues in lipid bilayers, which would facilitate the diazo-based esterification mechanism for labeling aspartate and glutamate residues [6]. Fortunately, this preference usually works to the advantage of those using lipid-based PAL probes, as most lipids reside within membranes. Collectively, these studies point to both carbene insertion and substitution of diazo intermediates as important mechanisms of diazirine photocrosslinking. Further, they provide important contextual information for interpreting the results of PAL studies in general, and lipid-based PAL studies in particular, in terms of both the identities of protein interactions and tandem mass spectrometry-based site mapping studies.

Finally, though diazirines are smaller than all available alternatives, no tag is without perturbation, and recent studies have highlighted the perturbations that diazirines can cause to lipids. Diazirines are often thought of as a rather nonpolar group, and therefore they are frequently installed within hydrophobic tails in the design of PAL probes. Recently, the Drescher lab revealed that the slight polarity of diazirines is sufficient to pull a flexible lipid tail to the membrane surface for diazirine-functionalized lipid tails [9]. When they examined the crosslinking behavior of a phosphatidylcholine (PC) analog (**3,** Figure 2) with a diazirine-functionalized acyl tail to either other PC molecules or to a model transmembrane helical peptide, all of the detectable crosslinking events were mapped to positions in the target at the membrane interface, as opposed to positions buried deep within the bilayer.

Whereas these results could be partially explained by the general labeling preferences of dialkyl diazirines, they nevertheless show that the photoactivated species is readily available on the membrane surface. From these studies, one must conclude that, though introducing a small diazirine group may seem innocuous (and indeed it is a minimalist substitution), it is critically important to verify that the probe behaves similarly to the native lipid of interest, either through verification that it binds to an established protein binder of the lipid, or through competition studies with the native lipid.

Photoaffinity labeling of bulk lipid species and metabolites

Many photoaffinity probes for bulk lipid species, such as PC and fatty acids, have been made in the past few decades. Considering the high concentration of these lipids in cells, it is difficult to verify the specificity of the identified lipid–protein interactions. As such, current research has focused on low-abundance lipids with higher likelihoods of engaging in specific interactions or utilizing clever experimental design to probe lipid–protein interactions in defined environments. In the following sections, we will highlight recent advances using lipid-based PAL probes for examining interactions of bulk phospholipids within liposomes, phosphoinositides, a phospholipid formed following alcohol consumption (phosphatidylethanol), sterols, posttranslationally lipidated proteins, and prokaryotic glycolipids from mycobacteria.

Bulk phospholipids within liposomes

One important clinical application of bulk phospholipids is as constituents of liposomes used for drug and vaccine delivery [10,11]. Though these species are chemically defined during their manufacturing, once introduced in vivo, they encounter serum and other protein-rich fluids, and stable interactions of proteins with the liposome that form a so-called protein corona can change the functionally relevant composition, and hence, properties of the liposome particle [12]. Pattipeiluhu et al. identified the protein corona of several clinically relevant liposomes by substituting a portion of the PC within the liposomes to a PAL probe of PC [13]. This method avoided the common artifacts of other methods to profile the liposome protein corona, and their study described an important role for apolipoproteins in the liposome corona.

Liposome binding assays are a common method for characterizing lipid–protein interactions. Jose and Pucadyil adapted a conventional liposome binding assay for use with PAL. In their study, they incorporated a fluorescent photocrosslinkable lipid into liposomes containing the lipid of interest [14]. The photocrosslinkable lipid had a diazirine moiety on the head group and the lipophilic fluorophore BODIPY covalently attached to one of the lipid tails, and it was synthesized from commercially available reagents. This method, termed proximity-based labeling of membrane-associated proteins, allowed for high-throughput detection of protein binding partners with high sensitivity without the aggregation artifacts that are commonly seen in liposome co-sedimentation assays.

Phosphoinositides

Phosphoinositides (PIPs) are a class of low-abundant, anionic signaling lipids that play key roles in many aspects of cell function. These lipids derive from phosphorylation of the head group of a bulk phospholipid, phosphatidylinositol (PI), and the specific phosphorylation patterns of the head group impact their localization, interactions, and bioactivities [15]. Building on years of expertise in designing PIP-based probes, the Schultz lab reported photocrosslinkable versions of PI [16] (**4,** Figure 2) and two oncogenic phosphoinositides, phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$) (**5,** Figure 2) and phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) (**6,** Figure 2) [17].

These probes featured a diazirine on the lipid tail for the photocrosslinking and a modified head group containing both acetoxymethyl esters to ensure membrane permeability and a coumarin photocage to mask the critical 3-phosphate. The photocage is particularly important, as PIPs have short half-lives, so temporal control of probe delivery and activation is crucial. The authors also built a version of the probe without the diazirine, which generates the native PIP after uncaging and allows for competition labeling experiments to verify the specificity of the PAL probes. Before uncaging, the probes were distributed in various intracellular membranes, but these lipids were observed to rapidly translocate to the plasma membrane after uncaging within a minute.

