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Review

Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins

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Abstract

Covalent attachment of myristate and/or palmitate occurs on a wide variety of viral and cellular proteins. This review will highlight the latest advances in our understanding of the enzymology of *N*-myristoylation and palmitoylation as well as the functional consequences of fatty acylation of key signaling proteins. The role of myristate and palmitate in promoting membrane binding as well as specific membrane targeting will be reviewed, with emphasis on the Src family of tyrosine protein kinases and α subunits of heterotrimeric G proteins. The use of myristoyl switches and regulated depalmitoylation as mechanisms for achieving reversible membrane binding and regulated signaling will also be explored.

Keywords: Fatty acylation; Myristoylation; Palmitoylation; Membrane proteins; Membrane rafts

Contents

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1. Introduction

Covalent attachment of fatty acids to proteins is now a widely recognized form of protein modification. Many fatty acylated proteins play key roles in regulating cellular structure and function. Within the past 3 years, several reviews have focused on fatty acylation of signaling proteins [1–4]. This review will highlight some of the newest advances in protein fatty acylation, particularly with regard to membrane binding and targeting of fatty acylated proteins.

The two most common forms of protein fatty acylation are modification with myristate, a 14-carbon saturated fatty acid, and palmitate, a 16-carbon saturated fatty acid. The enzymology of the myristoylation reaction is well understood. Proteins that are destined to become myristoylated begin with the sequence: Met–Gly. The initiating methionine is removed cotranslationally by methionine amino-peptidase, and myristate is linked to Gly-2 via an amide bond. As discussed below, *N*-myristoylation is catalyzed by *N*-myristoyl transferase, an enzyme that has recently been extensively characterized. In contrast, the enzymology of palmitoylation reaction(s) is more complex and is poorly understood. Nearly all palmitoylated proteins are acylated by attachment of palmitate through a thioester linkage to the sulfhydryl group of cysteine. (One exception is Sonic hedgehog, a protein that contains palmitate linked to the amino group of the N-terminal cysteine [5].) The location of these palmitoylated cysteine residues varies-some are present near the N- or C-termini of proteins, while others are located near transmembrane domains. Both myristoylation and palmitoylation can be dynamically regulated: the myristate moiety can be sequestered through the use of myristoyl switches, while palmitate can be removed by protein palmitoyl thioesterases.

2. Protein N-myristoylation

2.1. Structure and function of N-myristoyl transferase

N-myristoyl transferase (NMT) is a 50–60 kDa monomeric enzyme that catalyzes transfer of myristate from myristoyl-CoA to suitable peptide and protein substrates. To date, nearly one dozen NMTs from fungal and mammalian sources have been identified. Studies of NMT from the yeast S. cerevisiae reveal that the catalytic cycle exhibits the following Bi Bi reaction mechanism [6]: (1) myristoyl CoA binds to NMT; (2) the peptide substrate binds to NMT; (3) myristate is transferred to the N-terminal glycine of the peptide; (4) CoA is released from the enzyme; (5) myristoyl-peptide is released. In cells, *N*-myristoylation is a cotranslational process that occurs while the nascent polypeptide chain is still attached to the ribosome [7,8].

The requirements for both fatty acid and peptide substrate specificities of NMT have been characterized and have been extensively reviewed [9]. Myristate is attached specifically to the N-terminus of the acceptor protein via an amide bond. Longer or shorter fatty acyl CoAs are poor substrates, and their corresponding fatty acids are generally not transferred by NMT to acceptor proteins. It is interesting to note that palmitoyl CoA binds to NMT with approximately the same K_m as myristoyl CoA, although palmitate is not transferred by NMT to proteins. Since the concentration of palmitoyl CoA is 5–20-fold higher than myristoyl CoA in most cells, a mechanism must exist to prevent palmitoyl CoA from functioning as a competitive inhibitor of NMT in vivo. It has been proposed that due to its higher hydrophobicity, most of the palmitoyl CoA is sequestered in cell membranes and therefore is not accessible to cytoplasmic NMT [9,10].

Several *N*-myristoylated proteins, including recoverin, transducin and protein kinase A, have been shown to be heterogeneously fatty acylated, containing 12:0, 14:1 and 14:2 fatty acids in addition to 14:0 myristate [11,12]. Examination of *N*-myristoylated proteins in different tissues reveals that heterogenous *N*-acylation is primarily restricted to the retina [9,13]. The reason for the retina-specific nature of heterogenous acylation is not clear, as the fatty acyl CoA pools in retina appear nearly identical to those in other tissues [13].

The consensus sequence for NMT protein substrates is: Met–Gly–X–X–X–Ser/Thr–. The initiating Met is removed by methionine amino peptidase during translation and Gly-2 becomes the N-terminal amino acid. The requirement for Gly at the N-terminus is absolute; no other amino acid will substitute.

Table 1
N-Myristoyolated proteins: sequence and function

1. Protein kinases and phosphatases	
Src family tyrosine kinases	
Src	M **G** S S K **S** K P K D P S Q R R R
Yes	M **G** C I K **S** K E D K G P A M K Y
Fyn	M **G** C V Q C K D K E A T K L T E
Lyn	M **G** C I K **S** K R K D N L N D D E
Lck	M **G** C V C **S** S N P E D D W M E N
Hck	M **G** C M K **S** K F L Q V G G N T G
Fgr	M **G** C V F C K K L E P V A T A K
Yrk	M **G** C V H C K E K I S G K G Q G
Blk	M **G** L L S **S** K R Q V S E K G K G
Abl tyrosine kinases	
c-Abl	M **G** Q Q P G K V L G D Q R R P S
Arg	M **G** Q Q V G R V G E A P G L Q Q
Serine/threonine kinases and anchoring proteins	
cAMP-Dependent protein kinase, catalytic subunit	
Alpha	M **G** N A A A A K K G S E Q E S V
Beta-1	M **G** N A A **T** A K K G S E V E S V
Yeast protein kinase VPS15	M **G** A Q L **S** L V V Q A S P S I A
MPSK	M **G** H A L C V C S R G T V I I D
AKAP18	M **G** Q L C C F P F S R D E G K I
Phosphatases	
Calcineurin B	M **G** N E A **S** Y P L E M C S H F D
Yeast phosphatase PP-Z2	M **G** N S G **S** K Q H T K H N S K K
2. Guanine nucleotide binding proteins	
Gα proteins	
Gαi1	M **G** C T L **S** A E D K A A V E R S
Gαo	M **G** C T L **S** A E E R A A L E R S
Gαt	M **G** A G A **S** A E E K H S R E L E
Gαx	M **G** C R Q **S** S E E K E A A R R S
ADP-ribosylation factors	
Arf-1	M **G** L S F **T** K L F S R L F A K K
Arf-3	M **G** N I F G N L L K S L I G K K
Arf-5	M **G** L T V **S** A L F S R I F G K K
Arf-6	M **G** K V L **S** K I F G N K E M R I
3. Ca^{2+} binding/EF hand proteins	
Recoverin	M **G** N S K **S** G A L S K E I L E E
Neurocalcin	M **G** K Q N **S** K L R P E V M Q D L
Aplycalcin	M **G** K R A **S** K L K P E E V E E L
Visinin-like protein 3	M **G** K Q N **S** K L R P E V L Q D L
Ca^{2+} binding protein P22	M **G** S R A **S** T L L R D E E L E E
NAP-22	M **G** S K L **S** K K K K G Y N V N D
Hippocalcin	M **G** K Q N **S** K L R P E M L Q D L
GCAP 1	M **G** N V M E G K S V E E L S S T
GCAP 2	M **G** Q Q F **S** W E E A E E N G A V
S-Modulin	M **G** N T K **S** G A L S K E I L E E
Rem-1	M **G** K Q N **S** K L R P E V L Q D L

