IDENTIFICATION OF HUMAN DHHC20 AS A TRANSFORMING PALMITOYL ACYLTRANSFERASE

A Dissertation in Pharmacology by Jeremiah M. Draper

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Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The dissertation of Jeremiah M. Draper was reviewed and approved* by the following:

Charles D. Smith  
Professor and Charles and Carol Cooper Chair in Pharmacy  
South Carolina CoEE Endowed Chair  
Dissertation Adviser  
Chair of Committee

Kent E. Vrana  
Elliot S. Vessell Professor of Pharmacology  
Chair of the Department of Pharmacology

Michael F. Verderame  
Professor of Medicine  
Professor of Microbiology and Immunology  
Associate Dean for Graduate Studies

Jong K. Yun  
Associate Professor of Pharmacology  
Director, Pharmacology Graduate Program

Melvin L. Billingsley  
Professor of Pharmacology  
Professor of Biotechnology and Entrepreneurship  
President, Life Sciences Greenhouse of Central Pennsylvania

*Signatures are on file in the Graduate School.
ABSTRACT

Many important signaling proteins require the post-translational addition of fatty acid chains for their proper subcellular localization and function. One such modification is the addition of palmitoyl moieties by enzymes known as palmitoyl acyltransferases (PATs). Substrates for PATs include C-terminally farnesylated proteins, such as H- and N-Ras, as well as N-terminally myristoylated proteins, such as multiple Src-related tyrosine kinases. Since many of these proteins are involved in intracellular signaling pathways that drive cellular survival and proliferation, and their activity is predicated on specific subcellular localizations, the enzymes that catalyze their posttranslational modifications are attractive drug targets. However, the primary focus of selective drug development in this arena has been on the process of farnesylation, partly because the farnesyltransferases have been cloned, characterized, and used in screens to develop inhibitors. In contrast, the identification and characterization of human PATs has been hindered by the lack of defined assays to characterize these enzymes, thus preventing the direct validation of the involvement of these enzymes in diseases such as cancer.

Current assays to measure palmitoylation activity are functional; however, they employ fractionated cell lysates and are relatively low-throughput. These methods, though useful, are insufficient to directly measure PAT activity in intact cells, which would be useful in the identification of human PATs and studies of the regulation of these enzymes. Thus, we sought to develop quantitative and
efficient cellular assays to characterize PAT activity in intact cells. To develop these assays, we used cell-permeable, fluorescently-labeled lipidated peptides that mimic the PAT recognition domains of farnesylated and myristoylated proteins. These PAT substrate mimetics are accumulated by SKOV3 cells in a saturable and time-dependent manner. Although both peptides are rapidly palmitoylated, the SKOV3 cells have a greater capacity to palmitoylate the myristoylated peptide than the farnesylated peptide. Confocal microscopy indicated that the palmitoylated peptides co-localized with Golgi and plasma membrane markers, whereas the corresponding non-palmitoylatable peptides accumulate in the Golgi but did not traffic to the plasma membrane. These results indicate that the lipidated peptides provide useful cellular probes for quantitative and compartmentalization studies of protein palmitoylation in intact cells.

Exposure of SKOV3 cells to the peptides did not cause cytotoxicity at any concentration within the maximal 2 hr timeframe of the peptide uptake, palmitoylation, and intracellular localization experiments. However, when exposed to the peptides for 72 hrs cytotoxicity did occur in a palmitoylatable and motif-specific manner. Specifically, the non-palmitoylatable forms of both peptides caused minimal cytotoxicity even at the highest concentrations tested. In contrast, the palmitoylatable forms of both peptides exhibited cytotoxicity of nearly equal efficacy, as measured by the release of lactate dehydrogenase, when exposed to SKOV3 cells. However, the N-terminal myristoyl peptide exhibited greater potency for inducing cytotoxicity than the C-terminal farnesyl
peptide. Since these peptides mimic the palmitoylation motifs of C-terminally farnesylated and N-terminally myristoylated proteins it appears likely that they are acting as competitive inhibitors of the palmitoylation of these proteins. The fact that this inhibition is cytotoxic to SKOV3 cells validates the process of enzymatic palmitoylation, particularly of N-terminally myristoylated and palmitoylated proteins, as an interesting target for novel drug development.

Although a C-terminal farnesyl-directed human PAT has been identified and validated as an oncogene, no human N-terminal myristoyl-directed PAT has been identified and characterized with this activity. Therefore, we sought to identify a human N-terminal myristoyl-directed PAT and determine whether this enzyme plays a role in cancer. DHHC20 was chosen for characterization based on homology to the N-terminal myristoyl-directed yeast PAT Pfa3 and its localization to a significant site of intracellular N-terminal myristoyl-directed PAT activity, the plasma membrane. In order to characterize the gene, mammalian expression vectors with or without the human DHHC20-insert were transfected into NIH/3t3 cells. In vitro palmitoylation (IVP) assays demonstrated that DHHC20 has PAT activity specific for the N-terminal myristoyl motif with no detectable activity toward the C-terminal farnesyl motif, indicating that DHHC20 is a human PAT with activity toward proteins such as the Src-related tyrosine kinases. In addition, overexpression of the gene caused cellular transformation of NIH/3t3 cells as evidenced by foci formation and growth in soft agar. This indicates that expression of DHHC20 causes cells to lose contact-inhibition of proliferation and grow in an anchorage-independent manner. Also, cells
expressing human *DHHC20* demonstrated elevated proliferation in normal and serum-depleted environments as compared to control cells, indicating that the gene is involved in signaling pathways that drive cellular proliferation. Also, comparative analysis of multiple normal and tumor-derived tissue samples by quantitative polymerase chain reaction (qPCR) demonstrate that *DHHC20* is expressed in a tissue-specific manner, and that it is overexpressed in several human tumor types including ovarian, breast and colon. Therefore, these results characterize DHHC20 as a human N-terminal myristoyl-directed PAT and validate it as a novel target for anticancer drug development.
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Chapter 1

Literature Review
Proteins serve important roles as structural, enzymatic and signaling components within the intracellular and extracellular environments of living organisms. However, in order to effectively realize their active functionality, these proteins must retain structural and conformational integrity along with localization to the proper cellular compartments. One way in which proteins are processed to produce the eventual spatiotemporally functional product is through co- and post-translational protein modifications.

Proteins can be modified in a number of ways including the covalent attachment of a multitude of molecules including phosphoryl groups, oligosaccharides and fatty acids such as isoprenyl, myrisoyl and palmitoyl groups. The specificity of these modifications toward particular proteins is generally predicated on the presence of certain amino acid sequences and/or secondary or tertiary protein structure.

In recent years, the attachment of lipid groups to proteins has generated increasing attention. An assorted group of important proteins have been shown to be co- and/or post-translationally lipidated. Since many of these proteins are involved in the development of disease states, understanding the process by which these proteins are lipidated may allow us to develop better treatments for diseases associated with the bioactivity of these proteins.
1.1 LIPID MODIFICATIONS

Post- and co-translational lipid modifications are important processes that increase the hydrophobicity of proteins, promote their localization to specific cellular compartments, and/or alter their conformation (1-6). During the lipid modification process a long chain lipid or fatty acid is attached to a specific amino acid residue located within a protein's sequence. The addition of this group increases the hydrophobicity of the protein, thereby causing it to partition into cellular structures with similar characteristics. Depending on the type and number of lipid modifications, proteins can localize to specific membranes throughout the cell including the plasma membrane, golgi, and endoplasmic reticulum as well as unique microdomains within membranes including caveolae and lipid rafts.

Lipid modifications are defined by the lipid chain added to the protein and by the type of linkage produced between the lipid and the target amino acid (7). The attachment of lipids to proteins can occur through the formation of three different linkages: ester or thioester, ether or thioether, and amide linkages. A number of different lipid modifications exist in biological systems. However, only the three most common types, prenylation, N-myristoylation, and S-palmitoylation (Figure 1) will be discussed in order in the following sections.

Protein prenylation is the covalent post-translational attachment of a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid group to a protein at a conserved cysteine residue located near the C-terminus by way of a stable
S-isoprenyl:

Geranylgeranyl (C\textsubscript{20})

Farnesyl (C\textsubscript{15})

N-myristoyl (C\textsubscript{14})

S-palmitoyl (C\textsubscript{16})

Figure 1: Lipid Modifications of cellular proteins. Types of lipid chains that are added to intracellular proteins through prenylation, N-myristoylation, and S-palmitoylation are shown (modified from (2)).
thioether bond (8). The majority of proteins that undergo the process of prenylation are modified through geranylgeranylation in which a 20-carbon lipid chain is added to the protein. Proteins that undergo geranylgeranylation include many γ subunits of heterotrimeric G proteins, as well as Rap, Rac, Rho and other Ras-related small G proteins (8, 9). In addition, the covalent attachment of a farnesyl group to the backbone of a protein is termed farnesylation. This lipidation event occurs on many proteins including rhodopsin kinase and cGMP phosphodiesterase α (5, 10, 11), the γ subunits of some heterotrimeric G proteins (5, 10, 12), nuclear lamins A and B (13, 14), Ras-related proteins such as RheB and RhoB (15, 16), as well as all the Ras isoforms (17). Irrespective of the group attached, prenylation causes the localization of proteins to cellular membranes and plays a role in protein-protein interactions (18, 19).

The enzymes that drive prenylation, farnesyltransferases (FTases) and geranylgeranyltransferases (GTTases), were identified, cloned and purified in the early 1990s (18, 20). Both GTases and FTases catalyze the covalent and irreversible attachment of their lipid groups to cysteine residues located within a C-terminal CAAX motif. This CAAX motif is composed of the prenylated cysteine residue, two aliphatic amino acids, and an X which represents a particular amino acid (21, 22). GTases generally recognize and geranylgeranylate the cysteine residue of proteins that contain a CAAX motif with a C-terminal leucine (20). In contrast, FTases generally recognize and farnesylate the cysteine residue of
proteins that contain a CAAX motif with a C-terminal methionine, serine, glutamine or alanine (23, 24).

The primary biological role of protein prenylation is to serve as a membrane anchor and cause the localization of proteins to cellular compartments such as the plasma membrane, Golgi, endoplasmic reticulum as well as peroxisomes (25-29). In addition, many prenylated proteins, such as Ras, require prenylation for proper membrane localization in order to perform their function. To this end, it has been found that inhibiting the farnesylation of Ras impairs its function as well as Ras-related cellular transformation (16, 30). Based on this evidence and the fact that many important signaling proteins are prenylated and oncogenic, FTase has become a target for the development of anticancer therapeutics.

Initially, farnesyltransferase inhibitors (FTIs) were developed as CAAX tetrapeptides which acted as alternative substrates for the FTase enzyme (18, 31). However, these early tetrapeptide FTIs were limited by inefficient cellular uptake and rapid degradation. In order to overcome these limitations, several small molecule FTIs have subsequently been developed. Small molecule FTIs such as lonafarnib, tipifarnib, BMS-214662 and L-778,123 were initially developed to inhibit the farnesylation of the Ras isoforms. Indeed, studies have demonstrated that these compounds show great efficacy in inhibiting the farnesylation of these proteins (32-35). Although, these compounds inhibit the farnesylation of their predetermined targets, issues concerning their specific activity have come to light. It has been determined that K- and N-Ras, the two
isotypes most commonly constitutively activated in human cancers (36), are in fact substrates for GGTase-1(37). In cells treated with FTIs these proteins are alternatively prenylated by the addition of a geranylgeranyl group (38, 39) maintaining the activity of the proteins and their downstream signaling pathways (40, 41). Despite the inability of these compounds to inhibit the prenylation of K- and N-Ras, it has been demonstrated that FTIs inhibit tumor growth in preclinical models, including transgenic N- and K-Ras oncogenic mouse models (42, 43). These findings suggest that the antitumor activity of the FTIs in these models is dependent upon blocking the farnesylation of proteins other than K- or N-Ras, of which nearly 17 other known targets exist. Regardless of this lack of selectivity, these drugs have demonstrated efficacy in blocking tumor growth in preclinical in vivo models and have progressed into clinical trials in patients with various types of cancer including advanced breast cancer, tumors of the central nervous system, acute myeloid leukemia, and chronic myelomonocytic leukemia (44-46).

A second type of lipid modification is the process of N-myristoylation. During N-myristoylation a 14-carbon saturated fatty acid called myristic acid is attached to the N-terminal glycine of a protein via a stable amide bond (47, 48). Like protein prenylation, the enzymes that catalyze these reactions, N-myristoyltransferases (NMTs), have been identified and the mechanism of myristoylation has been extensively characterized (48, 49). However, in contrast to prenylation, which is purely a post-translational modification, the process of myristoylation is mainly co-translational (48, 49).
N-myrisotylated proteins are characterized by an N-terminal methionyl-glycyl sequence closely followed by a cysteine residue which composes the consensus recognition motif (1). The processing of N-myrisotylation involves the cleavage of the N-terminal methionine, thereby creating an N-terminal glycine which is modified by the addition of a myristoyl moiety through the action of N-myristoyltransferases. Similar to the prenylation reactions discussed above, certain amino acids in the consensus sequence of target proteins are required for myristoylation of the N-terminus. In this case, the glycine at amino acid position 2 is absolutely required for N-terminal myristoylation as site-directed mutagenesis of the glycine to an alanine abolishes myristoylation of substrate proteins (50, 51).

Two NMT isoforms, NMT-1 and NMT-2, are found in mammalian cells. These isoforms are ubiquitously expressed, share 77% sequence identity, and have similar substrate specificities (1). According to the analysis of NMT crystal structures these enzymes are compact, globular molecules that exhibit no structural homology to other proteins (52). Similar to protein palmitoylation, the mechanism of myristoylation is initiated by the binding of the lipid donor (52). Binding of myristoyl-CoA causes conformational changes in the NMT which forms a binding pocket for the target protein. Subsequently, NMT binds the target protein, transfers the myristoyl group to the protein’s N-terminal glycine by forming a stable amide bond, and releases the CoA followed by the myristoyl-modified protein.
Like prenylated proteins, several myristoylated proteins play important roles in signal transduction as well as cellular transformation and oncogenesis (53). N-myristoylated proteins include non-receptor Src-family kinases, G\(\alpha\) subunits of G proteins, as well as endothelial nitric oxide synthase (eNOS) (53-56). In addition, myristoylated proteins also play a role in the assembly, maturation and infectivity of virus proteins such as the polio VPO polypeptide precursor, as well as the human immunodeficiency virus 1 pr55\(^{gag}\) precursor (57, 58).

Myristoylation has two main biological functions. First, myristoylation can confer structural stability to proteins such as type IIB Calcineurin, the catalytic subunit of PKA, and the viral VP4 protein (1, 59, 60). Secondly, similar to prenylation, N-myristoylation causes membrane association as demonstrated by the inhibition of membrane association caused by site-directed mutagenesis of the N-terminal glycine residue of substrate proteins to alanine (50, 51). Although myristoylation is critical for the initial membrane association of these proteins, it has been determined that myristate produces weak membrane association, and that it is necessary, but not sufficient, to anchor proteins to cellular membranes (61, 62). Thus, myristoylated proteins are similar to prenylated proteins in that they require additional events or structures for stable association to cellular membranes.

Based on the important roles myristoylated proteins play in mammalian cells and the importance of myristoylation for the proper activity of these proteins, N-myristoyltransferases are attractive therapeutic targets to combat both human
pathogens, as well as cancer. NMTs are essential for the survival and growth of organisms such as *Candida albicans* and *Cryptococcus neoformans* (63, 64). Since these organisms are responsible for fungal infections in humans, NMTs are being targeted in new treatments for these types of infections (65-67). Also, based on the important role NMTs play in the replication of viruses, the enzymes have been targeted for the development of antiviral drugs specific for viruses such as HIV-1 (67). In addition, NMTs are also potential targets for anti-cancer therapy. Inhibiting myristoylation of Src-related tyrosine kinases by mutation of the N-terminal myristoylated glycine blocks the ability of these proteins to cause cellular transformation (50, 68). In addition, NMT is overexpressed in colonic tumors (69), and inhibiting myristoylation of p60-src in colonic cell lines causes decreased proliferation as well as colony formation (70). Based on this evidence, NMTs are potential targets for anti-cancer therapeutics.

### 1.2 PROTEIN PALMITOYLATION

Another lipidation process is thioesterification, also known as palmitoylation, in which a 16-carbon palmitoyl group is covalently attached to one or more cysteine residues in a target protein via a thioester bond (2). Although, other fatty acids such as myristate, stearate and arachidonate can also be S-linked to cellular proteins (71, 72), palmitate is the most commonly thioester-linked lipid. Palmitoylation is a particularly interesting process since it is unique among the various lipid modifications in that the thioester bond is reversible and regulatable (73). In addition, like many prenylated and myristoylated proteins,
many palmitoylated proteins are important signaling proteins that are involved in processes such as cellular survival, proliferation, and transformation. However, unlike the mechanisms of prenylation and myristoylation, the enzymes that catalyze palmitoylation have not been fully identified or characterized.

1.2.1 Protein Substrates Of Palmitoylation

A variety of proteins are palmitoylated within the cell. These proteins can be categorized into four subgroups based on their site(s) of palmitoylation. Table 1 (modified from (1, 74)) is a condensed list of palmitoylated proteins including their respective sites of palmitoylation (indicated as underlined and in bold font). Like prenylated and myristoylated proteins, many palmitoylated proteins are involved in important cellular signaling pathways and require membrane association for their proper activity. The addition of a palmitoyl group increases the hydrophobicity of proteins, in turn facilitating their proper cellular localization and activity.

Proteins categorized as type I palmitoylated proteins in Table 1 include transmembrane as well as integral-membrane proteins such as CD4 and the Dopamine D1 receptor. These proteins are not prenylated nor myristoylated (73), and palmitoylation occurs on cysteines located either adjacent to or just within the integral-membrane or membrane-spanning domain (1). Palmitoylation is influenced by the length and composition of the cytoplasmic tail and transmembrane sequences, with palmitoylation preferentially occurring on long
Table 1: Types of palmitoylated proteins with sites of palmitoylation
(modified from (1, 74))

<table>
<thead>
<tr>
<th>Type 1: Transmembrane, integral or peripheral membrane proteins</th>
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<tbody>
<tr>
<td>TGfα</td>
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<tr>
<td>Transferrin receptor</td>
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<tr>
<td>P-Selectin</td>
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<tr>
<td>SNAP-25</td>
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<tr>
<td>Viral proteins</td>
</tr>
<tr>
<td>Influenza HA, H1 subtype</td>
</tr>
<tr>
<td>Sindbis virus E2 protein</td>
</tr>
<tr>
<td>HIV-1 gp160</td>
</tr>
<tr>
<td>Seven transmembrane receptors</td>
</tr>
<tr>
<td>α2A adrenergic receptor</td>
</tr>
<tr>
<td>β2 adrenergic receptor</td>
</tr>
<tr>
<td>Serotonin receptor</td>
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<table>
<thead>
<tr>
<th>Type 2: Palmitoylated within an N- or C-terminal region</th>
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<tbody>
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<td>GAP43</td>
</tr>
<tr>
<td>PSD-95</td>
</tr>
<tr>
<td>GRK6</td>
</tr>
<tr>
<td>Gα subunits</td>
</tr>
<tr>
<td>αs</td>
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</tr>
<tr>
<td>α12</td>
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<table>
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</tr>
<tr>
<td>N-Ras</td>
</tr>
<tr>
<td>K-Ras(A)</td>
</tr>
<tr>
<td>Paralemmin</td>
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<table>
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cytoplasmic tails (75, 76). The sites of palmitoylation on these proteins usually lie within 12 amino acids of the membrane-spanning domain of the protein. However, since these proteins lack a defined consensus motif for recognition by PAT enzymes it appears likely that this group of proteins is palmitoylated in a non-enzymatic fashion, or in an enzymatically by PAT enzymes with an as yet uncharacterized motif specificity.

Type 2 palmitoylated proteins, such as GAP43 and PSD-95, are palmitoylated on cysteine residues located within an N- or C-terminal region that lacks a prenyl or myristoyl lipid attachment (1, 2, 73). Similar to type 1 palmitoylated proteins, proteins in this group also appear to lack a consensus motif for recognition by PAT enzymes, suggesting that they may be primarily non-enzymatically palmitoylated.

