Lipid switches in the immunological synapse

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Adaptive immune responses comprise the activation of T cells by peptide antigens that are presented by proteins of the Major Histocompatibility Complex (MHC) on the surface of an antigen-presenting cell. As a consequence of the T cell receptor interacting productively with a certain peptide-MHC complex, a specialized cell-cell junction known as the immunological synapse forms and is accompanied by changes in the spatio-temporal patterning and function of intracellular signaling molecules. Key modifications occurring at the cytoplasmic leaflet of the plasma and internal membranes in activated T cells comprise lipid switches that affect the binding and distribution of proteins within or near the lipid bilayer. Here, we describe two major classes of lipid switches that act at this critical water/membrane interface. Phosphoinositides are derived from phosphatidylinositol, an amphiphilic molecule that contains two fatty acid chains and a phosphate group that bridges the glycerol backbone to the carbohydrate inositol. The inositol ring can be variably (de-)phosphorylated by dedicated kinases and phosphatases, thereby creating phosphoinositide signatures that define the composition and properties of signaling molecules, molecular complexes, or whole organelles. Palmitoylation refers to the reversible attachment of the fatty acid palmitate to a substrate protein's cysteine residue. DHHC enzymes, named after the four conserved amino acids in their active site, catalyze this post-translational modification and thereby change the distribution of proteins at, between, and within membranes. T cells utilize these two types of molecular switches to adjust their properties to an activation process that requires changes in motility, transport, secretion, and gene expression.

Engagement of the TCR by peptide-MHC complexes presented on the surface of an APC leads to the formation of a stable cell-cell junction coined the immunological synapse (IS) (1). As a consequence, signaling pathways are initiated at the plasma membrane that leads, for example, to changes in cytoskeletal dynamics, protein transport, transcription, splicing, and translation. The initial events that trigger downstream events often comprise post-translational modifications that lead to conformational changes, altered compartmentalization, binding of adaptor proteins, or production of second messengers (2). Lipid switches constitute one class of switches engaged in the IS (3). The most common lipid switches modulating T cell function upon cell–cell couple formation are the interconversion of phospholipids (Fig. 1) and the S-acylation of cysteine residues in proteins by palmitate (Fig. 2). In several cases, these two types of switches act along the same pathway, as shown for Ca^{2+}-signaling in Figures 1 and 2. The two types of switches are also invoked on the side of the antigen-presenting cell (APC), referred to as reverse signaling in this context, but little is known about the more specific pathways that allow APC-specific adaptation after immune synapse formation (4). In this review we will focus on phosphoinositide and palmitoylation switches that are operative at the T cell side of the synapse once cells are stimulated. Furthermore, we will elaborate on the methodology that enables the identification of lipid moieties by mass spectrometry as a prerequisite for more quantitative interpretations of lipid modifications and concentration changes that accompany T cell activation.

Phosphoinositide specialization across the immunological synapse

The major phosphoinositide at the plasma membrane (PM) is $PI(4,5)P_2$, constituting 1% of all lipid species in the bilayer. It thereby shapes the biophysical properties of the PM and serves as a landing platform for $PI(4,5)P_2$-specific binding partners (5, 6). Activation of T cells trigger a profound change in the turnover and localization of $PI(4,5)P_2$ or its derivatives, $PI(3,4,5)P_3$ and diacylglycerol (DAG). The development of bioprobes generated from proteins with phosphoinositide-binding specificity led to the earliest images of membrane specialization at the immunological synapse (7–10). These early studies revealed a sustained accumulation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the immunological synapse, identifying the synapse not only as the site of signal transduction, but also of dynamic membrane changes (11, 12). Further studies soon revealed the immunological synapse as a focal point of diacylglycerol (DAG) accumulation where protein kinase D is recruited and activated (13). Protein kinase D has multiple roles including integrin activation that could contribute to synapse formation. Intriguingly, these studies also highlighted the dynamic nature of DAG accumulation as protein kinase D was only transiently associated with the immunological synapse.

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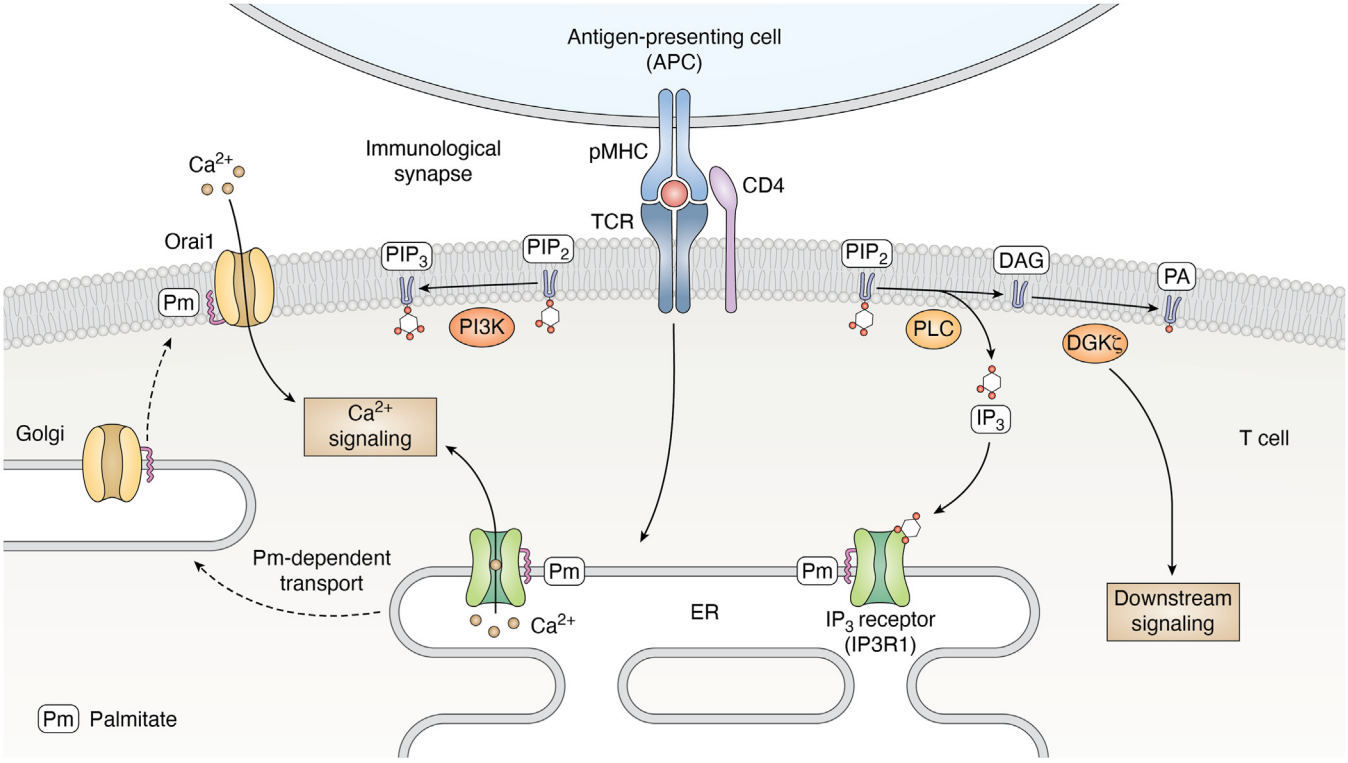

