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[10] A Live Cell, Image-Based Approach to Understanding the Enzymology and Pharmacology of 2-Bromopalmitate and Palmitoylation

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Abstract

The addition of a lipid moiety to a protein increases its hydrophobicity and subsequently its attraction to lipophilic environments like membranes. Indeed most lipid-modified proteins are localized to membranes where they associate with multiprotein signaling complexes. Acylation and prenylation are the two common categories of lipidation. The enzymology and pharmacology of prenylation are well understood but relatively very little is known about palmitoylation, the most common form of acylation. One distinguishing characteristic of palmitoylation is that it is a dynamic modification. To understand more about how palmitoylation is regulated,

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we fused palmitoylation substrates to fluorescent proteins and reported their subcellular distribution and trafficking. We used automated high-throughput fluorescence microscopy and a specialized computer algorithm to image and measure the fraction of palmitoylation reporter on the plasma membrane versus the cytoplasm. Using this system we determined the residence half-life of palmitate on the dipalmitoyl substrate peptide from GAP43 as well as the EC_{50} for 2-bromopalmitate, a common inhibitor of palmitoylation.

Introduction

Many proteins are concentrated on the plasma membrane (PM) by virtue of their lipid modifications. Most data show that lipid modifications of proteins may well be the primary physical determinant for targeting to and retention of some proteins to membrane lipid microdomains such as synapses and caveolae (El-Husseini *et al*., 2000; El-Husseini Ael *et al*., 2002; Kanaani *et al*., 2002; Loranger and Linder, 2002; Topinka and Bredt, 1998; Zacharias *et al*., 2002). Fusion of green fluorescent protein (GFP) to small peptide substrates for lipid modification (e.g., the N-terminal 12–18 residues of GAP43) has been shown to be sufficient to localize the fusion proteins to the PM in the absence of any other targeting signal (Zacharias *et al*., 2002). Similarly, mutagenic substitution of modifiable residues for ones that cannot be modified results in gross mislocalization and/or loss of function of the expressed proteins (Craven *et al*., 1999; Hiol *et al*., 2003b; Osterhout *et al*., 2003; Wiedmer *et al*., 2003). It has also been shown that different lipid moieties induce partitioning into different lipid environments or lipid microdomains of cells (Melkonian *et al*., 1999; Moffett *et al*., 2000; Zacharias *et al*., 2002). Specific associations of proteins within such microdomains, whether mediated by attractive, protein–protein interactions, forced proximity, or both, are critical components in the architecture of cellular communication, and lipid modifications undoubtedly play an important role in the creation and modulation of such protein associations. Among the lipid modifications, palmitoylation is the most poorly understood and may hold the most promise as a druggable process in human health and disease. It was only a short time ago that the general assumption was that palmitoylation was likely exclusively an autocatalytic process. However, a family of enzymes has been shown to be able to mediate the process.

This chapter reviews, compares, and contrasts the various common forms of protein lipid modifications. It then describes how to go about constructing fluorescent protein (FP)-based reporters for lipid modification

and live-cell based assays to study the processes that underlie protein lipid modification. It also describes advances in high-content or high-throughput microscopy that allow us to make very accurate measurements of even small changes in the subcellular distribution of lipid modified FPs. It also presents data demonstrating the utility of the methods described.

Types of Protein Lipid Modifications

Protein Prenylation

Prenyl and acyl groups are the most common forms of protein lipid modifications (Fig. 1). The two most common forms of prenylation are geranylgeranylation and farnesylation (Fig. 1A), whereas myristoylation and palmitoylation (Fig. 1B) are likely the most common forms of acylation. Most, if not all, of the biochemical steps regulating the prenylation of proteins have been deciphered (reviewed in Fu and Casey, 1999; Roskoski, 2003; Sinensky, 2000). In fact, the mechanistic pathway for farnesylation has been determined to the atomic level (Long *et al.*, 2002). This density of information

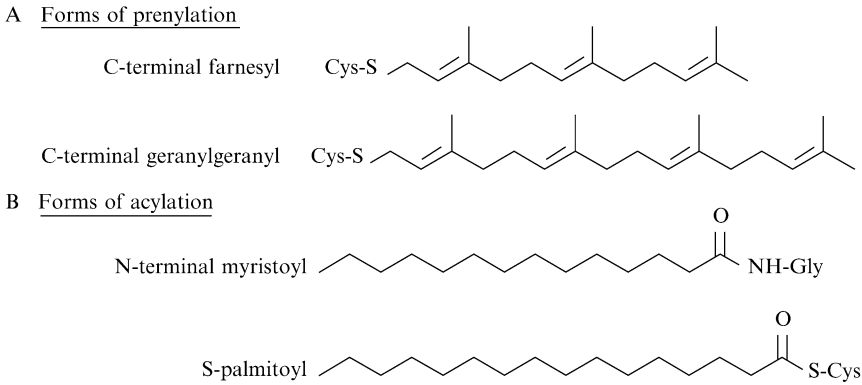

FIG. 1. Lipids that modify proteins: (A) prenylation and (B) acylation. Each class of modification targets proteins to which they are attached to unique subcellular locales (Melkonian *et al.*, 1999; Moffett *et al.*, 2000; Zacharias *et al.*, 2002). This ability is likely due to their different chain lengths, degree of saturation and branching, and their physical position on the proteins. Both forms of prenylation occur via stable thioether bonds on the final cysteine of a "CAAX" box at the C terminus of a protein. Myristoylation occurs via a stable amide bond to the N-terminal glycine of a protein, whereas the addition of palmitate occurs most commonly via a labile, but regulated, thioester bond to the side chain of a free, reactive cysteine on the cytoplasmic side of the PM.

for prenylation is due in part to the fact that prenyltransferases have been fairly successful therapeutic targets for the treatment of several types of cancer (Dinsmore and Bell, 2003; Ghobrial and Adjei, 2002), underscoring the importance of protein lipidation for human health and disease.

Protein Acylation

Among the types of acylation, enzymatic processes regulating myristoylation have been characterized best and are reviewed elsewhere (Farazi *et al*., 2001; Rajala *et al*., 2000; Resh, 1999). Briefly, proteins that will become myristoylated begin with a consensus sequence Met-Gly-X-X-X-Ser/Thr. The start Met is cotranslationally, proteolytically removed and the myristate is added to the exposed N-terminal glycine via a stable amide bond. The formation of this bond is catalyzed by *N*-myristoyl transferase with a high degree of selectivity for 14-carbon myristate (Farazi *et al*., 2001; Rajala *et al*., 2000). N-terminal myristoylation often exists in combination with palmitoylation, which can take at least two forms: *N*-palmitoylation (a much less common form) and *S*-palmitoylation (the most common). *N*-Palmitoylation, first described for the protein sonic hedgehog (Pepinsky *et al*., 1998), is the addition of palmitic acid to the α-amide of Cys-24, which is exposed proteolytically to become the N-terminal residue of the functional protein. Addition of a palmitoyl group by an amide bond to the N-terminal glycine has been shown to occur on the heterotrimeric G protein, Gαs (Kleuss and Krause, 2003).

Myristoylation frequently occurs in close spatial and often temporal proximity to palmitoylation on a protein. Cysteine residues (C) in close proximity to a myristoylated N-terminal glycine (G) are often modified by palmitate (Dunphy and Linder, 1998; Resh, 1999), as is the case for the Src family of protein tyrosine kinases, e.g., Fyn (Liang *et al*., 2004) and Lyn (Honda *et al*., 2000), but can also occur up to 21 residues away in a *de novo*-designed, GFP fusion protein (Navarro-Lerida *et al*., 2002) and 26 residues from the N terminus in eNOS (Robinson and Michel, 1995).

Palmitoylation may also occur in conjunction with prenylation (either farnesylation or geranylgeranylation), as is the case with many of the Ras superfamilies of signaling proteins (Hancock, 2003; Hancock *et al*., 1989, 1990). A C-terminal ''CaaX box'' provides the substrate recognition motif for prenyltransferases. In this motif, C is cys, a is aliphatic, and X denotes the residue that specifies either farnesylation or geranylgeranylation. Prenylation at the C-terminal cysteine is followed by proteolytic cleavage of the next three amino acids and carboxymethylation of the newly exposed, prenylated cysteine. Cysteine residues immediately N-terminal to the prenylated cysteine residue are frequently palmitoylated. Examples of such dual modification

can be found in H-Ras and N-Ras (illustrated here) (Apolloni *et al.*, 2000; Hancock, 2003; Hancock *et al.*, 1989).

Palmitoylation may also be the exclusive form of lipidation of a protein. In such cases, it is most common to find more than one palmitate attached to the protein [e.g., Gap43 (Liu *et al.*, 1993; Skene and Virag, 1989) and PSD-95 (Topinka and Bredt, 1998)]. Palmitoylated cysteines are often found in close proximity to basic residues, which provide additional membrane affinity by virtue of an electrostatic interaction with the negatively charged head groups of the membrane lipids.

