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Protein lipidation meets proteomics

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1. ABSTRACT

Protein lipidation is a crucial protein modification involving the attachment of hydrophobic carbon skeletons (C:14-C:60) of various lipid classes — fatty acids, sterols, glycero-, phospho- and glycolipids. The lipid-protein bond frequently (i) involves the N- or C-terminal ends of the target, (ii) requires amide, ether or ester bonds to a small aminoacid, usually Gly or Cys and (iii) depends on proteolytic events. Lipidation results in protein targeting to the membrane, with the protein behaving as a peripheral component and being oriented toward the inside or outside of the cell. The addition of a single lipid is not sufficient for membrane targeting and another signal, often involving additional lipidation, is required. The methods available for predicting lipid modifications to proteins highlight the importance of identifying short protein motifs in a field in which few data are currently available, due to the complex nature of the modification. Full proteome annotation is already feasible with these predictive tools. We show that lipidation may affect 2-4% of all proteins in a given proteome and that double-lipidation is widespread.

2. INTRODUCTION

Posttranslational modifications are known to expand the amino-acid code of proteins from the 20 to 22 natural amino acids to several hundreds (>300) of different building blocks (1). Posttranslational modifications have diverse functions but are widely recognized to be major modulators of protein activity, half-life and subcellular distribution. The study of posttranslational modifications is an expanding field within proteomics, thanks to a combination of the most recent developments in mass spectrometry and genomics (2). The analysis of posttranslational modifications is generally considered challenging. Some classic posttranslational modifications, such as phosphorylation, ubiquitinylation and acetylation, can now be examined with dedicated proteomic methods (3), but some modifications remain poorly described and predictive analyses are required to determine the likelihood of their occurrence in a protein. This is the case for lipid modifications, which result in association of the modified protein with the membrane. Membrane proteins may be integral or peripheral. Integral proteins span the membrane, interacting strongly with it through at least one

hydrophobic transmembrane α-helix. The insertion of integral proteins in the membrane often involves the secretion pathway and requires an N-terminal signal peptide. If this signal peptide is not cleaved by signal peptidase, its hydrophobic nature may also contribute to membrane binding. Integral proteins do not require posttranslational modifications for their anchoring in the membrane. Peripheral membrane proteins have no hydrophobic domains and interact with the membrane via only one domain. This interaction may involve amphipathic helices, hydrophobic loops and electrostatic interactions with the negatively charged lipid moieties of the membrane, or even protein-protein interactions. Lipidation is another interesting posttranslational modification, facilitating the anchoring in the membrane of various peripheral proteins. The non-covalent link formed between a typical membrane component and a functional protein appears to be particularly suitable for peripheral proteins with enzyme activity, as it ensures a high level of mobility and facilitates correct interactions with protein partners, which may not necessarily be either membrane-bound or hydrophobic. Moreover, the protein, although attached to the membrane, is in solution in either the intracellular or the extracellular compartment, ensuring its full activity and interaction with diffusive effectors, such as ATP or hormones. This review focuses on lipid modification, including, in particular, the methods used to predict its occurrence. The nature of the protein targets is not discussed in this review.

3. LIPID MODIFICATION OF PROTEINS

Protein lipidation involves the bonding of lipids to target proteins via a small number of different types of chemical bond. It results in the addition of between 210 Da (Myr) to more than 1300 Da (GPI-anchor) to the mass of the protein. The amino acids involved in covalent bonding are usually small, with Cys and Gly the most frequent (Figure 1). The lipid moiety is bound to the protein through thioether, amide or ester bonds (Figure 1). Most of the various types of natural lipids are found, from the simplest (fatty acids) to the most complex, in which two fatty acids are associated with a backbone. The addition of fatty acids is the most frequent type of lipid modification. Eukaryotic proteins display the largest diversity of lipid modifications, although such modifications also occur in bacteria.

3.1. Types of lipidation

Protein lipidation usually affects either the N- or C-terminal part of the protein, leaving the rest of the protein unconstrained. This enables the three-dimensional structure to fold freely, with the lipid moiety simply anchoring the protein to the membrane, attaching the protein to a given position or subdomain of the cell. If the protein is able to reverse membrane binding, then the three-dimensional fold remains the same. Finally, double lipidation is frequently observed close to the N- or C-terminus (N-Myr or C-Pre plus S-Palm) or at both ends of the molecule (N-Palm and C-cholesterol addition). Lipidation at both ends of the molecule is likely to result in significant restraint on the structure and mobility of the protein.

3.1.1. N-terminal lipidations

The N-terminal myristoylation (N-Myr) of proteins results in the irreversible addition of a saturated C:14 fatty acid to the free N-terminal Gly residue of some eukaryotic and viral proteins. Protein palmitoylation is the reversible addition of a C:16 fatty acid to the side chain of a Cys residue, via a thioester. N-terminal palmitoylation (N-Palm) has been described in the extracellular proteins “Hedgehog” and “Spitz” (4). These modifications have been described in both eukaryotic and viral proteins. Bacterial proteins can also undergo N-terminal lipidation. This process involves both N-Palm and the addition of diacylglycerol to the side chain of an N-terminal Cys residue (Figure 1). It results in di- or tri-acylated proteins, as observed in early studies with the outer membrane protein Omp (5) or with Toll-like receptors (6, 7). Palmitoyl acid seems to be the major fatty acid involved, but myristoyl and stearoyl acids have been implicated in some cases (8, 9).