Figure 1. Depiction of the T cell synapse highlighting important phosphoinositide switches. In particular, $PI(4,5)P_2$ conversion at the synapse leads to rapid changes in the second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), most prominently affecting Ca^{2+} signaling and protein kinase signaling. Note that the IP_3 receptor IP3R and the store-operated calcium entry channel Orai1 are required to be palmitoylated (Pm) for functionality.

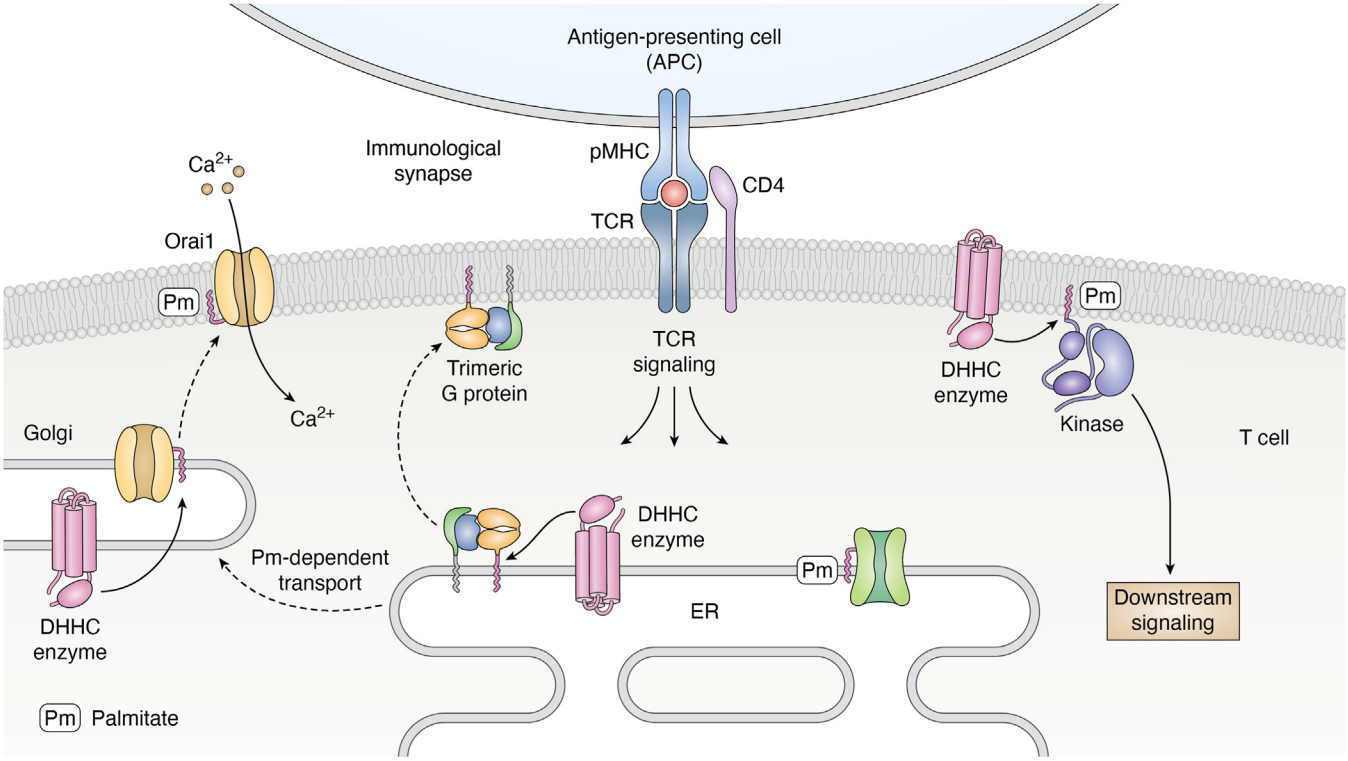

Figure 2. Depiction of the T cell synapse highlighting some of the most prominent palmitoylation targets in the synapse. The DHHC enzymes catalyzing palmitoylation (Pm) are located at the ER, the Golgi, or the plasma membrane. Palmitoylation may alter the transport, activity, or clustering of target proteins within membranes, as shown here for the G_α subunit of a trimeric G protein, the Ca^{2+} channel Orai1, or a kinase (Lck).

Given the rapid accumulation of DAG across the immunological synapse, the recruitment of the diacylglycerol kinases (DGKs) that convert DAG to phosphatidic acid (PA) and thereby limit DAG was also important to understand. Both α and ζ isoforms of DGKs were found to be expressed in T cells and recruited to the TCR complex (14). However, only DGKζ silencing reduced PA production, suggesting that DGKζ is the key isoform involved in limiting DAG accumulation across the synapse. In contrast to DGKζ rapidly localizing to the center of the synapse (15), DGKα was found to localize to the periphery (16), suggesting that this isoform is important in limiting the extent of DAG accumulation across the membrane after TCR activation. The interest in this localized burst of DAG was further stimulated by findings that suggested that DAG might drive polarization of the microtubule organizing center (MTOC) toward the synapse (16). The use of a photo-activatable DAG allowed localized light-driven production of DAG, which showed an increased polarization of the MTOC toward the focal point of DAG produced by peripheral DGKα. Further studies suggested that DAG-enriched organelles also polarized to this site, with stronger TCR signals eliciting stronger polarization (17).

A phosphoinositide signature across the CTL synapse during secretion: a PIP switch controlling secretion

The use of Total Internal Fluorescence microscopy imaging of T cells forming artificial synapses on supported lipid bilayers containing peptide MHC has been key to observing a peripheral localization of DGKα in the immunological synapse (16). This highlights the importance of being able to view T cells in 3D to capture areas of localized accumulation and depletion. This approach was taken further in experiments aimed at determining the membrane changes that occurred during secretion from cytotoxic T lymphocytes (CTLs) as they encountered antigen-specific target cells. In these experiments, imaging membrane changes in 4D (3D live imaging) proved essential in order to capture the rapid dynamic changes across the immunological synapse (18).