Table 1 (*continued*)

4. Membrane- and cytoskeletal-bound structural proteins	
MARCKS	M **G** A Q F **S** K T A A K G E A T A
MAC-MARCKS	M **G** S Q S **S** K A P R G D V T A E
Annexin XIII	M **G** N R H A K A S S P Q G F D V
Rapsyn	M **G** Q D Q **T** K Q Q I E K G L Q L
Pallidin (Band 4.2)	M **G** Q A L **S** I K S C D F H A A E
Hisactophilin 2	M **G** N R A F K A H N G H Y L S A
5. Viral proteins	
Gag proteins	
HIV-1	M **G** A R A **S** V L S G G E L D R W
Moloney murine sarcoma virus	M **G** Q T V **T** T P L S L T L D H W
Friend murine leukemia virus	M **G** Q A V **T** T P L S L T L D H W
Bovine leukemia virus	M **G** N S P **S** Y N P P A G I S P S
Baboon endogenous virus	M **G** Q T L **T** T P L S L T L T H F
Feline sarcoma virus (G-R)	M **G** Q T I **T** T P L S L T L D H W
FBR murine osteosarcoma virus	M **G** Q T V **T** T P L S L T L E H W
Mason–Pfizer monkey virus	M **G** Q E L **S** Q H E R Y V E Q L K
Mouse mammary tumor virus	M **G** V S G **S** K G Q K L F V S V L
Other viral proteins	
HIV-1 Nef	M **G** G K W **S** K S S V V G W P T V
Duck hepatitis B virus	M **G** Q H P A K S M D V R R I E G
Rhinovirus 16	M **G** A Q V **S** R Q N V G T H S T Q
Herpes simplex virus Ul11	M **G** L A F **S** G A R P C C C R H N
African swine fever virus pp220	M **G** N R G **S** S T S S R P P L S S
Chlorella virus Vp260	M **G** S Y F V P P A N Y F F K D I
6. Miscellaneous	
NADH cytochrome b_5 reductase	M **G** A Q L **S** T L S R V V L S P V
Nitric oxide synthase	M **G** N L K **S** V G Q E P G P P C G
Enteropeptidase precursor	M **G** S K R **S** V P S R H R S L T T
(2′,5′) OligoA synthetase	M **G** N G E **S** Q L S S V P A Q K L
NADH-Ubiquinone Oxidoreductase	M **G** A H L V R R Y L G D A S V E
BASP1	M **G** G K L **S** K K K K G Y N V N D
FRS2	M **G** S C C **S** C P D K D T V P D N
$cPLA_2$-γ	M **G** S S E V S I I P G L Q K E E

However, not all proteins with an N-terminal glycine are *N*-myristoylated and the ability to be recognized by NMT depends on the downstream amino acid sequence. In general, serine or threonine is preferred at position 6 and lysine or arginine is preferred at positions 7 and/or 8 (Table 1).

In fungi, deletion of the gene encoding NMT results in recessive lethality [14], suggesting that NMT may be an attractive anti-fungal target. Since fungal and human NMTs have overlapping but distinct substrate specificities, it should be possible to design compounds that inhibit fungal, but not human NMT. Depeptidized fungal NMT inhibitors that have recently been developed [15,16] show promise as fungicidal agents in *Candida albicans* and *Cryptococcus neoformans*, two organisms responsible for systemic fungal infections in immunocompromised individuals.

Although fungal NMT is encoded by a single copy gene, a recent report has documented the existence of two genes for NMT in mammalian cells [17]. The two isoforms, NMT-1 and NMT-2, share 77% identity, are both ubiquitously expressed, and exhibit similar substrate specificities. NMT-2 migrates as a single 65 kDa band on polyacrylamide gels, while NMT-1 exhibits multiple isoforms ranging from 49 to 68 kDa [17,18]. Some of these isoforms may be the result of alternative splicing [19].

In mammalian cells, the majority of the NMT activity is localized to the cytoplasm [17]. However, a significant proportion of the total NMT protein detected by Western blotting is evident in membrane fractions [20,21]. The amount of NMT activity in the membrane may be underestimated as the existence of membrane associated NMT inhibitors has been reported [22]. In addition, some of the membrane fraction may represent NMT bound to ribosomes. An amino-terminal sequence derived from an upstream start site within the NMT cDNA has been implicated in ribosomal targeting [23].

Within the past year, two fungal NMT structures were determined by X-ray crystallography [24,25]. Both studies reveal that NMT is a compact, globular molecule that exhibits no structural homology to other proteins. The structure of the yeast *Saccharomyces cerevisiae* NMT1p was solved as a ternary complex and reveals how myristoyl CoA and peptide substrates bind to the enzyme [25]. Myristoyl-CoA binds in a conformation similar to a question mark and, together with C-terminal regions of NMT, forms a portion of the peptide binding site. The structure allows identification of specific residues within NMT that serve to restrict substrate specificity to 14-carbon fatty acids and account for the preference for gly-2, ser-6, and basic amino acids at positions 7 and 8 of the peptide substrate. The C-terminal carboxylate was also shown to play a critical role in catalysis by binding to the N-terminal glycine ammonium group in the peptide substrate. The latter result explains why deletion of C-terminal residues inhibits NMT activity.