3.1.2. C-Terminal lipidations

C-terminal lipidations have been described only in eukaryotes. C-terminal prenylation (C-Pre) involves the addition of a non-linear unsaturated lipid (Figure 1), a farnesyl (C15:3) or geranylgeranyl group (C20:4), via a thioether bond, to a Cys located at or in the immediate vicinity of the C-terminal end of a protein (see below). The addition of cholesterol to the C-terminus has also been observed in proteins such as “Hedgehog”. Phosphatidylethanolamine-mediated fatty acylation (Figure 1) has been described in the autophagy protein Apg8. It requires the presence of a Gly residue at the C-terminus after the removal of the genuine C-terminal Arg residue (10). The fatty acids involved are saturated or monounsaturated: palmitic or stearic acid. This modification is sufficient for tight anchoring to the membrane (11). Finally, as shown in Figure 1, the core structure of the GPI anchor — the most complex lipid modification discovered to date — involves two phospholipids, phosphoethanolamine and diacylglycerophosphate, linked by a sugar pentamer. The GPI anchor may undergo additional, transient modifications, depending on the organism, and during biosynthesis and transfer from the endoplasmic reticulum to the membrane via the Golgi apparatus (12). The possible modifications include transient O-palmitoylation of the 2-OH moiety of inositol, preventing cleavage by phospholipase C. The addition of phosphoethanolamine to α-mannoses 2 and 3 is also observed.

3.1.3. Internal lipidations

S-Palmitoylation (S-Palm) occurs on internal Cys residues in eukaryotes. N*S*-Palm and C*S*-palm correspond to the palmitoylation of a Cys side-chain located in the vicinity of an N-Myr or C-Pre residue (13). The process is thought to depend on the initial lipidation. Truly internal S-Palm also occurs, generally involving a Cys residue located next to a transmembrane domain or on the cytosolic side of an integral membrane protein (14). Finally, together with S-Palm on a conserved Cys residue, the Wnt growth factor undergoes O-Palm (C16:1, see Figure 1) of the side-chain of a conserved Ser residue (15). This appears to be a second

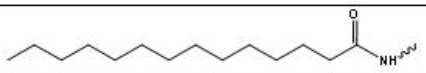
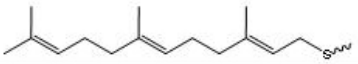
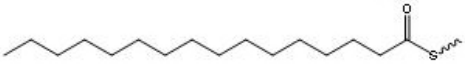
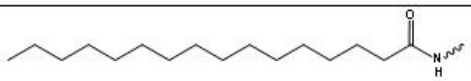
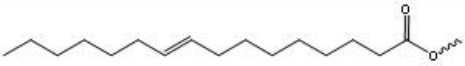
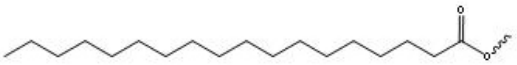
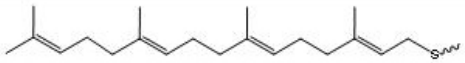
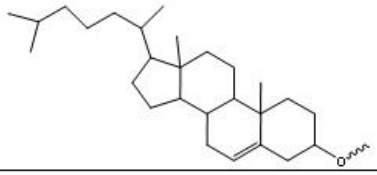
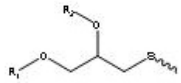
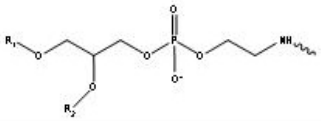
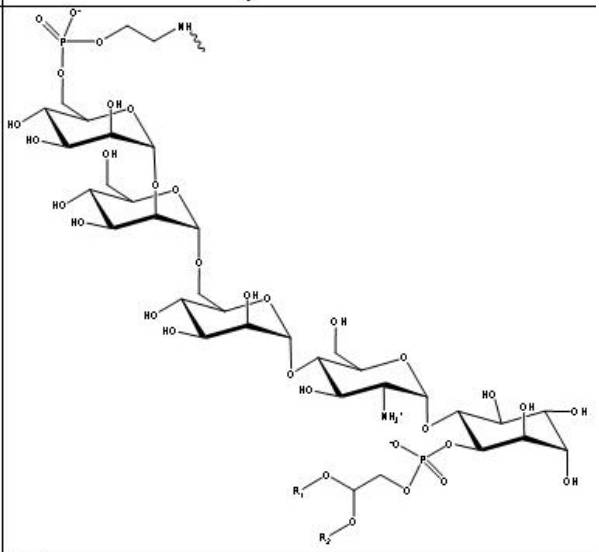
Lipid moiety type	Protein bond	Chemical structure
Myristoyl-N (C14:0)	Nt-Gly (amide)	
Farnesyl-S (C15:3)	Cys-S (thioether)	
Palmitoyl-S (C16:0)	Cys-S (thioester)	
Palmitoyl-N (C16:0)	Nt-Cys (amide)	
Palmitoyl-O (C16:0 or C16:1)	Ser-O, R_n (ester)	
Stearoyl-O (C18:0)	R_n (ester)	
Geranylgeranyl-S (C20:4)	Cys-S (thioether)	
Cholesterol-O (C27:1)	Gly-Ct (ester)	
Glycerolipid-S (C35:0)	Cys-S (thioether)	
Phosphatidylethanolamine or cephalin (C39:0)	Gly-Ct (amide)	
Glycosylphosphatidylinositol or GPI (C60:0), conserved core	ω-Ct (amide) ω=Ala, Asp, Asn, Cys, Gly or Ser	

Figure 1. The various lipid modifications occurring in proteins. The various types of protein lipidation are classified from the smallest to the largest. The size of each lipidation is determined by the number of carbon atoms involved. The chemical structures are shown. The type of bond to the protein is indicated in brackets and symbolized as a special bond in the chemical structure without any link. The number of unsaturated bonds is indicated. Nt, is N-terminal; Ct, is C-terminal

case of O-acylation, as the addition of C8:0 to the side-chain of the Ser3 residue of ghrelin, a 28-amino acid growth hormone, has also been observed (16).