CTLs are important in being able to destroy cancer or virally infected target cells that they recognize *via* the TCR. This initiates the polarized secretion of specialised secretory granules containing the pore forming protein perforin, together with a series of granzymes that enter the target *via* the perforin pore that triggers caspase cleavage and rapid apoptosis (19, 20). 3D imaging of the immune synapse revealed that actin was depleted at the point of secretion (21, 22). The use of 4D imaging revealed that the actin depletion across the synapse was dynamic, with F-actin reducing after cell recognition and prior to granule secretion, suggesting that the membrane-associated F-actin controlled secretion from CTLs (23). Using latrunculin to depolymerize actin across CTL synapses confirmed the role of actin in terminating secretion (24). These results raised the question of how the rapid depletion and recovery of actin might be linked to TCR signaling.

Multiple biological systems have identified the phosphoinositide PIP_2 in the recruitment of proteins that in turn direct F-actin to the plasma membrane (25–29). As TCR activation rapidly leads to the cleavage of PIP_2 by phospholipase-Cγ (PLCγ), this raised the possibility that PIP_2 depletion and recovery might, in turn, control actin depletion and recovery, in-tune with TCR signaling. A study using 4D imaging of CTLs encountering targets showed this to be the case (30). Using a panel of bio-probes to detect PI(4)P, PIP2, PI(3,4,5)P3, and DAG, dynamic changes were revealed within the first 2 min after cell recognition as the synapse formed in preparation for secretion. Using LifeAct-EGFP to identify changes in F-actin compared to changes in the phosphoinositide and lipid composition made it possible to define the "membrane signature" required for secretion from the CTL synapse (Fig. 3) (30).

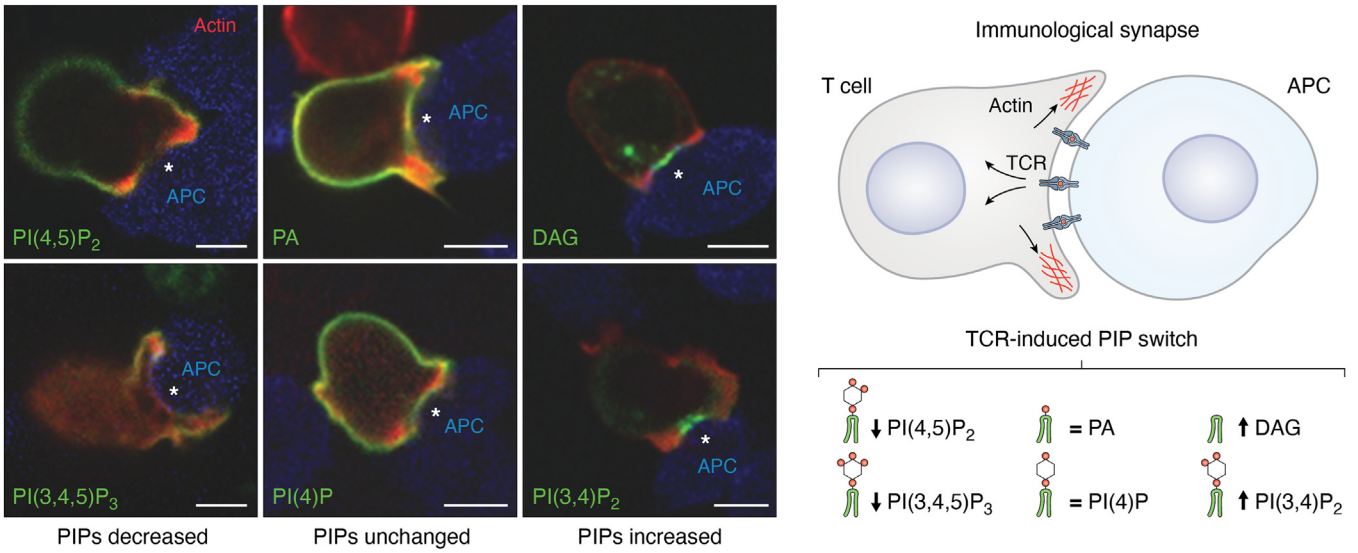

Figure 3. A TCR-induced PIP-switch triggers membrane specialization and actin depletion across the immunological synapse. Lipid and phosphoinositide-specific bio-probes (*green*) revealed the "membrane signature" across the immunological synapse (*) formed with the APC (*blue*) when actin (labeled with Lifeact, *red*) is depleted and granule secretion can occur. Images are single slices of 3D images as described in (30). Scale bars: 5 μm. The scheme on the *right side* shows the changes in lipid species that occur upon T cell stimulation, leading to TCR signaling and actin flow away from the center of the synapse.

A charge probe revealed a reduction in negative charge as PIP_2 was cleaved to form DAG. This accounted for the loss of phosphoinositide-4-phosphate-5-kinase (PIP5K) that can generate additional PIP_2 from PI4P, which also relies upon the negative charge of PIP_2, to associate with the membrane (31, 32). Loss of PIP5K not only exacerbates the PIP_2 depletion but also provides an experimental avenue to test the role of PIP_2 removal in controlling actin depletion across the synapse. By tagging a palmitoylation domain to PIP5K, it was possible to maintain localization of PIP5K across the synapse as it formed. Using this approach revealed that not only was PIP_2 retained across the synapse, but in addition, actin was also retained and secretion was inhibited (30). Thus, TCR signaling initiates a PIP switch across the synapse with the change in PIP_2 controlling the presence of actin and the ability of CTLs to secrete and kill.

Variations in a theme: the immunological synapse, cilia, and phagocytosis

After the initial finding that secretion is directed by the centrosome, which polarizes and docks at the immunological synapse, just as it occurs in ciliogenesis (22, 33), multiple parallels have been noted between the immunological synapse and cilia (34, 35). Remarkably, the membrane specialization observed at the secretory synapse closely resembles the phosphoinositide signature characterized across the primary cilium (36–38). Furthermore, experimentally increasing PIP_2 levels in the primary cilium inhibited Hedgehog signaling by the cilium, suggesting that there is a phosphoinositide mediated control of signaling by PIP_2. This is of particular interest as Hedgehog signaling is initiated at the immunological synapse after TCR activation (39). Whether there is a PIP_2 regulated control of TCR signaling is only just now being explored. Many different studies have explored the similarities between the immunological synapse and cilia that suggest that the synapse and cilia are "variations on a theme" (34, 35, 40–42). In both cases, PIP switches give rise to a dynamic change in membrane composition favoring specialized functions required for signaling and/or secretion.