2.2. Functions of protein N-myristoylation

2.2.1. Structural roles

X-Ray crystallography of several *N*-myristoylated proteins has revealed how myristate can play a structural role to stabilize three-dimensional protein conformation. Poliovirus is a non-enveloped virus that requires myristoylation of its VP4 protein for virus assembly. The electron density map revealed that the myristate moiety forms an integral part of the virion subunit structure [26]. In the catalytic subunit of PKA, myristate is positioned within a hydrophobic pocket and is required for structural and thermostability of the enzyme [27]. Crystal structures of recoverin and Arf-1 also reveal binding sites for myristate in hydrophobic pockets or grooves within the protein's three-dimensional structure (see below). These structures provide evidence that myristate does not always 'stick out' of a protein.

2.2.2. Membrane binding

Many *N*-myristoylated proteins are membrane bound and can be found in the plasma membrane or other intracellular membranes in eukaryotic cells. Abrogation of myristoylation, by mutation of Gly2 to Ala, generally results in reduction or loss of membrane binding. For example, studies of pp60v-src, the transforming protein of Rous sarcoma virus, have clearly established that myristoylation is necessary for directing the Src protein to the membrane. Non-myristoylated Src mutants do not bind to membranes and do not mediate cellular transformation [28,29]. Likewise, retroviral and lentiviral Gag proteins require myristoylation in order to bind to the

plasma membrane and mediate virus particle formation. Mutation of Gly-2 to Ala prevents myristoylation and inhibits membrane binding of Moloney murine leukemia virus, spleen necrosis virus and HIV-1 Gag proteins [30–33]; virus assembly by these mutants is blocked.

While myristoylation is clearly necessary for membrane binding of some proteins, it is not sufficient. Biophysical studies have established that the binding energy provided by myristate is relatively weak (approximately 10^{-4} M K_d) and not sufficient to fully anchor a peptide or protein to a cellular membrane [10]. A second signal within the *N*-myristoylated protein is therefore required for efficient membrane binding.

2.2.3. *The two-signal model for membrane binding of N-myristoylated proteins*

The second signal for membrane binding of *N*-myristoylated proteins has been defined as either a polybasic cluster of amino acids or a palmitate moiety (Fig. 1). It should be noted that a similar two signal model applies to membrane binding of farnesylated proteins, e.g., Ras [34,35]. Proteins that use a 'myristate plus basic' motif for membrane binding will be considered here, and those that use a 'myristate plus palmitate' signal will be considered in the next section.

The biophysical basis for membrane binding by 'myristate plus basic' motifs is now well established [36–39]. Myristate inserts hydrophobically into the lipid bilayer, and approximately 10 of the 14 carbons penetrate the hydrocarbon core of bilayer [39]. The basic amino acids form electrostatic interactions with the headgroups of acidic phospholipids [40]. In the plasma membrane, these acidic phospholipids (primarily PS and PI) are localized primarily to the inner leaflet of the bilayer, imparting a net negative charge to the cytoplasmic leaflet surface. Neither the hydrophobic nor the electrostatic interactions alone are sufficient to provide strong membrane binding. However, when both myristate and a basic motif are present within the protein, the hydrophobic and electrostatic forces synergize. For example, the presence of six basic residues in the N-terminal region of Src enhances its binding to membranes containing acidic phospholipids nearly 3000-fold. The myristate plus basic motif mediates binding of Src to membranes

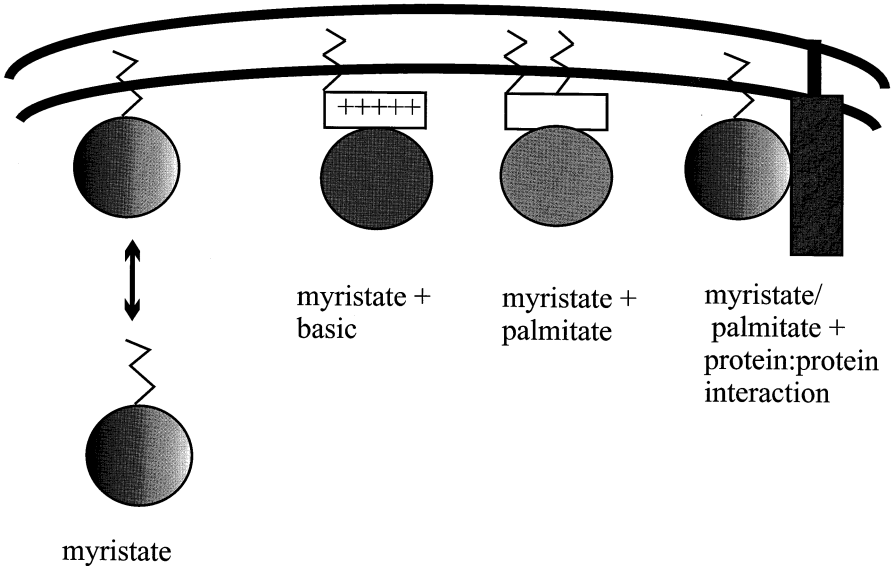

Fig. 1. The two-signal model for membrane binding of myristoylated proteins. In order to achieve stable membrane binding, myristoylated proteins require a second membrane binding signal. This can be provided in several ways. A cluster of basic residues can provide electrostatic interactions with acidic phospholipid headgroups in the inner leaflet of the bilayer (myristate+basic). One or two palmitate moieties can provide additional hydrophobic interaction with the bilayer (myristate+palmitate). Alternatively, membrane interaction of singly acylated proteins can be enhanced by protein–protein interactions with other membrane bound proteins.

in vitro and in vivo and is required for cellular transformation by v-Src [36,37].

Several other proteins have been shown to utilize a myristate plus basic motif for membrane binding, including the MARCKS protein (see below), HIV-1 Gag and HIV-1 Nef proteins [38,41]. The Gag polyproteins mediate assembly of type C retroviruses and lentiviruses at the plasma membrane and form the structural elements of the virion core. Like Src, HIV-1 Gag contains a myristate plus basic motif at its N-terminus that is necessary and sufficient for plasma membrane binding [38,42,43]. The myristate plus basic motifs of Src and Gag are functionally interchangeable: a Gag-Src chimera can mediate cell transformation [38], and a Src-Gag chimera can generate viral particles [44]. Examination of the myristate plus basic motif of HIV-1 Gag reveals that the cluster of basic amino acids forms an amphipathic β-pleated sheet, with nearly all of the positively charged residues oriented on the top surface of the molecule and poised to interact with acidic membrane phospholipids [38,45–47].