3.2. Modification mechanisms and the enzymes involved

Myristoyl-CoA:protein *N*-myristoyltransferase (NMT) catalyzes the transfer of myristate from myristoyl-CoA to a number of proteins (for a complete review on N-Myr, see Ref.(17)). There are two NMTs in higher eukaryotes and one in fungi and insects (18). NMT was thought to be specific to eukaryotes, but a similar activity has been detected in the bacterium *Pseudomonas aeruginosa* (19). N-Myr requires an N-terminal Gly residue and therefore cannot occur in proteins that have retained their genuine N-terminal Met residue. Two processes are known to be involved in protein processing to generate an appropriate substrate for NMT. N-terminal Met excision is the classical pathway responsible for early cleavage of the first Met residue (20). This process involves Met aminopeptidase activity; this enzyme has a substrate specificity allowing cleavage only if the side-chain of the second residue is small enough (21). All proteins with a Gly residue in position 2 are therefore appropriate substrates for N-Myr, although only a few of these proteins are eventually modified in this way (see below). The second process depends on proteolytic activities and allows secreted bacterial proteins to re-enter the eukaryotic host cell. Similar process may also apply to some viral proteins after proteolytic cleavage and processing of the precursor protein (22). If host- or bacterium-directed cleavage results in the exposure of a Gly residue at the N-terminus, then N-Myr may occur. In plants, this process may involve mechanisms of innate resistance to bacterial pathogens (23), such as AvrPphB (24, 25). Several studies in animals have indicated that an N-terminal Gly residue may also be exposed by caspase-mediated cleavage during apoptosis (26-29). Thus, N-Myr occurs both co- and posttranslationally provided that a proteolytic event unmasks an N-terminal Gly residue.

A dedicated palmitoyl *S*-transferase (PAT), Ski, is responsible for N-terminal palmitoylation (4, 30). For this reaction to occur, the signal peptide must be removed to unmask an N-terminal Cys residue. The addition of this lipid involves an initial rearrangement of the protein around the Gly-Cys residues. The thiol group reacts with the peptide bond to generate a transient thioether bond. Nucleophilic attack of the hydroxyl moiety of cholesterol then ensures both ester bonding of the lipid and release of the C-terminal intein autoprocessing domain (31).

N-Palm at an N-terminal Gly residue — rather than the usual N-Myr — has also been reported for the Gαi subunit of a heterotrimeric G-protein, but it remains unknown whether NMT or PAT is involved (32). However, the involvement of NMT is thought to be most likely because this enzyme is known to transfer various lipids (C8:0-C18:0 including C14:1, C14:2) and to allow efficient N-Palm (33-36).

It long remained unclear whether *S*-Palm was catalyzed by an enzyme. Indeed, some of the processes concerned appear to involve autoacylation (14, 37). Nevertheless, two-related PATs with a conserved Asp-His-His-Cys (DHHC, motif CX2CX3[RK]PXRX2HCX2CX2CX4DHHCXW[VI]XNC [IV]GX2NX3F (38)) domain have been described in yeast: Erf2 and Akr1 (39). The existence of other PATs has been suggested (40) and a longer list of these enzymes is now available (see Table 1 in Ref.(41)). Experiments involving the systematic deletion of the seven DHHC PAT genes in yeast have suggested that the members of this family are responsible for most, if not all of the S-Palm events occurring in the cell (42). The pfa4 PAT may be involved in the S-Palm of proteins with a C-terminal FWC motif. In humans, 23 different DHHC PAT genes have already been identified (43).

Bacterial lipoprotein modification occurs in three successive steps and results in the generation of bacterial lipoproteins (44). A diacylglycerol is first added to the side-chain of an internal Cys by a diacylglyceryl transferase. Cleavage by signal peptidase II then unmasks this diacylated Cys residue at the N-terminus of the protein, and the N-terminal diacylated Cys residue is then palmitoylated by a transacylase (Table 1).

Prenyltransferases are responsible for the addition of prenyl groups to the C-terminus of the protein. Three types of heterodimeric enzymes have been described so far (45). Protein farnesyltransferases (PFT) are responsible for the S-farnesylation of a Cys residue located three residues before the C-terminus (Table 1). Geranylgeranyl transferases (GGT) are responsible for the addition of a geranylgeranyl moiety. GGT1 recognizes the same protein motif and has an α subunit in common with PFT. 96% of the substrates of GGT1 are thought to correspond to substrates of PFT. Reciprocally, only 72% of the substrates of PFT are also substrates of GGT1 (see Fig.1 in Ref.(46)). This suggests that PFT has broader substrate specificity than GGT1. GGT2 has unique subunits and recognizes C-terminal motifs including a Cys residue. The five subunits required for the assembly of three different enzymes appear to be encoded by single genes in most organisms. Prenylation is often followed by further posttranslational modifications, including proteolytic cleavage of the last three residues, the Cys becoming the new C-terminal residue (Table 1). Two CaaX proteases with different, but overlapping substrate specificities have been described in the various phyla of the eukaryotic lineage (47-49). O-Methylation of the new C-terminus may also be observed (50).