Another variation on the same theme, in which phosphoinositide changes underpin dynamic localized membrane specialization, occurs during phagocytosis where membrane changes appear to mirror those occurring at the immunological synapse (43–45). In brief, phagocytosis initiated by kinases requires the hydrolysis of PIP_2 and depletion of PIP5K to modulate actin recruitment during phagocytosis. These changes have recently been shown to control forces required at the leading-edge during phagocytosis (46, 47).

A PIP-switch from endocytosis to ectocytosis

Although many of the findings surrounding the role of the PIP switch at the immunological synapse have focused on changes in membrane-associated F-actin driven by changes in PIP2, an important functional change at the membrane occurs as PIP_2 is cleaved and DAG generated. A recent study aimed at following the localization of TCR from recognition to detachment pointed to an important role for DAG as CTLs detach from their targets (48). The efficacy of CTLs is greatly enhanced by their ability to kill multiple targets in rapid succession. However, for serial killing to take place, CTLs not only need to recognize and attach to their targets but also let go so they can move from one target to the next. Using APEX-tagging of the CD3ζ chain of the TCR complex and electron microscope tomography, the localization of TCR could be viewed at high resolution across a depth of 1 μm of the immunological synapse. Among the surprising findings, this study revealed that TCR was not, as previously thought (49), undergoing endocytosis after it accumulated at the immunological synapse but was rather being shed into outward-budding vesicles from the plasma membrane of CTLs *via* a process termed "ectocytosis" (50). However, when considered in the context of the TCR-driven loss of PIP_2 at the synapse, the loss of endocytosis is not so surprising as structural studies have demonstrated that membrane PIP_2 is required for endocytosis (51, 52). What was more intriguing is that ectocytosis had been noted to involve DAG, a cone-shaped lipid able to induce negative curvature when inserted on the inner leaflet of the membrane (53, 54). Imaging with a bio-probe at the synapse revealed TCR-positive ectosomes also enriched in DAG, supporting an important role for DAG produced during the TCR-induced PIP switch that allowed TCR shedding that released CTLs from their targets.

The S-acylation/palmitoylation switch in the immunological synapse

General

Reversible S-acylation is a critical post-translational modification (PTM) that leads to the formation of a thioester bond between the cysteine residue of a protein and a fatty acid, most frequently palmitate (55). As a consequence, the biophysical properties of the respective proteins change, often with profound impacts on their physiological function and with implications for disease (56). Since the modified cysteines are typically close to or within the membrane of either peripheral or integral membrane proteins, S-acylation leads to the partitioning of the modified site into the membrane. Apart from membrane partitioning and altered sorting/transport in the ER and Golgi, S-acylation can also modulate the functional properties of a protein directly. An example is SNARE proteins, whose fusogenic capabilities can be influenced by S-acylation (57, 58). In many cases, however, deconvoluting the conformational effects on protein structure from the influence of acylation on membrane partitioning is almost impossible, and in most cases will occur in parallel.

The class of enzymes that are responsible for S-acylation for most target proteins are the so-called DHHC proteins (59, 60). A conserved zinc-finger domain characterized by the amino acid signature DHHC needs to act at a membrane-proximal protein-lipid interface, thereby transferring the acyl chain from coenzyme-A first to the cysteine of the DHHC motif and then further on to the cysteine of a target protein (61). The structure of two DHHC enzymes (DHHC15 and DHHC20)

was solved more recently (62), identifying the site of lipid attachment, but leaving open the question of how the substrate is recognized by the acylated DHHC protein. Interestingly, it seems that the transmembrane domains of DHHC20 perturb the membrane in a way that optimally exposes the catalytic cysteine to an incoming CoA-acyl molecule. Chain-length preferences for the acyl chain are distinct for different DHHCs (62, 63), but may also depend on the positioning of the target cysteine and the different distribution of lipids in the cell's organelles (64). The study by Greaves *et al.* showed that the most frequent fatty acids and acyl-CoA species are of the type C14:0 (myristate), C16:0 (palmitate), C18:0 (stearate), and C18:1 (oleate) (65). They then measured the preference of individual DHHC enzymes to the saturated C14:0, C16:0, and C18:0 species in an assay that uses fatty acid-azide/alkyne labeling of mammalian cells in combination with click chemistry-based detection. While DHHC-3, -5, -7, -11, and -15 prefer C14/C16 over C18, DHHC17 prefers C16/C18 over C14, and DHHC23 strongly favors C18. DHHC-2 and -4 show no clear chain length preference. Thus, while we will use the term palmitoylation in the following to describe DHHC enzyme mediated S-acylation, it implies that myristate, stearate, or other lipids can be attached to a given substrate protein, depending on the cell type, cellular compartment, and specific enzyme-substrate pair under investigation.

The second step of the palmitoylation reaction, the transfer of the lipid from the active site of the enzyme to the substrate, remains enigmatic. While in a few cases well-described protein recognition domains have been identified to confer specificity (64–66), the structural prerequisites for binding and acyl transfer are elusive. As a first goal, several DHHC enzymes relevant to T cells have been identified, and substrates of palmitoylation upon T cell activation are successfully identified and will be described in the subsequent paragraphs.

Palmitoyl transferases

A number of DHHC enzymes have been shown to be operative in T cells, in some cases at the proteomic level only (67, 68), in other cases by clearly assigned functionality (69, 70). However, most of this information is derived from Jurkat T cells and will certainly have to be expanded once primary T cells or even distinct subsets of the latter are profiled. For example, it has been shown that two isoforms of DHHC18 exist in primary human T cells, with the shorter isoform lacking the first 135 amino acids, probably because of alternative splicing-dependent use of an alternative promoter (71). The N-terminal 135 amino acids comprise the first two transmembrane helices in the long isoform (Fig. 4*A*), leading to a different architecture of the short isoform, which is the only isoform so far annotated to contain only 2 TM domains (Fig. 4*B*). Palmitoylation at the N-terminal cysteine 3 of the short isoform, which is far away from the active site DHHC motif, is required for activity *in vitro*, and interestingly, the short isoform is the predominantly palmitoylated variant in primary T cells (71). DHHC18 has been described as an HRAS palmitoylating enzyme, requiring the accessory protein GCP16 for its activity. It is structurally homologous to DHHC9, for which a structure in complex with GCP16 was recently solved (Fig. 4*C*). Interestingly, helix ^{62}G-^{73}L of GCP16 is in close contact to the C-terminal helix ^{279}I-^{288}C of DHHC9, an interface that is masked by an intramolecular interaction between the short helical motif EKKYW and the preceding C-terminal helix in DHHC20 (Fig. 4*D*).