2.2.4. Membrane targeting specificity

For some proteins, the myristate moiety provides not only membrane binding, but also membrane targeting functions. For example, when Src or Gag N-terminal sequences are attached to soluble proteins, the chimeras display specific localization to the plasma membrane [39,43]. Conversely, mutation of *N*-myristoylation sites in the yeast Gpa 1p protein or in HIV-1 Gag redirect the proteins to intracellular membranes, implying that myristate participates in plasma membrane targeting [48,49]. In Mason–Pfizer monkey virus, virion cores are assembled in the cytosol and myristoylation of Gag is required for intracellular transport to the plasma membrane [50]. How myristate plus basic motifs mediate plasma membrane targeting specificity is not known. It is possible that the plasma membrane contains higher concentrations of acidic phospholipids, compared to intracellular membranes, or that these phospholipids are enriched in plasma membrane microdomains. Alternatively, additional protein–protein interactions may occur at the plasma membrane that serve to preferentially enhance the binding of certain *N*-myristoylated proteins to the plasma membrane (Fig. 1).

2.3. Regulation of membrane binding of N-myristoylated proteins

2.3.1. Alteration of myristoylation levels

The amide bond that links myristate to the N-terminal glycine is relatively stable, and in general the half-life of myristate on a protein is equivalent to the half-life of the polypeptide chain backbone [51]. However, demyristoylation of the MARCKS protein in brain [52] and of a 68 kDa protein in *Dictyostelium discoideum* [53] has been described. Experiments in yeast have documented the existence of a nonmyristoylated pool of Gpa 1p [54]. It is not known how pools of nonmyristoylated proteins are created and maintained.

2.3.2. Myristoyl switches

The orientation of the myristoyl moiety relative to the protein to which it is attached is not always static. Some *N*-myristoylated proteins exist in two conformations. In one conformation, the myristate moiety is sequestered in a hydrophobic pocket within the protein. In the alternate conformation, myristate is flipped out and becomes available to participate in membrane binding. The transition between these two states is regulated by a mechanism known as the 'myristoyl switch'. Proteins that are subject to a myristoyl switch typically exhibit reversible membrane binding (Fig. 2). The triggers for the switch fall into three general categories: ligand binding, electrostatics, and proteolysis.

2.3.3. Myristoyl–ligand switches

Two of the best examples of ligand mediated myristoyl switch proteins are recoverin and Arf (ADP ribosylation factor). For both of these proteins, there is ample biochemical as well as structural evidence to substantiate a myristoyl switch mechanism of reversible membrane binding. Recoverin is a calcium binding protein in the retina that inhibits rhodopsin kinase and thereby regulates the phosphorylation level of photoexcited rhodopsin. In the Ca^{2+}-free state, the myristoyl group is sequestered in a hydrophobic pocket formed by hydrophobic and aromatic residues [55,56]. Binding of Ca^{2+} induces a conformational change within recoverin; the myristate is unclamped and ejected, thereby allowing membrane binding [55]. The residues important for Ca^{2+}-medi-

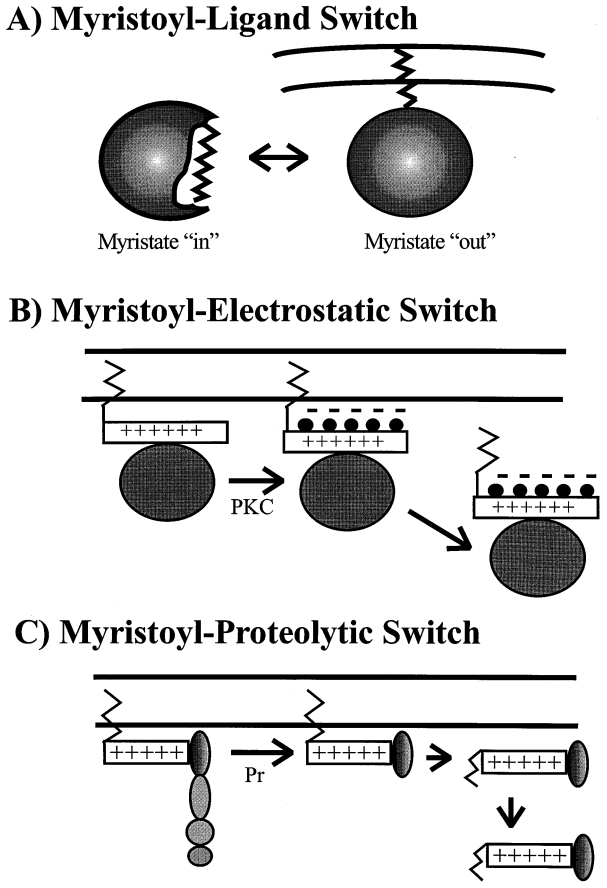

Fig. 2. Myristoyl switches are mechanisms for reversible membrane binding. (A) Myristoyl–ligand switch. Binding of ligand triggers a conformational change that regulates exposure of the myristate moiety. In one conformation, myristate is sequestered within a hydrophobic cleft of the protein. In the alternative conformation, myristate is 'flipped out' and promotes membrane binding. (B) Myristoyl–electrostatic switch. The MARCKS protein is bound to the plasma membrane by a myristate+basic motif. Phosphorylation by PKC within the basic region reduces the electrostatic contribution and results in release of the protein from the membrane. (C) Myristoyl–proteolytic switch. The HIV-1 Gag precursor, Pr55gag, binds to the plasma membrane via a myristate+basic motif. Cleavage of Pr55gag by HIV-1 Protease (Pr) triggers a myristoyl switch that results in formation of the p17MA product, sequestration of myristate, and release of p17MA from the membrane.

ated expulsion of myristate are conserved in other members of the recoverin family and several of these family members have been to shown to display a similar calcium–myristoyl switch. Arf proteins constitute another family of ligand-regulated myristoyl switch proteins in which guanine nucleotide binding regulates the exposure of the myristate moiety [57,58]. In the GDP-bound form, the myristoylated N-terminal helix is cradled in a shallow hydrophobic groove formed by loop λ3 within Arf-1 [59]. Binding of GTP triggers extrusion of loop λ3 and its continuous surfaces, resulting in expulsion of and subsequent membrane binding by the myristoylated N-terminus [60].