GPI is transferred to the protein by a membrane enzyme, GPI transamidase (12). The mammalian GPI transamidase consists of five subunits. The GPI8 subunit is probably the catalytic subunit, responsible for both GPI transfer and endoproteolytic cleavage, as it displays sequence similarity to Cys proteases.

To conclude, although there is strong convergence of both the function and the nature of protein modification by lipidation, the various enzymes involved are very heterogeneous in terms of enzyme mechanisms and lipid

Table 1. Short motifs useful for predicting the addition of a lipid to a protein

Lipid modification[1]	Catalyst for lipid transfer	Protein motif to be considered	PROSITE motif	Proteolytic process associated	Ref
N-Myr	NMT	<MG[^CDEKRWY][[^C]X[ACGF*N*RSTV][^DE][2]	PS00008, G[^EDRKHPFYW]X2[STAGCN][^P]	Met or propeptide cleavage	91, 96, 136
C-Pre	PFT GGT1 GGT2	CaaX>[3] CaaL>[3] CC>, CXC>, CCX>, CCXX> or CCXXX>	PS00294, C[^DENQ][LIVM]X	CaaX cleavage	90
N-Palm	PAT-ski	Signal peptide-CGPGR	-	Signal peptide cleavage	4
NS-Palm	DHHC PAT, autoacylation	<MG[C]XX[ACGF*N*RSTV][^DE] or <MG[^CDEKRWY][C]X[ACGF*N*RSTV][^DE]	-	-	96, 136
S-Palm	PAT	C, next to a transmembrane domain C, next to a C-Pre motif	-	-	37
O-Palm	O-acyltransferase (porcupine)	-	-	-	15
Cephalin	Atg7/Atg3, ubiquitin E1/-ligase like	GR>		Atg4 (Cys protease) cleavage	11, 137
Cholesterol	autocatalytic	Signal peptide-GCF	-	intein	4
GPI	GPI transamidase (GPI8 acts as the catalytic subunit)	[ACSND]X[AGS]X7[FILSV]$_9$	-	C-terminal cleavage (15-30 residues), GPI8-induced	12
Glycerolipid	Diacylglycyl transferase, Transacylase	Signal-peptide-C, [ILMFTV]X[AGS]C	PS00013 (no longer existing), [^DERK]6[LIVMFWSTAG]2X[LIVMFWSTAGCG][AGS]C PS51257 (matrix) <MVX13[RK][^DERKQ]6-20[LIVMFESTAG][LVIAM][IVMSTAFG][AG]C	Signal peptide cleavage	7, 98

[1] see Figure 1 for further details.[2] in fungi, the motif is restricted to <MG[^CDEFKRWY][[^C]X[ACGRSTV][^DE][^DE][^DE].
[3] a is aliphatic *i.e.* often I, L, V or M; X is usually A, E, M, S or T. Q was added in the *A. thaliana* search described in the text (see Text).

donor. As a result, the catalysts belong to proteins families in distinct clusters of orthologous groups (KOG, see Ref.(51)).

3.3. The second signal for membrane targeting: involvement of a second lipidation

The addition of a single short fatty acid, such myristoyl or farnesyl acid, to a protein is known to be insufficient to confer stable membrane binding, as it results in a membrane affinity of the order of only 0.1. mM (50). The constants for S-Palm or geranylgeranyl binding to the membrane are 10 times higher. This is probably why double acylation is frequently used to anchor proteins stably in membranes (Figure 2). Alternatively, a polybasic domain or a protein-protein interaction may also anchor the protein stably in the membrane (Figure 2). In either case, the second signal is reversible, whereas the first is not. These mechanisms involve effectors of protein-protein interaction (GTP hydrolysis), acidification by phosphorylation of the polybasic domain or depalmitoylation. An interesting situation is the members of the Ras family which can use the various modes (52).

Acylprotein thioesterases are involved in palmitate recycling (41). Two proteins with such activity have been described to date: APT1, a cytosolic enzyme and PPT1, a lysosomal lipase. Finally, the thioester bond is easily reversible, even in the absence of enzyme activity (31). Acidification by phosphorylation of a polybasic domain participating in membrane attachment was described of a number of N-Myr proteins including the catalytic subunit of the cAMP dependent protein kinase A (53-55). For instance, the Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) proteins may undergo phosphorylation by Protein Kinase C, which abrogates membrane binding (56). Similar mechanism was recently identified in the case of K-Ras, a C-Pre protein (57). Phosphorylation by Protein Kinase C of the polybasic region next to the C-Pre motif causes relocalization of the protein from the plasma membrane to the outer mitochondrial membrane where it induces cell death.

To conclude, most lipidation involve irreversible bonds to the protein targets. Nevertheless, reversible membrane binding in the cell is often ensured through the indirect reversibility of the second signal. As a result, the lipid acts as a key regulator in the intracellular traffic of the protein.