While these are probably the most common lipid species to occupy cysteine, other acyl groups have been shown to take residence on thiol side chains of cysteines. The best studied examples (Liang *et al.*, 2001, 2002, 2004) use matrix assisted laser desorption, time of flight (MALDI-TOF) mass spectrometry to positively identify the adducts. These studies showed modification of the N terminus of Src family kinases where the N-terminal glycine is consistently modified by myristate, while the adjacent cysteine residue may be *S*-acylated by palmitate, palmitoleate, stearate, or oleate (Liang *et al.*, 2004).

The most common way to attach palmitate to a protein is via a labile thioester bond to the thiol side chain of a cysteine residue (Bernstein *et al.*, 2004) (Fig. 1), but palmitoylation can also occur in other ways, for example, on an N-terminal cysteine, as is the case with Hedgehog (Pepinsky *et al.*, 1998), a secreted signaling protein. An example of palmitate modifying the weaker -OH nucleophile of threonine occurs on the carboxyl terminus of a spider toxin (Branton *et al.*, 1993). The ε-amino group of lysine can also be modified by palmitate via an amide bond. This occurs in several secreted proteins, including a bacterial toxin (Stanley *et al.*, 1998). Little if anything is known about the enzymes that may modify lysines or threonines in eukaryotic cells. While these forms of palmitoylation are of great interest, they lie beyond the scope of this chapter and as such the main focus of this chapter is exclusively palmitoylated proteins where the linkage is via a thioester bond to the thiol side chain of cytosolic, accessible cysteine residues.

There is no obvious sequence of residues that constitute a motif for palmitoylation among cytoplasmic proteins that are palmitoylated other than a requirement for the free thiol side chain of cysteine. Proteins may be palmitoylated near either the N terminus or, less frequently, the C-terminus or any one or more sites in the middle. Some examples are listed in Table I.

Palmitate Turnover

The residence half-life of the palmitoyl group on proteins can be considerably shorter than the half-life of the proteins to which it is attached (Lane and Liu, 1997; Loisel *et al.*, 1996; Wolven *et al.*, 1997), suggesting that

TABLE I
PROTEIN PALMITOYLATION MOTIFS OF CYTOPLASMIC PROTEINS OCCUR THROUGHOUT THE PROTEINS WITH NO DISCERNIBLE (AS OF YET) CONSENSUS SEQUENCE[a]

Protein	Relative location of the palmitoylation site within the protein	Palmitoylated sequence and the neighboring environment[b]	References
Gap43	N-terminal	^{N}-ML**CC**MRRTKQV-	Liu *et al.* (1993); Skene and Virag (1989)
PSD95	N-terminal	^{N}-MD**C**L**C**IVTTKKY-	Topinka and Bredt (1998)
RGS16	N-terminal	^{N}-M**C**RTLAAFPTT**C**LE-	Hiol *et al.* (2003)
Yck2	C-terminal	-FFSKLG**CC**-^{COOH}	Roth *et al.* (2002)
AtRac8 (*Arabidopsis*)	C-terminal	-G**C**LSNIL**C**GKN-^{COOH}	Lavy *et al.* (2002)
SNAP25	Internal	-^{83}KF**C**GL**C**V**C**P**C**NKL^{95}-	Lane and Liu (1997)
GAD65	Internal	-^{27}RAW**C**QVAQKFTGGIGN-KL**C**ALL^{48}-	Kanaani *et al.* (2002)
GRK6	Internal	-^{557}RQD**CC**GN**C**SDSEEE-LPTRL-^{576}	Stoffel *et al.* (1994)

[a] After el-Husseini Ael and Bredt (2002) and Smotrys and Linder (2004).
[b] Palmitoylated residues are in bold. Basic residues that may contribute to PM binding are underlined; superscript numbers indicate the relative position in the protein.

the process should be mediated by enzymes in a manner analogous to kinases and phosphatases for phosphorylation. These examples of a fast (minutes) dynamic response also occur on a timescale that is shorter than what should be required to deplete or replenish a pool of available palmitate. Such observations strongly suggest and led many to hypothesize that palmitoylation is regulated enzymatically. Reversible palmitoylation of PSD-95 is regulated by the activity of neurons. Depalmitoylation promotes the removal of PSD-95 from synapses (El-Husseini Ael and Bredt, 2002). Several other interesting reports of the regulated turnover of palmitate support a role for palmitoylation in the regulation of the structure and function of synapses that is highly dynamic (El-Husseini Ael *et al.*, 2002; Osten *et al.*, 2000; Rocks *et al.*, 2005).

Enzymes for Depalmitoylation

The relatively short residence half-life of palmitoylation of proteins is particularly suggestive of enzymatically mediated palmitate removal or

depalmitoylation. Indeed, such proteins were among the first enzymes to be identified as having an influence on the palmitoylation state of proteins. PPT1 (Camp and Hofmann, 1993; Camp *et al.*, 1994) is a lysosomal hydrolase that participates in the degradation of palmitoylated proteins by deacylating cysteine thioesters; acyl protein thioesterase 1 (APT1), a cytoplasmic protein first biochemically characterized as an acyl thioesterase by Duncan and Gilman (1998), is a member of the serine hydrolase, α/β fold family of lysophospholipases that has several additional substrate and lipid specificities. Regulated removal of the palmitoyl group from proteins is critical in human health as defects in PPT1 result in a severe neurodegenerative disorder known as infantile neuronal ceroid lipofuscinosis (ICNL) (Vesa *et al.*, 1995). The role of PPT1 in ICNL was confirmed by targeted disruption of the gene in a mouse resulting in a model of the human disorder (Gupta *et al.*, 2001).

Palmitoylation increases the hydrophobicity and thus the affinity of the protein for membranes. The unique chemistry and dynamic nature of palmitoylation make it unique among the various forms of protein lipidation, providing an important mechanism for cells to integrate, sort, regulate, or even generate various cellular stimuli. In neurons, it is involved in protein targeting, endocytosis, vesicle fusion, neurotransmitter release (vesicle exocytosis), clustering, and modulation of ion channels in the plasma membrane, cell adhesion, and others. Palmitate is the most common lipid species to occupy cysteine residues but it is not the only one. Liang *et al.* (2001, 2002, 2004) used MALDI-TOF mass spectrometry to identify the lipid moieties on the N terminus of Src family kinases. For this kinase, the N-terminal glycine is consistently modified by myristate, but the adjacent cysteine residue is modified most frequently by palmitate but also by palmitoleate, stearate, or oleate (Liang *et al.*, 2004) with a frequency of residence on the protein that is apparently related to their abundance.

Role of Palmitoylation in Development

The involvement of palmitoylation in development is exemplified by GAP43 and paralemmin, which are localized to filopodia and growth cones (Liu *et al.*, 1991; Skene and Virag, 1989; Strittmatter, 1991, 1992; Zuber *et al.*, 1989a,b) where each has been shown to influence the formation of filopodia and the branching of dendrites and axons. Interestingly, it has also been shown that simply expressing the palmitoylation motifs of these proteins fused to GFP is sufficient to induce the changes seen when overexpressing the native forms of GAP43 and paralemmin (Gauthier-Campbell *et al.*, 2004). This suggests that the critical factor in the morphogenic transformation of the membrane is due to insertion of the tightly packing and rigid

palmitates themselves. Similarly, examples of proteins involved in axon path finding and synapse formation are palmitoylated, including the cell adhesion molecule NCAM140 and the netrin-1 receptor DCC (deleted in colorectal cancer) (Herincs *et al*., 2005), both members of the NCAM family of proteins. Inhibiting palmitoylation disrupts raft localization of DCC, which is required for netrin-1-induced DCC-dependent ERK activation and netrin-1-dependent (Herincs *et al*., 2005) axon growth. Palmitoylated tetraspanins form unique lipid raft structures (called tetraspanin-enriched microdomains) that provide platforms for integrin-dependent cell adhesion (Yang *et al*., 2004).

During development, the ability of neurons to find their way to appropriate targets forming synaptic infrastructure is critical and, in many circumstances, dependent on the palmitoylation of key regulatory proteins. Subsequent to developmental morphogenic movements, formation, maintenance, and regulation of synaptic protein complexes are also dependent on palmitoylation. One of the first, most highly characterized and arguably most important palmitoylated synaptic protein is PSD-95, a PDZ domain-containing, scaffolding protein (Kim and Sheng, 2004). Retention of PSD-95 at the synapse is mediated by palmitoylation (Topinka and Bredt, 1998). A cycle of palmitoylation/depalmitoylation of PSD-95 facilitates the internalization of AMPA receptors (Ehrlich and Malinow, 2004) and presumably any other proteins bound to the scaffold at the time of internalization. In the case of the AMPA receptor, palmitoylation-related changes in the rate of internalization are closely tied to learning and memory in an *in vitro* preparation as well as during experience-driven synaptic strengthening by natural stimuli *in vivo* (Ehrlich and Malinow, 2004). The movement of ions through ionotropic channels sequestered at the synapse by palmitoylation-regulated scaffolding triggers exocytotic release of neurotransmitter. Not surprisingly, several of the critical components of the exocytotic apparatus are also palmitoylated, such as SNAP-25 (Hess *et al*., 1992) and members of the synaptotagmin family (Chapman *et al*., 1996; Heindel *et al*., 2003; Veit *et al*., 1996, 2000). The functional consequences of palmitoylation in these instances vary with the protein and range from sorting to presynaptic terminals (Han *et al*., 2005; Kang *et al*., 2004) to regulation of intracellular membrane flow and vesicle fusion (Fukasawa *et al*., 2004) in the case of Ykt6. Vesicles bound for the presynaptic membrane are loaded with the neurotransmitter. The neurotransmitter GABA is synthesized by gultamic acid decarboxylase 65 kDa (GAD65). Dual palmitoylation of GAD65 is required for moving the protein from the Golgi to presynaptic terminals. In this case, the palmitoylated cysteines are immediately adjacent to (1) a Golgi localization signal and (2) a membrane-anchoring motif (Kanaani *et al*., 2002). As is the case

with palmitoylated Ras proteins, cellular targeting signals, in addition to the palmitate, operate in a hierarchical way to determine the subcellular residence of the protein. In the case of GAD65, the dynamic, reversible nature of palmitate provides an elegant switch to regulate protein function or subcellular distribution as opposed to an adjacent, ''hardwired'' short stretch of amino acid residues.