Substrates of palmitoylation in T cells

The role of palmitoylation in T cell activation has recently been described by West *et al.* (68) in a comprehensive review. However, in most cases, palmitoylation occurs constitutively and not as a direct consequence of receptor stimulation. Thus,

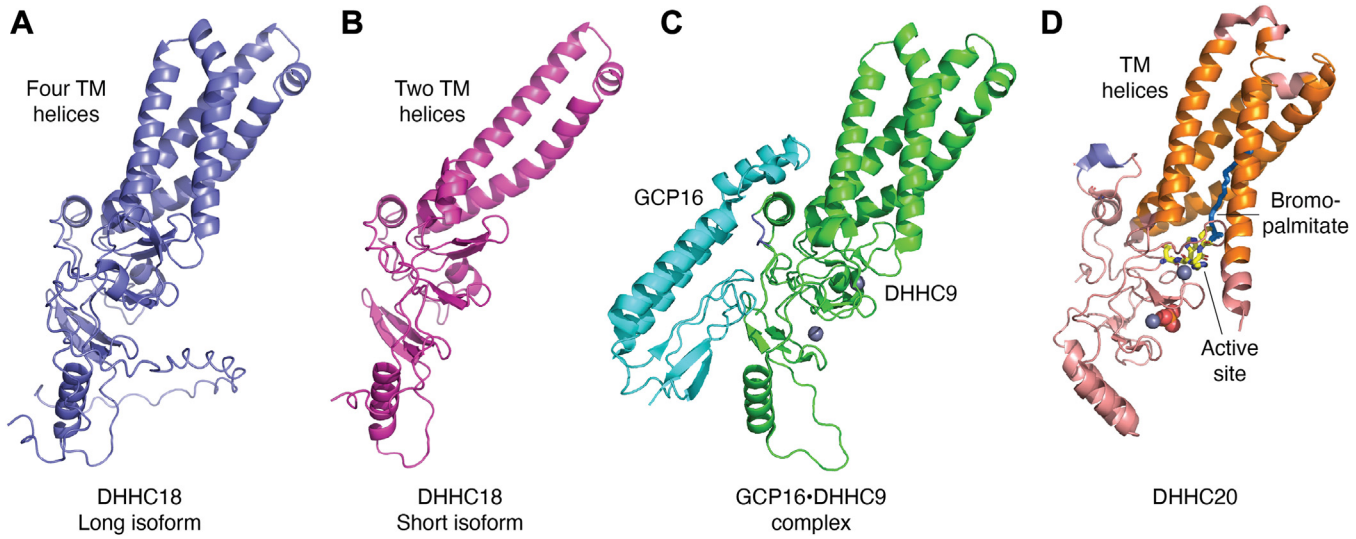

Figure 4. Structures and structural models of DHHC enzymes. *A* and *B*, comparison of the structures of the long and short isoforms of DHHC18 as predicted by Alphafold (124, 125). The long isoform (*A*) contains four transmembrane helices while the short isoform comprises two (*B*). *C*, electron microscopy structure of DHH9 (in *green*) in complex with the accessory protein GCP16 (in *cyan*) (126). The geometry of the GCP16 interaction site is conserved in the DHHC18 isoforms, while it is partially occupied by an internal short helix (shown in *blue*) in the crystal structure of DHHC20 (62) (*D*). In the DHHC20 structure the transmembrane helices are depicted in *orange* and the active site (DHHC) in *yellow* with the cysteine-attached bromo-palmitate in *marine blue*. Zinc ions are shown as *blue spheres*.

an activation-dependent palmitoylation switch has only been demonstrated in a few cases. For example, for the src kinase Lck it has been shown that a fraction of the protein becomes acutely palmitoylated by DHHC21 upon Fas receptor stimulation (70). In several other cases, palmitoylation has been shown to affect T cell signaling, but for the majority of these cases, this might be a consequence of abolished constitutive palmitoylation in the ER or Golgi. Such abrogation of palmitoylation might still influence the function of the activated T cell, but it is to be distinguished from altered palmitoylation *as a consequence* of T cell activation.

More recently, 70 proteins were identified that displayed higher overall palmitoylation in Jurkat T cells after stimulation by anti-CD3 and anti-CD28 antibodies for 10 min, compared to the non-stimulated control (71). Interestingly, only 18 proteins showed a stimulation-dependent decrease in palmitoylation, indicating that S-acylation of proteins is generally part of the activation program of the cell, similar to what is seen for tyrosine phosphorylation. The enhanced palmitoylation seen in the Acyl-Biotin-Exchange assay, that itself blurs spatial information, may stem from an increase in palmitoylation in the ER or Golgi compartment and thereby reflect the increased protein transportation requirements of the cell, or it could be the consequence of receptor-proximal activation at the immune synapse by a plasma membrane resident DHHC enzyme. A significant clustering of only a few functional types of proteins could be seen amongst the stimulation-induced palmitoylated proteins, as indicated in the following paragraph.

G proteins—A number of G proteins, namely GNAS, GNAO1, and GNAI1/2 were found to be inducibly palmitoylated, and interestingly, all of these subtypes also contain an N-terminal myristoylation motif. GNAI3 has been characterized as an inhibitor of chemokine-stimulated T cells, and its identification in the screen for activation-dependent palmitoylation may indicate a regulatory role in TCR-mediated signaling. So far, the GNAI2 and 3 proteins were described in the context of chemokine signaling, but the palmitoylation screen of activated T cells would also allow the identification of these proteins in TCR signaling. For example, it has been shown that GNAI2 is excluded from the center of the synapse upon activation of Jurkat T cells on supported bilayers (72). It is tempting to speculate that such compartmentation is modulated by the inducible palmitoylation of the GNAI2 protein upon activation, but other protein-protein interactions might well modulate the observed behavior.

Small G proteins and their regulators—For the small G proteins, it has been shown that NRas palmitoylation is required for T cell signaling, however, the T cell stimulation data suggest that KRas is inducibly palmitoylated. A differential role of Ras isoform for the production of either IFN-g or IL-4 has been discussed (73) and might indicate a special requirement of KRas in boosting T cell responses under conditions of strong stimulation by CD3/CD28 antibody regimen used in the Jurkat stimulation study. Moreover, the increased palmitoylation seen for RalA, RalB, and RCE1 indicates a yet underappreciated role of these small G proteins in T cell function.