3.4. Functional consequences of protein lipidation

3.4.1. Compartmentalization: membrane and lipid raft binding

Membrane proteins may be integral or peripheral to the membrane. Peripheral proteins are synthesized in the cytosol and targeted to the membrane, to which they are anchored via a lipid. The notion that there are discrete regions of the membrane, each with its own distinct set of

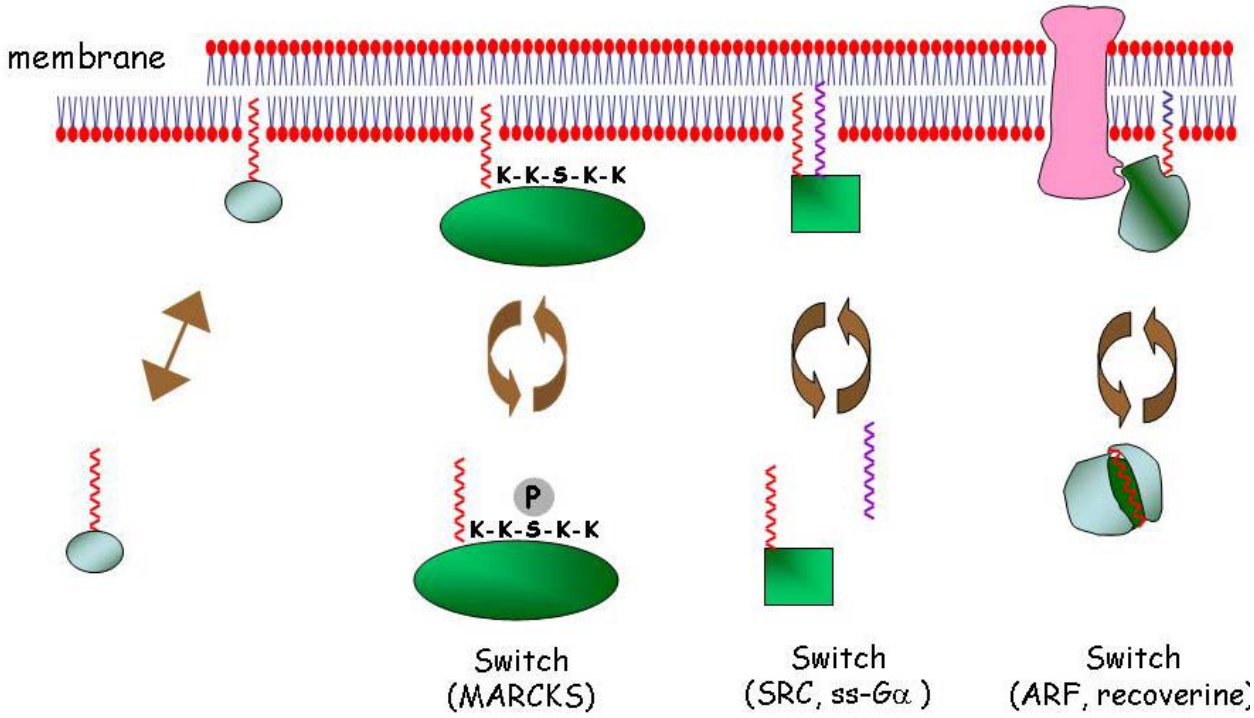


Figure 2. The second signal allows reversible membrane binding. The figures illustrate the three second signals described to date (13). "Switch" refers to a reversible mechanism involving phosphorylation, thioesterase or GTP hydrolysis. Examples of proteins undergoing such switching are indicated in brackets.

lipids and membranes has recently emerged from the fluid mosaic model of lipid organization. These microdomains include the lipid rafts, which can be isolated based on their solubility in nonionic detergents, such as Triton X-100. Several lipidation events may lead to accumulation of the modified protein in lipid rafts. Lipid rafts are small, dynamic and rich in sterol and sphingolipids. GPI anchors are the best known means of binding to lipid rafts (58). Palmitoylation via the O-acylated Wnt-1 growth factor (59) and S-Palm of the death receptor Fas (60) have also been shown to be important for raft anchoring. Generally, both N-Myr and NS-Palm result in binding to lipid rafts in higher eukaryotes (61-63). It has been predicted that 16% of the proteins identified in the plant raft proteome undergo N-Myr or S-Palm (usually both), whereas only 3% have GPI anchors (63).

Membrane networks also include other lipid subdomains. The NS-Palm of Vac8p, an N-Myr protein, has been shown to be crucial for vacuole membrane targeting (64).

3.4.2. Impact of lipid modification at cellular level

The activities of the various PAT enzymes do not seem to be essential and the deletion of up to five genes is feasible in yeast (41). The PAT family is large and includes proteins with overlapping substrate specificities. By contrast, acylprotein thioesterases are essential in humans, and their absence results in neurogenerative disorders. The "ski" PAT is also known to be required for "Hedgehog" signaling (30).