Type 3 palmitoylated proteins, including several Ras isoforms and paralemmin, are C-terminally prenylated and palmitoylated proteins. These proteins are first prenylated on the cysteine residue of the C-terminal CAAX box and subsequently palmitoylated on a cysteine adjacent to the site of prenylation (25). Unlike type 1 and 2 palmitoylated proteins, type 3 proteins contain a consensus motif which is composed of a C-terminal prenylated cysteine with an adjacent cysteine that may be recognized by PAT enzymes.

Lastly, type 4 palmitoylated proteins, such as certain Src-related tyrosine kinases, are first N-terminally myristoylated, and then palmitoylated on an adjacent cysteine. Similar to type 3 proteins, type 4 palmitoylated proteins contain an N-terminal palmitoylation consensus motif consisting of Met1-Gly2-
Cys$^3$, which is recognized by PAT enzymes (77). Following cleavage of the initiator Met$^1$, the new N-terminal Gly$^2$ is myristoylated (see section 1.1), which is required for subsequent palmitoylation of the adjacent Cys$^3$.

Both prenyltransferase and myristoyltransferase recognize their protein substrates based on specific C- or N-terminal sequences respectively. In contrast, palmitoylated proteins contain two different known motifs for recognition by PAT enzymes. Based on these differences in consensus motifs, it has been hypothesized that multiple motif-specific PATs exist to palmitoylate these different groups of proteins.

### 1.2.2. Enzymology Of Palmitoylation

In contrast to prenylation and myristoylation, whose mechanisms of action have been defined for well over a decade, the mechanism of protein palmitoylation has only more recently been characterized. Determining the mechanism of palmitoylation has been complicated due to a number of separate factors. First, the enzymes responsible for palmitoylation have been difficult to identify and characterize, which is further complicated by the existence of varying sites of palmitoylation. Second, unlike prenylation and myristoylation, palmitoylation is a dynamic, reversible process where palmitoylated proteins cycle between palmitoylated and non-palmitoylated forms as a result of PAT and thioesterase activities. Lastly, evidence exists for both enzymatic and non-enzymatic protein palmitoylation.
Several studies have demonstrated that some palmitoylated proteins including some Gα proteins, the Src-related tyrosine kinase Yes, and rhodopsin, can be palmitoylated in an environment lacking an enzyme source (78-80). This non-enzymatic palmitoylation reaction, also referred to as autoacylation, is facilitated by increasing a number of different factors including palmitoyl-CoA concentration, protein substrate concentration, pH, as well as time of the reaction (2). Although it is possible that some intracellular proteins may be palmitoylated in a non-enzymatic fashion, it is unlikely that autoacylation is the only mechanism of palmitoylation. This is particularly the case for proteins that are rapidly palmitoylated in response to cellular signaling events, since autoacylation can take from 30 minutes to the tens of hours (2).

There are three main lines of evidence for the existence of enzymatic palmitoylation. First, palmitoylation can be stimulated by signaling events within the cell (73, 81, 82). Although a protein can be palmitoylated in the presence of palmitoyl-CoA without an enzyme source if given enough time, many proteins undergo rapid processes of palmitoylation and depalmitoylation that cannot be explained by random non-enzymatic palmitoylation. For example, agonist activation of the β2-adrenergic receptor induces a substantial increase in [3H]-palmitate incorporation into the receptor (83), and mutation of the palmitoylated cysteine decreases the activation of the downstream effector protein adenylyl cyclase (84). In addition, the agonist-stimulated activation of several receptors influences the palmitoylation state of a number of other proteins including Gα proteins (85), eNOS(82), and the Src-related tyrosine kinase Lck (86). Second,
some proteins contain palmitoylation motifs for PAT recognition, binding and catalysis. As seen in Table 1, type 3 and type 4 palmitoylated proteins contain C- and N-terminal palmitoylation motifs, respectively. Type 3 proteins contain a C-terminal prenylcysteine residue with upstream palmitoylated cysteines, and prenylation is required before palmitoylation can occur (25). Thus, the C-terminally prenyl-modified type 3 protein acts as a recognition motif for PAT enzymes. In addition, type 4 proteins contain an N-terminal myristoylglycine residue with downstream palmitoylated cysteines, and myristoylation is required before palmitoylation can occur (77). Therefore, the N-terminally myristoyl-modified type 4 protein acts as a second recognition motif for PAT enzymes.

Third, enzymes that demonstrate PAT activity have recently been identified. The first verifiable PAT enzymes were identified in Saccharomyces cervisiae in 2002. In these studies, the yeast proteins Erf2/Erf4 and Akr1p were identified as PATs specific for Ras2 (87) and casein kinase2 (88), respectively. Comparison of the amino acid sequences of these two enzymes demonstrated that they shared a conserved Asp-His-His-Cys (DHHC) sequence located within a cysteine-rich domain (CRD), that was hypothesized to represent a signature catalytic motif for PATs since mutation of this domain blocked catalytic PAT activity of these enzymes (87, 88). The characterization of these enzymes and the identification of this domain has provided the starting point for the further identification of PATs in multiple species including Saccharomyces cervisiae (87-90), Mus musculus (91, 92) and Homo sapiens (93-95), each of which contain the DHHC-CRD consensus catalytic region. In addition, it has been
demonstrated that these enzymes exhibit substrate specificity. For example, proteins such as yeast Erf2/Erf4 (87) and Ak1rp (88), as well as human GODZ (93) and Hip14 (94) each have catalytic PAT activity toward proteins containing the C-terminal prenylcysteine substrate motif present in type 3 palmitoylated proteins, with no significant activity toward the substrate motif of type 4 palmitoylated proteins. In contrast, proteins such as yeast Pfa3 (89), and mouse DHHC17 and DHHC18 (91) show specific PAT activity toward proteins containing the N-terminal myristoylglycine substrate motif found on type 4 palmitoylated proteins. Thus, as previously hypothesized, PATs demonstrate catalytic activity in a substrate-dependent manner.

A total of 7 and 22 DHHC-CRD-containing proteins exist in yeast and humans, respectively (4, 96). In addition to the DHHC-CRD motif, this group of proteins shares other common characteristics. For example, these proteins are predicted to be integral membrane proteins and are found in the insoluble fraction of lysates (96, 97). Overall, DHHC-CRD-containing proteins are predicted to have between 3 and 6 transmembrane domains based on the individual protein sequences (96). Although many of these proteins have recently been characterized with PAT activity, the mechanism of palmitoylation is not fully understood due to the instability of these enzymes. The instability of this class of enzymes has also slowed the identification of PATs, and the enzymes responsible for these reactions are not fully identified and characterized.

The other side of the palmitoylation cycle is the process of de-palmitoylation, which is catalyzed by palmitoyl-protein thioesterases. Three
thioesterase enzymes have been identified. The first enzyme, protein-palmitoyl thioesterase-2 (PPT2) has been shown to remove the palmitate group from palmitoyl-CoA with no effect on the palmitoylation status of palmitoylated protein substrates (98). The second enzyme, protein-palmitoyl thioesterase-1 (PPT1) has been identified to depalmitoylate both Ras and Gα proteins (99). However, PPT1 is localized to the lysosomes (100), which precludes it from acting on Ras and Gα proteins to affect their activity and localization under normal physiological conditions. In fact, it appears that PPT1 is responsible for the depalmitoylation of proteins during the process of degradation (100) and since the protein is excreted when expressed in Sf9 and COS cells, its physiological substrates may also be excreted (101). The third enzyme, acyl-protein thioesterase (APT1), is a cytosolic protein that has been shown to depalmitoylate a number of proteins including Ras and Gα proteins (102), eNOS (103), and glycerol-3-phosphocholine (104). Based on its cellular distribution, APT1 is likely the thioesterase that depalmitoylates these proteins under physiological conditions that affect their cellular localization and membrane attachment.

1.3. BIOLOGICAL ROLES OF PALMITOYLATION

Palmitoylation is an important lipidation event that has many biological roles within the cell. When added to a substrate protein, the palmitate group acts as a membrane anchor and affects the localization of these proteins to various intracellular compartments, as well as specific microdomains within those compartments. In addition, palmitoylation affects protein-protein interactions as
well as protein stability. Since proper localization, stability, and contact with other proteins is important for the activity of many proteins, palmitoylation is essential for the activity of a number of proteins involved in a diverse array of intracellular signaling pathways that regulate processes such as cellular survival, proliferation, differentiation, vasodilation as well as protein aggregation. Due to the biological functions of palmitoylated proteins, the process of palmitoylation plays a role in multiple human diseases including neurological disorders, cardiovascular diseases and cancer.

1.3.1 Effects Of Palmitoylation On Target Proteins

The main function of protein palmitoylation is to increase hydrophobicity and facilitate the binding of proteins to membranes. Other lipid modifications including prenylation and myristoylation also play a role in this process. However, these modifications produce only transient membrane associations ($t_{1/2} \leq 1$ min) (62). In contrast, palmitoylation alone promotes more stable membrane association (61), with $\sim 15$ and $\sim 20$-fold greater affinity for membranes than myristoylation and farnesylation, respectively (52). A “kinetic membrane trapping model” has been proposed for proteins with dual acyl modifications (105). In this model, an initial modification is sufficient to transiently associate the protein to the membrane and palmitoylation subsequently anchors or “traps” the protein at the membrane (105). Proteins that undergo dual acylation of palmitoylation along with either prenylation or myristoylation demonstrate a very strong membrane association with $t_{1/2} \geq 70$ hr (105). The mutation of cysteine residues
that are palmitoylated is a common strategy to study the functional
consequences of preventing protein palmitoylation. Studies conducted in this
manner have consistently demonstrated the importance of palmitoylation for the
effective targeting of many proteins to the inner leaflet of the plasma membrane
(106-109). Conversely, the attachment of a palmitoylation motif to normally
cystosolic proteins such as p21ras (110) promotes membrane localization.

In addition to acting as a membrane anchor, the attachment of a palmitoyl
group also alters the trafficking of proteins between intracellular compartments.
For example, palmitoylation of the endogenous myrisotyl motif-containing Src-
related tyrosine kinase Fyn is necessary for localization of the protein from the
endoplasmic reticulum (ER) to the plasma membrane (PM). Also, wild-type Ras
proteins localize to multiple membrane compartments including the ER, the
Golgi, as well as the PM (111-115). However, studies have shown that the
farnesyl motif targets Ras isotypes to the ER and Golgi, whereas palmitoylation
is required to exit the Golgi complex (111, 112). In addition, it appears that the
localization of Ras proteins is affected by the recycling process of palmitoylation
and depalmitoylation. Ras proteins that are depalmitoylated traffic from the
plasma membrane to the Golgi complex, whereas palmitoylated Ras proteins
move from the Golgi complex to the plasma membrane (113, 115). The
importance of this pattern of recycling is further underscored by results showing
that irreversibly acylated N-Ras is non-specifically associated with internal
membranes, whereas reversibly palmitoylated N-Ras specifically distributes to
the Golgi and plasma membrane (115). This indicates that not simply
Palmitoylation not only anchors proteins to membranes and alters their localization to different cellular compartments, but also affects the localization of proteins to microdomains within these membranes. Evidence indicates that palmitoylation targets proteins to microdomains such as caveolea and lipid rafts in the plasma membrane (116, 117). In fact, many myristoylated and palmitoylated proteins such as Hck, Fyn, Lck (116-118), GAP-43 (119), and G proteins (120) are localized to lipid rafts. Also, the addition of a palmitoylation motif to proteins that do not normally distribute to lipid rafts, such as Src, induces palmitoylation of the protein and targeting to lipid rafts (117), which indicates that dual acylation sites are necessary for the association with plasma membrane microdomains. These microdomains contain high levels of sphingolipids and cholesterol and are resistant to solubilization with non-ionic detergents (121, 122). It is hypothesized that palmitoylated proteins are localized to these structures due to a high affinity for ordered domains such as those composed of tightly packed cholesterol and saturated phospholipids (5).

The addition of palmitate to a protein also facilitates protein-protein interactions and, in turn, intracellular signaling. Palmitoylation does not regulate these interactions through direct lipid-protein contacts, but rather by localizing proteins to the plasma membrane and microdomains within the membrane, so that protein-protein interactions can occur (2). This is particularly the case
because lipid rafts and caveolae are not only enriched with cholesterol and spingolipids, but also with signaling proteins (123-126). In fact, targeting of proteins to specific microdomains within the plasma membrane is particularly important for signaling proteins such as Src-related tyrosine kinases and Ras isoforms, since they require proper localization in order to have full activity and drive their intracellular signaling pathways (3, 73, 127-130). For example, mutating palmitoylated cysteines on Fyn (131) and Lck (132) inhibits the localization of these proteins to lipid rafts and blocks associated T cell activation. In addition, it has been demonstrated that palmitoylation is required to properly target H-, N- and (K2A)-Ras to the plasma membrane, and for activation of their transforming ability (2). Therefore, palmitoylation plays an important role in the proper localization of intracellular proteins, which directly affects the ability of these proteins to activate their downstream effectors and drive cellular signaling pathways.

Palmitoylation also modulates the stability and degradation of proteins. In some instances, palmitoylation affects protein stability by acting as a quality control checkpoint (133). For example, palmitoylation of the transmembrane yeast chitin synthase Chs3 protein appears to be an indicator of proper protein folding. Blocking palmitoylation of Chs3 prevents the protein from attaining an export-competent conformation and causes aggregation of the protein in the ER (134). Palmitoylation also modulates the stability and degradation of the yeast SNARE protein T1g1. Palmitoylation of this protein prevents its interaction with the E3 ubiquitin ligase Tu11. However, mutation of the palmitoylated cysteine
residue produces a resultant protein that interacts with Tu11, becomes ubiquitinated, and is targeted for degradation (90). In addition, the T1g1 mutants display a cellular distribution similar to wild-type T1g1 when the ubiquitin ligases were inactivated (90). These results infer that palmitoylation does not necessarily cause membrane targeting of T1g1, but simply prevents ubiquitination and degradation of the protein.

Overall, palmitoylation serves a number of important biological roles. Due to its hydrophobic nature, it acts as a membrane anchor allowing proteins to form more stable interactions with intracellular membranes. It also modulates the intracellular trafficking of proteins and induces their sequestration into specific membrane microdomains. By affecting the location of proteins as well as the stability of their interactions with intracellular membranes, palmitoylation in turn facilitates protein-protein interactions that modulate the ability of signaling proteins to activate their intracellular signaling pathways. Palmitoylation also appears to modulate protein stability and degradation by acting as a quality control checkpoint and inhibiting ubiquitination of some proteins. Thus, palmitoylation plays many important roles in the biological function of cells.

1.3.2 Roles Of Palmitoylation In Human Diseases

Huntington’s disease is a neurological disorder characterized by abnormal body movements, lack of coordination, as well as mental and behavioral abnormalities. The disease is caused by the expansion of polyglutamine repeats in the huntingtin protein which results in the accumulation of intracellular protein
aggregates (135), that are thought to arise as a result of the misfolded protein (4), that leads to neuronal toxicity. It has been subsequently demonstrated that the huntingtin protein is palmitoylated, and that palmitoylation has functional consequences for the protein (12). Mutation of the palmitoylated cysteine of the mutant huntingtin protein caused an increase in the number and speed of formation of intracellular inclusions with an associated elevation in neuronal toxicity (12). Thus, these results indicate that palmitoylation inhibits the aggregation of mutant huntingtin proteins, in turn protecting cells from the toxicity associated with this aggregation.

Another neurological disease associated with palmitoylation is infantile neuronal ceroid lipofuscinosis (INCL). INCL is a neurodegenerative disease with an incidence of 1 in 12,500 that is characterized by early visual loss, rapid mental deterioration, and death between 8 and 11 years of age (136). Unlike huntington’s disease, where the problem may be associated with reduced protein palmitoylation, INCL appears to be associated with a buildup of palmitoylated proteins. It has been demonstrated that the majority of patients diagnosed with INCL have silencing mutations in the gene for PPT1 (136), the lysosomal protein palmitoyl thioesterase. In addition, deletion of the PPT1 gene in mice produces the same phenotypic characteristics of the human disease including the accumulation of storage material in the brain, neuronal loss, and early death (137). Since PPT1 is responsible for the depalmitoylation of proteins localized to the lysosomes, it appears that INCL is caused by the intracellular buildup of palmitoylated proteins. This causes the accumulation of palmitoylated proteins in
the ER which activates the unfolded protein response, which in turn causes the activation of caspase-12 and caspase-3, apoptosis, and neurodegeneration (138).

Palmitoylation potentially plays a role in many other neurological disorders since important neuronal proteins such as GAP-43 (139), PSD-95 (140), the serotonin receptor (1), Src-related tyrosine kinases (116, 117), Gα proteins (108, 141), and many G protein-coupled receptors (142) are palmitoylated. For instance, it appears that dynamic palmitoylation is important for the formation of synaptic connections (143). During the development of these connections, the tips of growing axons migrate to their targets and become specialized for information transmission. Following formation of these connections the circuitry is functional; however, an “extended critical period” of time remains where remodeling of the synapses is sensitive to neural activity (144, 145). During this time, synaptic connections are potentiated due to this synaptic plasticity (143), and it has been demonstrated that the palmitoylation of GAP-43 and other growth cone proteins is decreased (143). Based on these data, it has been proposed that a decrease in the palmitoylation of these specific proteins may cause the disengagement of molecular machinery so that axonal extension can occur, and that defects in the dynamic palmitoylation system may have devastating consequences on the neurological system (143).

Palmitoylation may also play a role in cardiovascular diseases by affecting endothelial notric oxide synthase (eNOS). eNOS is a type 4 N-terminally myrisotylated and palmitoylated protein that is localized to the Golgi complex as
well as microdomains of the PM (146-148). This protein mediates blood pressure, vascular remodeling and angiogenesis through the production of nitric oxide (NO) (149). Mutation of the palmitoylated cysteine residues within the protein prohibits membrane targeting of eNOS and impairs both basal and agonist-stimulated NO release (147, 150). In addition, in vivo deletion of the eNOS gene produces several cardiovascular phenotypes including elevated blood pressure and accelerated atherosclerosis (151). Since eNOS is responsible for the regulation of so many important activities of the cardiovascular system, and its activity is predicated on palmitoylation-dependent intracellular localization, it seems that the process of palmitoylation is likely involved in multiple cardiovascular diseases.

Palmitoylation also likely plays a role in disorders of the immune system, particularly T cell-mediated diseases such as inflammatory bowel disease, psoriasis, multiple sclerosis, type-1 diabetes, rheumatoid arthritis, and organ graft rejection (152). T cell receptor (TCR) signaling requires the formation of a TCR complex in lipid rafts that contain signaling proteins such as Src-related tyrosine kinases (i.e., Lck, Fyn), LAT, and the TCR (117, 118, 153). Since palmitoylation is required for Lck, Fyn, and LAT to localize to lipid rafts, and the activity of the TCR is dependent on the presence of these proteins, this suggests that palmitoylation plays a role in TCR signaling. Indeed, it has been demonstrated that mutation of the palmitoylation sites of LAT prevents lipid raft localization, activation, and recruitment of LAT-binding proteins for signal transduction (154). In addition, mutations of the palmitoylation sites of Fyn (131) and Lck (132) block
the localization of these proteins to lipid rafts, subsequently inhibiting T cell activation. Therefore, since overactivity of the TCR is involved in multiple autoimmune disorders, and the activity of the TCR is dependent on the palmitoylation of its associated proteins, palmitoylation plays a role in these autoimmune disorders.