Cysteines: Primary Sites of Palmitoylation

The nature of cysteines, their thiol side chains, the types of reactions they undergo, and the nature of the bonds they form all figure very prominently into interest in palmitoylation. Cysteine residues are among the most nucleophilic agents in a cell (Bernstein *et al.*, 2004) and are the most common site of palmitoylation. However, palmitoylation can also occur in other ways, for example, on an amine of an N-terminal cysteine, as is the case with Hedgehog (Bijlsma *et al.*, 2004; Pepinsky *et al.*, 1998), a secreted signaling protein. An example of palmitate modifying the weaker -OH nucleophile of threonine occurs on the carboxyl terminus of a spider toxin (Branton *et al.*, 1993). The ε-amino group of lysine can also be modified by palmitate linked by an amide bond. This occurs in several secreted proteins, including a bacterial toxin (Basar *et al.*, 1999). Little is known about the enzymes that may catalyze the modification of lysines or threonines in eukaryotic cells. While these forms of palmitoylation are of great interest, they lie beyond the scope of this chapter and as such the main focus of this chapter is exclusively palmitoylated proteins where the linkage is via a thioester bond to the thiol side chain of a free, reactive cysteine residue.

Thiols of cysteine residues are also targets of other types of modifications (Di Simplicio *et al.*, 2003; Hershko and Ciechanover, 1998; Lipton *et al.*, 2002). The redox potential of different cellular compartments varies, but in general, in healthy cells, the cytoplasm is a reducing environment, meaning that solvent-exposed cysteine side chains are not typically disulfides (Sitia and Molteni, 2004). Thiols are the most potent nucleophilic amino acid residue side chains with rates of reaction that depend on the pK_a of the thiol, with the pK_a being dependent on the local environment of the side chain within the context of the whole protein. Unlike other residues with side chain nucleophilic moieties (-OH or -NH_2), thiol side chains undergo conjugations, redox, and exchange reactions (Di Simplicio *et al.*, 2003). Conjugation reactions include nitric oxide (NO) or *S*-nitrosylation, fatty acids such as palmitate, reactive oxygen species, and reactive nitrogen species forming bonds that are not susceptible to cleavage by hydroxylamine, a reagent used to quantitatively remove thioester-linked palmitate (Munro and Williams, 1999). Another source of thioester bonds in cells is the transient association

between ubiquitin and the E1, E2, and certain E3 ubiquitination enzymes (Hershko and Ciechanover, 1998; Passmore and Barford, 2004). Based on the physical characteristics of ubiquitin-related thioesters it is highly unlikely that they are ever in a position to be palmitoylated.

Palmitoyl Acyl Transferases (PATs)

It is clear that palmitoylation is intricately tied to signaling pathways by a variety of mechanisms, giving the distinct appearance of being a regulated process. However, despite years of searching, only recently has the molecular identity of enzymes capable of palmitoylation been discovered. A family of at least 23 enzymes called palmitoyl acyl transferases (designated ZDHHC 1–23), capable of catalyzing the addition of palmitate to cysteines, was identified. Three of these have already been linked to human diseases. Erf2p and Akr1p were identified as the first PATs in yeast (Lobo *et al*., 2002; Roth *et al*., 2002). These were the first reports that provided the molecular identity of a protein associated with or having PAT activity. One very important piece of evidence also gleaned from these reports was the likelihood that the DHHC-CRD (Asp-His-His-Cys-cysteine-rich domain) motif was a signature for this type of activity. The identification of GODZ, a DHHC-CRD protein, as a PAT that specifically palmitoylates the $\gamma 2$ subunit of the $GABA_A$ receptor provided evidence that PATs exist in vertebrates (mammals) and that the DHHC-CRD motif is the hallmark of at least one type of enzyme that can palmitoylate proteins *in vivo* (Keller *et al*., 2004). One known, critical function of a DHHC-CRD protein is the transfer of palmitate to a cysteine residue substrate. When comparing the number of potentially palmitoylated proteins (hundreds to thousands?) to the current number of PATs (23), it is evident that at least some PATs will have more than a single substrate. The consequences of a nonfunctional PAT should, in theory, result in nonpalmitoylated substrates that are unable to function appropriately in their normal capacity. It is also probable that a particular substrate can be palmitoylated by more than one PAT, in which case, hypopalmitoylated substrates may be the result of a nonfunctional PAT. In any event, unless the palmitoylation-related ''gain'' (analogous to the gain in an electronic circuit) inherent in the signaling cascade that dictates one particular endpoint is known, the relationship between decremented palmitoylation and outcome cannot be determined a priori. Identification of PATs cleared a critical roadblock in our understanding of palmitoylation. Our ability to identify the substrates of PATs and to understand how PATs are regulated has continued to proceed, albeit slowly. Development of agonist and antagonist assays for the process will be important in the development of new pharmacological tools to

study the process as well as pharmacological tools that are directly relevant to human health and disease.

Links between Palmitoylation and Disease

Immediately following their discovery, members of the DHHC-CRD family were linked to human diseases. Chromosome 22q11 is associated with a complex syndrome sometimes referred to as velocardiofacial syndrome or, more commonly, as 22q11 deletion syndrome (22q11DS). One of the several diseases associated with the syndrome is schizophrenia. Several genes and 72 identified single nucleotide polymorphisms (SNPs) (Bassett *et al*., 2003; Karayiorgou *et al*., 1995) have been identified in the affected region. Among the SNPs, rs175174 was tightly associated with susceptibility to schizophrenia (Mukai *et al*., 2004). This SNP causes improper splicing of the gene ZDHHC8, retention of intron number 4, and introduction of an early stop codon resulting in early termination of translation. This sets up a situation where the truncated version behaves as a dominant-negative regulator of palmitoylation. Mukai *et al.* (2004) generated *Zddhc8* knockout mice that expressed impaired sensorimotor gating, a fear of new spaces, and reduced motor activity (Jablensky, 2004). These mice also proved resistant to the NMDA receptor blocking drug MK801, which is known to activate locomotor activity, suggesting that ZDHHC8 affects behavior in part by interfering with glutamatergic transmission (Mukai *et al*., 2004). These results correlate with other studies showing that palmitoylation modulates several neurotransmitter systems, including the glutamatergic system (Drisdel *et al*., 2004; el-Husseini Ael and Bredt, 2002; Hayashi *et al*., 2005).

HIP14 (huntingtin interacting protein 14) is a human DHHC-CRD-containing protein that interacts with htt, the protein that incurs hyperexpanded stretches of polyglutamine in Huntington's disease (Huang *et al*., 2004; Singaraja *et al*., 2002). Importantly, HIP14 expression is limited to the neurons (medium spiny neurons in the striatum) impacted most severely in the disease (Singaraja *et al*., 2002), thereby providing a potential mechanism for the puzzling and important phenomenon of cell-type specificity so often observed in neurodegenerative diseases. ZDHHC15, one of the PATs that palmitoylates PSD-95 and GAP43 most efficiently (Fukata *et al*., 2004), has been linked to nonsyndromic X-linked severe mental retardation (Mansouri *et al*., 2005). There is a breakpoint in the regulatory portion of ZDHHC15 resulting in gene transcription failure. If any of the known mutations in PATs are acting as the sole genetic cause underlying the pathology of the disease they are associated with, it suggests that the degree of functional redundancy may be lower than it appears (Fukata

et al., 2004) or, alternatively, that specificity is the result of selective expression in a certain cell type or even a distinct subcellular domain.

These early examples of the profound consequences of misregulated palmitoylation illustrate the importance of the modification and suggest that other malfunctioning PATs will be associated with human disease.