Prenyltransferases are crucial for development but their activity is otherwise not truly essential (65). In the fungus *Candida albicans*, abnormal morphology was the only effect observed when both GGT1 and PFT were inactivated (66). By contrast, these genes have been shown to be essential in the yeast *S. cerevisiae* (67). In mice, PFT was shown to be crucial for embryonic development but dispensable in adults (68). Inactivation of the farnesyltransferase or GGT1 causes only slightly delays development or has mild effects on the hormonal response in the higher model plant *Arabidopsis thaliana* (69). Inactivation of the gene encoding the α subunit common to PFT and GGT1 (resulting in the inactivation of both PFT and GGT1) induces more severe developmental defects, but still has only a limited impact (70). This may be due to the overlapping specificities of the three types of enzyme, with GGT2 able to compensate for the absence of PFT and GGT1. In mice, inactivation of GGT2 leads to a genetic disease known as the Hermansky-Pudlak syndrome with series of defects including partial albinism, and prolonged bleeding (71). In animals, PFT inhibitors have anticancer effects due to their effect on the C-Pre of two small G-proteins (i) the Ras protein (which is absent from plants), a crucial protein in cell cycle progression and (ii) RhoB, a protein with a short half-life (72, 73).

Unlike PFT or GGT1, NMT1 has been shown to be essential in animals, plants, several protists and fungi (74-78). By contrast, the NMT2 gene appears to be redundant in plants at least (75) and possibly in animals (76).

In yeast, inactivation of the GP8 GPI transamidase gene results only in a slow-growth phenotype (79). By contrast, expression of this gene appears to be essential in trypanosomes (80), a class of protozoan parasites with an unusually large amount of GPI-anchored protein at their surface (81).

This review does not aim to detail the cellular functions associated with lipidated proteins. However, it should be noted that lipidated proteins, such as small G proteins and protein kinases, are often key players in signal transduction pathways (18), accounting for the importance of lipid modifications. Among major lipidated proteins, one should necessarily quote the oncoproteins of the Ras family, the Arf regulators of cellular traffic, Rho GTPases, heterotrimeric G proteins, the nuclear lamins, most calcium-dependent protein kinases, the tyrosine kinases of the c-Src family, kinetochore proteins, the heat shock protein DNA J, cGMP phosphodiesterases, protein phosphatases etc… Longer lists of lipidated proteins are available for C-Pre in Table 1 of Refs (82, 83), for N-Myr in Ref.(84) and for GPI-anchors in Table 3 of Ref.(85).

4. PREDICTION OF PROTEIN LIPIDATION

Lipidated proteins are associated with membranes. The investigation of membrane proteomes is challenging in terms of both direct analyses (e.g. biochemical fractionation, proteomic analyses) and *in silico* approaches. Predictive analyses based on bioinformatics have proved to be a powerful technique for membrane proteome analysis, due to the existence of structural features, such as a signal peptide or transmembrane α-helices. For instance, 20 to 30% of the predicted open reading frames of a typical animal or plant proteome are generally predicted to contain at least one transmembrane helix (86). A combination of various criteria, based on both the amino-acid sequence of an open reading frame protein, including the prediction of endoplasmic targeting, and the occurrence of transmembrane helices, led to the suggestion that as many as 25% of the open reading frames of the plant *Arabidopsis thaliana* correspond to potential integral membrane proteins (87). The identification of peripheral membrane proteins, including lipidated proteins, is much more problematic.

4.1. Short Motifs used to predict lipidation: their advantages and limitations

4.1.1. Introducing protein patterns (or motifs) and associated syntax

Protein patterns were introduced early in the development of proteomics, for the identification of proteins with similar functions (88). Motifs also play an important role in the identification of putative lipidated proteins. The pattern syntax used in this manuscript is: "<M" constrains the pattern to the N-terminal residue (*i.e.* an initiator M), "X>" constrains the pattern to the C-terminal residue, "[^Y]" means that residue Y (or a subset list) is excluded and "[Z]" means that residue Z (or a subset list) is included. X indicates any normal amino acid. By definition, the initiator M residue is considered to be M(1). PattinProt http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_pattinprot.html is a useful dedicated search engine making it possible to scan a protein sequence or protein database for one or several patterns.

The PROSITE database compiles a large number of sequence signatures useful for the prediction of lipidation (89). However, a number of these sequence signatures no longer seem to be accurate (Table 1). Below, we will review and update the motifs known to predict lipid modification accurately (Table 1). Some modifications involve too few known targets and can therefore neither be analyzed nor even truly retrieved. N- or C-terminal motifs strongly constrain searches because of their unique position at the extreme end of the protein. This results in these motifs being better predicted than other motifs in less well defined positions in the amino-acid sequence.

4.1.2. Lipidation motifs

The first motif identified was the CaaX motif (90). This motif was defined based on an alignment of known prenylated G-proteins of the Ras family (90). It remains useful for the rapid prediction of prenylation by either PFT or GGT1 (Table 1). GGT2 prenylation can also be predicted with a highly simplified motif comprising two Cys residues (Table 1).

The substrate specificity of *S. cerevisiae* NMT has been studied *in vitro* (91) with various reduced substrates starting with a Gly residue and comprising at least eight residues, the minimal size for this reaction. The consensus motif G-[^EDRKHPFYW]-X2-[ACGNST]-[^P] emerged from analyses of the various peptides that did and did not act as effective substrates (see data summarized in Ref.(92)). Based on this pattern, 71 *S. cerevisiae* proteins have been predicted to undergo N-Myr; however, only a small number of these proteins appear to be genuine substrates, demonstrating the inefficiency of prediction based on this motif (93). The substrate specificity of the two human NMTs differs from that of fungal and protozoan enzymes in several respects (94), reducing the reliability of predictions based on the consensus sequence. Studies of the substrate specificity of the plant enzyme have led to the identification of a new relaxed motif (Table 1 and Ref.(95)). This motif is also useful for the prediction of N-Myr by either NMT1 or NMT2 in animals (75, 96). The yeast enzyme has a more restricted motif, with acidic residues not permitted in remote positions (95).