### 1.3.3 Palmitoylation in Cancer

Palmitoylation also plays a role in the development of cancer by affecting the localization and activation of several type 3 and 4 palmitoylated proteins, including H-, N-, and K-Ras(A), as well as the Src-related tyrosine kinases. It has been well demonstrated that activated type 3 palmitoylated Ras proteins drive several signaling pathways that contribute to the malignant phenotype including cellular survival, proliferation, and differentiation (155, 156). As discussed above (1.3.1), dynamic palmitoylation is important to the proper localization of several Ras isotypes in that the depalmitoylated forms traffic from the plasma membrane to the Golgi complex, and the palmitoylated forms move from the Golgi complex to the plasma membrane (113, 115, 157). For many years, the scientific community had a long-held view that Ras proteins operated only at the plasma membrane; however, recent studies have shown the importance of Ras compartmentalization for signal transduction (14, 158, 159) as it has been demonstrated that the Ras proteins are able to activate signaling while localized in compartments other than the PM, including the ER (14) and Golgi complex (14, 159, 160). In addition, studies have demonstrated that H-
and N-Ras activation at the PM stimulates depalmitoylation, resulting in a redistribution to the ER and Golgi, where they can be repalmitoylated (113, 115, 157). Regardless of the compartment of activity, it has been demonstrated that mutations of Ras that affect the palmitoylation status of the protein impair the activity of the enzymes as measured by activation of signaling pathways such as the mitogen-activated protein kinase (MAPK) pathway (158, 161). Since, approximately 20-30% of all human tumors contain activating mutations of the ras genes (36), and palmitoylation is necessary for the proper activity of these proteins, palmitoylation plays a role in the development of tumors containing ras mutations.

Palmitoylation also plays a role in cancer through type 4 palmitoylated proteins such as the Src-related tyrosine kinases. These kinases have been shown to activate pathways that drive cellular proliferation, inhibition of apoptosis and cellular transformation (162-165). In addition, studies have demonstrated that the expression and activity of Src-related tyrosine kinases such as Lck, Yes and Lyn are upregulated in multiple types of primary human cancer and tumor-derive cell lines including lymphoma (163, 166), leukemia (167), melanoma (168), glioblastoma (169), as well as carcinomas of the lung and colon (170-172). In addition, many of these kinases appear to play a role in metastasis since they exhibit elevated expression and activity in in vivo metastases and metastatic cell lines (57, 166, 170, 173). The activity of these proteins induce pro-metastatic phenotypic characteristics such as increased cellular motility, matrix metalloproteinase production and invasiveness, as well as decreased binding to
the extracellular matrix through the disassembly of focal adhesions (169, 174-177). Since palmitoylation is imperative for the effective localization of these proteins to the plasma membrane and in turn their effective signal transduction, this lipidation event appears to be an integral component of the machinery that allows these proteins to drive cellular transformation.

1.4 ASSAYS OF PALMITOYLATION

As noted previously, the enzymes responsible for protein palmitoylation have only recently begun to be elucidated. These enzymes have historically been difficult to identify because classical biochemical approaches of purification and characterization have been unable to definitively identify these proteins. This difficulty is brought about by the nature of the proteins. As integral membrane proteins, attempts to express and isolate mammalian DHHC proteins in bacterial and yeast cells has been met with little success (94), and when isolated from mammalian cells there is a rapid loss of enzymatic activity. However, advancements have been made in the last several years that have allowed the identification of palmitoylated proteins and PATs. In this section, the types of palmitoylation assays will be covered, including the advantages and disadvantages of each.

Historically, the most frequently used protein palmitoylation assays have used metabolic labeling of cultured cells with radiolabeled forms of palmitate such as $^3$H-palmitate (87, 88, 91-93, 178, 179). Generally, cells are grown on cell culture plates and the labeled palmitate is added to the culture medium,
taken up by the cells, and subsequently metabolically incorporated into the palmitoylation sites of proteins. The cells are then lysed, and the labeled proteins are generally purified and/or analyzed by SDS-PAGE. The radioactive emission signal is subsequently detected by fluorography when the dried gels are exposed to X-ray film. There are three advantages to using this method for the detection of palmitoylated proteins and PATs. First, palmitoylation can be monitored in live cells. Second, the palmitoylation of specific full length proteins can be determined. Third, rates of palmitoylation can be determined by performing pulse-chase analysis. However, there are also several disadvantages to using the radiolabeling technique. First, investigators must take special care in utilizing precautionary measures when working with radioactive materials. Second, this method requires long labeling and exposure times in order to obtain a sufficient signal for measurement. In general, cells are exposed to the radiolabeled palmitate for at least 4 hrs, and detection of palmitate incorporation into the protein requires from 1 to 2 weeks of exposure for the fluorogram. Lastly, the amount of radiolabeled palmitate incorporation and detection are dependent on multiple factors, such as the ability of the palmitate to enter cells, the ratio of labeled and unlabeled palmitate that is produced within the cells, and the amount of palmitoylation that occurred before the labeling period began (179). Thus, this method cannot provide quantitative data of the extent of acylation.

Another method to study the palmitoylation of proteins is by utilizing matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
(MALDI-TOF MS) (178, 179). This is usually carried out on metabolically labeled palmitoylated proteins (discussed in the previous paragraph) that have been purified by chromatography or through SDS-PAGE and subsequently digested with protease. The palmitoyl group can then be removed by hydroxylamine, which shifts the mass of the depalmitoylated peptide. In this way, the identification of a palmitoylated protein can be confirmed along with the molecular identity of the acyl group (180). The advantages of using MS are that it allows the estimation of the stoichiometry of the palmitoylation of a particular protein of interest and provides the investigator with the exact mass of the modifying group (178, 181). However, the disadvantages of using MS for the analysis of palmitoylation are that thioester-linked palmitate is susceptible to alkali hydrolysis and can therefore be easily lost. Also, palmitoylated peptides are extremely “sticky”, making them very easy to lose during the process of peptide preparation.

An alternate method to study protein palmitoylation is fatty acyl exchange chemistry. In this method, the fatty acid on the palmitoylation site is exchanged for another, more readily detectable, label (179). In the first step of the process all pre-existing free sulfhydryls are blocked by N-ethylmaleimide (NEM). Next, hydroxylamine is added to cleave the thioester bond that attaches the fatty acid to the cysteine at the site of palmitoylation; thus removing the fatty acid group. This cleavage leaves the previously acylated cysteine residue with a free sulfhydryl group. In the third step, the newly created free sulfhydryl group is labeled with thiol-specific reagents such as $^3$H-NEM or non-radioactive biotin-
conjugated 1-biotinamido-4-[4-(maleimidomethyl)cyclohexanecarboxamido] butane (Btn-BMCC). Once the palmitoylation site is labeled, palmitoylation of the protein can be purified by SDS-PAGE or affinity for streptavidin resin and detected by either fluorography or chemiluminescence, depending on the label (179). There are a number of advantages to using fatty acyl exchange chemistry to study protein palmitoylation. First, this method increases the sensitivity of detection by up to 12 times that of standard $^3$H-palmitate incorporation (179). This allows the detection of palmitoylated proteins that are expressed at relatively low levels that may not be detected by using $^3$H-palmitate incorporation. Second, since the hydroxylamine treatment removes virtually all the palmitates from proteins, it provides a more accurate measurement of the stoichiometry of palmitoylation. Third, the method can employ non-radioactive labels, allowing investigators to avoid the precautions necessary to work with radioactive materials. Lastly, palmitoylation can be assayed on non-living preparations from tissue or cell samples.

The last method to study protein palmitoylation is termed *in vitro* palmitoylation (IVP) and employs fluorescently labeled lipopeptides that mimic the specific palmitoylation motifs of type 3 and type 4 palmitoylated proteins (94, 182, 183). In this method, membrane fractions containing the enzyme source are mixed with non-labeled palmitoyl-CoA and the fluorescently labeled lipopeptides. The PAT enzymes in the membrane fractions subsequently catalyze the addition of the palmitoyl group to a palmitoylatable cysteine on the lipopeptides. This modification causes a shift in the hyrdophobicity of the
peptide, which can be detected by high performance liquid chromatography (HPLC). Like fatty acyl exchange chemistry, one advantage of this method is that it does not require the use of radioactive materials. In addition, this assay is much faster than other palmitoylation assays, making it more amenable to screening for potential inhibitors of palmitoylation. The main disadvantage to this method is that it only demonstrates the activity of a PAT toward the motif of type 3 and 4 palmitoylated peptides. Thus, the specific proteins targeted by a given PAT cannot be determined using this method because the peptides are short and lack the secondary and tertiary structures associated with those proteins.

1.5 PROTEIN PALMITOYLATION AS A THERAPEUTIC TARGET

The lipid modification processes of proteins have attracted increasing interest as potential therapeutic targets in recent years. This interest is based on the necessity of the attachment of these lipid groups for the proper localization and activity of important intracellular signaling proteins. The majority of research in this area has focused on the Ras proteins, and in particular, on the process of post-translational farnesylation. This is partly due to the fact that farnesyltransferases have been cloned (184-186) and used to identify inhibitors through drug screening (38, 187, 188). In contrast, the identification of selective inhibitors of palmitoylation has been obstructed by identification of the PAT enzymes themselves, and the lack of assays suitable for the high-throughput screening of potential inhibitors. This section provides a brief history and
As noted previously, the Ras proteins activate pathways that are involved in cellular proliferation, survival, and differentiation, and are constitutively activated in a large number of human tumors. The activity of these proteins is predicated on localization to specific cellular compartments, which is facilitated by lipidation events such as farnesylation and palmitoylation, and inhibiting these processes impairs the function of these proteins. Based on this information, several groups have devoted a significant amount of time and resources toward the development of FTIs to inhibit the farnesylation of the Ras isoforms (189). Many of these compounds demonstrated effective inhibition of Ras farnesylation (32-35) as well as antitumor activity (42, 43), and were taken into clinical trials (189). However, the advancement of FTIs to the clinic has been hindered by several issues, including the cystostatic nature of their effects and a lack of clinical efficacy (155), some of which may not occur if the process of palmitoylation were selectively targeted instead of farnesylation.

Palmitoylation is a better target for the development of anticancer agents for several reasons. First, unlike the irreversible process of farnesylation, palmitoylation is a dynamic and reversible process (73). Therefore, blocking the farnesylation of Ras only affects newly synthesized proteins that have not been farnesylated, but it has no affect on the activity of previously farnesylated proteins. Since the $T_{1/2}$ of the Ras isotypes can reach 27 hr (190), the signaling pathways activated by the proteins will remain activated far beyond the initiation
of treatment. In contrast, palmitoylation is a dynamic process where the $T_{1/2}$ of the palmitoyl group on Ras is less than 10 min (191). Since the process is rapidly reversible, inhibition of palmitoylation will affect all forms of the proteins including both the non-palmitoylated and palmitoylated forms. In addition, it has been determined that the turnover of palmitate on oncogenic H-Ras is more rapid than that observed on wt H-Ras (191), which may allow for the selective targeting of cells containing Ras mutations versus normal endogenous cells. Second, targeting palmitoylation may provide greater selectivity and a lower chance of non-specific activity. The human farnsyltransferase enzyme is known to farnesylate at least 17 different target proteins (189). Therefore, inhibition of farnesyltransferase does not selectively block farnesylation of the Ras proteins, but instead inhibits the farnesylation of a wide array of proteins. For example, it has been demonstrated that the farnesylated protein RhoB has a $T_{1/2}$ of approximately 2 hr (192), and it has been suggested that the effects observed by FTI treatment are not caused by inhibitory effects on the Ras proteins, but are rather a result of the inhibition of RhoB (192). In contrast, multiple motif-specific PATs have been identified in yeast (87-90), as well as mammals (91-95). These PATs have been demonstrated to be specific for either type 3 or type 4 palmitoylated proteins, and may have greater specificity within those groups based on sequence or structural characteristics outside of the C- and N-terminal PAT recognition motifs. Since multiple enzymes exist with specificities for different motifs, and thus proteins, it seems likely that selectively inhibiting individual PATs will more specifically inhibit targeted pathways, and produce
fewer non-specific effects. Third, unlike inhibiting farnesylation, selectively inhibiting PATs may block mutant N- and K-Ras activity. It has been demonstrated that K- and N-Ras, the most prevalently mutated Ras isoforms in human tumors (189), are alternatively prenylated by GGTase-1 in cells treated with FTIs (39, 193), and maintain their activation (40, 41). Thus, the antitumor activity observed by these compounds is due to effects on proteins other than K- and N-Ras. In contrast, palmitoylation is not known to be substituted, and as discussed above, PATs likely exist with specificity toward proteins including the Ras isoforms. Thus, compounds developed to selectively inhibit PATs specific for these Ras isoforms could directly block their activity and be useful in the treatment of 20-30% of human tumors. Lastly, blocking palmitoylation may produce a greater cytotoxic effect than the inhibition of farnesylation. In general, FTIs are cytostatic agents that induce G2/M accumulation in a variety of human tumor cell lines, with G1 arrest occurring only in cells containing an H-Ras activating mutation (58, 194). These drugs are generally only able to induce apoptosis when combined with a second signal such as serum deprivation (195), or when used in combination with other inhibitors of signal transduction (196, 197). In contrast, the treatment of SKOV3 cells with a lipopeptide mimetic that acts as a competitive inhibitor for the palmitoylation of type 4 N-terminally myristoylated and palmitoylated proteins causes cytotoxicity at relatively low concentrations (section 2.4.5). This indicates that selective inhibitors of palmitoylation, in particular the palmitoylation of N-terminally myristoylated and
palmitoylated proteins, may be cytotoxic agents that can be used to treat multiple forms of cancer including solid tumors.

There are two broad categories of palmitoylation inhibitors: Lipid-based and non-lipid palmitoylation inhibitors. The lipid-based palmitoylation inhibitors include compounds such as 2-bromopalmitate (2BP), cerulenin, and tunicamycin (Figure 2). Although these compounds can be used to inhibit the palmitoylation of proteins, none are particularly selective agents. The use 2BP, a palmitate analog that inhibits the incorporation of palmitate into proteins (198), has recently gained popularity. The inhibitory effects of this compound on palmitoylation have been verified in several proteins including the Src-related tyrosine kinases, Rho family kinases and H-Ras (198-200). Therefore, 2BP acts as a broad range inhibitor of palmitate incorporation, but does not selectively inhibit the palmitoylation of specific palmitoylatable protein substrates. Inside the cell, 2BP is converted to 2BP-CoA, a non-metabolizable analog of palmitate (178). However, once metabolized, the mechanism by which 2BP inhibits palmitoylation is unknown, although a number of possible mechanisms have been suggested (178). First, 2BP-CoA may bind to a PAT and form an inhibitor:enzyme complex. Second, 2BP may be transferred to the target protein, but the decreased hydrophobicity may reduce binding of the protein to the lipid bilayer. Lastly, 2BP may alter lipid metabolism in such a way as to reduce the levels of intracellular palmitoyl-CoA that is available for palmitoylation. In fact, this compound inhibits a number of enzymes involved in lipid metabolism including carnitine palmitoyltransferase-1, fatty acid CoA ligase, glycerol-3-phosphate
Figure 2. Lipid-based inhibitors of protein palmitoylation. The chemical structures of lipid-based compounds used to inhibit the palmitoylation of proteins (modified from (176)).
acyltransferase as well as triacylglycerol biosynthesis (201, 202). Therefore, it is important to note that although 2BP inhibits palmitoylation, the effects of 2BP treatment are not necessarily due to its effects on protein palmitoylation.

Cerulenin (2,3-epoxy-4-oxo7,10 dodecadienoylamide), another lipid-based inhibitor shown in Figure 2, and certain analogs have also been shown to inhibit palmitoylation (203) of proteins including the myelin proteolipid protein (204) and CD36 (205). Like 2BP, the mechanism by which cerulenin inhibits protein palmitoylation is unknown. However, it has been suggested that cerulenin acts by chemically modifying sulfhydryl groups, in turn forming chemical adducts on the cysteines of either the protein acceptor or the PAT and inhibiting the process of palmitoylation (204, 206). Like 2BP, cerulenin also has effects on cellular lipid metabolism. The compound binds to β-keto-acyl-ACP synthase, thus inhibiting the biosynthesis of fatty acids. Cerulenin also inhibits HMG-CoA synthetase, which causes decreased cholesterol biosynthesis. Thus, like 2BP, cerulenin is a non-selective inhibitor of protein palmitoylation with additional effects on the metabolism of cellular lipids.

Another lipid-based palmitoylation inhibitor is the nucleoside antibiotic tunicamycin (Figure 2). This compound is primarily known for its ability to inhibit the N-linked glycosylation of proteins; however, it has also been demonstrated to inhibit the palmitoylation of proteins such as GAP-43, the estrogen receptor variant α, certain Ca^{2+} channels, and the myelin proteolipid protein (129, 204, 207, 208). Like 2BP and cerulenin, the mechanism by which tunicamycin inhibits palmitoylation is unknown, however, it has been suggested that it functions by
competing with palmitoyl CoA for binding to PATs. Also, like the other palmitoylation inhibitors, tunicamycin has non-specific activities associated with the inhibition of protein glycosylation.

In addition to the lipid-based inhibitors of palmitoylation, several non-lipid inhibitors have also been recently identified (209) (Figure 3). It has been their ability to inhibit the localization of fluorescently-labeled endogenous proteins to the plasma membrane in intact cells. In addition, these compounds have been demonstrated to abrogate signaling through the Raf/Mek signaling pathway and decrease human tumor cell line proliferation \textit{in vitro}. The \textit{in vivo} antitumor activity of compounds I through IV were also characterized, and it was observed that treatment of Balb/c mice bearing JC cell tumors with each of these compounds lead to a decrease in tumor volume as compared to vehicle-treated mice. Unlike the lipid-based palmitoylation inhibitors, whose mechanisms of action are not known, the selectivity of these agents suggests a specific mechanism of action of these compounds. Since they are selective for C-terminal farnesyl-directed or N-terminal myristoyl-directed palmitoylation, they likely do not act as palmitoyl-CoA competitors, but rather may target the protein binding site. This mechanism would make it unlikely that they would disrupt fatty acid biosynthesis or other palmitoyl-CoA-dependent reactions.

Overall, palmitoylation is an important lipidation event that affects the activity of many proteins involved in multiple human diseases, and should be considered an important target for selective inhibition. Palmitoylation is a more
<table>
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<th>Compound Structure and Name</th>
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<th>Motif Specificity</th>
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**Figure 3.** Non-lipid inhibitors of palmitoylation. The chemical structures and selectivity of non-lipid compounds palmitoylation inhibitors (modified from (209)).
demonstrated that compounds listed here as I through IV are selective inhibitors of C-terminal farnesyl-directed palmitoylation, whereas compound V is a selective inhibitor of N-terminal myristoyl-directed palmitoylation (209). These compounds have been shown to inhibit palmitoylation both quantitatively and qualitatively through use of the IVP assay covered previously, and by observing attractive target for the development of anticancer drugs because the reaction is reversible, provides a greater chance for selectivity, provides a greater opportunity to target tumors with K- and N-Ras mutations, and is likely to produce compounds with cytotoxic effects. There are two broad categories of palmitoylation inhibitors: Lipid-based and non-lipid palmitoylation inhibitors. The lipid-based compounds (2BP, cerulenin and tunicamycin) are widely used to inhibit palmitoylation in vitro. However, the mechanism of action of these compounds is still unknown, they lack defined substrate specificity, and affect other aspects of cellular function including lipid metabolism. Alternatively, the non-lipid palmitoylation inhibitors (compounds I through V) are not widely used. However, they demonstrate substrate specificity, which suggests a specific mechanism of action, and they appear less likely to non-specifically affect lipid metabolism. Therefore, these molecules may serve as lead compounds in the further refinement and development of future selective PAT inhibitors. In addition, the recent identification of motif-specific PATs provides the opportunity to develop selective inhibitors of individual PATs, which may have an even greater potential to help in the fight against many widespread human diseases.
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Chapter 2

Cellular Palmitoylation and Trafficking of Lipidated Peptides

Jeremiah M. Draper, Zuping Xia and Charles D. Smith

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2.1 ABSTRACT

Many important signaling proteins require the post-translational addition of fatty acid chains for their proper subcellular localization and function. One such modification is the addition of palmitoyl moieties by enzymes known as palmitoyl acyltransferases (PATs). Substrates for PATs include C-terminally farnesylated proteins, such as H- and N-Ras, as well as N-terminally myristoylated proteins, such as many Src-related tyrosine kinases. The molecular and biochemical characterization of PATs has been hindered by difficulties in developing effective methods for the analysis of PAT activity. In the present studies, we describe the use of cell-permeable, fluorescently-labeled lipidated peptides that mimic the PAT recognition domains of farnesylated and myristoylated proteins. These PAT substrate mimetics are accumulated by SKOV3 cells in a saturable and time-dependent manner. Although both peptides are rapidly palmitoylated, the SKOV3 cells have a greater capacity to palmitoylate the myristoylated peptide than the farnesylated peptide. Confocal microscopy indicated that the palmitoylated peptides co-localized with Golgi and plasma membrane markers, whereas, the corresponding non-palmitoylatable peptides accumulate in the Golgi but did not traffic to the plasma membrane. Overall, these studies indicate that the lipidated peptides provide useful cellular probes for quantitative and compartmentalization studies of protein palmitoylation in intact cells.
2.2 INTRODUCTION

A major mechanism by which cells regulate the subcellular localization of proteins is through post-translational lipid modifications which increase the hydrophobicity of proteins, promote their localization to specific cellular compartments, and/or alter their conformation (reviewed in (1-6)). Many important signaling proteins, including Ras proteins and Src-related tyrosine kinases, require localization to the plasma membrane to activate their downstream effector proteins (2, 7-12). Accumulating evidence demonstrates that targeting of these signaling proteins to the plasma membrane is facilitated by post-translational lipidation reactions, and that blocking the enzymes that catalyze these reactions is an effective means of blocking signaling through these pathways (13-17).