Using Fluorescent Proteins to Study Protein Lipid Modifications

Over the last decade, fluorescent proteins have become an indispensable tool for understanding so many details in cell biology, signaling, and physiology, among others. Our understanding of the function and effects of lipid modifications of proteins is a prime example how enabling FPs have been. Substrates for the various forms of lipid modification are encoded by relatively short peptides and therefore easily fused to either the N or the C terminus of a FP. Such a fusion allows, at the very minimum, the ability to determine the effect of a lipid moiety on the subcellular distribution of an otherwise inert protein. In the case of *Aqueorea* FPs, there are no other known signal or targeting sequences existing in FPs that dictate a specific subcellular distribution; the monomeric proteins diffuse freely throughout the cytoplasm and nucleus unless directed to do otherwise by fusion with a targeting signal.

Monomeric Fluorescent Proteins: A Critical Feature for Studying Palmitoylation

Not all fluorescent proteins are created equally. Currently, *Aequorea* GFP is the only fluorescent protein known that is not an obligate homo-oligomer in its natural state; the GFP from *Renilla* is an obligate dimer (Ward, 1998; Ward and Cormier, 1979) and RFP (dsRED from coral) is an obligate tetramer (Baird *et al.*, 2000), as are all other characterized fluorescent proteins from corals. Quite some time before a rigorous determination of the homoaffinity was made, it was known that even *Aequorea* GFP dimerized to some degree in solution (Yang *et al.*, 1996). GFP crystallizes either as a dimer (Battistutta *et al.*, 2000; Palm *et al.*, 1997; Yang *et al.*, 1996), or as a monomer (Brejc *et al.*, 1997; Ormo *et al.*, 1996; Wachter *et al.*, 1997, 1998, 2000). In the dimeric crystal structure, the unit cell consists of two monomers associated in a slightly twisted, head-to-tail fashion via many hydrophilic contacts, as well as several hydrophobic contacts. The very different solvent conditions used by each group are sufficient to explain the differing results (Ward, 1998). Residing within a large hydrophobic patch, residues A206, L221, and F223 are sufficient to cause formation of the dimer at relatively low concentrations in solution and in

living cells. However, changing these residues singly or in combination to positively charged residues such as A206K, L221K, and F223R effectively eliminated the interaction of the monomers (Zacharias *et al*., 2001); the resulting monomeric GFPs are called mGFPs. To determine the strength of the interaction in solution, Zacharias *et al.* (2001) used sedimentation equilibrium analytical ultracentrifugation to characterize the affinity of GFPs with the wild-type interface as well as the mGFPs. In significant contrast to X-ray crystallography, the experimental conditions used in the analytical ultracentrifugation experiments approximated cellular physiological conditions and were able to provide definitive information (McRorie and Voelker, 1993) about the affinity of the complex. Other mutations are also thought to affect the state of GFP aggregation. F99S and M153T first described in relation to aggregation by Crameri *et al.* (1996) reduce obvious patches of surface hydrophobicity and could inhibit aggregation, but no dissociation constant has yet been determined for their Cycle3 mutant. Indeed, the triple mutant (F99S, M153T, V163A) (Crameri *et al*., 1996) has a diffusion coefficient inside mammalian cells one order of magnitude higher than that of wild-type GFP, implying a corresponding reduction in binding to other macromolecules (Yokoe and Meyer, 1996). Because V163 points into the interior of the protein (Ormo *et al*., 1996) and because F99S and M153 face outward, the latter two are most likely the culprits in wild-type GFP. However, the triple mutation did not alter the overall speed of fluorescence development at 37° compared to wild type (Crameri *et al*., 1996). Mutations V163A and S175G together actually slow the final aerobic development of fluorescence (Siemering *et al*., 1996), even though they improve greatly the yield of the properly matured protein.

The crystal structure of cyclized Cycle3 GFP was determined (Hofmann *et al*., 2002). The authors found a crystallographic dimer interface different than those reported previously and concluded that various polar and nonpolar patches on the surface of GFP could serve to dimerize the proteins in ways previously undescribed. However, it is unlikely that the dimer interface described in this work exists under physiological conditions with great affinity in non-Cycle3 versions of GFP because it is clear from sedimentation equilibrium analytical ultracentrifugation experiments on GFP containing A206K (and other mutations) (Zacharias *et al*., 2001) that there is virtually no remaining affinity when the more commonly observed dimer interface is altered. Further biophysical characterization of Cycle3 and mutants derived from it is clearly warranted.

The issue of GFP oligomerization is significant for several reasons. Most of the potential for trouble arises when GFP or its spectral mutants

are fused to a host protein to track protein localization or expression or to measure interactions by fluorescence resonance energy transfer (FRET) (most often mCFP and mYFP). If GFP dimerizes in the context of being part of a fusion protein, it could also foreseeably dimerize the host protein to which it is fused. The situation could become even stickier if the host protein is itself an oligomer. When measuring the interactions of molecules by FRET, it is imperative that the fluorophores used to report the interactions do not themselves in any way influence, or worse yet, create the interactions being measured. Obviously, if the fluorophores have affinity for each other, then doubt is cast on the accuracy of any measurement made to the presumed interaction of the host proteins. The problems associated with GFP dimerization are most troublesome when measuring intermolecular FRET in a two-dimensional space such as a membrane (Dewey and Datta, 1989; Dewey and Hammes, 1980; Fung and Stryer, 1978; Snyder and Freire, 1982; Wolber and Hudson, 1979; Yguerabide, 1994; Zimet *et al*., 1995) where the interest in lipid modified proteins is greatest. In this situation, we found that wild-type GFPs were very likely to dimerize even when expressed at very low surface densities (Zacharias *et al*., 2001). Because the monomerizing mutations alter nothing but the homoaffinity of GFP, we recommend including them (preferably A206K) in all GFP expression constructs where dimerization is not desirable.

The issue of weak dimerization of *Aequorea* FPs is not as serious when the objective of the study is to look simply at proteins localized to the plasma membrane by lipid modification. In general, the subcellular distribution of dimerizing FPs on a gross level is indistinguishable from the monomeric versions. However, this is not the case with tetrameric coral proteins. The oligomeric state of many of them extends beyond the obligate tetramer to the formation of relatively high-affinity ($<100\ \mu M$ dissociation constants) dimers of tetramers, setting up a situation in which large subplasmlemmal lattices can form. We found that in most cell types, lipid-modified oligomeric versions of FPs give poorly defined PM localization, a very aggregated or patchy appearance with a great deal more fluorescence in perinuclear membranes.

High-Throughput Microscopy (HTM)/High-Content Screening (HCS) to Study Lipid-Modified Proteins

We have spent a lot of energy on the development and use of high-content screening (also called high-throughput microscopy because of the incredibly large amount of information that can be obtained from any number of single cells) for use as a tool for pharmacology, cell biology,

and functional genomics. There is now a realized and growing need for the development of novel HCS algorithms to answer fundamental questions in cell biology and signaling. The use of such algorithms on our automated imaging system, the EIDAQ100 (later versions were called the IC100 after Beckman Coulter Instruments bought the EIDAQ100 and began development of the instrument for mass production), can provide highly quantitative analyses of cellular phenomena that could not be obtained easily using traditional microscopy techniques and analysis tools. The usefulness of HCS extends significantly beyond applications in drug discovery; the capability to objectively and quantitatively image millions of cells rapidly provides an unparalleled analytical advantage to cell biologists. Teasing apart signaling networks in live cells using image-based, functional assays is inherently more information dense than in traditional isolated, cell-free systems. Answering very basic biological questions using HCS also allows a seamless transition to an assay for an intermediate- to high-throughput screen of small molecule libraries in search of compounds with the potential to be therapeutic agents.

Morphometric Analysis of Palmitoylation with HCS

Existing HCS algorithms are capable of discriminating minute changes in many types of cell morphology or subcellular protein distribution (Conway *et al.*, 2001; Ghosh *et al.*, 2000; Minguez *et al.*, 2002; Morelock *et al.*, 2005). There is essentially no limitation on the types of morphology that can be used, alone or combinatorially, as criteria for a unique marker (Boland and Murphy, 2001; Price *et al.*, 1996, 2002; Roques and Murphy, 2002). Multiple criteria ranging from the location or concentration of a fluorophore in a cell (Boland and Murphy, 1999a,b, 2001; Boland *et al.*, 1998; Markey *et al.*, 1999; Murphy *et al.*, 2000; Price *et al.*, 2002; Roques and Murphy, 2002) to a physical change in the shape of a cell or a redistribution of cellular contents such as chromosomes, transcription factors (Ding *et al.*, 1998), microtubules (Minguez *et al.*, 2002), and membrane protrusions (e.g., neurites, ruffles) (Price *et al.*, 1996; Roques and Murphy, 2002) can be used individually or combined to enhance the sensitivity and accuracy (Boland and Murphy, 2001; Price *et al.*, 1996; Roques and Murphy, 2002). These algorithms provide flexible and unbiased reports of the existence and degree of interesting, visible changes in cells allowing for high-resolution determinations of pharmacological efficacy tied to such a change (Conway *et al.*, 2001; Ding *et al.*, 1998; Ghosh *et al.*, 2000). It is the exquisite sensitivity for subtle change and the objectivity of HCS that should make possible the use of cell lines for a wide variety of assays, including functional genomics, proteomics, and pharmacology.