N-Palmitoylation in the extracellular proteins "Hedgehog" and "Spitz" has been reported to result from S-to-N rearrangement, leading to the attachment of the palmitate moiety to the N-terminal Cys residue at a signal peptide-CGPGR motif (4). Cholesterol is added to the Gly residue in the conserved GCF motif (4, 97). GPI anchors are bound to a new C-terminus located 15 to 30 residues downstream from the genuine C-terminus, within the [ACSND]X[AGS]X7[FILSV]9 motif (the binding site is underlined, see Table 1).

Finally, a very long motif (<MVX13[RK][^DERKQ]6-20[LIVMFESTAG][LVIAM][IVMSTAFG][AG]C) has

been identified as responsible for lipidation in Gram-positive bacteria (98).

4.2. Other motif-based strategies

The low prediction efficiencies of several of the motifs in Table 1 led Eisenhaber's group to propose new tools for predicting the most frequently observed lipid modifications: C-pre (PrePS), N-Myr (Myr predictor) and GPI (big-PI Predictor). These authors propose a method combining the use of sequence motifs identifying the regions in which modifications are likely to take place with physical features involved in enzyme catalyst interactions, amino-acid bulkiness or remote sequences to calculate a score, which is then classified on the basis of a threshold (99-101). These tools are among the most reliable currently available online (see Table 1). These authors also introduced the idea that the features of protein lipidation may be species-specific. For instance it appears that the lipidation enzymes from Lower and Higher Eukaryotes do not share exactly the same specificity. This was clearly demonstrated in the case of NMTs (94, 95, 102, 103). These features are clearly linked to subtle variations in the substrate binding pocket of the various NMTs, as suggested by molecular modeling (95, 100).

Other programs, not necessarily using motifs, but instead based on learning from compiled sequences, can also be used to predict lipidation in bacterial proteins or GPI modification (Table 1).

5. TOWARD WHOLE PROTEOME ANNOTATIONS

Efforts to achieve genome-wide predictions of lipid modification by N-myristoylation (N-Myr), C-prenylation (C-Pre) or glycosylphosphatidylinositol (GPI) anchors have begun, with the aim of identifying peripheral proteomes. The proportion of peripheral proteins modified by lipids is estimated at less than 4%, with the issue of the exact location of these proteins with respect to the membrane yet to be resolved. Other types of peripheral membrane proteins lacking specific features in their amino-acid sequences are difficult to identify through predictive approaches. Mass spectrometry-based proteomics can only rarely provide the information required for *de novo* determination of the occurrence of such modifications. Large-scale proteomic strategies have proved effective for obtaining information about proteolytic events, but have systematically failed to provide information about crucial — often rare or challenging —lipidations, and peptides with such modifications have never clearly been characterized for any protein in large-scale proteomic analyses.

5.1. Proteomic analysis of lipid modifications

It has recently become possible to investigate major lipid modifications at the proteomic level using various methods, involving either large peptide libraries mimicking possible substrates or new affinity methods coupled to mass spectrometry analysis.

5.1.1. The power of *in vitro* studies

Most lipid modifications can be studied with a number of methods (reviewed in Ref.(104)). Prenylation is often studied by fusing glutathione-*S*-transferase to C-terminal tetrapeptides, making large-scale analysis difficult. The prenylation of short tetrapeptides encompassing the CaaX motif or the myristoylation of octapeptides starting with Gly is feasible *in vitro* (91, 105). The availability of medium-throughput non-radioactive quantitative assays therefore makes it possible to analyze the extent to which such peptides are modified *in vitro* by purified enzymes. Such assays are now available for both prenyltransferases and NMT (106, 107). With such methods available and dozens of peptides studied, prediction can be improved. This has made it possible to refine and relax the original N-Myr motif in fungi, plants (95) and animals (108, 109). The data obtained also suggest that N-Myr was underestimated with the motifs originally used for its analysis. Stringent assessments in various proteomes suggested that 0.5. to 1.7.% of the proteins present in the proteome underwent this modification (95, 110).

5.1.2. Dedicated purification

GPI-anchored proteins in plants and animals can be selected using a combination of biochemical, spectrometric and bioinformatic methods (111). Such methods have led to the retrieval of nine GPI proteins from humans and 44 from the model plant *A. thaliana*. Most of these GPI proteins are correctly predicted by the various bioinformatics tools available, indicating that GPI protein prediction is reliable.

5.1.3. Protein tagging technologies

A tagging technique based on farnesyl analogs (FPP-azide or F-azide-OH) has been developed for the identification of naturally farnesylated proteins (112). Cells are supplied metabolically with the reactive analog. The modified proteins are captured and the biotinylated conjugated analog is affinity-purified on streptavidin beads. The proteins are then identified by mass spectrometry. The application of this method to animal cells has provided new information on farnesylation, with the identification of 21 farnesylated proteins, including various Ras proteins. It has been shown, contrary to expectations based on motifs alone, that annexins with CX5 or ending with the CKQQ motif can be modified (Table 1). Nevertheless, most of the proteins identified contained the CaaX motif. This method could be applied to geranylgeranylation. An antibody against farnesyl-Cys residues can be used to purify farnesylated proteins by immunoprecipitation (113). This technique is potentially useful for proteomic analysis.