One such lipidation process is thioesterification, also known as palmitoylation, in which a 16-carbon palmitate group is covalently attached to one or more cysteine residues in a target protein. A variety of proteins are known to be palmitoylated (3, 7, 18) and many of these proteins can be separated into groups based on motifs adjacent to the site(s) of palmitoylation (3). One group of palmitoylated proteins contains a C-terminal farnesyl motif, while a second group contains an N-terminal myristoyl motif. Both types of proteins undergo multi-step post-translational modification in order to effectively localize to the plasma membrane. The farnesylated group, which includes proteins such as H-, N- and K2A-Ras (3, 18), contains a C-terminal CAAX motif that is required for palmitoylation of a cysteine located near the C-terminal farnesylcysteine (19).
Following farnesylation of the cysteine of the CAAX motif by farnesyltransferase, the “AAX” residues are proteolytically removed and the new C-terminus is carboxymethylated. The final modification consists of the attachment of palmitate groups to cysteines near the C-terminal farnesylcysteine through a thioester linkage to the sulfhydryl of the target cysteine, which is catalyzed by a palmitoyl acyltransfease (PAT) enzyme (13, 19). This final lipid modification is required to target these Ras isoforms to the membrane and for complete activation of their transforming ability (13, 20, 21). The myristoylated and palmitoylated group of proteins includes several Src-related kinases such as Lck and Fyn, and is characterized by an N-terminal methionyl-glycyl sequence closely followed by a cysteine residue. In this case, the processing involves the cleavage of the N-terminal methionine, thereby creating an N-terminal glycine which is modified by the addition of a myristate moiety through the action of N-myristoyltransferase. Similar to the reactions discussed above, the myristoylation modification is necessary for the subsequent palmitoylation of one or more cysteine residues near the N-terminus of the protein via a PAT (3, 22, 23). As with the C-terminal modified proteins, the palmitoylation modification is required for effective localization of these proteins to the plasma membrane and for effective signal transduction (14-16).

Since distinct motifs at the C- or N-termini of these proteins direct their palmitoylation, we (24-27) and others (3) have hypothesized that different PAT enzymes recognize and palmitoylate these classes of proteins. The existence of motif-specific PATs is of interest because of the roles of palmitoylated proteins in
the cell. Many of these proteins, such as the Ras proteins and Src-related tyrosine kinases, are involved in signaling pathways that drive cellular survival and proliferation (28-31). The fact that the functions of these proteins are predicated on their specified subcellular localizations makes the enzymes that catalyze their post-translational modifications attractive drug targets. In fact, the enzymes involved in the post-translational modifications of Ras proteins are considered prime targets for anticancer drugs (32, 33). However, the primary focus for the development of anti-Ras agents has been on the process of farnesylation. This is partly because the farnesyltransferases have been cloned (34, 35) and used in screens to identify and develop inhibitors (36, 37). In contrast, the identification and characterization of human PATs has been hindered by the lack of defined assays to characterize these enzymes and to identify small molecule inhibitors.

We have previously described indirect cellular assays that can be used to screen chemical libraries for PAT inhibitors (27) and in vitro PAT assays that can be used for low-throughput confirmation of PAT inhibitory activity (24, 25). However, these methods are still insufficient to directly measure PAT activity in intact cells, which would be useful in the molecular identification of human PAT enzymes and biochemical studies of the regulation of these PATs. Therefore, in these studies we sought to develop quantitative and efficient cellular assays to characterize PAT activity in intact cells using peptides that mimic the palmitoylation motifs of farnesyl and myristoyl-directed PATs. The data demonstrate that synthetic lipidated peptides provide excellent tools to conduct
such studies on PAT activity, as well as studies of the role of palmitoylation in the subcellular trafficking of lipidated proteins.

2.3 MATERIALS & METHODS

Materials

Palmitoyl-CoA, 2-mercaptoethanol (βME) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Alexa Flour 594-conjugated wheat germ agglutinin (W11262), Hoechst 33342 and BODIPY TR C5-Ceramide (B34400) were purchased from Molecular Probes (Eugene, OR). The fluorescent peptides (NBD)-CLC(OMe)-Farn, and Myr-GC(NBD) were synthesized as t-butyl-disulfide-protected precursors by solution-phase chemistry using mild conditions to maintain chemically labile functional groups, e.g. the farnesyl-cysteine thioether linkage, as previously described (24, 38). These peptides (1 or 5 mM in DMSO) were stored under argon at -80 °C, and deprotected immediately prior to their use by incubation with βME/DMSO (2.7:97.3 or 12:88, v/v, respectively) containing 50 mM Tris, pH 8.0 at 55 °C for 20 min with agitation. Peptides containing alanine residues in the place of the cysteine residues, i.e. (NBD)-ALC(OMe)-Farn and Myr-GA(NBD), to be used as non-palmitoylatable controls were synthesized by parallel methods.

Cell culture

All cell culture reagents were obtained from Gibco Co. (Grand Island, N.Y). SKOV3 cells (ATCC HTB-77) were maintained in Dulbecco’s Modified
Eagle’s Medium (DMEM) containing 10% fetal bovine serum, 1 mM sodium pyruvate and 50 µg/ml gentamycin at 37 °C in an atmosphere of 5% CO₂/95% air.

**Cytotoxicity assays**

To determine the potential cytotoxic effects of the peptides, SKOV3 cells were plated into 96-well microtiter plates and allowed to attach for 24 hr. The peptides were resuspended in DMSO as a vehicle and diluted in standard culture media to various concentrations. The cells were subsequently incubated with the peptides for 2 hr at 37°C. Toxicity was determined by measuring the amount of lactate dehydrogenase (LDH) released, as an indication of cell permeability, using the CytoTox-One Homogeneous Membrane Integrity Assay kit from Promega (Madison, WI).

**Cellular palmitoylation assay**

In dose-response studies, SKOV3 cells were grown to confluence and incubated for 60 min at 37 °C with one of the test peptides (or DMSO as the vehicle) in culture media at final concentrations varying from 0.1 - 80 µM. In time-course studies, the cells were grown to confluence and incubated with one of the test peptides at 1 µM in culture media at 37 °C for times varying from 5 - 120 min. For all experiments, the parent peptides and the palmitoylated peptide products were collected from the cells using the following standard extraction protocol. After incubation with the peptide, the cells were washed twice with ice-
cold PBS, killed by the addition of ice-cold 50% methanol, scraped and transferred to a glass test tube. The peptides were extracted into the organic phase by the addition of potassium carbonate-buffered dichloromethane / water / methanol (4:3:1, by volume) and low-speed centrifugation. The organic phase was transferred to another tube, and the remaining aqueous phase was extracted once more with buffered dichloromethane. The organic fractions were combined, dried under nitrogen, and analyzed immediately by HPLC as indicated below. As controls for the retention times of the non-plamitoylated and palmitoylated peptides, each peptide (10 µM) was incubated with 100 µM palmitoyl-CoA in high-pH acylation buffer (50 mM citrate, 50 mM phosphate, 50 mM Tris and 50 mM 3-(cyclohexylamino) propane-1-sulphonic acid, pH 8.2) at 37 °C for 10 min with agitation. The samples were extracted as described above and subjected to HPLC analysis as indicated below.

**HPLC method for quantifying peptide palmitoylation**

Assay extracts were dissolved in 27 µl of DMSO and the peptides were resolved on a reversed-phase Chromolith RP-8e column (2 µm; 300 Å; 4.6 mm x 100 mm) using a methanol gradient at a flow rate of 1 ml/min. Initially, the mobile phase was maintained at 30% methanol / 70% water for 1 min, followed by a linear gradient of 30-100% methanol over 5 min. The mobile phase was maintained at 100% methanol for 6 min, and decreased on a linear gradient to 30% methanol over 2 min. The peptides were detected by the fluorescence of their fluorophore (7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)) at its optimal
excitation and emission wavelengths of 465 and 531 nm, respectively. Peptides that eluted at a retention time of Peak B (Figure 3) were shown by mass spectroscopy to be palmitoylated, whereas peptides eluting at any other time were non-palmitoylated. The mean values of the area under the curve (AUC) for each treatment were converted to fmole of peptide using a standard curve generated by direct injection of known quantities of the peptides. The percentage of peptide palmitoylated was calculated as the mass of Peak B divided by the total peptide mass in the sample.

**Confocal laser microscopy**

Confocal microscopy was performed using a Zeiss LSM 510 NLO Laser Scanning Microscope with Multiphoton Excitation and Zeiss software (Confocal Microscopy Core at MUSC). To monitor the trafficking of the peptides in SKOV3 cells over time, the cells were incubated with Hoechst 33342 (2 µg/ml) for 10 min at 37°C in DMEM without phenol red or serum, washed twice with warm media, then incubated with peptide (1 µM). The cells were maintained at 37 °C in 5% CO₂ and images were captured at times from 15 to 120 min. To compare the localization of the peptides with the plasma membrane, the cells were incubated with peptide (1µM) in DMEM without phenol red or serum at 37 °C. After 15 min, AF594-WGA (15 µg/ml) was added and the cells were further incubated for an additional 15 min at 37 °C. The media was then removed, and the cells were washed twice with fresh media and directly observed by confocal microscopy. Localization of the peptides to the Golgi apparatus was determined by incubating
the cells with BODIPY TR C₅-ceramide (0.17 mg/ml) on ice for 30 min (39). The cells were then washed twice with ice-cold HBSS (Gibco), and warm media containing 1 µM peptide was added. The cells were incubated for 30 min at 37 °C, washed twice and visualized as above. All images presented are single sections.

2.4 RESULTS

2.4.1 Cytotoxicity of lipidated peptides.

The (NBD)-CLC(OMe)-Farn peptide (Figure 1) is a farnesylated tripeptide that mimics the C-terminus of H-Ras and provides the putative recognition motif for PATs which palmitoylate the cysteine residue of C-terminally lipidated proteins. The (NBD)-ALC(OMe)-Farn peptide is identical to (NBD)-CLC(OMe)-Farn except that it has a nonpalmitoylatable alanine substituted for the cysteine residue. Similarly, the Myr-GC-(NBD) peptide (Figure 1) is a myristoylated dipeptide that mimics the N-termini of several Src-related tyrosine kinases and provides the putative recognition motif for PATs which palmitoylate the adjacent cysteine residue. The corresponding nonpalmitoylatable peptide Myr-GA-(NBD) has an alanine substituted for the cysteine residue. Because of the hydrophobicity of the lipidated peptides, it was necessary to determine their toxicities toward SKOV3 cells under conditions that were utilized in subsequent experiments. Therefore, the toxicities of the peptides after 2 hr were measured using the LDH release assay. As indicated in Figure 2A, there was no induction
**Figure 1: Structures of lipidated peptides.** The (NBD)-CLC(OMe)-Farn peptide is a farnesylated tripeptide that mimics the C-terminal recognition motif of farnesylated and palmitoylated proteins such as H-Ras and Paralemmin. The Myr-GC-(NBD) peptide is a myristoylated dipeptide that mimics the N-terminal recognition motif of myristoylated and palmitoylated proteins such as the Src-related tyrosine kinases.
Figure 2. Cytotoxicity of lipidated peptides. SKOV3 cells were exposed to the indicated concentrations of either (NBD)-CLC(OMe)-Farn (■) or (NBD)-ALC(OMe)-Farn (□) in Panel A, and either Myr-GC-(NBD) (▲) or Myr-GA-(NBD) (△) in Panel B for 2 hr. The release of LDH into the medium was then measured as an indication of toxicity of the peptides as described in the Materials and Methods section.
of LDH release from cells treated for 2 hr with either the (NBD)-CLC(OMe)-Farn or (NBD)-ALC(OMe)-Farn peptide. In addition, treatment with the Myr-GC-(NBD) and Myr-GA-(NBD) peptides caused only a small increase in LDH release at high peptide concentrations (Figure 2B).

2.4.2 Cellular palmitoylation of lipidated peptides.

The elution times of parent and palmitoylated peptides were determined using reversed-phase HPLC analyses. Pure parental (non-palmitoylated) peptide eluted at the positions defined in the top row of Figure 3. In vitro incubation of the peptides with palmitoyl-CoA at pH 8.2 allows non-enzymatic palmitoylation. This treatment resulted in the conversion of Peak A to Peak B (middle row of Figure 3), which were confirmed by mass spectroscopy to be the palmitoylated forms of the peptides. As expected, replacement of the cysteines with alanines in the (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) peptides blocked their abilities to be palmitoylated. In addition, since the retention times of the non-palmitoylated and palmitoylated cysteine-containing peptides are well-resolved, the method allows for the quantification of the amount of palmitoylated and non-palmitoylated peptide. Therefore, in further experiments, the AUC of the peak at a retention time equivalent to that of Peak B was considered to represent the palmitoylated peptide, and the AUC of Peak A was considered to be non-palmitoylated peptide.

Determination of the ability of the peptides to be palmitoylated by intact cells was examined by incubating SKOV3 cells for 60 min at 37 °C with a 1 µM
Figure 3: Chromatograms of (NBD)-CLC(OMe)-Farn, (NBD)-ALC(OMe)-Farn, Myr-GC-(NBD) and Myr-GA-(NBD) under multiple conditions. Panel Peptide Only. Pure peptide was treated with β-Mercaptoethanol and extracted and resolved by HPLC as described in the Materials and Methods section. Panel Chemical Palmitoylation. Pure peptide was treated with β-Mercaptoethanol, incubated in vitro with palmitoyl-CoA to induce chemical palmitoylation and extracted and resolved as above. The non-palmitoylated substrate peptides elute at the position of peak A, while the palmitoylated product peptides elute at the position of peak B. Panel Cellular Palmitoylation. Peptide was treated with β-Mercaptoethanol, incubated at a 1 µM peptide concentration with SKOV3 cells at 37°C for 1 hour and extracted and resolved as stated above.
concentration of each peptide. The cells were subsequently washed and the cellular lysates were extracted and subjected to reversed-phase HPLC analysis (bottom row of Figure 3). The results indicate that the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides are palmitoylated by SKOV3 cells, manifested as a decrease in Peak A and a corresponding increase in Peak B. In contrast, the (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) peptides remain non-palmitoylated, which allows them to be utilized as non-palmitoylatable peptides in cellular localization studies described below.

2.4.3 Kinetics of peptide palmitoylation by SKOV3 cells.

To examine the kinetics of peptide palmitoylation in SKOV3 cells, dose-response curves were generated by incubating the cells for 60 min at 37 °C with peptides at concentrations of 1 - 80 µM. As demonstrated in Figure 4A, the cellular uptake of the (NBD)-CLC(OMe)-Farn peptide increased in a linear manner until the peptide concentration reached 20 µM; whereas, the cellular uptake of the Myr-GC-(NBD) peptide increased in a roughly linear fashion until a concentration of 40 µM. At concentrations of 2.5 µM and lower, the uptake of the two peptides was not significantly different; however, at concentrations of 10 µM and greater, the uptake of the Myr-GC-(NBD) peptide was significantly greater than that of the (NBD)-CLC(OMe)-Farn peptide.

Figure 4B indicates that significant amounts of non-palmitoylated (NBD)-CLC(OMe)-Farn accumulates in cells treated with peptide concentrations of 2.5 µM or greater. In contrast, nearly all of the Myr-GC-(NBD) peptide is
Figure 4: Dose-response curves for lipidated peptide uptake and palmitoylation by SKOV3 cells. SKOV3 cells were incubated for 60 min at 37 °C with the indicated concentration of (NBD)-CLC(OMe)-Farn or Myr-GC-(NBD) peptide. Samples were then extracted and analyzed as described in the Material and Methods section. Panel A. The total amount of (NBD)-CLC(OMe)-Farn (■) or Myr-GC-(NBD) (▲) peptide accumulation in the cells was determined as the total fluorescence in the samples. Values represent mean ± sd fmol of peptide in triplicate samples. Panel B. The amounts of non-palmitoylated (NBD)-CLC(OMe)-Farn (□) and palmitoylated (NBD)-CLC(OMe)-Farn (■) are shown. Panel C. The amounts of non-palmitoylated Myr-GC-(NBD) (△) and palmitoylated Myr-GC-(NBD) (▲) are shown. Panel D. The percentages of palmitoylated (NBD)-CLC(OMe)-Farn (■) or Myr-GC-(NBD) (▲) peptide are shown. For Panels A-C, values represent the mean ± sd for triplicate samples in a typical experiment. For Panel D, values represent the mean ± SEM for 3 – 9 experiments.
palmitoylated, even when it is given at high concentrations (Figure 4C). This is summarized in Figure 4D, which demonstrates that the majority of Myr-GC-(NBD) peptide is palmitoylated regardless of peptide concentration; whereas, maximal palmitoylation of the (NBD)-CLC(OMe)-Farn peptide occurred at peptide concentrations up to 1.0 µM and decreased significantly at higher concentrations. Since maximal palmitoylation was observed for both peptides at a concentration of 1 µM, this concentration was chosen for all further experiments.

To determine the effects of exposure duration on peptide uptake and palmitoylation, SKOV3 cells were incubated with a 1 µM concentration of either the (NBD)-CLC(OMe)-Farn or Myr-GC-(NBD) peptide at 37 °C for times of 5 to 120 min. As indicated in Figure 5A, both peptides were accumulated by the cells in a linear manner until approximately 30 min when uptake of the peptides reached a plateau. The Myr-GC-(NBD) peptide was rapidly palmitoylated by the cells such that more than 60% of the peptide was modified at the earliest measurable time point, and levels of non-palmitoylated peptide did not increase over the 60 min time course. In contrast, only 38% of the (NBD)-CLC(OMe)-Farn peptide was palmitoylated at the 5 min time point, and the extent of palmitoylation plateaued at approximately 30 min.