HCS Machine Vision Algorithms to Quantify Reporter Density on the Plasma Membrane

We have focused on generating an application, a machine vision algorithm, for HCS that will accurately and objectively quantify the amount of a fluorescence localized to the plasma membrane of cells, as well as translocation between the cytoplasm and the PM in either direction. The approach we have used is to extract information on a per-cell basis rather than a field-based measurement, meaning that a much finer determination of the biological event can be extracted from original data. Algorithms that analyze images on a per-cell basis require significantly more effort to program and validate but our effort is being rewarded. The algorithm used to determine the plasma membrane localization (with respect to the amount of the fluorophore in question in any other subcellular domain) is named Thora (Vala Biosciences). Palmitoylation has served as one of the model biological systems during the development of Thora. Reporters of palmitoylation are FPs fused either to peptide substrates for palmitoylation or to whole proteins that are palmitoylated. In both cases, the palmitoylated versions of the proteins are localized to the PM. When palmitoylation is blocked or palmitates are removed pharmacologically, the reporter becomes displaced from the PM, diffusing to the cytoplasm, to a degree that depends on the efficiency of the block provided by the pharmacological tool. Upon displacement, the reporter diffuses to the cytoplasm where its accumulation can be measured and ratioed against the decrease in intensity on the plasma membrane. Increases in cytoplasmic abundance or decreases in the abundance of the reporters on the plasma membrane can also be compared to many other metrics that are determined automatically when the cell images are analyzed by algorithms such as Thora.

GAP43:YFP is Palmitoylated, Localized to the Plasma Membrane, and Is a Stereotypical Reporter of the Cellular Capacity for Palmitoylation

The N terminus of GAP43 (neuormodulin) is one of the most intensively studied models for acylation of a cytoplasmic protein (Arni *et al.*, 1998; Fukata *et al.*, 2004; Gauthier-Campbell *et al.*, 2004; Hess *et al.*, 1993; Liang *et al.*, 2002; Skene and Virag, 1989). It is particularly useful for many reasons, including the fact that palmitoylation is the predominant form of lipidation and that the short N-terminal peptide substrate for palmitoylation can function efficiently in isolation of the remainder of the protein (Zacharias *et al.*, 2002). The N terminus of GAP43 is doubly palmitoylated on two adjacent cysteine residues (Table I). When a 12-residue palmitoylation

substrate peptide from the N terminus GAP43 is fused to the N terminus of YFP, this peptide, by virtue of its palmitoylation, traps YFP on the PM (Fig. 2). Conversely, if palmitoylation of GAP43:YFP is blocked or inhibited, the protein diffuses freely throughout the cell, including the nucleus (Fig. 2D), as is the case when GFP is not fused to any other peptide or protein (Fig. 2B). GAP43:YFP is free of such ancillary signals for PM targeting or any enzymatic activity present in the parent protein.

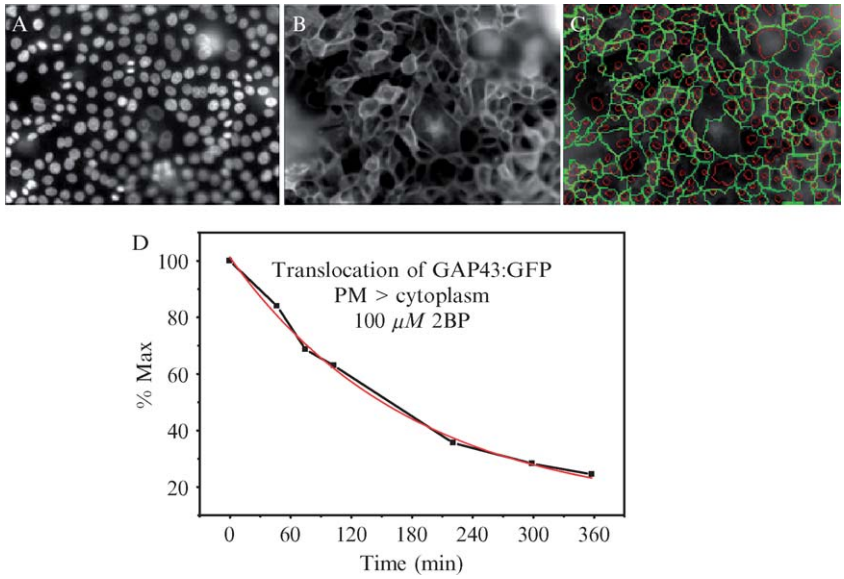

FIG. 2. Residence half-life of palmitate. Quantitative analysis of the time course of translocation of GAP43:YFP from the plasma membrane in response to 100 μM 2BP. MDCK cells stably expressing GAP43:YFP were exposed to 100 μM 2BP for 6 h. During this time, the same field of view of cells was imaged repeatedly in two channels: Hoechst/nucleus (A) and GFP/PM (B) on the EIDAQ100 (the three images, A, B, and C are at time 0). The PM mask (green lines) defined by Thora is shown (C); the nucleus is delimited by red lines; green lines mark the area identified as PM. By determining the average fluorescence intensity of the area defined by the PM mask (green in C), PM labeling was reduced by 80% from the maximum, or starting density during the 6-h period (D). Nonlinear least-squared fit of data to a single exponential decay curve (red line 4D) described data accurately and gave a decay constant, equivalent in this assay to the residence half-life of palmitate on this substrate of 179 min or ~3 h, the same as other published estimations for the residence of palmitate on cytosolic proteins (Lane and Liu, 1997; Wolven *et al.*, 1997). Data were fit and the curve generated using MicroCal Origin.

Determination of the Residence Half-Life of Palmitate on GAP43:YFP Using HCS

Palmitoylation is a dynamic posttranslational modification. The thioester bond is labile, but regulated, and as a result, the turnover of palmitate residing on proteins is generally shorter than the rate of turnover of the protein that is modified by palmitate (El-Husseini Ael *et al.*, 2002; Lane and Liu, 1997; Loisel *et al.*, 1996; Wolven *et al.*, 1997). Using HCS we were able to determine the residence half-life of palmitate on GAP43:YFP expressed stably in MDCK cells (Fig. 2). While we expect that the residence half-life of palmitates will be context specific, the fact that we have identified a time that corresponds roughly to other measurements determined biochemically demonstrates the utility of the method. Another significant advantage provided by using this technology for such determinations is the fact that the measurement was generated in a living cell system over the course of 6 h rather than by incorporation of radiolabeled palmitate (tritiated palmitic acid; ^{3H}PA). Analysis of the incorporation of ^{3H}PA is relatively labor and cost intensive. The turn-around time for experiments using ^{3H}PA can be incredibly slow unless the system has incorporated a significant bias, such as stable overexpression of the substrate of interest. Even under ideal circumstances, the proteins must be separated by some means, typically SDS–polyacrylamide gel electrophoresis (one or two-dimensional SDS-PAGE) prepared for autofluorography and exposed to film for periods of up to 6 months. Typical times for a measurable autofluorographic signal from GAP43:YFP following incorporation of ^{3H}PA into the GAP43:YFP stable cell line following two-dimensional SDS-PAGE was 10 to 24 weeks. Using HCS to determine the state of palmitoylation provides mechanism for determining the kinetics for the palmitoylation of a substrate, as well as the activity of an associated PAT in the context of a living cell. Having the reporter of palmitoylation constitutively in the palmitoylated state lends itself best to an antagonist mode assay such as the ones illustrated in Figs. 2 to 5. In contrast, the disease states associated with PATs appear to be cases in which there is a state of hypopalmitoylation of substrates associated with a nonfunctional PAT. This suggests that in at least these cases, the assay mode needed for a drug discovery program would be agonist mode. There are several approaches to developing agonist mode assays for palmitoylation, each with advantages and drawbacks. Conceptually the simplest is to introduce a nonpalmitoylated, fluorescent substrate into the cytoplasm and track the translocation of the reporter from the cytoplasm to the plasma membrane as it becomes palmitoylated. Introduction of such a reporter on a large scale is somewhat problematic so methods to unmask a protected palmitoylation site fused to a stably expressed fluorescent reporter of palmitoylation are under development.

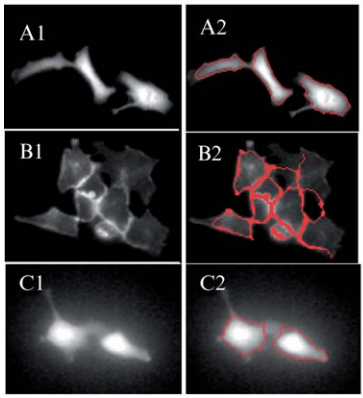

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Image	Avg. intensity cytoplasm	Avg. intensity PM	Ratio
A	212.12	91.26	0.43
B	104.15	145. 65	1.40
C	181.83	89.57	0.49

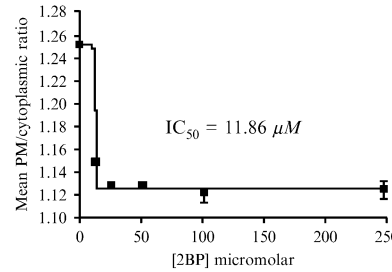
FIG. 3. Determining the simple case of whether GAP43:YFP is on the PM or in the cytoplasm using the HCS algorithm. In this analysis, MDCK cells (same images as in Fig. 2) transiently transfected with (A) GFP alone, (B) GAP43:YFP, and (C) GAP43:YFP in the presence of 100 μM 2BP. Using masks that define the plasma membrane and the cytoplasm, the algorithm determined that the PM/cytoplasm ratio was significantly different between PM and cytoplasm localization (D). The localization of GFP alone (B) is described by a ratio similar to that of GAP43:YFP under conditions where its localization to the PM has been inhibited by incubation in 100 μM 2BP (C).