Another interesting case of an activation-induced palmitoylated protein is the guanine nucleotide exchange factor (GEF) Arl13B, a protein for which is has been shown that its palmitoylation is required for cilia formation (74). While T cells don't make cilia, they share many features as discussed in a separate section of this review. Strikingly, Arl3 localizes to the immunological synapse where it acts as a GEF for Arl3, a small G protein that releases the tyrosine kinase Lck and presumably its homolog Fyn at the immune synapse (40). More specifically, Arl3 is thought to act in concert with the protein UNC119A that extracts myristoylated Lck from a donor membrane for delivery to the plasma membrane. Interestingly, there is a preference for activated Lck, phosphorylated at Y394, to be delivered to the plasma membrane. Since it has been demonstrated that Lck is inducibly palmitoylated upon Fas receptor stimulation (70), it will be interesting to follow the role of Arl13 B palmitoylation in Fas signaling and to probe its role in T cell activation, where, for example, an increase in Fyn palmitoylated upon stimulation was observed (71).

Intramembrane proteases—The two proteins, presenilin-1 and SPPL3, are part of the family of mammalian aspartyl intramembrane proteases that cleave single-passe transmembrane proteins in the process of signaling and secretion of substrates (75, 76). Both proteins were found to be palmitoylated upon T cell stimulation (71), and both proteins contain membrane-proximal cysteine residues that are candidates for acylation. In the case of SPPL3, these cysteines are at the N-terminal part of a transmembrane helix that also contains active site residues and therefore might well modulate the enzymatic function. Interestingly, SPPL3 has been implicated in the stimulatory potential of peptide-MHC-class I complexes by acting on the B3GNT5 glycosyltransferase (77). A change in the glycosphingolipid repertoire of the antigen-presenting cell is seen that markedly changes the potential of peptide-MHCI to activate T cells. In T cells, a protease-independent function of SPPL3 has been demonstrated in the activation of Nuclear Factor for the Activation of T cells (NFAT), and it might be worth following the influence of the corresponding Cys mutations in the context of NFAT-dependent cytokine production (78).

Receptors, transporters, and channels—Palmitoylation plays an exquisite role in Ca^{2+} channel signaling. The regulatory Kvβ2.1 subunit of the voltage-dependent potassium channel is driven into the lipid microdomains that are thought to present signaling entities within the IS (79). Similarly, the localization of Orai1 channels was shown to depend on palmitoylation at Cys-143 by DHHC20, and impaired recruitment of the C143A mutant to the immunological synapse was observed (69). The Orai1 C143A mutant displays diminished calcium signals, NFAT activation, and IL-2 production, and the number of T cell receptors delivered to the synapse was reduced. However, for both channels it is not clear whether palmitoylation is increased upon TCR stimulation, at least

they were not found amongst the targets of stimulation-dependent palmitoylation in Jurkat T cells.

Another receptor that was increased in palmitoylation levels upon stimulation is the scavenger receptor SCARB1, which is the major entry point for binding and uptake of high-density lipoprotein and which plays a role during T cell development (80). Similarly, stomatin, another lipid-binding protein, which is palmitoylated at Cys-29 (81) displays increased palmitoylation in activated T cells. For stomatin-like protein 2, modulation of T cell activity has been shown (82), but the role of stomatin has not been investigated in this context.

Activation-dependent depalmitoylation—While the majority of proteins showing significantly changed palmitoylation levels show an increase upon T cell activation, there are proteins that display decreased bulk palmitoylation levels upon stimulation (71). One interesting candidate is the scaffolding protein CKAP4, also named CLIMP-63, a mostly ER-resident type II transmembrane protein important for shaping ER morphology in an acylation-dependent manner (83). In the human pancreatic cancer-derived cell line S2-CP8, it was shown that a fraction of CKAP4 is localized to the plasma membrane, where it serves as a receptor for different ligands, including Dickkopf 1 (DKK1). In the case of interaction with DKK1, CKAP4 was reported to be de-palmitoylated in the presence of a Wnt ligand, and the palmitoylation state was suggested to control microdomain localization (84). Interestingly, CKAP4, among other proteins, has recently been identified as susceptible to competitive acylation of C16:0 *versus* C18 (85). Mechanistically, an acylation switch can be associated with a lateral movement between different types of microdomains, again pointing at a profound impact of the palmitoylation/de-palmitoylation switch for organizing the signaling events initiated at the plasma membrane. It will be interesting to see whether CKAP4 plays a similar role in the immune synapse and also what impact it has on Ca^{2+} signaling in T cells, given its described function in interacting with the anion channel VDAC2 and the Ca^{2+}-channel IP3 receptor, thereby modulating intra-mitochondrial Ca^{2+} concentrations (86).

SNARE proteins—the immunological versus *the neuronal synapse*

In neurons, fast exocytosis is required to allow signal transmission to ensue with high speed (in the ms time range). SNARE proteins constitute the last and decisive molecular complex to be formed prior to membrane fusion of the synaptic vesicles with the plasma membrane. A typical neuronal SNARE complex is formed by syntaxin1A/B, SNAP25, and the vesicular protein VAMP2 (Syb2) (87). SNAP-25 is constitutively palmitoylated, and this modification is mandatory for its function since SNAP-25 localization to the plasma membrane depends on acylation (58). More recently, it was found that palmitoylation of two membrane-proximal cysteines in the Stx1A localized within the TM domain, alters spontaneous vesicle fusion in the synapse (57). Interestingly, the retinal ribbon-specific STX3B homolog of STX1A does not contain the TM cysteines required for palmitoylation. Transfection of STX3B into STX1A knockout cells does not rescue the electrophysiological phenotype, showing compromised Ca^{2+}-evoked and spontaneous release. However, forced palmitoylation by exchanging the STX3BTM with the STX1ATM domain restores the wt phenotype. Thus, palmitoylation in this case is utilized as a facilitator of membrane fusion, probably by locally disrupting the target membrane structure.

For the synapse of CD8 T cells, the SNARE complex is formed by Stx11, SNAP-23, and VAMP7 (88, 89), and it has been shown that VAMP7 palmitoylation is of critical importance for localization to the Golgi and the immune synapse in Jurkat T cells (71). VAMP7 contains a so-called longin domain that acts autoinhibitory with respect to the SNARE helix and constitutes a landing platform for adaptor proteins to bind and potentially regulate the vesicle fusion process (90, 91). In CD8 T cells, VAMP7 was shown to be required for the exocytosis of lytic granules, but whether palmitoylation is required for efficient membrane fusion is not known (89). Interestingly, Syntaxin-11 was shown to be palmitoylated in an activation-dependent manner (71). Stx11 does not contain a transmembrane helix but six cysteines in its C-terminus that can be palmitoylated and have been shown to be important for vesicle exocytosis in platelets (92). The increased palmitoylation of VAMP7 and Stx11 in activated T cells would thus be compatible with a model where relatively rapid adjustments in the palmitoylation status of at least two out of the three SNARE proteins enable effector function.