### 2.4.4 Subcellular trafficking of palmitoylated peptides in SKOV3 cells.

To examine the intracellular fate of the lipidated peptides, SKOV3 cells were incubated for 30 min at 37 °C with 1 µM of (NBD)-CLC(OMe)-Farn (Figure 6 panels A – C), (NBD)-ALC(OMe)-Farn (panels D – F), Myr-GC-(NBD) (panels G
Figure 5: Time course of peptide palmitoylation by SKOV3 cells. SKOV3 cells were incubated for the indicated times at 37 °C with 1 µM (NBD)-CLC(OMe)-Farn or Myr-GC-(NBD) peptide. Samples were then extracted and analyzed as described in the Material and Methods section. Panel A. The total amount of (NBD)-CLC(OMe)-Farn (■) or Myr-GC-(NBD) (▲) peptide accumulation in the cells was determined as the total fluorescence in the samples. Values represent mean ± sd fmol of peptide in triplicate samples. Panel B. The amounts of non-palmitoylated (NBD)-CLC(OMe)-Farn (□) and palmitoylated (NBD)-CLC(OMe)-Farn (■) are shown. Panel C. The amounts of non-palmitoylated Myr-GC-(NBD) (△) and palmitoylated Myr-GC-(NBD) (▲) are shown. Panel D. The percentages of palmitoylated (NBD)-CLC(OMe)-Farn (■) or Myr-GC-(NBD) (▲) peptide are shown. For Panels A-C, values represent the mean ± sd for triplicate samples in a typical experiment. For Panel D, values represent the mean ± SEM for 3 – 9 experiments.
I) or Myr-GA-(NBD) (panels J – L) peptide, and monitored by fluorescence confocal microscopy. At this time point, at least 75% of the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides are palmitoylated (Figure 5), whereas none of the (NBD)-ALC(OMe)-Farn or Myr-GA-(NBD) peptides are palmitoylated (Figure 3). Staining with WGA was used to localize the plasma membranes of the cells (Figure 6, Panels A, D, G and J), and produced the expected peripheral staining pattern. Panels B, E, H and K indicate that each of the peptides readily entered the cells. Strong peptide fluorescence co-localized with WGA in Panels C and I, indicating that a high percentage of the palmitoylated (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides were associated with the plasma membrane. However, minimal peptide fluorescence co-localized with WGA in Panels F and L, indicating that the non-palmitoylated (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) peptides were not associated with the plasma membrane. In addition, perinuclear peptide fluorescence resembling the Golgi apparatus was observed by all peptides, but was much more pronounced with the non-palmitoylated (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) peptides (panels E and K) than the palmitoylated (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides (panels B and H).

The localization of the peptides to the Golgi apparatus was confirmed by further confocal microscopy studies in which SKOV3 cells were incubated with 1 µM of the (NBD)-CLC(OMe)-Farn (Figure 7 Panels A – C), (NBD)-ALC(OMe)-Farn (Panels D – F), Myr-GC-(NBD) (Panels G – I) or Myr-GA-(NBD) (Panels J –
Figure 6: Localization of lipidated peptides to the Plasma Membrane.
SKOV3 cells were incubated for 30 min at 37 °C with 1 µM of (NBD)-CLC(OMe)-Farn (Panels A – C), (NBD)-ALC(OMe)-Farn (Panels D – F), Myr-GC-(NBD) (Panels G – I) or Myr-GA-(NBD) (Panels J – L) peptide. The cells were then quickly washed and imaged by confocal microscopy. The distributions of the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) fluorescent peptides (which are essentially completely palmitoylated at this time and concentration) are shown in Panels B and H. The distributions of the (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) fluorescent peptides (which are completely non-palmitoylated) are shown in Panels E and K. Plasma membranes were visualized with AF594-WGA (Panels A, D, G and J). Panels C, F, I and L show overlays of the two labels in cells exposed to (NBD)-CLC(OMe)-Farn, (NBD)-ALC(OMe)-Farn, Myr-GC-(NBD) and Myr-GA-(NBD) peptides respectively.
L) peptide for 30 min at 37 °C. The Golgi apparatus was concurrently labeled by the addition of BODIPY TR C5-ceramide (Panels A, D, G and I). Overlays of the images (Panels C, F, I and L) demonstrated that the bulk of the non-plasma membrane-associated peptide fluorescence of the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides (Panels C and I) was associated with the Golgi apparatus, and nearly all of the peptide fluorescence of the (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) peptides (Panels F and L) was associated with the Golgi apparatus.

The kinetics of intracellular trafficking of the lipidated peptides were evaluated in studies depicted in Figure 8. SKOV3 cells were incubated with Hoechst for 10 min at 37 °C, then with 1 µM of the (NBD)-CLC(OMe)-Farn (Figure 8 Panels A – D), (NBD)-ALC(OMe)-Farn (Panels E - H), Myr-GC-(NBD) (Panels I – L) or Myr-GA-(NBD) (Panels M – P) peptide at 37 °C for 15 to 120 min. Fifteen minutes was taken as the earliest time point to ensure that the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides were maximally palmitoylated. Significant amounts of each peptide were observed in the cells at the earliest time point; however, the distribution of (NBD)-CLC(OMe)-Farn (Panel A) included both the plasma membrane and the Golgi; whereas Myr-GC-(NBD) (Panel I) was essentially exclusively localized to the plasma membrane. In contrast, at 15 min both the (NBD)-ALC(OMe)-Farn (Panel E) and Myr-GA-(NBD) (Panel M) peptides were localized in the Golgi apparatus. As time progressed, the (NBD)-CLC(OMe)-Farn peptide (Panels A – D) maintained localization to
Figure 7. Trafficking of lipidated peptides to the Golgi apparatus. SKOV3 cells were incubated for 30 min at 37 °C with 1 µM of (NBD)-CLC(OMe)-Farn (Panels A – C), (NBD)-ALC(OMe)-Farn (Panels D – F), Myr-GC-(NBD) (Panels G – I) or Myr-GA-(NBD) (Panels J – L) peptide. The cells were then quickly washed and imaged by confocal microscopy. The distributions of the fluorescent peptides are shown in Panels B, E, H and K. The Golgi apparatus was visualized with BODIPY TR C₅-ceramide (Panels A, D, G and J). Panels C, F, I and L show overlays of the two labels in cells exposed to (NBD)-CLC(OMe)-Farn, (NBD)-ALC(OMe)-Farn, Myr-GC-(NBD) and Myr-GA-(NBD) peptides respectively.
Figure 8: Time course of lipidated peptide trafficking in SKOV3 cells. SKOV3 cells were incubated for 10 min at 37 °C with Hoechst (2 µg/ml) and washed. The cells were subsequently incubated with 1 µM of either (NBD)-CLC(OMe)-Farn (Panels A – D), (NBD)-ALC(OMe)-Farn (Panels E – H), Myr-GC-(NBD) (Panels I – L) or Myr-GA-(NBD) (Panels M – P) peptide. The cells were then imaged by confocal microscopy at 15 (Panels A, E, I, M), 30 (Panels B, F, J, N), 60 (Panels C, G, K, O) or 120 (Panels D, H, L, P) min.
both the plasma membrane and Golgi, whereas the Myr-GC-(NBD) peptide (Panel I –L) increased Golgi localization and became both Golgi- and plasma membrane-associated. In contrast, both the (NBD)-ALC(OMe)-Farn (Panel E - H) and Myr-GA-(NBD) (Panel M - P) peptides remained primarily localized to the Golgi for at least 120 min.

2.5 DISCUSSION

The importance of protein lipidation for the proper localization and activity of many signaling proteins has been extensively discussed (2, 7-11, 13-16). Since these proteins are involved in important signaling pathways including cell survival, proliferation and activation (28-31), inhibition of their lipidation has been considered to be an attractive area for new drug development. In this context, most effort has focused on developing inhibitors of farnesyltransferases. Inhibitors of PATs have been slower to develop because the identities of the enzymes are not yet well characterized, although significant progress has occurred over the past few years (27, 40, 41). Seminal studies in yeast identified Efr2/Erf4 as a PAT for yeast Ras (42), and Akr1 as a PAT for yeast casein kinase (43). The conserved DHHC domain of these proteins was hypothesized to represent a signature catalytic motif for PATs, and has provided a starting point for characterization of human PATs. For example, we recently demonstrated that human HIP-14 (DHHC17) is a farnesyl-directed PAT that causes oncogenic transformation of fibroblasts when overexpressed (26). Similarly, Swarthout et al. have demonstrated that CGI-89 (DHHC9) palmitoylates N- and H-Ras, but not N-myristoylated Ga1 or GAP-43 (44). However, these studies do not provide
definitive identification of the predominant PAT(s) acting within the cellular environment.

Lipidated peptides that mimic palmitoylation motifs have been used by us to quantify palmitoylation in vitro (24-27), and by others to study trafficking, localization and membrane interactions in live cells (45-49). In particular, studies by Schroeder et al. (45, 46) were instrumental in demonstrating that lipopeptides that mimic protein palmitoylation sites are useful tools for the analyses of PAT activity and protein trafficking. In the present study, we sought a quantifiable intact-cell assay to characterize cellular PAT activities using fluorescent peptides that mimic the two putative recognition motifs for enzymatic palmitoylation. We have previously used HPLC following in vitro assays to separate fluorescent lipidated PAT substrate peptides from the corresponding products (24-27). The current data clearly demonstrate that the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides can be palmitoylated by intact SKOV3 cells, while the (NBD)-ALC(OMe)-Farn and Myr-GA-(NBD) peptides remain non-palmitoylated. Well-resolved peaks representing the substrate peptides and the palmitoylated products of the Cys-containing peptides were verified by mass spectroscopy, and can be quantified by their fluorescence. Therefore, the Cys-containing lipopeptides provide biochemical tools for quantifying both farnesyl and myristoyl-directed PAT activity within intact cells and the Ala-containing lipopeptides can serve as non-palmitoylated controls for localization in confocal analyses.

To further characterize cellular PAT activities, dose-response and time course studies were performed with each substrate peptide. Dose-response
studies indicate that the Myr-GC-(NBD) peptide accumulates in the cell in a significantly greater amount than the (NBD)-CLC(OMe)-Farn peptide, which may be due to the somewhat smaller size of the Myr-GC-(NBD) peptide (MW = 738) compared to the (NBD)-CLC(OMe)-Farn peptide (MW = 920). In addition, data shown in Figure 4 indicate a difference in the ability of SKOV3 cells to palmitoylate the Myr-GC-(NBD) and (NBD)-CLC(OMe)-Farn peptides. Specifically, SKOV3 cells are able to maintain nearly-complete palmitoylation of the Myr-GC-(NBD) peptide regardless of its concentration in the medium. In contrast, the cells could maintain maximal palmitoylation of the (NBD)-CLC(OMe)-Farn peptide to a concentration of only 1 µM. This suggests that SKOV3 cells have a greater capacity to palmitoylate proteins containing the N-terminal MyrGC recognition motif than the C-terminal CLC-Farn motif. There are several possible reasons for this including differences in the abundancy and efficiency of the different PAT enzymes as well as differences in thioesterase activity. In any case, the lower cellular capacity of farnesyl-directed PAT activity suggests that this class of PATs may be more amenable to pharmacologic inhibition than the myristoyl-directed PATs.

The time course studies indicate that the uptake of the Myr-GC-(NBD) peptide is faster than the uptake of the (NBD)-CLC(OMe)-Farn peptide, and that cellular levels of both peptides plateau after approximately 30 min. The differences in the kinetics also provide some clues as to the location of the different PAT activities. For example, the majority of the Myr-GC-(NBD) peptide was located at the plasma membrane at 15 and 30 min. Since the palmitoylation
of the peptide is essentially complete at those times, the myristoyl-directed PAT activity is most likely located at the plasma membrane. This finding is consistent with previous studies in which the palmitoylation of N-myrsitoylated proteins increased localization to the plasma membrane, especially within lipid rafts (29, 30, 50). Therefore, targeting of these proteins appears to occur through a kinetic trapping process in which the protein is palmitoylated at its final destination. It is less clear, however, where the farnesyl-directed PAT activity is located because there is a temporal lag before maximal palmitoylation is achieved, and a significant amount of the peptide is internalized by 15 min. Therefore, it is unclear if the (NBD)-CLC(OMe)-Farn peptide was palmitoylated in the plasma membrane, ER, Golgi or trans-Golgi network, all of which have been reported to have PAT activity (51, 52).

As previously shown by others (45, 46), we confirm that the intracellular localizations of the palmitoylated and non-palmitoylated forms of the farnesyl-motif peptides are markedly different. For example, the non-palmitoylated (NBD)-ALC(OMe)-Farn peptide localizes exclusively to the Golgi; whereas, the palmitoylated (NBD)-CLC(OMe)-Farn peptide localizes to the Golgi and the plasma membrane. This difference indicates that palmitoylation of the (NBD)-CLC(OMe)-Farn is required for localization to the plasma membrane and that the lack of palmitoylation confines the peptide to the Golgi. This is similar to the intracellular trafficking of wild-type Ras proteins which are localized to intracellular compartments including the ER and Golgi complex and the plasma membrane (53-57). Additional reports indicate that the farnesyl-motif targets Ras
to the ER and Golgi complex, whereas palmitoylation is required to exit the Golgi complex (53, 54). Furthermore, the recycling of dephosphorylated and palmitoylated Ras appears to affect the localization of the protein, in that dephosphorylated Ras traffics from the plasma membrane to the Golgi complex, whereas palmitoylated Ras moves from the Golgi complex to the plasma membrane (55, 57). The importance of this cycle to the localization pattern of farnesylated and palmitoylated proteins was further described by Rocks, et al. (57), who showed that irreversibly acylated N-Ras is non-specifically associated with internal membranes; whereas, reversibly palmitoylated N-Ras localizes to the Golgi and plasma membrane. Overall, our results support the hypothesis that the PAT responsible for the palmitoylation of Ras is located in the Golgi complex.

The localization of the palmitoylated and non-palmitoylated myristoyl-motif peptides is also different. The non-palmitoylated Myr-GA-(NBD) peptide remains localized to the Golgi; whereas, the palmitoylated Myr-GC-(NBD) peptide traffics to the plasma membrane. These results are supported by data from Schroeder, et al. who used palmitoylatable Myr-GCG-(NBD) and non-palmitoylatable Myr-GSG-(NBD) peptides in CV-1 cells (46). They found that the palmitoylatable form of the myristoyl-motif peptide was primarily localized to the plasma membrane, and the non-palmitoylatable form localized to the Golgi. In addition, N-myristoylated and palmitoylated GFP-chimeras have been found to localize to Golgi and plasma membrane (58), which is consistent with the localization of endogenous myristoyl-motif-containing proteins such as the Src-related-tyrosine-
kinase Fyn (59). Overall, the localization results obtained in this study indicate that the (NBD)-CLC(OMe)-Farn and Myr-GCG-(NBD) peptides mimic the localization patterns of C-terminally farnesylated and palmitoylated proteins, and N-terminally myristoylated and palmitoylated proteins, respectively. In each case, the cellular distributions of the palmitoylated and non-palmitoylated forms of the peptides are markedly different.

In conclusion, we have developed quantitative cell-based model systems to monitor human PAT activities using peptides that mimic the N-terminal myristoyl- and C-terminal farnesyl-palmitoylation motifs. These peptides are cell permeable and are rapidly palmitoylated by endogenous PATs. The data indicates that SKOV3 human ovarian carcinoma cells have a substantially higher myristoyl-directed PAT activity than farnesyl-directed PAT activity. In related studies, we have recently described the identification and characterization of selective inhibitors of farnesyl-directed and myristoyl-directed PATs that may provide new therapeutic agents (27). The present model systems also provide means to identify additional human PATs, e.g. using gene-specific siRNA knockdown of potential targets.

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**Abbreviations:**

AUC, Area Under the Curve; DMEM, Dulbecco’s Modified Eagles Medium;

HPLC, High Performance Liquid Chromatography; LDH, lactate dehydrogenase;

NBD, (7-nitro-2-1,3benzoxadiazol-4-yl)-; PAT, Palmitoyl acyltransferase; PBS,

phosphate-buffered saline; WGA, Wheat Germ Agglutinin.
2.6 REFERENCES

Chapter 3

Rationalization of DHHC20 as a Human Myristoyl-Directed Palmitoyl Acyltransferase
Palmitoylation is integral to the proper localization, conformation and activation of many important signaling proteins. However, the specific enzymes that drive these reactions are not fully characterized, and there are no motif-specific inhibitors of palmitoylation in common use. This chapter outlines the rationalization of characterizing DHHC20 as a human myristoyl-directed PAT based on homology, intracellular localization, and the use of lipopeptides as selective competitive inhibitors of palmitoylation.

As seen in section 2.4.1, exposing SKOV3 cells to the palmitoylatable and non-palmitoylatable forms of both the C-terminal farnesyl and N-terminal myristoyl peptides causes minimal cytotoxicity over the 2 hr. duration of those experiments. However, to determine the long term propensity of these peptides to induce cytotoxicity SKOV3 cells were treated with multiple concentrations of either palmitoylatable or non-palmitoylatable forms of the C-terminal farnesyl and N-terminal myristoyl peptides for a duration of 72 hrs. Following the 72 hr treatment the release of LDH was measured according to section 2.3 and the results are shown in Figure 1. As the figure shows, the non-palmitoylatable forms of the peptides induce very low levels of cytotoxicity, regardless of the concentration used. In contrast, the palmitoylatable forms of both peptides induce nearly complete cytotoxicity. However, a difference does exist in the potencies of the (NBD)-CLC(OME)-Farn and Myr-GC-(NBD) peptides to induce cytotoxicity in this cell line. The EC_{50} of the (NBD)-CLC(OME)-Farn and Myr-GC-(NBD) peptides are 29.6 and 0.67 µM respectively, demonstrating that the Myr-
Figure 1. Cytotoxicity of lipidated peptides in SKOV3 cells after 72 hrs. SKOV3 cells were exposed to the indicated concentrations of palmitoylatable (■) or non-palmitoylatable (□) (NBD)-CLC(OMe)-Farn, or palmitoylatable (▲) or non-palmitoylatable (△) Myr-GC-(NBD) for 72 hr. The release of LDH into the medium was then measured as an indication of toxicity of the peptides as described in the Materials and Methods section. The values represent the mean ± SEM of 3 experiments.
GC-(NBD) peptide is more potent than the (NBD)-CLC(OMe)-Farn peptide at inducing cytotoxicity in SKOV3 cells. Since these peptides mimic the likely C- and N-terminal palmitoylation motifs, they compete with endogenous proteins that contain those motifs for binding to specific cellular PATs. In this way, the (NBD)-CLC(OMe)-Farn and Myr-GC-(NBD) peptides may act as competitive inhibitors of C-terminal farnesyl-directed and N-terminal myristoyl-directed PATs respectively. Thus, competitively blocking the enzymatic activity of C-terminal farnesyl-directed and N-terminal myristoyl-directed PATs independently may cause cytotoxicity in SKOV3 cells, with the inhibition of N-terminal myristoyl-directed PATs demonstrating much greater potency.

This data provides two points of important information. First, the selective inhibition of PATs, specific for both the C-terminal farnesyl and N-terminal myristoyl motifs, can induce cytotoxicity in a human ovarian adenocarcinoma cell line. This gives credence to the hypothesis that PATs, specific for given motifs, are interesting targets for anticancer drug development. Second, the Myr-GC-(NBD) peptide is a more potent inducer of cytotoxicity than the (NBD)-CLC(OMe)-Farn peptide in this cell line. Although these peptides demonstrate a concentration-dependent difference in cellular uptake, this cannot fully explain the difference in cellular cytotoxicity as cells with lower overall uptake of the Myr-GC-(NBD) peptide at lower peptide concentrations (2.4.3, Figure 4A) exhibit greater cytotoxicity than cells with higher overall uptake of the (NBD)-CLC(OMe)-Farn peptide at higher peptide concentrations. However, these cells may be more affected by the inhibition of N-terminal myristoyl-directed PATs due to a
greater reliance on those signaling pathways for survival. It has been demonstrated that tumor cells can become “addicted” to their mutated signaling pathways and that deactivation of those pathways can lead to dramatic apoptosis as well as tumor regression (1-6). In addition, as seen in section 2.4.3, SKOV3 cells appear to have a greater propensity to drive and maintain the palmitoylation of the Myr-GC-(NBD) peptide than the (NBD)-CLC(OMe)-Farn peptide, which may indicate that these cells contain a mutation, or express higher levels of activity, in one or multiple signaling pathways driven by N-terminally myristoylated and palmitoylated proteins, and that selective inhibition of that pathway is causing apoptosis. Regardless of the mechanism, it appears that N-terminal myristoyl-directed PATs play an integral role in the survival of these ovarian adenocarcinoma cells, and these enzymes are interesting targets for the development of anticancer drugs.