The analysis in Fig. 2 represents the case for a palmitoylated cytoplasmic protein. The residence half-life for palmitate on integral membrane (TM) proteins may be different (Loisel *et al*., 1999). Because depalmitoylation of integral membrane proteins does not usually result in an immediate translocation of the protein from the PM into the interior of the cell, this particular assay is not suitable for quantifying depalmitoylation of TM-type proteins (e.g., G protein-coupled receptors [GPCRs]). Rather, depalmitoylation of TM proteins is likely to cause a lateral redistribution within the membrane into or out of lipid various lipid microdomains (Loisel *et al*., 1999), in which case, monitoring changes in the associative properties of such protein in the two-dimensional space of the PM is better achieved using intermolecular FRET (Zacharias *et al*., 2002).

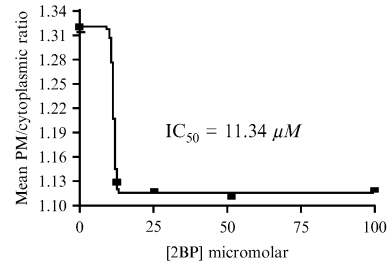
Thora Can Measure Precisely the Subcellular Distribution of GAP43:YFP: IC_{50} of 2BP

In a stepwise demonstration of the utility of Thora we show that it is able to make a simple binary decision of whether GAP43:YFP is on the PM or in the cytoplasm (Fig. 3) and subsequently that Thora can make fine

Inhibitor [μM]	Mean	SD	CV [%]
0	1.2508	0.028	2.24
12.5	1.1499	0.027	2.37
25	1.1289	0.034	3.01
50	1.1288	0.021	1.87
100	1.1209	0.050	4.43
250	1.1294	0.0751	6.65



Inhibitor [μM]	Mean	SD	CV [%]
0	1.3242	0.055	4.18
12.5	1.1324	0.049	4.28
25	1.1188	0.043	3.85
50	1.106	0.023	2.10
100	1.112	0.0004	0.036



Inhibitor [μM]	Mean	SD	CV [%]
0	1.1924	0.034	2.83
12.5	1.0814	0.028	2.57
25	1.0747	0.017	1.57
50	1.0068	0.011	1.06
100	0.999	0.021	2.07
250	1.0069	0.020	1.89

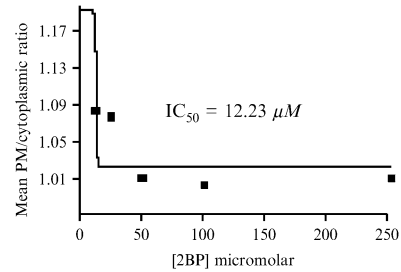


FIG. 4. Determination of the IC_{50} of 2BP using HTM. Inhibiting palmitoylation of GAP43:YFP with 2BP causes translocation of the reporter from the PM to the cytoplasm, allowing the determination, in live cells, of the IC_{50} of 2BP (the concentration at which 2BP reaches half its maximal effect) for this substrate. The IC_{50} determination was done simultaneously in three separate plates; 16 individual wells of cells were exposed to each concentration of 2BP. MDCK cells stably expressing GAP43:YFP were plated at 10,000 cells per well in three 96-well imaging plate (Costar) and allowed to adhere for at least 6 h. Pairs of columns were exposed to 2BP at concentrations ranging from 0 to 250 μM (16 wells/concentration). After a 2-h exposure, cells were imaged on the EIDAQ100 (Q3DM/Beckman Instruments) at 32°. The IC_{50} determined from each of these plates was approximately the same, even though the range of ratio values was shifted slightly for each plate (Fig. 3 tables and graphs). The PM distribution of GAP32:YFP was analyzed using Thora. PM fluorescence was ratioed against the fluorescence in the cytoplasm and plotted versus the concentration of 2BP. Z' for the assays was 0.2 to 0.22. The mean, SD, and CV (%) were cumulative determinations from individual cells in all wells. The identity of the PAT specific for GAP43 has not been confirmed but it is likely that HIP14 may contribute to its state of palmitoylation (Huang *et al.*, 2004). Even though the IC_{50} values are almost identical, information density around the IC_{50} value is low. For this reason, we increased the number of 2BP concentration, surveyed around the IC_{50} to make a finder determination.

Inhibitor [μM]	Mean	SD
0	1.34	0.052
0.1	1.32	0.057
0.5	1.27	0.061
1	1.32	0.044
2.5	1.29	0.078
5	1.29	0.089
10	1.24	0.11
15	1.09	0.02
20	1.003	0.191
50	0.916	0.106
100	0.872	0.170

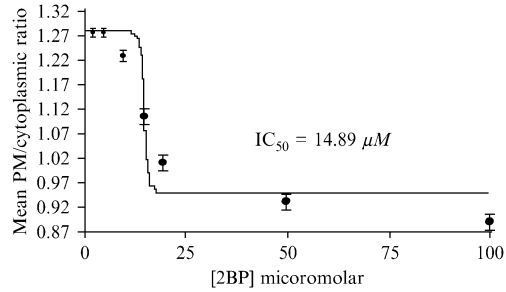


FIG. 5. Determination of the IC_{50} of 2BP using HTM. Because IC_{50} determinations in Fig. 3 had very few data points surrounding the IC_{50}, we increased the number of concentrations surveyed around the IC_{50} value to fill the gap. The IC_{50} value obtained was almost the same; however, the confidence in the value is increased dramatically when the value is supported by a greater concentration of data in this area.

determinations (Figs. 4 and 5) of the quantity of fluorescence on the PM, providing a level of sophistication great enough to make incremental determinations of the localization of the reporter. Two important questions are answered here. Data in Fig. 3 illustrate that the algorithm is sufficient to ensure success in an assay that is attempting to determine the simple question of whether the probe has moved from the membrane. Additionally, the second level of sophistication, the fractional localization of the reporter to the PM, provides an unprecedented degree of precision at high throughput that makes it possible to determine when there has been only a partial redistribution of a reporter (Figs. 4 and 5). This provides a tool that allowed us to determine the IC_{50} value (concentration at which the effectiveness of the pharmacological reagent is at half-maximum) of 2BP, the most commonly used, most specific inhibitor of 2BP known from the literature. 2BP has been used at many different concentrations to inhibit palmitoylation, apparently without regard to its efficacy. The expense and time involved in determining the IC_{50} of 2BP using radioactive methods and biochemistry would be significant. HCS offers a relatively inexpensive and reproducible way to determine this value.

Once we were sure that simple determinations could be made using artificially induced extreme conditions (i.e., all PM versus all cytoplasm), we focused on determining the IC_{50}, a more delicate measurement. Our first data set illustrates that the reproducibility among cells, wells, and plates is fairly good. We were able to generate almost identical IC_{50} values

in all three plates. The first set of experiments illustrates that there is a ratio change in the PM/cytoplasm ratio when using this technique. The second important point clearly illustrated is the importance of having many data points surrounding the IC_{50}. If the value for a compound is not known, then a standard, broad range of concentrations is a good place to start. Once a general idea of where the IC_{50} will lie is obtained it is important to repeat such experiments using a larger number of concentrations hovering at either side of the IC_{50}, as in Fig. 5. Our narrowed, more focused attempt revealed that our original IC_{50} values were fairly accurate given the paucity of information in the IC_{50} value range of the concentrations used in Fig. 4. Data in Fig. 5 also provide a greater deal of confidence in the IC_{50} value.

Determination of the Compatibility of the Cellular Reporter System with Dimethyl Sulfoxide (DMSO)

Data from experiments using 2BP were done by diluting a concentrated stock solution of 100 m*M* 2BP in DMSO (a ''1000×'' stock solution) to the final concentrations indicated. Under these conditions, exposing the cells to 100 μM 2BP resulted in an exposure to 0.1% DMSO. It is important in experiments like this to maintain a constant final concentration of DMSO. The DMSO concentration should be below the level where there are detectable effects of DMSO. Rows of DMSO-only controls (0 and 1%) should be included on the same plate at least until the assay has been completely validated. Figure 6 illustrates a dose–response experiment for DMSO alone using concentrations ranging from 0.01 to 10%. From these data it is clear that even at DMSO concentrations much higher than normally used in a HTS screen of a small molecule library, there is little, if any, effect on this particular reporter system; it is encouraging that despite its common use in such experiments, we found no mention in the literature of DMSO having any effect on palmitoylation.