2.3.4. *Myristoyl–electrostatic switches*

The MARCKS protein is a protein kinase C (PKC) substrate whose membrane binding is regulated by a myristoyl electrostatic switch [61]. As discussed above, binding of nonphosphorylated MARCKS to membranes is mediated by its myristate plus basic motif. Phosphorylation of MARCKS by PKC occurs within the MARCKS basic domain, resulting in introduction of negative charges into the positively charged region. This reduces the electrostatic interactions with acidic phospholipids and results in displacement of MARCKS from the membrane and into the cytosol [62]. The myristoyl switch and membrane release can be prevented by replacing the N-terminal sequence of MARCKS with that of GAP-43, a dually palmitoylated protein [63]. The GAP43/MARCKS chimera remains at the membrane, even when phosphorylated by PKC, presumably because insertion of two palmitates into the bilayer provides stable membrane association. Fibroblasts expressing the GAP43/MARCKS chimera are impaired in cell spreading, a finding that illustrates the functional importance of the myristoyl switch [64].

An additional example of a myristoyl–electrostatic switch is provided by hisactophilin, a histidine-rich actin binding protein in *Dictyostelium*. Hisactophilin has a cluster of basic residues including seven histidines near the N-terminal myristate. Increasing the pH above 7 titrates the charge of the histidines and triggers a myristoyl-histidine switch which reduces membrane binding affinity [65].

Another potential candidate for a myristoyl-electrostatic switch is Src. The myristate plus basic motif of the Src protein contains sites for phosphorylation by PKC and PKA. Phosphorylation of either site reduces the partitioning of model Src peptides onto lipid bilayers by 10-fold [39]. However, unlike MARCKS, translocation of Src from the membrane to the cytosol upon phosphorylation has not been

consistently demonstrated [39,66]. Since the partition coefficient for monophosphorylated Src is about 10^4 M^{-1} and the accessible lipid concentration in a typical cell is estimated to be 10^{-3} M, most of the monophosphorylated Src would be expected to remain membrane bound [39].

2.3.5. A myristoyl–proteolytic switch

HIV-1 Gag provides the most recent example of a protein whose membrane binding is regulated by a myristoyl switch. Gag is synthesized as a polyprotein precursor (Pr55gag) that is directed to the plasma membrane by a myristate plus basic motif [38]. During virus assembly, Gag is cleaved by the viral protease. The N-terminal cleavage product, p17MA, retains the same N-terminal sequence as Pr55gag, yet p17MA binds weakly to membranes [43,67]. The basis for the differential membrane binding of p17MA compared to Pr55gag has been attributed to a myristoyl switch in which myristate is accessible within Pr55gag but sequestered within p17MA. Cleavage of Pr55gag by HIV-1 protease has recently been shown to be the trigger for the myristoyl switch [68]. Release of p17MA from the membrane is important for viral infectivity, as the matrix protein forms a component of the preintegration complex that translocates to the nucleus upon HIV-1 infection [69].

3. Protein palmitoylation

3.1. Enzymology of palmitoylation

3.1.1. Palmitoyl acyl transferase (PAT)

In contrast to *N*-myristoylation, the identity(ies) of the enzyme(s) responsible for protein palmitoylation have not yet been firmly established. This is directly related to the extreme difficulty that investigators have experienced in attempting to obtain adequate amounts of purified PAT protein for analysis. To complicate matters, there are numerous reports documenting the existence of mechanisms for nonenzymatic palmitoylation of proteins. The pros and cons of enzymatic versus nonenzymatic protein palmitoylation will be discussed below.

Two groups have reported partial purification of enzyme activities responsible for palmitoylation of Src family kinases and Gα protein subunits [70,71]. Both PAT's exhibit a preference for myristoylated protein substrates and for palmitoyl CoA over other fatty Acyl CoA substrates. These PATs are membrane bound and the PAT that palmitoylates Gα proteins is enriched in the plasma membrane [71]. Three additional reports have appeared recently in which PATs have been purified to homogeneity. A 70 kDa PAT was described that palmitoylates red blood cell spectrin; it is not known whether other proteins will serve as substrates [72]. Using a nonprenylated *Drosophila* Ras peptide as substrate, a PAT activity was purified and its cDNA cloned from Bombyx mori [73]. This PAT consists of two polypeptides that both contain structural features found in fatty acid synthase, an enzyme that catalyzes palmitate synthesis. Gelb and coworkers also used Ras as a substrate and purified a PAT that transfers palmitate from palmitoyl-CoA to C-terminal cysteines in H-Ras [74]. Further purification and sequencing revealed that the PAT activity was catalyzed by thiolase A, an enzyme that participates in the terminal steps of β-oxidation of fatty acids [75]. Since thiolase is localized in organelles (mitochondria and peroxisomes) and typically catalyzes hydrolysis rather than transfer of palmitate, its relevance for palmitoylation of plasma membrane bound Ras is not yet apparent.

The sulfhydryl group of cysteine is a good nucleophile and so it is not surprising that under appropriate conditions one can observe nonenzymatic palmitoylation of proteins (Gα) and peptides (c-Yes) in vitro [76,77]. Many of the characteristics of nonenzymatic palmitoylation are similar to those expected for PAT, including a dependence on time, substrate concentration, and the presence of myristate on the protein or peptide substrate. However, two lines of evidence argue against a nonenzymatic mechanism for protein palmitoylation in vivo. First, the reaction kinetics for nonenzymatic acylation appear to be too slow to account for rapid palmitoylation of signaling proteins. Transacylation of Gα subunits in vitro occurs with a time scale of tens of minutes. Estimates for spontaneous acylation of proteins in vivo are in the tens-of-hours range. This estimate is based on the existence of acyl CoA binding proteins that sequester long chain fatty acyl CoAs and severely reduce their intracellular concentration and therefore the rate of nonenzymatic transfer to proteins [78]. Second, se-

quence specificities for protein *S*-acylation exist that are difficult to rationalize with a nonenzymatic acylation process. For example, hemagglutinin (HA) and hemagglutinin esterase fusion (HEF) proteins are plasma membrane bound glycoproteins from the same virus (influenza). In mammalian cells, HA is palmitoylated, whereas HEF is primarily modified with stearate. Replacement of the C-terminal tail of HEF with that of HA results in a chimeric protein that is palmitoylated. Moreover, when HEF is expressed in insect cells it is mostly palmitoylated, despite the fact that stearoyl CoA is present in the insect cells [79]. These findings of acyl chain selectivity cannot readily be explained by an uncatalyzed mechanism. Studies with the Fyn tyrosine kinase also indicate a clear difference between nonenzymatic palmitoylation in vitro and the palmitoylation reaction occurring in cells. A Fyn mutant in which cysteines 3 and 6 are changed to serine can be palmitoylated nonenzymatically in vitro (A. Wolven and M.D. Resh, unpublished results), but little or no palmitoylation of this mutant occurs in vivo [51,80]. Mutation of the same cysteines to alanine residues blocks palmitoylation of Fyn in vivo and in vitro. This implies that nonenzymatic fatty acylation of Fyn does not occur to any significant extent in vivo. Taken together, the available evidence strongly suggests that PATs exist, but in the absence of definitive identification, it remains possible that nonenzymatic mechanisms may contribute to fatty acylation of certain proteins.