This intriguing result suggests that cells may possess dedicated transport machinery, that is, lipid transfer proteins, to ferry these PIPs between organelles. Indeed, their PAL-based proteomics studies revealed several proteins with lipid-binding domains, and knockdown of two of these hits, MPP6 and ATP11A, delayed the rate of plasma membrane translocation. These studies, and future studies inspired by it, will add to the rapidly growing compendium of proteins responsible for inter-organelle lipid transport by non-vesicular mechanisms [18].

Phosphatidylethanol: A phospholipid formed following alcohol consumption

Phosphatidylethanol (PEth) is a non-native lipid that is formed after ingestion of alcohol, through a conserved side-reaction, transphosphatidylation, of a ubiquitous enzyme, phospholipase D (PLD), whose natural function is to hydrolyze PC to form the signaling lipid phosphatidic acid [19,20]. Because of its specificity to ethanol consumption and its long serum half-life, PEth has recently been widely adopted clinically as a biomarker for acute or chronic alcohol consumption [21,22]. Interestingly, PEth has a long in vivo residence time, prompting us to hypothesize that PEth may engage in interactions with specific protein partners to alter human physiology, as suggested by some *in vitro* studies. To shed light on the poorly understood functions of this lipid, we designed a PAL probe for PEth [23]. Using a bifunctional diazirine alkyne alcohol as a PEth mimic, a bifunctional lipid probe (**7,** Figure 2) is formed chemoenzymatically by PLD enzymes in the same manner and at the same organelle locations within live cells as PEth. By generating **7** in cells and performing photocrosslinking in this native environment followed by click chemistry-enabled enrichment, we identified hundreds of proteins as potential interactors of **7** in a manner that was dependent on both UV light and PLD activity. Critically, we found that the proteome-wide crosslinking of **7** was effectively competed by addition of PEth to cells or its precursor ethanol, which is converted in situ to PEth by PLDs. Finally, we validated a top hit, the single-pass transmembrane protein basigin/CD147, as a strong **7**/PEth interactor and further mapped residues responsible for the crosslinking to narrow in on a lipid binding site within the protein. These studies suggest that PEth can engage in specific lipid–protein interactions and highlights a path toward uncovering potential pathophysiological roles for PEth in diseases of alcohol abuse.

Cholesterol and oxysterols

In addition to glycerophospholipids, the PAL strategy has also been applied to sterols, a class of lipids encompassing the abundant cholesterol as well as its lower abundance oxidized derivatives. Cholesterol has been a popular target for PAL, with pioneering work by the Cravatt lab using an aliphatic diazirine photocrosslinker [24] and more recent probes with alternate linkages and placement of alkyne and diazirine groups developed by the Covey, Schaffer, and Mennerick labs [25–28]. Recently, the Covey lab designed a group of sterol probes bearing the alternate trifluoromethylphenyl diazirine (TPD) crosslinker, as potential probes for both cholesterol itself and oxysterols, which are regioselectively and stereoselectively oxidized forms of cholesterol that have distinct signaling functions [29,30]. In cellular assays, these TPD-based sterol probes showed some similarities to the activities of cholesterol but not of oxysterols. Comparing these probes to previous aliphatic diazirine-based cholesterol probes, they found that the TPD probes formed distinct labeling patterns of protein binding sites, as detected by mass spectrometry.

Cholesterol can be metabolized to a vast array of bioactive steroids, and these metabolic pathways start with the oxidation of cholesterol to oxysterols, which are present in cells at very low concentrations [31]. In addition to being intermediates on the way to steroids and other hormones, the oxysterols themselves can also serve as potent signaling molecules, with the precise configuration of the oxygen atoms serving as a type of barcode. The Ondrus lab recently designed a probe for one such oxysterol, 20(*S*)-hydroxycholesterol (20(*S*)–OHC), which introduced both a diazirine

photocrosslinker and an alkyne for affinity purification [32]. Their probe design preserved the subtle structural information that distinguishes oxysterols from one another, and it was prepared using a clever, concise synthetic route (**8,** Figure 2) [32].

They first verified the specificity of the probe as a faithful reporter of 20(*S*)—OHC interactions by demonstrating its ability to crosslink to the only known 20(*S*)—OHC-binding protein, Smoothened, which is part of the Hedgehog signaling pathway [33]. They subsequently generated a detailed analysis of the proteome-wide binders of their probe. In particular, they compared the protein binders of the 20(*S*)—OHC probe both in the absence and presence of the native target, 20(*S*)—OHC, as a competitor, which yielded a much smaller and targeted group of potential interactors. Intriguingly, the top interactor, TMEM97, is an ER-localized transmembrane protein recently identified as the long-sought after sigma-2 receptor [34], which has a storied history in pharmacology and connections to cancer and neurological disease [35,36]. Though many sigma-2 receptor modulators have been developed, an endogenous ligand for this protein had long been elusive [37].

By using the 20(*S*)—OHC probe in a competition-based binding assay, Ondrus et al. showed that TMEM97 can distinguish between oxysterols with subtle differences in oxygenation. Using structural approaches, they identified a 20(*S*)—OHC binding site within TMEM97. Finally, the authors found that the 20(*S*)—OHC—TMEM97 interaction enhanced a key protein—protein interaction of TMEM97, with the Niemann-Pick C1 protein, a major regulator of intracellular cholesterol trafficking, pointing to a new plausible molecular function for 20(*S*)—OHC beyond its role in Hedgehog signaling. More generally, this study highlights the power of using optimal PAL probe design and competition-based proteomics to identify high-value targets in the universe of lipid—protein interactions.