Although a great deal of progress has been made toward the identification and characterization of PATs in recent years, one glaring hole remains; the identification of a human N-terminal myristoyl-directed PAT. The majority of these studies have focused on identifying PATs with activity toward either transmembrane, prenylated, and/or non-myristoylated N- and C-terminally palmitoylated proteins (7-13). In contrast, PATs specific for N-terminally myristoylated and palmitoylated proteins have thus far remained the least characterized, and studies in this area have focused primarily on yeast (14, 15) and mouse proteins (16). Thus, no human N-terminal myristoyl-directed PAT
has been identified. Therefore, we set out to identify the first human PAT with N-terminal myristoyl-directed activity.

As noted in section 1.2.2, a total of 7 and 22 proteins exist in yeast and humans, respectively, that contain the DHHC-CRD catalytic domain (17, 18). To narrow the search from a total of 22 candidates, the putative human PATs were analyzed by sequence and structural homology as well as intracellular localization. The yeast protein Pfa3 was the first protein of any species characterized to have PAT activity toward N-terminally myristoylated and palmitoylated proteins (14, 15). Thus, yeast Pfa3 was used in homology searches to identify highly homologous human DHHC-CRD-containing proteins. Homology searches of the overall protein structures as well as the DHHC-CRD region sequences demonstrated that a group of 5 human DHHC-CRD-containing proteins exhibited high homology with the yeast PAT Pfa3 (18, 19). The human proteins identified were DHHC-2, 3, 7, 15 and 20. This homology is illustrated in Figure 2, where the amino acid sequences of these proteins are aligned with that of yeast Pfa3 using the DNassist software. This alignment demonstrates that there is homology between the proteins, particularly in the catalytic DHHC-containing cysteine-rich domain (DHHC-CRD). The list of potential human N-terminal myristoyl-directed PATs was further narrowed by analyzing the intracellular localization of the proteins. Although PAT activity has been observed in many intracellular compartments (20, 21), the kinetic and localization data presented in section 2.4 demonstrate that the primary site of N-terminal myristoyl-directed PAT activity in SKOV3 cells resides in the plasma membrane.
Figure 2. Aligned amino acid sequences of Pfa3-homologous human DHHC-CRD-containing proteins. The amino acid sequences of yeast Pfa3 and human Pfa3-homologous proteins were aligned using DNassist. Identical amino acids across all 6 proteins are shaded in pink, amino acids with similar charge and polarity are shaded in green, and the cysteine-rich domains of the sequences are contained within the box.
In addition, in localization studies carried out by Ohno et. al., the only human DHHC-CRD-containing protein with high homology to yeast Pfa3 that demonstrated significant plasma membrane localization was DHHC20 (18). Subsequently, individual selective alignment of DHHC20 to Pfa3 (Figure 3) demonstrated that the proteins share a great deal of sequence homology in the DHHC-CRD. In addition, analysis of these proteins using the SMART sequence analysis website (Figure 4) suggests that Pfa3 and DHHC20 both contain a highly homologous catalytic DHHC-CRD domain that is located at roughly the midpoint of the amino acid sequence. In Pfa3, a single transmembrane domain lies to the carboxyl side of the DHHC-CRD domain; whereas, in the DHHC20 protein the DHHC-CRD domain is flanked on both sides by transmembrane domains. Therefore, the predicted topology of these proteins is similar in that both contain a roughly centrally located DHHC-CRD domain with transmembrane domains; however, they differ in the number of transmembrane domains. Overall, this information demonstrates that DHHC20 is homologous to Pfa3 in its amino acid sequence, particularly within the DHHC-CRD domain, and it is the only Pfa3-homologous human DHHC-CRD-containing protein that is properly localized to the plasma membrane. Thus, DHHC20 was chosen as a putative N-terminal myristoyl-directed human PAT.

In summary, the selective competitive inhibition of C-terminal farnesyl and N-terminal myristoyl-directed PAT activity causes cytotoxicity in SKOV3 cells, with the inhibitory affects of N-terminal myristoyl-directed activity demonstrating far greater potency. These results further substantiate PATs, and in particular N-
Figure 3. Amino acid sequence alignment of yeast Pfa3 and human DHHC20. The amino acid sequences of yeast Pfa3 and human DHHC20 are aligned using DNassist. Identical amino acids are shaded in pink, amino acids with similar charge and polarity are shaded in green, and the cysteine-rich domains of the sequences are contained within the box.
Figure 4. Topology of yeast Pfa3 and human DHHC20. The topology of yeast Pfa3 and human DHHC20 as predicted by Swiss-Prot. The proteins each contain a PAT catalytic domain composed of a DHHC-containing cysteine-rich domain flanked by multiple transmembrane domains (TM) along with N- and C-terminal regions that are variable between the proteins.
terminal myristoyl-directed PATs, as potential targets for anticancer drug development. The yeast protein Pfa3 has been characterized to be specific for N-terminally myristoylated proteins and this activity has been demonstrated to reside primarily in the plasma membrane of human SKOV3 cells. Homology searches and localization analysis have demonstrated that the human DHHC20 protein is the only DHHC-CRD-containing protein to exhibit both a high homology to Pfa3 as well as localization to the plasma membrane. Based on this information, DHHC20 is a potential human N-terminal myristoyl-directed PAT, and the characterization of this protein is carried out in the next chapter.
References

Chapter 4

DHHC20:  A Human N-Terminal Myristoyl-Directed Palmitoyl Acyltransferase That Causes Cellular Transformation

Jeremiah M. Draper and Charles D. Smith

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4.1 ABSTRACT

Posttranslational enzymatic palmitoylation is required for several cancer-associated proteins to activate downstream signaling pathways. Therefore, the palmitoyl acyltransferase (PAT) enzymes that catalyze these reactions are interesting potential targets for anticancer therapeutics. However, mammalian PATs, and particularly human PATs specific for N-terminally myristoylated and palmitoylated proteins, are not fully characterized. Therefore, in this study, we sought to identify and characterize a human PAT specific for N-terminally myristoylated and palmitoylated proteins. To carry out these studies, we stably transfected NIH/3t3 cells with the human DHHC20 gene. Cells expressing the exogenous DHHC20 gene displayed a 42% increase in palmitoylation activity toward a peptidomimetic of N-terminally myristoylated and palmitoylated proteins with no change in activity toward a peptidomimetic of C-terminally farnesylated and palmitoylated proteins, indicating that DHHC20 is a human N-terminal myristoyl-specific PAT. In addition, expression of DHHC20 in NIH/3t3 cells caused phenotypic changes consistent with cellular transformation. For example, cells expressing DHHC20 displayed a 23- to 39-fold increase in soft agar colony formation as well as a 120- to 210-fold increase in foci formation. In addition, cells expressing DHHC20 exhibited elevated rates of proliferation as compared to control cells. Lastly, quantitative polymerase chain reaction analysis of human tissue samples demonstrated that DHHC20 is expressed in a tissue-specific manner, and is overexpressed in several types of human tumors, including ovarian, breast and colon. Overall, these results demonstrate that
DHHC20 is a human N-terminal-myristoyl-specific PAT involved in cellular transformation, and that it is overexpressed in several human tumor types.

4.2 INTRODUCTION

Palmitoylation is the covalent attachment of a 16-carbon palmitate group to cysteine residues on a target protein. Like other post- and cotranslational lipid modifications, such as farnesylation and myristoylation, palmitoylation plays an integral role in the regulation of intracellular proteins by increasing protein hydrophobicity, regulating subcellular localization, and/or altering protein conformation (1-3). A multitude of important signaling proteins, such as the Src-related tyrosine kinases and Ras proteins, require plasma membrane localization in order to activate their intracellular signaling pathways (4-6). It has been demonstrated that targeting of these signaling proteins to the plasma membrane is facilitated by post-translational lipidation events and that blocking the activity of their lipidating enzymes is an effective way to block signaling through these pathways (7, 8).

A diverse set of proteins are known to be palmitoylated (6, 9, 10) several of which can be categorized based on motifs next to their site(s) of palmitoylation (9). One group of palmitoylated proteins, which includes proteins such as H-, N-, and K2A-Ras, contains a C-terminal farnesyl motif (9, 10). In this group, the palmitoylation motif is composed of a post-translationally farnesylated C-terminal cysteine which is required for palmitoylation to occur at a cysteine adjacent to the primary lipidation event, and the attachment of palmitate groups through a
thioester linkage is catalyzed by a palmitoyl acyltransferase (PAT) enzyme (11). In addition, it has been demonstrated that this palmitoylation event is required to target these proteins to the plasma membrane and allow complete activation (11-13). A second group of enzymatically palmitoylated proteins contain an N-terminal myristoyl motif. This group, which includes several Src-related kinases such as Lck and Fyn, has a recognition motif composed of an N-terminal myristoylated glycine with an adjacent cysteine(s) residue. This myristoylation modification is required for palmitoylation, via a PAT, to occur at cysteines adjacent to the N-terminus (9). Again, it has been shown that this palmitoylation modification is imperative for the effective localization of these proteins to the plasma membrane as well as their effective signal transduction (14).

Many enzymatically palmitoylated proteins, such as the Ras isotypes and Src-related kinases, are involved in signaling pathways that drive cellular proliferation and survival (15, 16). Since the activity of these proteins is predicated on localization to the plasma membrane, enzymes that catalyze these reactions are attractive targets for novel therapeutics. In fact, enzymes that catalyze the post-translational lipid modifications of Ras are considered particularly interesting targets for anticancer drug development (17). However, the development of anti-Ras agents has been primarily focused on inhibiting the process of farnesylation. This is mainly due to the fact that farnesyltransferases have been cloned and used in screens to identify inhibitors (18, 19). In contrast, the discovery of inhibitors of enzymatic palmitoylation has been slow, due primarily to difficulties involved in identifying and characterizing human PATs.
Progress has been made in identifying PATs through seminal studies in *Saccharomyces cervisiae* that identified Erf2/Erf4 (20) and Akr1p (21) as PATs specific for yeast Ras2 and casein kinase2, respectively. These two enzymes were found to share a common Asp-His-His-Cys (DHHC) motif located within a cysteine-rich domain (CRD). Subsequently, several groups have used this homologous domain to identify yeast (22, 23) as well as mammalian PATs (24, 25). PATs specific for the N-terminal myristoyl motif remain the least investigated group, and no human enzyme with this specificity has been identified. In addition, although many N-terminally myristoylated and palmitoylated proteins drive signaling pathways involved in cancer, the role of their palmitoylating enzymes is not well characterized, and there is no direct evidence that PATs specific for this motif are able to cause cellular transformation.

In this study, we sought to characterize DHHC20 as a human N-terminal myristoyl-specific PAT, and assess its role in cellular transformation. DHHC20 was chosen based on sequence homology as well as intracellular localization. Although PAT activity has been observed in several compartments including the Golgi, trans-Golgi network, and endoplasmic reticulum (26), studies conducted by our lab and others point to the plasma membrane as the likely site of N-terminal myristoyl-specific PAT activity in mammalian cells (26, 27). In addition, the vacuole-localized yeast protein Pfa3 has been shown to palmitoylate the N-terminal myristoylated region of Vac8 (22). Human DHHC20 shares significant sequence homology with yeast Pfa3 and displays plasma membrane localization
(28). Thus, this homology and specific intracellular localization makes DHHC20 a human N-terminal myristoyl-specific PAT candidate. Here, we use previously described (29) fluorescently labeled peptides that mimic the C-terminal farnesyl and N-terminal myristoyl motifs to demonstrate that DHHC20 is a human PAT specific for the N-terminal myristoyl palmitoylation motif and not the C-terminal farnesyl motif. The results also show that DHHC20 is overexpressed in samples from several human tumor tissues, and that overexpression of the gene causes cellular transformation in vitro. These results validate DHHC20 as a new target for the development of anticancer therapeutics.

4.3 MATERIALS & METHODS

Materials

Palmitoyl-CoA, 2-mercaptoethanol (β-ME) and dimethyl sulfoxide (DMSO) were purchased from Sigma. The fluorescent peptides 7-nitro-2-1,3-benzoazadiazol-4-yl (NBD)-CLC(OMe)-Farn, and Myr-GC-(NBD) were synthesized as t-butyl-disulfide-protected precursors by solution-phase chemistry using mild conditions to maintain chemically labile functional groups (e.g., the farnesyl-cysteine thioether linkage), as described previously (30). The protected peptides (1 mM in DMSO) were stored at -80°C under argon, and deprotection occurred immediately prior to use by incubation with 2-mercaptoethanol/DMSO (2.7:97.3 or 12:88, v/v, respectively) containing 60 mM Tris, pH 8.0 at 55 °C for 25 min with agitation. All other reagents were purchased from Sigma unless otherwise stated.
**DHHC20 Construct**

Human *DHHC20* cDNA (accession # NM_153251) was obtained from Invitrogen in an Ultimate ORF Clone (Catalogue # HORF01, clone I.D. IOH5758). *DHHC20* cDNA was cloned into the pDEST-26 mammalian expression vector (Invitrogen) using the Gateway LR Clonase II Enzyme Mix (Invitrogen). The resultant vector was subsequently sequenced to determine integrity of the clone. Details of the TOPO cloning reactions and the expression vector can be found at [www.invitrogen.com](http://www.invitrogen.com).

**Cell Culture**

All cell culture reagents were obtained from Gibco (Invitrogen) unless otherwise noted. NIH/3t3 cells (ATCC – CRL-1658) were maintained at 37 °C in an atmosphere of 5% CO2/95% air in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% bovine serum (BS), 50 µg/ml gentamycin, and 1mM sodium pyruvate.

**Generation of Stably Transfected Clones**

NIH/3t3 cells were transfected with an expression vector containing the *DHHC20* gene insert or an identical vector without the gene. Transfection of the cells was carried out in Opti-MEMI containing 2 µg/ml vector and 2.5 µg/ml Lipofectamine 2000 (Invitrogen) for 5 hours at 37°C. Following transfection, the cells were maintained in DMEM containing 10% BS without antibiotics for 48 hr at 37°C. After 48 hr, the transfectants were subjected to selection in media
containing 400 μg/ml geneticin for 10 days at 37 °C in an atmosphere of 5% CO₂/95% air. Individual colonies were harvested using sterile cloning disks (Scienceware) and maintained in selection media containing geneticin at a reduced concentration (100 μg/ml). Several colonies transfected with each vector were expanded and subjected to RT-PCR to determine expression of human DHHC20.

**Reverse Transcription Assay**

Total RNA from NIH/3t3 cells transfected with the non-DHHC20-containing vector (3t3-V) and cells transfected with the DHHC20-containing vector (3t3-D20-1, 2 and 5) was isolated from each clone using the RNeasy RNA isolation kit (Qiagen, # 74104). 1 μg of total RNA was amplified in each RT-PCR reaction using the one step RT-PCR kit (Qiagen, # 210210). DHHC20 mRNA was amplified using primers designed to span multiple introns. The 5’ primer is 5’-GTG GAG CTC TGC GTG TTT ACTA -3’ and the 3’ primer is 5’- CGT GTA TCT GTC AGT TCA TTC GTC C-3’ (Invitrogen). The 511-bp product was resolved on a 1% agarose gel and visualized using ethidium bromide. As a control 1μg of mRNA from each sample was subjected to amplification under the same conditions using primers specific for 18S RNA. The 5’ primer is 5’ – TTG GAG GGC AAG TCT GGT G-3’ and the 3’ primer is 5’- CCG CTC CCA AGA TCC AAC TA-3’ (Invitrogen). These samples were run on an adjacent agarose gel and visualized as above.
**Membrane Fractionation**

Subcellular fractions were isolated as described previously (30). Briefly, cellular clones were grown in 150 mm tissue culture dishes to confluence and collected by centrifugation. The cells were swollen for 30 min on ice in a hypotonic lysis buffer consisting of 10 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, and 5 µM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by homogenization through a 21 gauge needle and the nuclei and debris were removed by centrifugation at 5,600 x g for 10 min at 4°C. The supernatant was subsequently centrifuged at 100,000 x g for 1 hr at 4°C. The pellet produced by ultracentrifugation was collected as the membrane fraction and resuspended in lysis buffer for use in the *in vitro* palmitoylation (IVP) assays or RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris pH 8.0) for western blot analysis.

**Western Analysis**

SDS was added to membrane fractions in RIPA buffer to a 1% concentration, and the samples were incubated at 72°C for 15 minutes. Subsequently, 20 µg of protein from the membrane fraction of each clone was loaded on a gel, electrophoresed, transferred to a PVDF membrane (BioRad), exposed to antibodies reactive to the 6x-His epitope tag (Abcam, ab9108), and visualized.
IVP Assay

The IVP assay was performed in a similar fashion as previously described in several studies (25, 29, 30). Briefly, IVP assays using either the NBD-CLC(OMe)-Farn or MyrGC-(NBD) substrates independently consisted of 10 µM deprotected peptide, 60 µM palmitoyl-CoA, 33 µg membrane protein, and acylation buffer (50 mM citrate, 50 mM phosphate, 50 mM Tris, and 50 mM CAPS at pH 7.2) in a total volume of 100 µl. Initiation of the reaction was carried out by incubating the peptide substrates with membrane fractions derived from the indicated cell lines in acylation buffer for 8 min at 37ºC with agitation. To start the reaction, palmitoyl-CoA was added, gently vortexed, and the mixture was incubated for a further 15 min at 37ºC with agitation. The reaction was terminated by addition of 2 ml of CH₂Cl₂: methanol: water (2:1:1), in turn extracting the peptide into the organic phase. The organic phase was subsequently dried under N₂ and stored at -80ºC until analyzed by high-performance liquid chromatography (HPLC) as previously described (29, 30). The mass of the peptide palmitoylated in each reaction was determined based on the ratio of the palmitoylated to the total native peptide in each reaction. Enzymatic palmitoylation was calculated by subtracting the amount of background palmitoylation (reactions containing no enzyme) from the amount of total palmitoylated peptide in each assay. Statistical analyses were performed using the InStat data-analysis program by two-tailed unpaired t-Test. A value of p ≤ 0.05 was considered statistically significant.
**Soft Agar Assay**

Twenty-thousand stably transfected human *DHHC20*-expressing cells (3t3-20-1, 2, 5) and non-DHHC20-expressing cells (3t3-V) cells in exponential growth phase were plated in DMEM containing 10% bovine serum and 0.6% Bacto-Agar (Difco) in 35 mm plates on day 1 and incubated in the atmosphere noted above. Colonies were scored as roughly greater than or equal to 50 cells/colony and photographed at 40x magnification 21 days later.

**Foci-Formation Assay**

Two-hundred-thousand stably transfected human *DHHC20*-expressing cells (3t3-20-1, 2, 5) and non-*DHHC20*-expressing cells (3t3-V) cells in the exponential growth phase were plated in each well of a six-well dish in selection media and atmosphere as noted above. The cells were allowed to grow to confluence and the media was changed every 3-4 days. At 14 days past confluence, wells were photographed at 40x magnification. The wells were subsequently aspirated and inverted over a white light source. Foci were scored as objects that were clearly opaque to the naked eye under these conditions.

**Cellular Proliferation Assays**

Stably transfected human *DHHC20*-expressing cells (3t3-20-1, 2, 5) and non-*DHHC20*-expressing cells (3t3-V) were plated in 96 well plates (2,000 cells per well) in selection media. Twenty-four hours, later the media was removed, cells were washed with PBS, and media containing 0, 2 or 10% bovine serum
was added. At time point zero (day 1), and at 24 hour intervals thereafter, the cells were fixed with 10% TCA directly added to the culture media. The cells were subsequently washed with water, stained with sulforhodamine B, washed with 1% acetic acid and destained with 10mM Tris. The samples were then analyzed for absorbance at 560nm using the Spectramax M5 spectrophotometer (Molecular Devices). The mean and standard deviation for each sample were calculated and the graphs shown are representative of three independent experiments.

qPCR of DHHC20 expression in normal and tumor-derived tissue

Human cDNA from multiple normal and tumor tissues was obtained in the Human Cancer Survey TissueScan Real-Time Panel I from Origene (CSRT101). Quantitative PCR was carried out on β-actin-normalized cDNA using a DHHC20-specific Taqman Gene Expression Assay (Applied Biosystems Hs00863895_g1) on the MyiQ Single Color Real-Time PCR Detection System (BIO-RAD). To determine the relative DHHC20 expression between different normal tissues the average Ct value for each normal tissue group was determined and the % relative expression was calculated using the equation: % relative expression = \((2^{\Delta Ct}) \times 100\), where \(\Delta Ct\) represents the difference in Ct for the tissue group vs. the overall Ct for all tissue types. To determine the difference in DHHC20 expression between tumor and matched normal tissue the average Ct value for each normal tissue and tumor-derived group was determined and the % relative expression was calculated using the equation: % relative expression = \((2^{\Delta Ct}) \times \)
100, where $\Delta$Ct represents the difference in Ct for the tumor-derived samples from the Ct for the matched normal tissue samples. The average expression level was calculated from 4 separate experiments and graphed using GraphPad Prism. Statistical analyses were performed using the InStat data-analysis program by two-tailed unpaired t-Test. A value of $p \leq 0.05$ was considered statistically significant.