3.1.2. Palmitoyl protein thioesterases

Two thioesterases, PPT1 and APT1, have been identified that deacylate Ras and Gα proteins in vitro. PPT1 is unlikely to mediate depalmitoylation of plasma membrane bound proteins in vivo as it is localized to the lysosome [81]. Further analysis revealed that PPT1 is mutated in a specific lysosomal storage disease, infantile neuronal ceroid lipofuscinosis. Individuals with PPT1 mutations accumulate small lipid thioesters that are the likely substrates for PPT1 [82]. A related palmitoyl thioesterase, PPT2, was recently described [83]. PPT2 will only remove palmitate from palmitoyl CoA, not from palmitoylated protein substrates. In contrast, Duncan and Gilman have purified an acyl protein thioesterase (APT1) that depalmitoylates Gα subunits and Ras in vitro and Gαs in vivo [84]. Interestingly, APT1 had originally been identified by others as a lysophospholipase, although the enzyme clearly prefers palmitoylated protein substrates over lipid substrates. It is likely that additional palmitoyl protein thioesterases exist and further studies will be needed to elucidate the substrate specificities of these enzymes for different classes of palmitoylated proteins.

3.1.3. Substrates

3.1.3.1. Fatty acids. The preferred substrate for PAT appears to be palmitoyl CoA [70,71]. However, several 'palmitoylated' proteins have also been shown to incorporate other long chain fatty acids, including stearate, oleate and arachidonate. Thus, the term '*S*-acylation' provides a more accurate description of this type of fatty acid modification [85]. For example, base hydrolysis of total heart and liver proteins reveals the presence of detectable levels of thioester linked 14:0, 18:0, 18:1, 18:2 fatty acids in addition to C16:0 [13]. Gα subunits [86], myelin [87], P-selectin [88], asialoglycoprotein receptor [89] and various platelet proteins [90] have been shown to heterogeneously *S*-acylate with stearate and arachidonate. Whether acylation with different fatty acids affects protein function has not yet been determined.

3.1.3.2. Protein sequences. A previous review [1] categorized palmitoylated proteins into four types (Table 2). Type I sequences consist of plasma membrane receptors and other membrane proteins that are typically palmitoylated on one or several cysteine residues located adjacent to or just within the transmembrane domain. Both the length and composition of the transmembrane sequences as well as the length of the cytoplasmic tail appear to influence the choice of fatty acid for acylation. Proteins with short basic tails are modified with stearate, while those with longer tails are primarily palmitoylated [91–93]. Type II proteins include members of the Ras family that are modified with a farnesyl moiety in their C-terminal CAAX box. Prenylation of Ras is required for subsequent palmitoylation of cysteines in the C-terminal region [94]. A third group of proteins (Type III) are palmitoylated at cysteine residues near the N- or C-

termini. Type IV proteins are dually fatty acylated with myristate and palmitate and nearly all (the exceptions being eNOS and AKAP18) contain the consensus sequence Met–Gly–Cys at their N-termini. Myristoylation of Gly-2 facilitates palmitoylation of Cys-3, but the requirement for myristate can be bypassed by targeting the protein to the plasma membrane via other means [95,96].

Table 2
Palmitoylated protein sequences

Type I: Transmembrane, integral or peripheral membrane proteins		
	TGFα	... EKPSALLKGRTA**CC**HSETVV
	Transferrin receptor	...KANVTKPKR**C**SGSICYGT...
	P-Selectin	...KDDGK**C**PLNPHS
	CD4	...GIFF**C**VR**C**RHRRRQ...
	CD36	MG**C**DRN**C**..... **C**A**C**RSKTIK
	Caveolin-1	...VP**C**IKSFLIEIQ**C**ISRVYSIYVHTF**C**DPFF...
	Band 3	...FTGIQII**C**LAVLWVV...
	SNAP-25	...LGKF**C**GL**C**V**C**P**C**NKLKSSDA...
Viral proteins		
	Influenza HA, H1 subtype	...M**C**SNGSLQ**C**RI**C**I
	Sindbis virus E2 protein	...PTSLALL**CC**VRSANA
	Sindbis virus 6K protein	...R**CC**SC**C**LPF
	VSV G protein	...RVGIHL**C**IKLKHTKK...
	HIV-1 gp160	...DDLRSL**C**LFSYHRLRD...
	Rift valley fever virus G2	...FSSIAII**C**LAVL...
Seven transmembrane receptors		
	α2A adrenergic receptor	...RRAFKKIL**C**RGDRKRIV
	β2 adrenergic receptor	...FQELL**C**LRRSSLK...
	Dopamine D1 receptor	...LGCYRLCPAT...
	LH/hCG receptor	...LLSRFG**CC**KRRAELYRRK...
	Endothelin A receptor	...FQS**C**L**CCCC**YQSKS...
	Endothelin B receptor	...KRFKNCFKSCL**CC**W**C**QSFEE...
	Vasopressin receptor	...SVSSELRSLL**CC**ARGRTPPS...
	Neurokinin B receptor	...RAGFKRAFRW**C**PFIQVSSYD...
	Serotonin receptor	...HKLIRFK**C**TS
	Somatostatin receptor 5	...FRQSFQKVL**C**LRKGSGAKDA...
	Rhodopsin	...MVTTL**CC**GKNPLGD...
Type II: Prenylated, palmitoylated proteins		
	H-Ras	...SGPG**C**MS**C**KCVLS
	N-Ras	...GTQG**C**MGLPCVVM
	K-Ras(A)	...TPG**C**VKIKKCVIM
	Paralemmin	...DMKKHR**C**K**C**CSIM
Type III: Palmitoylation within an N-terminal or C-terminal region		
Gα subunits		
	αs	MG**C**LGNSKTEDQRNE
	αq	MTLESIMA**CC**LSEEAKEA
	α12	MSGVVRTLSR**C**LLPAEAG
	α13	MADFLPSRSV**C**FPG**C**VLTN
	α16	MARSLRWR**CC**PW**C**LTEDEKAA
GAP43		ML**CC**MRRTKQVEKNDDDQKIEQDGI
Ca^{2+} channel β2a subunit		MQ**CC**GLVHRRRVRV
PSD-95		MD**C**L**C**IVTTK KYRYQDEDTP
RGS4		M**C**KGLAGLPA S**C**LRSAKDMK
GRK6		...FSRQD **CC**GN**C**SDSEE ELPTRL