Mass spectrometric analysis of phosphoinositides and palmitoylated proteins

Phosphoinositide analysis

Bio-probes to detect PI(4)P, PIP_2, and $PI(3,4,5)P_3$ are powerful tools to study dynamic changes of these signaling lipids within the membranes of immunological synapses. The possibility of combining these methods with direct quantitative analysis of phosphoinositides allows these processes to be studied at a molecular level. This includes the determination of stoichiometries of protein-PIP interactions and lipid species distributions for each phosphoinositide class. Whereas a growing number of headgroup-specific interactions of phosphoinositides with proteins are being described at the IS, little is known about the specificities of PIP-binding proteins of the IS for PIP species with a particular fatty acid composition. Quantitative mass spectrometric analysis of phosphoinositides is very challenging, as evidenced by the fact that these lipid classes are not usually included in global lipidomics approaches. The reasons for this are manifold and of both biological and analytical nature. Phosphoinositides are very low-abundant membrane lipids and can account for less than 1% of the lipid repertoire of mammalian cells. Compared to other lipid classes, phosphoinositides are characterized by a highly dynamic interconversion from mono-to di-to tris-phosphorylated PIs due to rapid and site-specific enzymatic de- and re-phosphorylation as well as rapid fatty acid remodeling (93). Capturing the phosphoinositide profile of a

cell at a given time accordingly requires very fast and careful sample processing in the presence of phosphatase inhibitors. In combination with advanced mass spectrometric data acquisition and analysis modes, vigorous analysis of phosphoinositides has now become possible (Fig. 5).

A two-step extraction was shown to be efficient in removing the bulk of less to non-polar lipids by a first neutral extraction (94, 95). A subsequent extraction with acidified organic solvent facilitates the dissociation of phosphoinositide-protein interactions on the one hand, and on the other hand leads to a better distribution of phosphoinositides from the aqueous to the organic phase *via* the protonation of the phosphate residues of the inositol head groups. A disadvantage of the two-phase extraction is that phosphoinositides are increasingly distributed into the aqueous phase as the degree of phosphorylation increases (96), so this must be taken into account in the quantitative determination. The choice of reaction vessels for extraction also plays a major role, as phosphoinositides tend to bind more strongly than other membrane lipids to different surfaces. Silanized glass surfaces or polypropylene-based tubes have proven to be suitable choices here. Initially, mass spectrometric analysis was mostly performed *via* shotgun or LC approaches in combination with multiple reaction monitoring or headgroup-specific scan modes, targeting positively charged cation adducts or neutral loss fragments (see for example Refs. (95, 97, 98)). Highly phosphorylated phosphoinositides exhibit poor chromatographic behavior, ionization, and transfer efficiencies, which must be taken into account in the analytical approach. A breakthrough was the introduction of chemical derivatization with diazomethane, which leads to methylation of the phosphate groups of the inositol ring. This permethylation significantly improves the stability, ionization mode, and chromatography of phosphoinositides and leads to a significant increase in the sensitivity with which these lipids can be detected (94, 99–103). Using this methodology, phosphoinositide quantification of a variety of samples of diverse origins (*e.g.*, cells, organelles, and viruses) have been performed, but until recently were limited to class specificity and did not include analysis of regioisomers. Because phosphoinositide regioisomers are characterized by distinct subcellular localizations and functions (93, 104, 105)), regioisomer-specific analysis is essential for understanding the role of phosphoinositides in the context of T cell signaling and beyond. While regioisomer analysis is well established for deacylated glycerophosphoinositides using radioactive or MS-bases methods (106, 107), separation of intact, acylated phosphoinositide regioisomers remained a challenge. Recent work has made significant progress in the analysis of phosphoinositide regioisomers through the application of additional separation techniques. Specifically, regioisomer separation of intact phosphoinositide species was achieved by combining phosphate methylation with chiral or supercritical fluid chromatography (108–111) which enabled quantitative analysis of up to 104 different lipid species covering PI3P, PI4P, PI5P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ in HEK293T cells (110). In the context of the IS, mass spectrometric phosphoinositide profiling is inherently a very challenging task that depends on either biochemical enrichment of these membrane structures or lipid imaging. While the first approach is difficult to implement and may depend on affinity purifications from cells expressing (endogenously) tagged proteins that localize to the immunological synapse, the second approach is limited by the resolution of imaging systems and depends on matrices optimized for phosphoinositide detection.

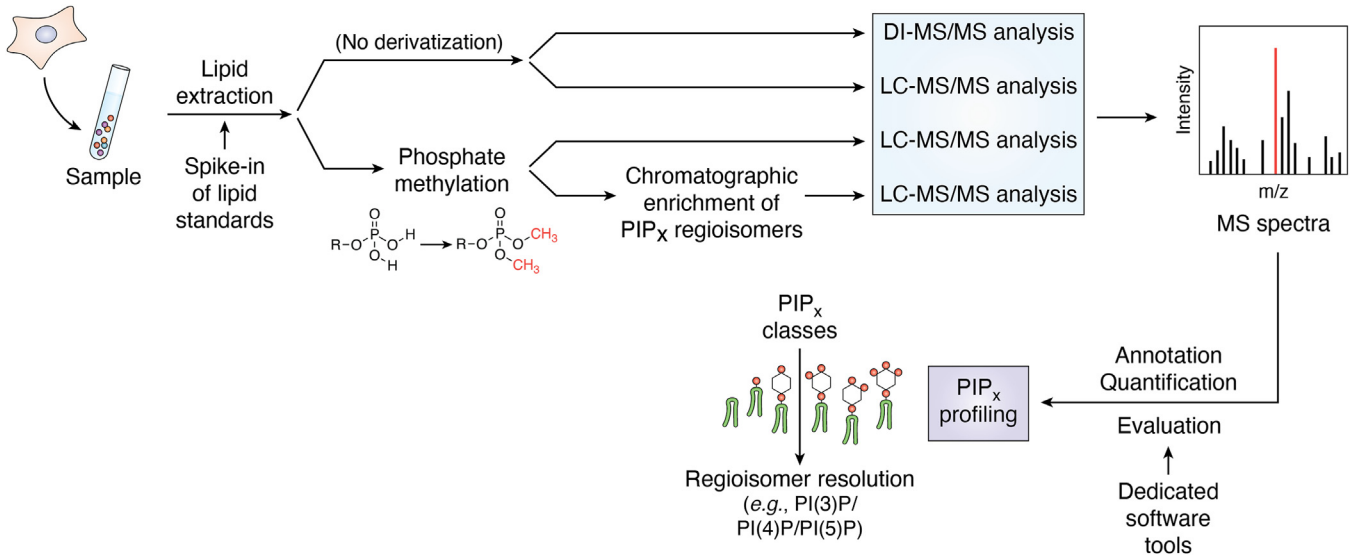

Figure 5. Workflows for MS-based phosphoinositide analytics. For the relative quantification of phosphoinositides, specific PIP_x-class standards are spiked in. The lipid extract can be analyzed either directly by direct injection (DI)-MS/MS or by liquid chromatography (LC)-coupled MS/MS analysis. Phosphate methylation of the lipid extracts significantly increases the sensitivity of the PIP_x analysis. In addition to LC-MS/MS analysis of methylated lipids, recent work has shown that PIP_x regioisomers can be separated using chiral chromatography. A preceding chromatography step can further improve the sensitivity of the analysis. MS spectra evaluation is done with dedicated software tools. PIP_x, mono-, di- or triphosphorylated phosphatidylinositol.