Cytotoxic Effects of Antagonists of Palmitoylation

As discussed previously, the single best characterized and the most commonly used pharmacological inhibitor of palmitoylation is 2BP. However, there are few reports about the effects of 2BP other than its ability to block palmitoylation. One report (Cnop *et al*., 2001) has shown a correlation between the concentration of palmitate and 2BP in cytotoxicity of preparations of rat β-cell preparations and that the mechanism of toxicity was due to necrosis and apoptosis. It has also been suggested that palmitate-related cytotoxicity may be due to the impairment of mitochondrial membrane (de Pablo *et al*., 1999). We frequently observed cellular

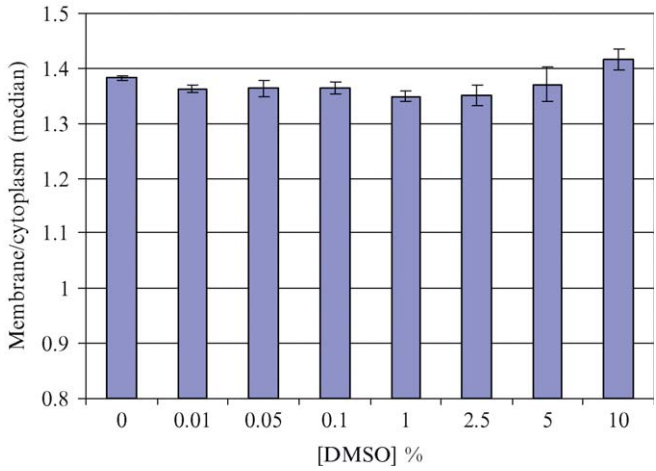


FIG. 6. DMSO does not have an effect on the displacement of GAP43:YFP from the plasma membrane. Cells were plated as in Figs. 4 and 5. DMSO was added to the cells at a range of concentrations that greatly exceeds what is introduced with the addition of 2BP. Even at 100 μM 2BP, the final percentage of DMSO is 0.1%.

toxicity that appeared to be correlated to the cell plating density when each well was exposed to the same concentration (100 μM) of 2BP (Fig. 7). It is likely that lower cell density results in a greater effective cellular membrane concentration of the 2BP, elevating it to the point where toxicity can be observed easily; the lipophilic 2BP will partition out of the aqueous culture medium and into cellular membranes—fewer cells will concentrate available 2BP to a greater degree than the same amount being distributed among more cells. This finding is critical in the development of assays to characterize PATs because cell plating density and homogeneity are critical, standard parameters that must be known to conduct all cell-based assays. Likewise, it is critical to understand any toxic effects of compounds that modulate palmitoylation. Specifically, in the toxicity ''mechanism-based'' meaning, is there something inherent in inhibiting all PATs or specific PATs that is responsible for cell death or is there some alternative reason, such as disruption of normal membrane integrity or the uncontrolled induction of one or more lipid-mediated signaling pathways in a cell, that is responsible for cell death? With efficacy and molecular mechanism of action, toxicity is a critical issue in the development of a high-throughput screen to find small molecule modulators of palmitoylation or

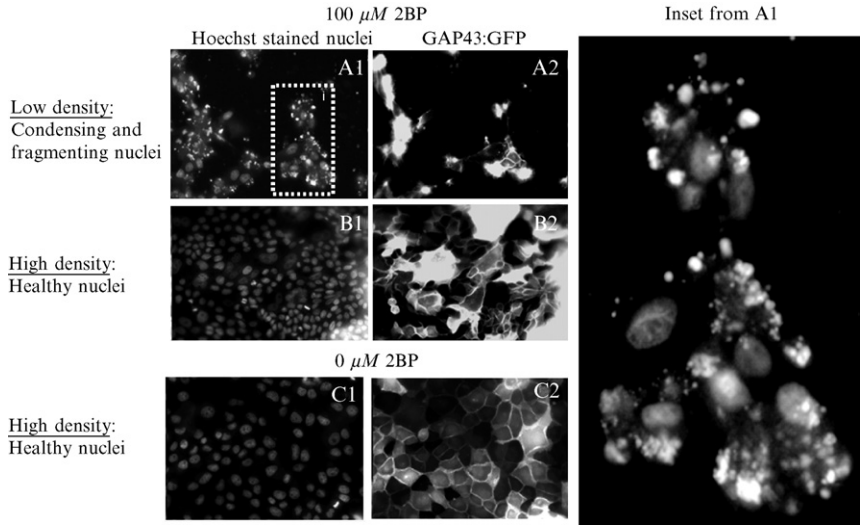


FIG. 7. Cytotoxic effects of 2BP. MDCK cells stably expressing GAP43:YFP were plated at (A) low (1000 cells/well) and (B and C) high/normal density (10,000 cells per well) and then exposed to 100 μM 2BP (A and B) or no 2BP (C). The low-density plating (A1) shows widespread nuclear condensation and fragmentation suggestive of cells undergoing apoptosis (Robertson *et al.*, 2000); see also the inset from A1 for a better view of the cells. This morphological change from normal nuclear integrity (see B1 and C1) occurred in as few as 6 h following addition of 2BP to the low-density culture. Cells at high-density culture were resistant to such changes for at least 18 h following exposure to 2BP (B and C). Interestingly, 2BP successfully caused translocation of GAP43:YFP from the PM in cells plated at high and low density (A2 and B2). Cells not treated with 2BP (C) had normal nuclei and GAP43:YFP remained localized to the PM (C2). Cells were allowed to adhere for at least 6 h before any treatment. 2BP (100 mM, in DMSO) was added to culture medium with 2% serum and vortexed just prior to addition to the cells in culture.

any other high-throughput screen. Such information will provide a critical foundation for monitoring the toxic effects of compounds that modulate palmitoylation, the most critical factor, closely following efficacy, of a high-throughput screen for small molecule modulators of palmitoylation. Observations like this are easy to make when developing an HCS, live-cell or image-based assay. Most cell biologists will be familiar with many important changes in cell morphology that differ from normal and as such should be very vigilant for such occurrences. Using a vision-based system such as the EIDAQ100 to make these determinations provides an unprecedented opportunity to make better decisions about what constitutes a healthy assay.

Considerations for Designing HCS, Cell-Based Assays with an Emphasis on Palmitoylation

Choosing a Palmitoylated Protein or Peptide

Many of the points made here can be applied generally to other types of assays, but we present them with lipid-modified proteins in mind. The first step is developing a biosensor system that will report on the activity of known and unknown modulators of the ability of a cell to palmitoylate a specific substrate. Our preferred substrate for palmitoylation is the N-terminal 12 or 18 residues of GAP43. This substrate represents only one of many possible substrates for palmitoylation; other substrates (see Table I) can be substituted but GAP43 is one of the most widely used and most extensively characterized of all substrates for palmitoylation. We utilize many other substrates of palmitoylation to answer specific questions about individual proteins, but for general questions, we use GAP43 most commonly.

Choosing a Fluorescent Protein

If fluorescent protein-based sensors are to be used, the choices are now significantly greater than they were a few years ago. This choice is made much in the same manner as choosing a small molecule fluorophore to label a particular cellular component. The excitation and emission spectra should be unique from other existing or future coexisting fluorophores. The fluorescent proteins should be bright (highest possible extinction coefficient and quantum yield) and should be truly monomeric. In general, the more red shifted the FP is, the better, as there is less cellular autofluorescence at redder wavelengths. Among these factors, perhaps only the oligomeric state of the FP is a unique consideration. Many frequently encountered FPs from corals are obligate tetramers and will cause a distinct patchy distribution that does not necessarily reflect the endogenous situation for the experimental peptide (e.g., GAP43).

Creation of Stable Reporter Cell Lines

There are also many factors that must be taken into account when choosing a cell line for developing these assays, ranging from the organism of origin (e.g., human, mouse, or dog) to the morphology of the cells. The fluorescent signal from a stable line expressing any FP-based reporter must be robust. Generating a robust cell line is not always a simple matter. In a practical sense the challenge is to express enough of the reporter so that image contrast is sufficient while retaining low image exposure times and, at the same time, not expressing so much of the reporter that normal cellular processes are overwhelmed. While the EIDAQ100 can and often

does use brightly stained nuclei to establish an initial focal plane, the second image, often the FP-based reporter, must be bright enough to generate sufficient contrast so that the EIDAQ100 autofocus system is able to refocus if necessary. Large signal-to-noise ratios are also helpful in the subsequent analysis of any image data using Thora where image contrast is a key factor in the identification and quantification of the PM. In general, stable lines expressing the minimum amount of reporter necessary for efficient image acquisition and analysis are the best. This must be determined empirically for each line.

Drift is a problem encountered occasionally in such reporter cell lines. In our experience with reporter lines expressing lipid-modified FPs, the most common type of drift is a slow diminution of the fluorescent signal over many passages level but never so far in the subcellular distribution. For this reason it is important to expand any useful reporter cell lines and freeze down many tubes of the cells at early passages.