Table 2 (*continued*)

Type IV: Myristoylated, palmitoylated proteins	
Src family tyrosine kinases	
Yes	MG**C**IKSKEDKGPAMKY
Fyn	MG**C**VQ**C**KDKEATKLTE
Lyn	MG**C**IKSKRKDNLNDDE
Lck	MG**C**V**C**SSNPEDDWMEN
Hck	MG**C**MKSKFLQVGGNTG
Fgr	MG**C**VF**C**KKLEPVATAK
Yrk	MG**C**VH**C**KEKISGKGQG
Gα subunits	
αi1	MG**C**TLSAEDKAAVERS
αo	MG**C**TLSAEERAALERS
αz	MG**C**RQSSEEKEAARRS
AKAP18	MGQL**CC**FPFSRDEGK
eNOS	MGNLKSVGQEPGPP**C**GLGLGLGLGL**C**GK

Palmitoylated cysteine residues are in bold and underlined.

3.2. *The role of palmitoylation in membrane binding and plasma membrane targeting*

The function of the palmitate moiety in fatty acylated proteins has primarily been inferred by mutating the modified cysteine residue(s) to serine or alanine and observing the behavior of the mutant protein. While mutational analysis is extremely useful, the possibility remains that an observed phenotype may be due to the loss of the cysteine residue per se, rather than loss of the palmitate [2–4].

3.2.1. *Dually fatty acylated proteins*

The myristate plus palmitate two-signal motif encoded by the Met–Gly–Cys sequence is both necessary and sufficient to direct Src family kinases as well as Gα proteins to the plasma membrane [51,80,97–101]. Mutation of Gly-2 and/or Cys-3 reduces or eliminates membrane binding and plasma membrane targeting. Moreover, attachment of the N-terminal sequences of Fyn, Lck or Gαi to heterologous cytosolic proteins redirects the chimeras to the plasma membrane. The biochemical basis for membrane binding by the myristate plus palmitate signal is clear: two fatty acids are better than one [102]. This explanation also pertains to dually palmitoylated proteins that are not myristoylated but contain two sites for palmitoylation (e.g., GAP43 and certain Gα subunits), as well as palmitoylated, farnesylated proteins such as H-Ras [103].

An excellent model has recently been advanced to account for the ability of palmitoylated proteins to be specifically targeted to the plasma membrane. The 'kinetic bilayer trapping' hypothesis [102] proposes that proteins with a single lipophilic group (myristate or farnesyl) transiently interact with multiple intracellular membranes. Acylated protein that reaches the plasma membrane will be rapidly palmitoylated by a plasma membrane bound PAT and remain stably attached to the plasma membrane because of the strong hydrophobicity and the slow kinetic off rate of the dual fatty acyl anchor. Several lines of evidence support this model. First, a PAT that palmitoylates Gα subunits has been shown to be enriched in the plasma membrane [71]. Second, myr–gly–cys-containing peptides, as well as farnesylated N-Ras peptides, are specifically palmitoylated at and remain bound to the plasma membrane [104,105]. Third, the N-terminal myr–gly–cys motif has been shown to promote rapid plasma membrane targeting of the Src family kinase Fyn. Mutation of the Cys-3 palmitoylation site reduces the rate of membrane binding and redirects the protein to intracellular membranes [51,98].

Proteins that are acylated with two or more palmitates rather than myristate plus palmitate, appear to take a different route to the plasma membrane. For example, SNAP25 and GAP43 require nearly 20 min for palmitoylation and plasma membrane binding [106]. Unlike Fyn, which goes directly to the plasma membrane within 2–5 min after biosynthesis, GAP43 and SNAP-25 travel through the secretory

pathway [106]. Replacement of the N-terminal SH4 motif of Fyn with the N-terminal 10 amino acids of GAP43 results in a chimeric protein with slow membrane binding kinetics [98]. These results indicate that at least two different pathways operate for membrane targeting and plasma membrane binding of palmitoylated proteins.

3.2.2. *Targeting of palmitoylated proteins to plasma membrane rafts*

It is now recognized that rather than being a vast continuous 'sea' of lipid, the plasma membrane contains discrete microdomains enriched in specific types lipids and proteins. There has recently been much interest in microdomains which are referred to as 'rafts'. Rafts are characterized by a relative resistance to extraction with cold, nonionic detergents, enrichment in sphingolipids and cholesterol, and the presence of GPI-anchored proteins. They have also been called DRMs, detergent resistant membranes, or DIGs, detergent insoluble glycosphingolipid enriched membranes and the absolute distinction among and between these terms has been reviewed [107,108]. Several recent reports have confirmed that small submicron domains do exist at the surface of living cells and that these rafts contain GPI-anchored proteins [109–111].

Lipids in rafts appear to be present in a liquid ordered phase, and primarily contain saturated fatty acyl chains [108]. Many proteins that are acylated with saturated fatty acids such as myristate and palmitate have been reported to be localized to raft-like membranes. However, these proteins are localized to the cytoplasmic surface of the plasma membrane, and the inner leaflet of the plasma membrane bilayer has a different lipid composition compared to the outer leaflet. Myristoylated and palmitoylated proteins such as the Src family kinases, eNOS, and Gα subunits appear to be enriched in membrane microdomains known as caveolae [112,113]. Caveolae are 50–70 nm microinvaginations of the plasma membrane that are detergent resistant and enriched in a marker protein, caveolin.