Protein lipidation with myristoyl groups

Apart from their roles as individual molecules and constituents of membranes, lipids also modulate cellular functions by their cotranslational or posttranslational modification of proteins. One example of protein lipidation is the acylation of N-terminal glycine residues with 14-carbon myristic acid groups, known as N-myristoylation. Because this process is covalent, chemical myristoylation probes do not require photocrosslinking to reveal the identity of the lipidated proteins. However, derivatizing a myristate group with a diazirine does enable analysis of protein—protein interactions that are directly mediated by the lipidation. The Tate group designed three diazirine-bearing myristoylation probes, where the diazirine group was installed at three different positions [38].

Several studies were performed to validate that these diazirine-modified myristate groups behaved similar to existing myristoylation probes and to native myristoylation itself. First, without UV treatment, these probes all labeled proteins similarly, in a manner dependent upon N-myristoyltransferases (NMTs), as evaluated by SDS-PAGE and bottom-up mass spectrometry-based proteomics. The amounts of proteins enriched by each probe were strongly correlated with each other. Further, a co—crystal structure of the coenzyme A thioester of one diazirine probe (**9,** Figure 2) with human NMT1 revealed a very similar conformation of the probe to natural myristoyl groups. Finally, the researchers used this diazirine probe to identify the interactors of several myristoylated proteins. In these studies, an affinity-tagged myristoylated protein was expressed in each sample and, following UV-mediated crosslinking, proteins that were crosslinked by the myristoylation probe were identified by comparative proteomics. Interestingly, a few interactors of the myristoylated proteins were identified, and the interaction of the myristoylated FSP1 and TOMM40 was dependent upon myristoylation. Thus, this approach holds promise for using diazirine-based PAL to identify lipidation-dependent protein—protein interactions of lipidated proteins, and we envision that it could be extended to other forms of lipidation (e.g., S-acylation/palmitoylation, N-palmitoylation, prenylation, etc.).

Mycobacterial glycolipids

Up to this point, we have discussed probes for lipids present in human cells. Yet, bacterial pathogens have unique lipids, some of which play important roles in infection and thus can have a major impact on human health. In particular, mycobacteria, which can cause diseases such as tuberculosis, have a thick and multilayered cell wall that has several unique lipid species [39]. Notably, the outer membrane of the mycobacterial cell wall is rich in mycolate glycolipids that function in bacterial survival and represent an important potential drug target [39]. Because of the extremely hydrophobic environment in the mycobacterial outer membrane, it has been quite challenging to profile the proteins residing within and interacting with this structure.

The Swarts lab recently addressed that challenge by developing a PAL probe (**10,** Figure 2) of a mycolate glycolipid, wherein a mycolate was conjugated to a diazirine-containing fatty acid [40]. Fluorescence-activated cell sorting analysis and extraction assays verified that the probes specifically localized to these “mycomembranes” (i.e., mycobacterial outer membrane). Under conditions of PAL photocrosslinking, this probe enabled pulldown and identification of proteins in a probe- and UV-dependent manner. Known mycomembrane proteins Ag85 and MspA were detected, and the plasma membrane-resident protein MptA served as a negative control, validating that this method could

specifically capture mycomembrane-resident proteins. The researchers then used this method to identify mycomembrane proteins during different growth phases. Interestingly, they found significant differences in the mycomembrane protein profile between bacteria in log phase and in early stationary phase.

These results suggest that the mycobacterial outer membrane undergoes dynamic remodeling of its proteome during growth and further highlight the ability of these lipid-based PAL probes, which are compatible with live cells, to reveal changes in lipid–protein interactions that can accompany physiological changes. It is interesting to imagine their application in more complex settings, such as in various models of mycobacterial infection, including its active and latent phases in different hosts [41,42].

Conclusions

Photoaffinity labeling represents a powerful approach to capture transient interactions between lipids and their protein interactors. In this review, we have discussed the most recent advances in our understanding of the properties and behavior of diazirines, the most commonly used photoaffinity labeling tag, and applications of this strategy to answer important biological questions about specific types of lipid–protein interactions. In most studies, researchers were able to establish that their PAL probes behaved similarly to the native lipid through competition assays and binding affinity toward known protein targets. Nevertheless, the number of potential protein interactors that exceed statistical significance in the proteomics identification studies, despite these stringent criteria, still often tends to be intractably large.

Unfortunately, biological validation, which involves careful selection of candidate hits for downstream analysis, can remain the bottleneck. Cleverly designed experiments such as competition proteomics, as well as strategies to further narrow target selection, will need to be employed to translate these proteomics hits into proven biochemical and biologically significant interactions. We envision that future studies in this arena will harness PAL alongside noncovalent affinity purification [43] and non-affinity approaches to identify and elucidate biologically important interactions across the proteome of a growing list of physiologically important lipid species.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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