Cell Plating

Determining and maintaining optimal plating conditions are the most critical parameters for reducing the noise in a cell-based assay system. Automated cell dispensation systems such as the Multidrop systems from Thermo Electron, among others, are not prohibitively expensive and will soon pay for themselves by reducing error and frustration. The optimal cell densities must be determined on a per-assay basis. For all lipid-modified FPs studied to date, the subcellular distribution is defined most clearly when the cells are at or near confluence or in islands of tens to hundreds of cells. Within such areas, the best definition is at sites of cell–cell contact. For palmitoylation, islands of less than 10 cells provide sufficiently distinct morphology and high enough contrast to give robust measurements.

Choice of Cell Type

The choice of cell type for studying lipid-modified proteins is absolutely critical, as the variability of the expression pattern of such proteins can vary substantially among the cell type. For palmitoylated proteins, we find that MDCK and U2OS work very well, whereas HeLa, COS, and HEK are poor, each for different reasons. Both MDCK and U2OS are flat and, when confluent relatively columnar with high, fairly vertical walls between cells creating strong, bright, distinct boundaries where the lipid-modified FPs accumulate. These cells have small nuclei relative to their cytoplasm compartment, leaving sufficient room for clear observation of the reporter signal when it moves between the plasma membrane and the cytoplasm. Cell types other than MDCK and U2OS may work well for similar applications but such determinations must be made empirically. Generally the

cells should be flat, column shaped, have relatively small nuclei, be sufficiently adherent to a wide variety of substrates, and remain monolayers rather than growing into amorphous piles as the cells reach confluence.

Control Compounds

One of the primary impediments to developing assays for protein palmitoylation has been the near absence of specific pharmacological tools to characterize the system. It has been known for quite some time that only very few compounds inhibit palmitoylation of proteins in tissue and cell lines; these include cerulenin (DeJesus and Bizzozero, 2002; Hiol *et al.*, 2003a; Lawrence *et al.*, 1999), polyunsaturated fatty acids, and 2BP (Webb *et al.*, 2000). Of these compounds, 2BP is used most frequently and successfully and is now perceived as the pharmacological benchmark agent to understanding palmitoylation (Webb *et al.*, 2000). The molecular mechanism of action of 2BP is not known but it is believed it may act by forming an irreversible bond with PATs. The molecular mechanism of action of cerulenin is believed to be by the inhibition of fatty acid synthase (Menendez *et al.*, 2004), specifically 3-ketoacyl ACP synthase, a fairly early point in the palmitoylation pathway well upstream of the physical addition of palmitate to a protein. Cerulenin inhibition of fatty acid synthesis is also known to induce apoptosis in a variety of cancer cells in as little as 2 h (Thupari *et al.*, 2001; Zhou *et al.*, 2003).

Evaluation of Assay Quality by Z'

Z' is a dimensionless statistical characteristic and is used for comparison and evaluation of assay quality (Zhang *et al.*, 1999). The Z' factor is

$$Z' = 1 - \frac{(3\sigma_{+control} + 3\sigma_{-control})}{\mu_{+control} - \mu_{-control}}$$

and can be calculated using only control data. A Z' factor equal to 1.0 is a perfect assay. $Z' > 0.5$ indicates a good assay. This coefficient takes into account the dynamic range of the assay signal, as well as the variation associated with the reference control measurements. Because the Z' factor is dimensionless, it is suitable for comparison between assays.

Troubleshooting

Data presented in Fig. 3 illustrate that the variability of the IC_{50} determination for 2BP plate-to-plate basis is remarkably small. Likewise, the standard deviations (σ in the formula just given) and %CVs are very

good. However, the Z' factors were poor. The main problem with these data is that the change in fluorescence intensity under the mask identified by the algorithm as being the PM does not drop significantly during the period of time the cells were exposed to 2BP. The most likely explanation for this is that the time of exposure to 2BP was not long enough to achieve the maximum level of depalmitoylation of the GAP43:YFP reporter. What this means in a practical sense is that a significant proportion of the GAP43:YFP reporter did not translocate from PM to the cytoplasm, resulting in a small change in the PM/Cyt ratio. This hypothesis is supported by the experiment shown in Fig. 8 where after 6 h approximately an 80% reduction in overall PM fluorescence intensity was obtained. Our rationale for shortening the exposure time to determine the IC_{50} for 2BP was that cells were not able to withstand a 6-h exposure to 250 μM 2BP; after 2 h cells in wells at this concentration were dying and floating away. Together these results suggest that we have started at a point where the instrument and algorithm noise are at a sufficiently low level and the variability of the biology is remarkably low. The primary problem being that the dynamic range of the assay is too small. In our experience, this component of the Z' equation is the one most frequently being inadequately robust when developing cell-based assays with FP reporters of some activity.

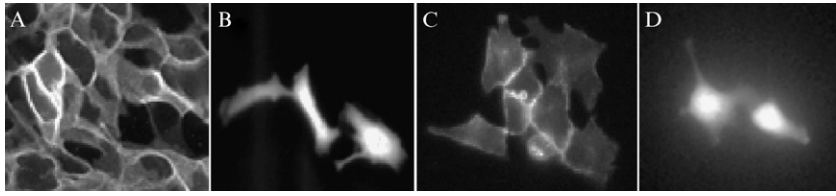


FIG. 8. GAP43:YFP is a reporter of palmitoylation. (A) Stably expressed GAP43:YFP is localized to the PM of MDCK cells, illustrating the remarkable homogeneity in the expression pattern that has been achieved in this stable line. (B) GFP not fused to any subcellular targeting motif is expressed throughout the interior of the cell, including the nucleus, illustrating that GFP alone has no known inherent targeting signals. (C) Transiently transfected GAP43:YFP is also expressed on the plasma membrane of cells except when palmitoylation is inhibited (D) by preincubation of transiently transfected cells in 2-bromopalmitate (2BP) (100 μM) (Webb *et al.*, 2000), illustrating that palmitoylation of the adjacent cysteines on the 12-residue peptide from the N terminus of GAP43 (NH2-MLCCMRRTKQV-YFP) fused to the N terminus of YFP is sufficient to retain the protein at the PM (Arni *et al.*, 1998; Liu *et al.*, 1993); there is nothing else inherent in the GAP43 peptide or YFP that will localize this protein to the PM. Cells in B, C, and D are representative of many having the same morphology.

System Error Identification

In addition to plate-to-plate variability of measurement such as the IC_{50} for 2BP, we will determine the day-to-day stability of repeated measurements of the same plates. Such measurements will illustrate the consistency and stability of the hardware and software components of our assay system. Based on previous experience with this instrument we believe that the variability or noise generated from this component will be minimal (Moran, 2005). Day-to-day and plate-to-plate determinations of the consistency and stability of the biological component will also be performed. Plating cells and dispensing reagents in a consistent manner will be enabled using dedicated multichannel pipettors and a Multidrop 384 automated cell-dispensing system.

Data Tracking

All variability from all sources should be presented in a tabulated matrix in a way that will clearly illustrate the source and extent of all variabilities. Such tabulation provides a convenient tracking system allowing one to manage assay development. Information tracking can be established easily using MS Excel spreadsheets. Data and analyses in the spreadsheets can be hyperlinked easily to original imaging data, allowing one to drill down to individual original data points, including images of single cells and eventually link out to individual compounds. This connectivity will enhance our ability to identify strengths and weaknesses of each step in the process efficiently.

Image Organization and Analysis

This aspect of HTM or HCS is certainly one of the most problematic. There is a great deal of effort being made to develop programs that allow researchers to catalog and store image and associated data. The enormous volume of data that can be generated in a very short time was almost unmanageable just a few years ago. The expense of collecting and saving data from a few experiments was almost prohibitive. However, the price of storage media has dropped to the point where it is no longer the issue it once was. The bigger problem is managing image data so that subsequent analyses within and between data sets are efficient and that archived data are not lost or overlooked due to poor documentation and organization.

Even modest use of HTM or HCS will generate, in a short time, very large amounts of data. A plan to manage these data should be integrated

into the experimental design at the earliest stages. HCS systems often include software that aids in the organization of data, but these are most frequently platform specific. Efforts are also being made at the commercial level to fill the need for efficient data management systems. The most significant open source effort to our knowledge is the open microscopy environment (Goldberg *et al.*, 2005). This increasingly comprehensive system offers many advantages, including cross-platform compatibility. The project already has significant momentum, becoming an important community resource that is worth supporting.

Conclusions

Palmitoylation is a key determinant of the subcellular localization of proteins to regions of membrane specialization, including lipid rafts and synapses. Many synaptic proteins are palmitoylated and are the anchor for the formation of multimolecular signaling complexes, as is the case with PSD-95. Palmitoylation is a dynamic modification, making it unique among the various lipid modifications and a posttranslational modification that likely works like other reversible modifications such as phosphorylation. The process has been shown to be mediated by a family of enzymes, PATs, that contain ZDHHC-CRD motifs. Links between PATs and human diseases are still tentative but represent promising avenues for understanding the molecular basis for diseases such as Huntington's disease, mental retardation, and schizophrenia. Understanding the changes that occur in the substrates for PATs in disease states will be fertile ground for the development of HTS assays to discover pharmacological agents to correct the problem. The assay that may be the most important is an agonist mode assay, the more difficult of the forms to design.

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