The mechanism responsible for accumulation of dually acylated proteins to microdomains has not yet been elucidated. Kinetic studies have established that targeting of dually acylated Src kinases to the plasma membrane occurs first, followed by a slower partitioning to detergent-resistant microdomains [98]. Palmitoylation is required for localization of myristoylated Src kinases, eNOS and Gα proteins to caveolae, whereas myristoylation alone appears to be insufficient. One could speculate that insertion of the dual fatty acyl anchor into a liquid ordered lipid domain would be energetically favorable [114], and that this would drive partitioning of myristoylated, palmitoylated proteins into caveolae. This hypothesis is supported by the findings that dually acylated proteins, which prefer the liquid ordered phase, are enriched in rafts, whereas prenylated proteins, which have a bulky branched structure that does not partition well into the liquid ordered phase, are excluded from rafts [115] Alternatively, protein–protein interactions with caveolin could drive fatty acylated proteins into caveolae. Caveolin is a palmitoylated protein, but palmitoylation is not required for its localization to caveolae [116]. Instead, palmitoylation of caveolin appears to facilitate its interaction with Gαi subunits [101]. Interactions between caveolin and eNOS (see below) and between caveolin and Fyn [117] have also been reported. Caveolin appears to provide a link between activated integrins and Fyn, and serves as a mechanism for propagating integrin-mediated signaling [117].

The functional significance of protein localization to rafts is best illustrated in the case of T cells. Many of the critical signaling components involved in T-cell receptor-mediated signaling are localized to rafts, including the Src family kinases Fyn and Lck and the palmitoylated, transmembrane protein LAT [118]. Activation of the T-cell receptor results in recruitment of other signaling molecules to rafts, including the zeta chain of the T-cell receptor, ZAP-70, Vav, and PLCγl [119,120]. Disruption of raft structure by agents such as filipin or nystatin also disrupts early steps of T-cell receptor activation. Likewise, mutation of the palmitoylation sites within LAT prevents LAT partitioning to rafts, and reduces LAT tyrosine phosphorylation as well as recruitment of LAT-binding proteins such as Vav and PLC-γl to rafts [121]. These findings indicate that accumulation of tyrosine phosphorylated T-cell proteins in rafts is important for T-cell receptor-mediated signal transduction.

An additional function of raft localization appears to be to increase local protein concentrations in order to facilitate protein–protein interactions. For ex-

ample, myristoylation and palmitoylation of Fyn serves to localize Fyn to plasma membrane rafts and to position its SH2 and kinase domains in close proximity to the zeta chain of the T-cell receptor. Phosphorylation of the zeta chain by Fyn occurs, followed by an interaction between the Fyn SH2 domain and tyrosine phosphorylated residues in the ITAM motifs of the zeta chain [122]. A chimeric Fyn molecule which is plasma membrane bound, but not associated with rafts, is impaired in interacting with the zeta chain. Likewise, a transmembrane version of Lck, that is plasma membrane bound, but not fatty acylated and not raft associated, is impaired in transducing some of the late events in T-cell receptor-mediated signaling [123]. Rafts may therefore serve a scaffolding function for organizing signaling molecules into effective macromolecular signaling complexes.

3.2.3. *Membrane localization by palmitoylation can be replaced by other membrane targeting signals*

Although protein palmitoylation does play a key role in raft localization for some proteins, there are numerous examples where the membrane binding function of palmitate can be replaced by another membrane targeting signal. For example, myristoylation can substitute for palmitoylation in conferring membrane association and transforming activity to the oncogenic $G\alpha_{12}$ subunit [124]. The hormone responsive activity of nonacylated Gαz can be restored by coexpression with βγ subunits. Presumably the prenyl group on the γ subunit provides the hydrophobicity required for membrane association and signaling by an associated α subunit [96]. Fusion to transmembrane proteins can also restore membrane binding and signaling. For example, fusion of the nonpalmitoylated Gαi subunit to the α2A-adrenergic receptor restores membrane binding and agonist mediated signaling [125]. Likewise, when nonpalmitoylated Lck is fused to the transmembrane protein CD16:7 the chimera can support T-cell receptor-mediated increases in tyrosine phosphorylation and Ca^{2+} flux, although transcriptional activation and activation of MAP kinase are reduced [123].

3.2.4. *Regulated depalmitoylation*

It is well established that activation of Gαs through stimulation of the β-adrenergic receptor or by mutation results in increased palmitate turnover [126–128]. However, the functional significance of this event has been a source of controversy. The following model attempts to incorporate the currently available information. Activation of G protein leads to dissociation of the αs subunit from βγ and depalmitoylation of αs by a thioesterase [128,129]. Following GTP hydrolysis, αs reassociates with βγ and is rapidly repalmitoylated. Quantitative analyses of the stoichiometry of αs palmitoylation reveals that the overall percentage of Gαs that is palmitoylated before and after receptor stimulation does not change [130]. What is the consequence of this increased palmitate turnover? Some investigators have observed translocation of Gα subunits from the membrane to the cytosol upon receptor activation [128,131], while others maintain that Gα subunits remain membrane bound but become concentrated in plasma membrane subdomains [132]. Further studies will be needed to clarify the meaning of these different observations.

Another example of a protein whose palmitoylation levels are regulated is the endothelial form of nitric oxide synthase (eNOS). eNOS is a myristoylated, palmitoylated protein that is localized to caveolae and interacts with caveolin [133,134]. In resting cells, caveolin-bound eNOS is inactive. Treatment with agonists or intracellular Ca^{2+} mobilizing agents results in dissociation of eNOS from caveolin and caveolae and enzyme activation [135]. Agonist treatment also stimulates depalmitoylation of eNOS [136]. Since dual fatty acylation is required for localization of eNOS to caveolae, it is tempting to link the depalmitoylation event with the change in eNOS localization and its interaction with caveolin. However, the situation is apparently more complex, as nonacylated eNOS can still interact with caveolin [137] and may remain membrane bound as well [138].

4. Conclusions and perspectives

It is now clear that myristoylation and palmitoylation influence a wide spectrum of structural and functional features of proteins. This review has primarily focused on the role of fatty acylation in promoting membrane binding and targeting. Myristoyl-

ated proteins require a second signal for stable membrane binding, and this signal is provided by a polybasic domain or palmitate. For palmitoylated proteins, the situation is more complex. Transmembrane proteins do not require palmitoylation for membrane association and palmitoylation generally exerts more subtle effects on the structure and function of these integral membrane proteins. Many palmitoylated proteins are associated with membrane microdomains ('rafts') and partitioning of palmitoylated proteins into rafts has emerged as an important aspect of signaling in T cells. Finally, it is important to remember that protein–protein interactions also contribute to membrane binding and targeting of fatty acylated proteins. Moreover, fatty acid insertion into cellular membranes facilitates protein–protein interactions and allows the lipid bilayer to serve as a molecular scaffold for organization of membrane-bound signaling complexes.

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