### 4.4 RESULTS

#### 4.4.1 Expression of Human *DHHC20* in transfected NIH/3t3 cells

To characterize the activity of DHHC20, vectors with or without the *DHHC20* gene were transfected into NIH/3t3 cells and stable clones were selected. As seen in Figure 1A, verification of *DHHC20* expression was determined by RT-PCR analysis of the clonal mRNA. The figure shows that the human *DHHC20* gene is not expressed in the empty-vector control clone (3t3-V), whereas the gene is expressed in each of three *DHHC20*-containing clones (3t3-20-1, 2, & 5). In addition, as seen in Figure 1B, 6x-His tag immunoreactivity is observed by Western analysis at a size consistent with the molecular weight of human DHHC20. Thus, the *DHHC20*-transfected clones express the gene of interest at the RNA and protein levels, and can be used to monitor changes in morphologic and phenotypic characteristics caused by the expression of human DHHC20.
Figure 1: DHHC20 expression in stable NIH/3t3 clones. (A) mRNA from stably transfected 3t3 clones was isolated, reverse transcribed and amplified using primers specific for human DHHC20 and mouse 18S RNA as a loading control. The products were run on a 1% agarose gel and visualized by staining with ethidium bromide. (B) Protein from cellular membrane fractions was subjected to western analysis using antibodies specific to the 6x-His epitope tag.
4.4.2 PAT activity of DHHC20

In order to determine the catalytic PAT activity and substrate-specificity of DHHC20, *in vitro* palmitoylation (IVP) assays were conducted on membrane fractions of 3t3-V and 3t3-D20-1 cells. The reactions contained 33 µg of membrane proteins, 60 µM palmitoyl-CoA, and 10 µM of either the C-terminal farnesyl-mimicking NBD-CLC(OMe)-Farn or N-terminal myristoyl-mimicking MyrGC-(NBD) peptides, for 15 minutes. As seen in Figure 2, there was no difference in the PAT activity toward the NBD-CLC(OMe)-Farn peptide. However, palmitoylation of the MyrGC-(NBD) peptide was significantly elevated when exposed to membrane fractions of 3t3-D20-1 cells compared to 3t3-V cells. The myristoyl-directed PAT activity of these cell lines were 111 ± 5.8 and 78.5 ± 7.9 pmoles peptide palmitoylated per milligram protein per minute, respectively. This represents a 42% elevation in the total amount of palmitoylated MyrGC-(NBD) peptide. These results demonstrate that DHHC20 is a human protein with specific enzymatic palmitoylation activity towards the N-terminal myristoyl motif with no detectable activity toward the C-terminal farnesyl motif.

4.4.3 DHHC20-mediated cellular transformation

In order to determine whether the expression of DHHC20 can confer the ability of cells to grow in an anchorage-independent manner, the clones were plated in soft agar. Twenty-one days after plating, the cells were photographed and the number of colonies formed per plate was determined. Photographic evidence seen in Figure 3 indicates that control cells lacking human DHHC20
Figure 2: Enzymatic Palmitoylation Activity of DHHC20. *In vitro* palmitoylation assays were performed using 3t3-V (-□-) or 3t3-D20-1 (-■-) membrane fractions. The assays consisted of either 10 µM of the NBD-CLC(OME)-Farn or MyrGC-(NBD) peptides with 60 µM palmitoyl-CoA, 33 µg membrane protein, and acylation buffer in a total volume of 100 µl. Initiation of the reaction was carried out by incubating the peptide substrates with membrane fractions derived from the indicated cell lines in acylation buffer for 8 min at 37°C with agitation. To start the reaction, palmitoyl-CoA was added, gently vortexed, and the mixture was incubated for a further 15 min at 37°C with agitation. Palmitoylated peptides were resolved by HPLC using a reverse phase wide pore butyl column and the amount of the peptide palmitoylated in each reaction was calculated based on the ratio of the palmitoylated to the total native peptide in each reaction. Statistical analyses were performed using two-tailed unpaired t-Test and the data shown represents the mean of 3 experiments ± SEM. (*) indicates p ≤ 0.05
Figure 3: DHHC20 expression induces foci formation and growth in soft agar. Panel I: Suspensions of (A) 3t3-V, (B) 3t3-D20-1, (C) 3t3-D20-2 or (D) 3t3-D20-1 cells in exponential growth phase were plated in DMEM containing 0.33% Bacto-Agar overlaid in 35 mm plates with 0.6% agar gel. The cells were fed fresh media every 3 to 4 days and pictures were taken at 40x magnification 21 days after plating. Panel II: (A) 3t3-V, (B) 3t3-D20-1, (C) 3t3-D20-2 or (D) 3t3-D20-5 cells in exponential growth were plated in each well of a six-well dish in selection media. The media was changed every 3-4 days, and at 14 days past confluence the wells were photographed at 40x magnification. Panel III: The average number of colonies or foci formed per plate for each cell line was determined. The data represent the mean ± SEM from two separate experiments.
DHHC20-expressing cell lines 3t3-D20-1 (Figure 3, Panel IB), 3t3-D20-2 (Figure 3, Panel IC) and 3t3-D20-5 (Figure 3, Panel ID) each formed colonies. Quantification of this visual data in Figure 3, Panel III, shows that 3t3-V cells produced 0.5 ± 0.2 colonies per plate. In contrast, 3t3-D20-1, 3t3-D20-2 and 3t3-D20-5 cells grew an average of 20.0 ± 4.7, 11.8 ± 0.2 and 12.7 ± 4.4 colonies per plate, respectively. Thus, DHHC20-expressing clones produced a 39, 23 and 24-fold increase respectively in soft agar colony formation compared to the control cells. These data indicate that DHHC20 induces cells to grow in an anchorage-independent manner.

The expression of DHHC20 also allows cells to escape from contact inhibition. Figure 3, Panel IIA shows that 3t3-V cells grow only as a monolayer and they cease proliferation once saturation is achieved. In contrast, 3t3-D20-1 (Figure 3, Panel IIB), 3t3-D20-2 (Figure 3, Panel IIC) and 3t3-D20-5 (Figure 3, Panel IID) cells each form large foci in tissue culture plates. These visual data were quantified in Figure 3 Panel III, where it can be seen that 3t3-V cells produced an average of 0.2 ± 0.2 foci per plate, whereas 3t3-D20-1, -2 and -5 cells formed 42.5 ± 2.2, 24.2 ± 1.2 and 27.0 ± 0.3 foci per plate, respectively. This represents a 211, 121 and 134-fold elevation in foci formation with the expression of DHHC20. Overall, these data demonstrate that NIH/3t3 cells expressing human DHHC20 are able to proliferate past saturation, thus indicating that DHHC20 is able to suppress contact inhibition of proliferation in this cell line.
Increased proliferation in serum-deprived conditions is another phenotypic characteristic of cellular transformation. To determine if DHHC20 causes an increase in cellular proliferation each clone was grown in media containing reduced (2%) or standard (10%) bovine serum over the course of 3 days. Samples were fixed each day and stained with SRB to quantify total protein accumulation over time. The absorbance over time for each cell line is presented in the graphs of Figure 4. As seen in Figure 4A, each DHHC20-expressing clone proliferated more rapidly in a 2% serum environment than cells lacking the gene. Also, as Figure 4B demonstrates, DHHC20-expressing cells proliferated slightly more rapidly than cells lacking the gene in a standard 10% serum environment. Thus, it appears that DHHC20 drives proliferation through serum-dependent and independent pathways. Overall, these results demonstrate that the expression of DHHC20 induces phenotypic characteristics associated with cellular transformation.

4.4.4 Expression of DHHC20 in human tissue samples

As noted previously, palmitoylated proteins play an integral role in driving proliferation and survival signaling pathways in cancer cells. In addition, palmitoylation of these proteins is required for proper cellular localization as well as full activation. Therefore, proteins that drive the palmitoylation of these targets have the potential to be oncogenic. This appears to be the case since overexpression of the C-terminal farnesyl-directed PAT DHHC17 (25) and this
Figure 4: DHHC20 expression causes elevated proliferation in NIH/3t3 cells. 3t3-V (□), 3t3-D20-1 (■), 3t3-D20-2 (▼) or 3t3-D20-5 (♦) cells were seeded onto 96 well plates (2,000 cells/well) in standard media on day 0. On day 1, the media was changed to DMEM containing 2% (Panel A) or 10% (Panel B) Bovine Serum. Plates were fixed at 24 hour intervals and subjected to SRB staining and an absorbance reading at 560 nm. The data shown is the mean ± SD of the absorbance at each time point, and is representative of three separate experiments.
newly characterized N-terminal myristoyl-directed PAT DHHC20 have been shown to cause transformation of NIH/3t3 cells. Due to the activity of these proteins in cellular models, it appears likely that DHHC20 could play a role in human cancer as well. To determine the expression pattern of the gene in humans, we analyzed transcript levels from several normal and tumor tissues by quantitative-PCR. As seen in Figure 5A, DHHC20 (normalized to β-actin) shows a tissue-specific pattern of expression; with transcript levels varying greatly between tissue types. The normalized expression of DHHC20 in prostate tissue is elevated nearly 750% compared to ovarian tissue and roughly 650% compared to breast tissue. In addition, DHHC20 is expressed in colon tissue at levels roughly 600% and 500% greater than those observed in breast and ovarian tissue respectively. Overall, these results indicate that DHHC20 is expressed at relatively low levels in ovary, breast and kidney tissues, and at relatively high levels in thyroid, liver, colon and prostate tissue. To determine if the expression of DHHC20 is modified in tumor tissue the overall transcript levels of tumor-derived tissues were compared to those of matched normal tissues in Figure 5B. The results indicate that DHHC20 expression is elevated at the transcript level in a number of tumor tissues. Statistically significant overexpression of the gene is observed in several tumor tissues including prostate, kidney, colon, breast, and ovarian tissues. Tumor tissues with particularly elevated levels of DHHC20 include colon, breast, and ovarian tumors which show expression of the gene at 380%, 440% and 540% of control tissue expression, respectively. Overall, these
Figure 5: Expression of *DHHC20* in normal and tumor-derived human tissue. Lyophilized β-actin-normalized cDNA obtained in a 96 well format from Origene Technologies was amplified using a *DHHC20*-specific primer/probe. (Panel A): Comparative expression of *DHHC20* in normal tissue samples. The average Ct value for each tissue group was determined and the % relative expression was calculated using the equation: % relative expression = (2^{-\Delta Ct}) \times 100, where ΔCt represents the difference in Ct for the tissue group vs. the overall Ct for all tissue types. (Panel B): Expression of *DHHC20* in tumor vs. matched normal tissue. The average Ct value for each normal tissue and tumor-derived group was determined and the % relative expression was calculated using the equation: % relative expression = (2^{-\Delta Ct}) \times 100, where ΔCt represents the difference in Ct for the tumor-derived samples from the Ct for the normal tissue samples. Values represent the mean ± SEM. of 4 experiments. For Panel B, (*) indicates p ≤ 0.05 and (**) indicates ≤ 0.01.
results indicate that DHHC20 expression is greatly elevated in several human tumor tissues.

4.5 DISCUSSION

Posttranslational lipidation events are important mediators of the proper localization and activity of many signaling proteins (2-6). Due to the integral roles of these proteins in important signaling pathways such as cellular activation, survival and proliferation, inhibition of the enzymatic lipidation of these proteins has been considered an attractive area for novel drug development. To date, the majority of effort has focused on the development of farnesyltransferase inhibitors. Inhibitors of PATs have not been developed because the enzymes that catalyze these reactions have not been fully identified and characterized, although recently significant progress has been made (1, 2). This progress can be traced back to the identification of Erf2/Erf4 and Akr1p in yeast as PATs specific for Ras2 (20) and casein kinase2 (21), respectively. These two enzymes share a conserved Asp-His-His-Cys (DHHC) domain that was hypothesized to represent a signature catalytic motif for PATs. This domain has provided the starting point for the identification of mammalian PATs (24, 25). The majority of these studies have focused on identifying PATs with activity toward either transmembrane, prenylated, and/or non-myristoylated N- and C-terminally palmitoylated proteins (24, 25, 31). Conversely, PATs specific for N-terminally myristoylated and palmitoylated proteins have thus far remained the least characterized, and studies in this area have focused primarily on mouse genes
In addition, although many palmitoylated proteins drive signaling pathways involved in cancer, only DHHC17, a C-terminal farnesyl-directed PAT, has been directly shown to cause cellular transformation (25). Therefore, in this study, we sought to identify the first human N-terminal myristoyl-specific PAT and assess its potential role in cancer by determining its ability cause cellular transformation in vitro and its pattern of expression in human tissues and tumors.

In the current study, we used fluorescently-labeled lipopeptides to characterize the PAT activity of DHHC20. Lipopeptides that mimic protein palmitoylation sites have proven to be useful tools for the analysis of protein trafficking, localization, membrane interactions, as well as PAT activity (33-35). In the past, we have used peptides that mimic the palmitoylation motifs of C-terminally farnesylated or N-terminally myristoylated proteins to quantify palmitoylation in vitro based on the differential retention times of the palmitoylated and non-palmitoylated forms of the peptides when analyzed by HPLC (25, 27, 29, 30). This approach was used to identify and characterize DHHC17 as a PAT with specificity toward the C-terminal farnesyl motif (25). The current data, generated using the same peptides, clearly indicate that human DHHC20 causes an elevation in the palmitoylation of the Myr-GC-(NBD) peptide with no significant affect on the level of palmitoylation of the (NBD)-CLC(OMe)-Farn peptide. Overall, these data indicate that DHHC20 is a PAT with substrate specificity for the N-terminal myristoyl palmitoylation motif. This motif occurs in several proteins such as the Src-related tyrosine kinases and some $G_\alpha$ subunits. Since DHHC20 and all previously identified PATs in yeast and mammals show
specificity toward particular substrates, it appears that specific structural components of the substrate protein (or peptide) are recognized by the PATs. Furthermore, this confirms that different PATs are responsible for the palmitoylation of proteins expressing the C-terminal farnesyl or the N-terminal myristoyl motif as we previously hypothesized on the basis of differential stability and pharmacologic sensitivities of the enzymatic activities toward the lipopeptide substrates (29, 36).

To determine if overexpression of DHHC20 promotes cellular transformation, we expressed the human gene in NIH/3t3 cells and monitored the cells for phenotypic changes. The results indicate that cells expressing DHHC20 were able to grow in soft agar and grow beyond confluence to create foci on plastic. Anchorage-independent growth pattern and growth beyond confluence are both phenotypic characteristics associated with cellular transformation, indicating that overexpression of DHHC20 is sufficient to cause transformation of NIH/3t3 cells in vitro. In addition, cellular proliferation studies demonstrate that expression of DHHC20 causes an elevation in proliferation particularly in a low-serum environment. These results suggest that DHHC20 promotes the activation of signaling pathways that drive cellular proliferation, and are consistent with the phenotypic characteristics produced by activation of many N-terminal myristoyl-motif-containing proteins, particularly the Src-related tyrosine kinases. For example, activation of these kinases drives cellular proliferation, inhibition of apoptosis, as well as transformation (16, 37, 38). Thus, it appears likely that DHHC20 drives cellular proliferation and transformation by
palmitoylating N-terminally myristoylated and palmitoylated proteins, such as the Src-related tyrosine kinases.

To further characterize DHHC20, we sought to determine its expression in both normal and tumor-derived human tissues. Quantitative PCR studies demonstrate that \textit{DHHC20} has a tissue-specific expression pattern. Organs such as the thyroid, liver, colon and prostate express relatively high levels of \textit{DHHC20}, whereas \textit{DHHC20} is expressed at relatively low levels in the ovary and breast. This tissue-specific expression runs contrary to the ubiquitous expression pattern exhibited by some DHHC genes such as \textit{DHHC4}, -5, -7, and -10, but is consistent with previous findings for this gene and some others within the family (28). Particularly, Ohno et al. observed that \textit{DHHC20} was expressed at high levels in the testis and placenta, at somewhat lower levels in the brain, heart, liver, lungs, thymus and leukocytes, and was not expressed at all in skeletal muscle and the small intestine. These results corroborate our findings of \textit{DHHC20} expression in the lung and liver. Interestingly, Ohno et al. observed no expression of the gene in the small intestine, whereas we found the gene expressed in colon tissue samples, indicating a difference in expression in parts of the lower digestive tract. In addition, it is interesting to note that Ohno et al. observed the highest expression of \textit{DHHC20} in highly proliferating and differentiating tissues such as the testis and placenta. They also found that \textit{DHHC20} was expressed in certain tissues, such as leukocytes and brain tissues, that specifically overlap with the expression pattern of some Src-related tyrosine kinases, such as Lyn, Hck, Fgr, Blk and Lck, that are not ubiquitously expressed
(Reviewed in (39)). In addition, we herein demonstrate that DHHC20 expression is significantly upregulated in ovarian, breast, colon, kidney and prostate tumors in comparison with organ-matched normal tissues. Of these, the greatest increase was observed in ovarian, breast and colon tumors. Interestingly, exploration of pathology reports associated with the ovarian and breast tumor samples demonstrated no correlation between the expression of DHHC20 and the expression of HER2 (data not shown) suggesting that the expression of the gene and activity of its product are independent of HER2. This is not surprising considering many potential targets of DHHC20 are tumorigenic and potently transforming when constitutively activated in breast cancer with or without an overexpression of HER2 (40, 41). It is also of interest to note that several potential targets of DHHC20 palmitoylation, such as Lck and Yes, have elevated expression and activity in colorectal carcinomas as well as their metastases (42-45). Overall, these findings show that human DHHC20 is expressed in a tissue-specific manner and expression is upregulated in several tumor tissues in a pattern seemingly consistent with the expression of its likely intracellular targets. These results implicate DHHC20 in the development of human cancer and give further evidence linking the gene with its potential enzymatic targets.

In conclusion, we have identified DHHC20 as the first human PAT with specificity to the N-terminally myristoyl PAT recognition motif. Also, expression of the gene in NIH/3t3 cells caused cellular transformation as evidenced by an increase in proliferation, foci formation and growth in soft agar. In addition, we have determined that DHHC20 is expressed in a tissue-specific manner and that
the gene is overexpressed in several human tumors including breast, ovary and colon, implicating it not only in cellular transformation, but also in the development of human cancer. Each of the phenotypic characteristics caused by the expression of DHHC20 are consistent with those produced by the activity of N-terminally myristoylated Src-related tyrosine kinases, suggesting that DHHC20 functions by activating one or more of these kinases. These results make N-terminal myristoyl-directed PATs, and DHHC20 in particular, interesting targets for the development of novel anticancer therapeutics.