Palmitoylation analysis

Monitoring changes in the lipid composition of activated T cells upon cell contact formation has been successfully achieved by optical probes and light microscopy as beautifully shown for the PIP switches in this review (see Fig. 2 for an example). Probe development has been successful for several of the PIP moieties based on the distinctive headgroup features of individual phosphoinositides and their recognition by genetically encoded lipid sensors (112).

As described before for phosphoinositides, such optical methods to assess lipid changes are complemented by techniques that assess lipid moieties by mass spectrometry-based methods. In the case of palmitoylation, where ideally the identity of the acylated protein as well as the site of modification can be determined, direct methods, especially at the proteomic level, are difficult to achieve. While direct acylation has been reported *via* LC-MS (113) or MALDI-TOF (114) analysis for isolated proteins or mixtures of reduced complexity, the hydrophobic nature of the resulting lipopeptides often prohibits their enrichment and unambiguous assignment, especially when sample amounts cannot be scaled easily, as it is for example the case for many primary cell samples. Thus, indirect methods relying on click chemistry or Acyl-Biotin-Exchange have been the most successful methods to determine palmitomes at the proteomic level (115, 116). For example, using the Acyl-Biotin-Exchange method, it could be shown that the short isoform of the DHHC18 enzyme is the predominantly palmitoylated variant in primary T cells, with subsequent biochemical experiments demonstrating that palmitoylation is required for enzymatic activity (67, 71).

Despite the success of these methods, they also carry their limitations. For example, it is typically not possible to derive the site of palmitoylation from these experiments, and an unambiguous assignment to a particular cysteine has to be derived from mutational analysis. Even then, mutating cysteine to alanine may affect protein functional states in ways not anticipated by the experimentalist. Moreover, while palmitoylation is the most widely found S-acylation, an increasing variety of this type of modification has to be taken into account (117), in particular, when the cleavage of the thioester bond is employed as the sole specificity step of a given procedure. Moreover, DHHC enzymes have been shown to display different degrees of promiscuity towards acyl chain length (63), implying that depending on the abundance of a certain lipid, they may catalyze the modification of a protein substrate by lipids other than palmitate. In platelets, it was shown that an exogenous supply of lipids such as palmitate or stearate can shift the repertoire of S-acylated proteins towards the excess lipid (118). Similarly, another study demonstrated that feeding cells with stearate can lead to altered Cys-3 acylation of GNAI proteins, with the subsequent formation of the unsaturated oleate as a signal that drives the G protein out of cell membrane detergent-resistant fractions where they potentiate EGFR signaling (119). Both examples indicate that more global changes in lipid composition, either by exogenous supply or potentially *via* altered synthesis, can affect the acylation pattern of cellular proteomes.

Given the changes in lipid composition of the T cell membrane upon dietary changes or metabolic rewiring upon T cell activation, assessing changes in the lipidomes of T cells is mandatory.

Future challenges and opportunities

Determining the compositional changes and spatiotemporal patterning of phospholipids has become possible with the advent of advanced technologies and is of general importance beyond the analysis of T cells. Lipid extraction and enrichment methods in combination with increasingly sophisticated mass spectrometry and data analysis tools enabled to delineation of changes in headgroup identity, acyl chain length, and saturation in several cases (108–111). However, determining regioisomers of phosphoinositides in primary cells, for example, blood-derived T cells, remains a rewarding but extremely challenging aim given their dynamic changes by phosphorylation and dephosphorylation and by their low abundance. Targeted search algorithms together with tailored separation by chiral chromatography lead the way towards this goal and will hopefully pave the way for the methodology to become a routine procedure, ideally even in clinical settings. For example, mutations in genes that drive phosphoinositide conversion are causative for several, mostly neurological disorders. Thus, utilizing mass spectrometric identification could serve as a meaningful tool for diagnosis, in particular when it is combined with spatial information about the phosphoinositide's whereabouts in the cell.

In the case of palmitoylation, a similar approach cannot be foreseen. Monitoring the subtle changes in acylation chain length and saturation level in a given lipidated protein by direct methods is often impeded by the available amounts and unfavorable mass spectrometric properties of acylated peptides. Similarly, raising antibodies against acylated proteins has rarely been reported, thus precluding indirect detection in this manner. Routinely monitoring palmitoylation as a post-translational modification similar to phosphorylation is therefore not a realistic aim for the near future, especially given the fact that enrichment and MS analysis most likely must be optimized for each individual protein. It might thus be more promising for now to follow an alternative approach that is based on a mechanistic understanding and accurate bioinformatic prediction of acylation sites. Defining the rules that govern the acylation of target proteins by the DHHC enzymes or the de-acylation by thioesterases or hydrolases would be an important step for the improvement of the already existing *in silico* toolsets (120–123). While not substituting the experimental validation of the biological significance of protein S-acylation, such an understanding would certainly help to draw conclusions of where, by which enzyme, and for how long a given protein is modified within the cell.

Within the framework of these more general approaches to detect changes in lipid composition or lipidation of proteins, the immunological synapse may serve as an excellent model system to synergize the findings on these molecular switches and their cellular consequences. Given the intimate knowledge of the critical protein and lipid players in the IS, further

knowledge of the localization and mode of action of the enzymes that drive the respective interconversions will add to a more complete picture of how the changes in this specialized contact zone are organized over time. Ultimately, more comprehensive and quantitative knowledge will feed into mathematical conceptualizations that capture the essential features of this fascinating cellular ultrastructure.

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Abbreviations—The abbreviations used are: CTL, cytotoxic T lymphocytes; DAG, diacylglycerol; DGKs, diacylglycerol kinases; PIP, phosphatidylinositol-3,4,5-trisphosphate.

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