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4.6 REFERENCES


Chapter 5

Perspectives on the role of DHHC20 in cellular signaling, human disease, and its potential as a therapeutic target
The co- and post-translational lipid modifications of proteins have garnered increasing attention as potential processes to selectively target in order to treat several human diseases. Since many important signaling proteins involved in various diseases require these modifications for their proper localization, stability, and activity, it has been hypothesized that selectively inhibiting the lipidation of these proteins could inhibit their activity and yield positive therapeutic results in the clinic. Based on this hypothesis, many academic and industrial groups have invested time and resources in the development of selective lipidation inhibitors. These groups have directed the majority of their attention toward the processes of myristoylation and farnesylation. In particular, the process of farnesylation has been extensively studied and multiple inhibitors have demonstrated efficacy in *in vitro* and *in vivo* cancer models. Some of these drugs have made the leap into clinical trials; however, they have had difficulty in gaining acceptance from the FDA primarily due to a lack of clinical efficacy. This lack of efficacy is likely due to a number of factors associated with the process of farnesylation that may be overcome by selectively targeting a more dynamic lipidation event such as palmitoylation. However, the arena of protein palmitoylation has remained a relatively untapped source of therapeutic intervention.

One of the main reasons that the process of palmitoylation has not been extensively investigated as a therapeutic target is due to the lack of defined human proteins characterized to demonstrate PAT activity. The identification of PATs across multiple species began in 2002 with the characterization of two
yeast enzymes, and has subsequently grown to include multiple PAT enzymes in mammals. However, these studies have generally focused on the identification of PATs specific for either C-terminally farnesylated and palmitoylated proteins or N- or C-terminally palmitoylated proteins that lack a specified and required primary lipidation event. Due to this focus, PATs specific for N-terminally myristoylated proteins have remained relatively neglected, and no human enzymes with this activity have previously been identified.

The current studies use comparative homology, localization, and a lipopeptide based IVP assay to identify a potential N-terminal myristoyl-directed human PAT and characterize its activity. It was demonstrated that DHHC20 is a human PAT with activity toward the type 4 N-terminal myristoyl motif, with no measurable activity toward the type 3 C-terminal farnesyl motif. This indicates that DHHC20 has specified PAT activity toward N-terminally myristoylated proteins such as the Src-related tyrosine kinases, eNOS and certain Gα proteins, with no activity toward C-terminally farnesylated proteins such as the Ras isotypes. In addition, it was demonstrated that expression of this protein in NIH/3t3 cells induces phenotypic characteristics consistent with cellular transformation including elevated proliferation, anchorage-independent growth and a loss of contact inhibition. Also, a survey of DHHC20 expression determined that the gene is overexpressed in multiple human tumor tissues including ovarian, breast and colon. Overall, the results derived from these studies implicate DHHC20 in the development of cancer and suggest that it is a potential target for the development of novel anticancer agents.
Based on the results obtained from these studies in conjunction with the scientific literature, a probable model of DHHC20-associated localization, activity, and possible disease states that are implicated as a result of that activity can be generated (Figure 1). It appears probable that DHHC20 resides as a transmembrane protein intercalated into the plasma membrane. At the plasma membrane, DHHC20 catalyzes the covalent transfer of the palmitoyl group from palmitoyl-CoA to a cysteine residue adjacent to the myristoylated N-terminal glycine via a thioester bond of the target protein that is transiently associated with the plasma membrane due to the hydrophobic nature of the myristoyl group. The attachment of the palmitoyl group to the target protein further increases the hydrophobicity of the protein, causes it to form a more stable association with the plasma membrane, and causes it to partition into cholesterol-rich microdomains such as lipid rafts and caveolae. These microdomains are enriched with signaling proteins that can either facilitate the activation of the protein or become activated by the protein. The microdomain-localized protein is subsequently able to activate its downstream signaling pathways which lead to the resultant signaling outcome and, in abnormal situations, the progression of certain diseases. These signaling outcomes vary based on the targeted type 4 protein that is palmitoylated, and are likely subject to differences based on cell type. As noted previously, there are a total of 12 proteins known to be N-terminally myristoylated and palmitoylated. This group includes important signaling proteins such as the Src-related tyrosine kinases,
Figure 1. Potential activity, substrates and disease states associated with DHHC20.
endothelial nitric oxide synthase (eNOS), as well as several Gα subunits.

G proteins are plasma membrane localized signal transducers that connect receptors to effectors, and in turn drive intracellular signaling pathways (1). N-terminally myristoylated and palmitoylated Gα proteins such as Gαi1 and Gαo are activated by various receptors including dopamine receptors (2), serotonin receptors (2), and the proteinase-activated receptor 1 (PAR1) (3). These G-proteins affect cellular activities including transcription, motility/contractility, secretion, and ion channel signaling (2). The role of palmitoylation in the regulation of these proteins is less well characterized than that of other type 4 proteins, and evidence exists supporting two separate roles for palmitoylation. First, it has been demonstrated that palmitoylation is required in order to localize Gαi to caveolin-enriched domains of the plasma membrane (4). Secondly, studies have demonstrated that palmitoylation of Gαo increases aggregation of the protein, and causes a compaction of the GTP-binding domain which may function to block the activation of the protein by inhibiting the addition binding of GTP (5, 6). Thus, it appears that palmitoylation may facilitate the activation of some Gα subunits by allowing localization to the plasma membrane, however, it may also inhibit activation by preventing the binding of GTP. These activities may be substrate specific, or may apply to all N-terminally myristoylated and palmitoylation Gα proteins; however, more investigation into the roles that this process plays on regulating the activity of these proteins is necessary. Since
DHHC20 potentially palmitoylates these proteins, it may play a role in many diseases associated with the activity of the dopamine, serotonin and PAR1 receptors. However, since the role of palmitoylation in the activity of these proteins is still unclear, it is too early to know how DHHC20 may act in these diseases.

eNOS is a peripheral membrane protein expressed in endothelial cells that is localized to the Golgi and microdomains of the plasma membrane. This subcellular localization is facilitated by the addition of myristoyl and palmitoyl groups. Blocking the palmitoylation of this enzyme inhibits its proper localization and drastically reduces its activity \textit{in vivo} \cite{7, 8}. eNOS is an important enzyme because it catalyzes the production of nitric oxide (NO) by the vascular endothelium. NO, in turn, regulates a number of fundamental cellular processes such as differentiation, growth, migration, as well as mitochondrial respiration \cite{9}. The importance of this activity is underscored by the generation of mice containing genetic deletions of eNOS. These mice demonstrate a number of cardiovascular phenotypes including accelerated atherosclerosis, elevated blood pressure, aberrant vascular modeling, and impaired angiogenesis \cite{9}. Since, DHHC20 potentially palmitoylates eNOS, whose activity is predicated on palmitoylation, DHHC20 may play an important role in the regulation of proper cardiovascular activity. As such, DHHC20 may play a protective role in the regulation of the cardiovascular system, and selectively activating this enzyme may prevent the development of atherosclerosis and reduce blood pressure.
Potential DHHC20-targeted proteins of possibly the greatest interest are the Src-related tyrosine kinases. The Src-related tyrosine kinases are implicated in the regulation of a number of important signaling pathways and, as such, the misregulation of their activity is implicated in multiple disease states. Under normal circumstances, these proteins demonstrate differing expression patterns with some being expressed ubiquitously throughout the body, while others demonstrate tissue-specific expression patterns toward the brain and immune system (10). In addition, many of these proteins are overexpressed in various forms of cancer (10). Thus, these proteins play important roles in the brain and immune system, and are associated with disease progression in these areas, as well as cancer on a wider scale. Thus, since DHHC20 potentially palmitoylates these proteins it may be involved in multiple Src-related tyrosine kinase-associated diseases and, thus, is potentially a novel drug target for the treatment of those diseases.

The Src-related tyrosine kinase Fyn is implicated in the development of Alzheimer’s disease. The abnormal accumulation and deposition of amyloid-β peptides is a hallmark of Alzheimer’s disease, a disease that causes degeneration of synapses and neurons, and in turn a progressive decline in cognitive abilities (11). The exact relationship that exists between the buildup of amyloid-β, synaptic impairment, and the resultant decline in cognitive abilities, however, remains uncertain (12). However, in transgenic mice with high levels of amyloid-β, which serve as models for Alzheimer’s disease, it has been determined that the ablation of Fyn decreases, and the overexpression of Fyn
increases, amyloid-β-induced synaptotoxicity and premature mortality in these mice (13). Fyn is in an excellent position to modulate the effects of amyloid-β since it is located at the postsynaptic density of glutamatergic synapses (11). In addition, it has been determined that Fyn becomes activated during the engagement of integrins and glutamate receptors (14), which have been demonstrated to be involved in the disruption of synaptic function that is associated with amyloid-β (15). Thus, it appears that Fyn is likely activated by the buildup of amyloid-β and facilitates signaling pathways that are involved in the phenotypic and/or morphologic characteristics associated with Alzheimer’s disease. Since Fyn requires palmitoylation, possibly by DHHC20, for its proper localization and activity, abrogating this activity by inhibiting DHHC20 may be a novel mechanism to treat or prevent Alzheimer’s disease.

DHHC20 may also be integrally involved in the activity of lymphocytes and may be a potential target with therapeutic value in many lymphocyte-associated diseases. For example, as noted in section 1.3.2, T cell receptor (TCR) signaling requires the formation of a TCR complex in lipid rafts which is composed of the TCR, LAT, and type 4 N-terminally myristoylated and palmitoylated Src-related tyrosine kinases such as Lck and Fyn. Also, palmitoylation of Fyn and Lck is required for lipid raft localization, and inhibition of this process inhibits T cell activation and proliferation. The overactivity or misregulation of the T cell response is involved in many autoimmune diseases such as psoriasis, inflammatory bowel disease, rheumatoid arthritis, type-1 diabetes, as well as organ graft rejection. Since the palmitoylation of Src-related tyrosine kinases is
necessary for the activation and proliferation of T cells, blocking the activity of DHHC20 may abrogate the anti-self immune response and yield positive therapeutic results in the treatment of many autoimmune diseases.

The Src-related tyrosine kinases are also implicated in the development of cancer. The expression and activity of these kinases are upregulated in several types of primary human cancer and tumor-derived cell lines including lymphoma, leukemia, melanoma, glioblastoma, as well as carcinomas of the lung and colon (16-23). In addition, the expression of Src-related tyrosine kinases such as Yes (24), Hck (25), and Fyn (26) induce cellular transformation. Thus, this group of kinases appears to be integrally involved in neoplastic progression, and DHHC20 may induce the cellular phenotypes consistent with transformation observed in this study by driving the activation of one or more of these proteins. In addition, many N-terminally myristoylated and palmitoylated Src-related tyrosine kinases play a role in metastasis as well. The expression and activity of Src-related tyrosine kinases such as Lck, Lyn and Yes are elevated in \textit{in vivo} metastases, as well as metastatic cell lines (18, 22, 27, 28). Their activity causes pro-metastatic phenotypic characteristics such as increased cellular motility, matrix metalloproteinase production and invasiveness, as well as decreased binding to the extracellular matrix through the disassembly of focal adhesions (19, 29-32).

Also of interest is the confluence of data surrounding the colon. According to our results, \textit{DHHC20} is expressed in normal human colon tissue samples, and is greatly elevated in tumor tissues derived from the colon. Several N-terminally myristoylated and palmitoylated Src-related tyrosine kinases, some ubiquitously-
expressed and some not expressed in normal tissue of the colon, are frequently overexpressed or activated in colorectal cancer tissues (17, 18, 20, 27). Therefore, it does not seem to be a coincidence that NIH/3t3 cells expressing DHHC20 metastasize to the intestines to a greater extent than control cells (Appendix 1, Figure 1B), and demonstrate elevated invasiveness to organs of the digestive tract. Since many of potential protein targets of DHHC20 are overexpressed in colorectal cancer tissues, the activity of these proteins may confer a competitive advantage to the cells when located in or around the environment of the digestive tract. For example, DHHC20 may palmitoylate and allow the activation of Src-related tyrosine kinases that drive morphological changes that increase the localization of the cells to the digestive tract, possibly through an increased attraction toward digestive tract specific chemokines, or an increase in the binding of the cells to the organs themselves. Although the answer to this question is unknown, overall, this information associates the activity of DHHC20 with the activation of Src-related tyrosine kinase signaling pathways, in turn linking DHHC20 to cellular transformation, tumor formation and metastasis. Therefore, since the Src-related tyrosine kinases require palmitoylation, potentially by DHHC20, for their proper localization and activity, abrogating this activity by inhibiting DHHC20 may be a novel mechanism to treat cancer.

The potential importance of DHHC20 as target for anticancer drug development is further underscored by the confluence of data surrounding this PAT and ovarian cancer. As demonstrated in Chapter 3, human SKOV3 ovarian
cancer cells are killed when treated with a peptide that competes with endogenous N-terminally myristoylated proteins for binding to N-terminal myristoyl-directed PATs. In addition, it has been shown that DHHC20 is a PAT specific for N-terminally myristoylated proteins, and it is overexpressed in human ovarian tumor tissue samples. Therefore, it can be hypothesized that the cytotoxicity exhibited upon treatment with the competitive PAT inhibitor is due to the inhibition of DHHC20. This suggests that DHHC20 is a more attractive target for the development of anticancer agents than farnesyltransferase inhibitors, that are cytostatic and lack clinical efficacy, since its inhibition is directly cytotoxic to cancer cells.

In addition, the importance of the Src-related tyrosine kinases in many disease states is well known, particularly in the areas of autoimmune disorders and cancer. Due to this fact, much effort has been exerted to develop inhibitors of these kinases. However, it is proposed that many of these compounds function by binding to the ATP sites of these kinases (10, 33), which poses problems with the selectivity of these compounds since the ATP binding sites of many kinases are highly similar. Since DHHC20 likely has relatively few proteins with similar structure and activity, it may prove to be a more advantageous target for inhibition than the kinases themselves.

In conclusion, the characterization of DHHC20 has demonstrated that the protein is an N-terminal myristoyl-directed human PAT that produces phenotypic characteristics associated with cellular transformation. This gene demonstrates a tissue-specific mode of expression and is overexpressed in multiple types of
human tumor tissue. Since there are 12 known N-terminally myristoylated and palmitoylated proteins, DHHC20 may be a central component that affects the activation of many cellular signaling pathways that are involved in various diseases including Alzheimer’s disease, artherosclerosis, rheumatoid arthritis, type-1 diabetes, and cancer. Thus, DHHC20 is potentially a novel therapeutic target for the treatment of multiple human diseases.
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Appendix

Supplemental Data
DHHC20-associated tumor formation and metastasis

Since cells expressing DHHC20 exhibited phenotypic characteristics consistent with transformation, the tumorigenicity of these cells was evaluated in mice. However, since the DHHC20-expressing clones were phenotypically consistent, the 3t3-D20-1 cells were used as representative of the group. Female NOD/SCID mice, approximately 6-7 weeks old, were subcutaneously injected with 5 x 10^6 DHHC20-expressing (3t3-D20-1) or control (3t3-V) cells in 200µl PBS. The tumor volume in each mouse was determined on days 22, 25, 28 and 31 post-injection and the average tumor volume from three independent experiments was calculated. As seen in Figure 1A, 3t3-D20-1 cells produced significantly larger tumors than 3t3-V cells. In fact, the average tumor volume in 3t3-D20-1-injected mice was 1180 ± 150 mm³ 31 days post-injection compared to 415 ± 197 mm³ in 3t3-V-injected mice, a nearly 2-fold increase in tumor volume. On day 31 post-injection, many mice injected with the 3t3-D20-1 cells exhibited distended abdomen, whereas no 3t3-V-injected mice exhibited this morphology. Subsequent to this observation the mice were sacrificed and the abdomen of the animals were inspected. Figure 1B shows representative pictures of exposed abdomen from each group. In general, mice injected with 3t3-V cells showed relatively minor morphological abnormalities, in the form of small growths, within the abdomen. However, most 3t3-D20-1-injected mice showed severe and extensive abnormalities in the form of very large growths. These growths often contained extremely ulcerative regions and were found throughout the abdomen, but particularly associated with the intestines.
Figure 1: DHHC20 causes *in vivo* tumor growth and metastasis formation.
Six female NOD/SCID mice approximately 6-7 weeks old were subcutaneously injected in the right hind flank with 5 x 10^6 DHHC20-expressing 3t3-20-1 (-■-) or control 3t3-V (-□-) cells. The length (L) and width (W) of tumors were measured using calipers 22, 25, 28 and 31 days post-injection. Tumor volume was calculated using the formula: (L x W^2)/2. On day 31, the animals were euthanized, a gross necropsy was performed, and pictures of the abdominal cavity were taken. The data shown represents the mean of 3 experiments ± SEM. (*) indicates p ≤ 0.05.
In order to determine if the masses observed were indeed tumors and subsequent metastases rather than other non-related abnormal growths, the mice were submitted to Dr. K.L. Helke for full pathological workup. The pathology reports indicated that the masses found in both treatment groups were in fact unencapsulated tumors and associated metastases. This presents a problem in using the 3t3-V cell-treated mice as true controls since it is not normal for wt NIH/3t3 cells to produce tumors and metastases \textit{in vivo}. However, these abnormal results may be the result of two separate factors. First, wt NIH/3t3 cells are known to spontaneously transform due to various factors including growth to confluence and elevated passage number (personal communication with ATCC representative). Thus, the 3t3-V cells may have spontaneously transformed over the time period of selection or within the mice themselves. Second, the NOD/SCID mice used for these experiments have severely compromised immune systems due to a lack T and B cell development. Thus, it is possible that a combination of spontaneous transformation with an immune-compromised host lead to the formation of tumors by control cells in these animals. In addition, in the future it would be advisable to use a syngeneic mouse model to remove the questions associated with a compromised immune system. In any case, the results from these experiments cannot be used to unambiguously ascertain whether DHHC20 induces tumor formation and metastasis \textit{in vivo}.

Although the 3t3-V cells cannot be used directly as controls to determine if the expression of DHHC20 induces tumor formation and metastasis, they can be
used as a control to determine some other affects of the expression of DHHC20. Since both clones were derived from the same initial vial of wt NIH/3t3 cells and they were selected under the same conditions over the same period of time, the only difference between the clones is the expression of DHHC20. Therefore, any differences in phenotypic or morphologic characteristics observed are due to the expression of DHHC20.

Although tumors and metastases were found in mice injected with both clones, differences were observed in the characteristics of the masses produced. As noted above the 3t3-20-1 tumors and metastases were much larger than those produced by 3t3-V cells. This indicates that the DHHC20-expressing cells have a higher rate of proliferation than the 3t3-V cells in vivo, which is consistent with results observed in vitro (section 4.4.3). This elevation in proliferation is further demonstrated in the pathology reports by a roughly doubling of the number of mitoses observed per field in 3t3-20-1 (20 per field) tumors over 3t3-V (11 per field) tumors. In addition to differences in proliferation, differences in the morphological characteristics and location of neoplasia were also evident in the pathology reports. For example, although both cell lines produced unencapsulated neoplasms, 3t3-V neoplasms were well circumscribed, whereas 3t3-20-1 neoplasms were poorly demarcated. In addition, both cell lines produced metastases, however, there was a distinct difference in the invasiveness observed. Both cell lines produced invasive metastases of the pancreas, however, 3t3-V cells exhibited only limited invasiveness toward other organs. In contrast, 3t3-20-1 cells were also highly invasive to tissues of the
digestive tract. These invasive metastases caused constriction of portions of the digestive tract and caused the stomach to distend with ingesta. In addition, some of these mice presented with abdominal cavities that contained blood tinged fluid, a possible result of these invasive metastases.

Overall, this data cannot be used to demonstrate that DHHC20 induces tumor formation and metastasis in vivo, however, it does provide information on phenotypic characteristics associated with DHHC20 expression. Expression of DHHC20 causes elevated proliferation in vivo, and induces an increase in the invasiveness of cells toward tissues of the digestive tract. Thus, it appears that DHHC20 confers an affinity to areas of the digestive tract to these cells. The implications of these findings are discussed in Chapter 5.

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VITA

Jeremiah M. Draper

Education

2008  Doctor of Philosophy, Department of Pharmacology
       Pennsylvania State University, College of Medicine, Hershey, PA

2006  Master of Business Administration
       Pennsylvania State University, Middletown, PA

2003  Master of Science, Department of Biology
       Bucknell University, Lewisburg, PA

2001  Bachelor of Science, Department of Biology
       Bucknell University, Lewisburg, PA

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Jeremiah M. Draper, Charles D. Smith. Protein acyltransferase assays and
inhibitors. (In press to Molecular Membrane Biology)

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