N-Myr proteins can be selected based on Staudinger ligation. The cells are fed with azidomyristate, which can serve as a substrate of NMT. Azidomyristate reacts with triarylphosphine that has been biotinylated or tagged with any epitope and the reacting proteins can be affinity-purified. This method can be used to identify unexpected NMT substrates, such as those generated by posttranslational proteolytic cleavage (114).

The acyl-biotinyl exchange method leads to replacement of the S-Palm moiety by biotinyl groups from protein extracts (115). Further purification on streptavidin

Table 2. Online software for predicting protein lipidation

Prediction software	Modifications predicted	Prediction method	Reference and Website
LipoP 1.0.	N-Palm in Gram-negative bacteria	Hidden Markov models	Ref.(138) http://www.cbs.dtu.dk/services/LipoP/
LipPred	Lipoproteins in Gram-positive bacteria	Naive-Bayesian networks	Ref.(139) http://www.jenner.ac.uk/LipPred
CSS-Palm	S-Palm	Similarity scores	Ref.(140) http://bioinformatics.lcd-ustc.org/css_palm/
The MYR predictor	N-Myr	Protein patterns and scoring	Ref.(100) http://mendel.imp.ac.at/myristate/SUPLpredictor.htm
Myristoylator	N-Myr	Neural network	Ref.(141) http://www.expasy.org/tools/myristoylator/
PlantsP	N-Myr (plants)	Hidden Markov model	Ref.(142) http://plantsp.genomics.purdue.edu/plantsp/html/myrist.html
Termi*N*ator3	N-Myr, NS-Palm together	Protein patterns and scoring	Ref.(96, 144) http://www.isv.cnrs-gif.fr/terminator3/index.html
PrePS	C-Prenylation	Protein patterns and scoring	Ref.(101) http://mendel.imp.ac.at/sat/PrePS/index.html
big-PI Predictor	GPI anchors	Protein patterns and scoring	Ref.(143) http://mendel.imp.ac.at/sat/gpi/gpi_server.html
DGPI	GPI anchors	-	*Link not working anymore*
GPI-SOM:	GPI anchors	unknown	Fankhauser & Maeser, unpublished http://gpi.unibe.ch/

beads, followed by elution and mass spectrometry analysis, leads to the identification of S-Palm proteins (116, 117). The use of this method led to the identification of 35 new S-palmitoylated proteins in yeast (42). Further analysis in various PAT-deficient yeast backgrounds made it possible to match substrate and PAT specificity. This new approach to S-Palm is very important, as this type of lipidation remains difficult to characterize, particularly as it is highly labile and reversible.

5.2. Towards complete annotation of lipidated proteins

Efforts towards prediction of the complete set of lipidated proteins have been made possible using dedicated tools in various organisms.

5.2.1. Current state of knowledge of the lipidated proteome in animals

To our knowledge, the very first attempts in this emerging field aimed at defining GPI-anchored proteins in the worm *Caenorhabditis elegans* (118). In this pioneering study, 86 putative proteins (i.e. 0.4.5% of the proteome) were proposed to undergo the modification. This effort was next extended to other organisms (99). The extent of N-myristoylated was also studied in various Eukaryotes and shown to reach 0.8.% in Humans (110). Full lists of N-Myr candidates are available (http://mendel.imp.ac.at/myristate/SUPLpredictionlists.htm #Eu-F+V). Concerning prenylated proteins, 248 sequences (0.8.% of the proteome) could be defined in Humans (see http://mendel.imp.ac.at/sat/PrePS/HumanPRENbase/).

Advances at defining sequence clusters for C-Pre and their conservation in closely related organisms were also made recently available (46). For instance, the 248 sequences in humans define 238 distinct clusters.

Together the data suggests that more than 2% of the proteins of the human proteome may undergo a lipid modification. So far, genome annotation of lipid modifications is not yet available in animals but all tools are available.

5.2.2. A case study, degree of lipidation of the *arabidopsis thaliana* proteome

The recent compilation of a list of plasma membrane proteins from *Arabidopsis thaliana* effectively identified by proteomic analyses retrieved about 1,000 proteins, corresponding to about 20% the expected total number of proteins (119). Prediction based on the most reliable predictors of C-Pre, N-Myr, S-Palm and GPI-anchored proteins identified 17% of the proteome as lipidated with any of the three modifications, in most cases (90%), involving double acylation (Figure 3*A*). GPI anchors seem to be widespread in the proteome. The continuation of recent efforts, based on various methods and aiming to improve the description of plasma membranes should result in more complete experimental proteomes and more accurate data on the prevalence of lipidated proteins in membranes being obtained (120-123).

Applying predictive tools to the complete proteome (27,000 entries) and comparing the results obtained with data from recent experimental and predictive analyses of GPI-anchored proteins (85, 124), a complete set of N-Myr, C-Pre and GPI-anchored proteins can be retrieved for *A. thaliana*. The results obtained are very similar, with 4% of the proteome considered likely to undergo a lipidation event (Figure 3*B*). This value conforms perfectly to expectations if we consider that (i) 25% of a given proteome corresponds to membrane proteins (87) and (ii) 17% of membrane proteins are predicted to be lipidated (see above). However, N-Myr appears to be prevalent and monoacylation more frequent among all lipidated proteins (30%) than among the proteins of the plasma membrane proteome. This analysis suggests that membrane fractionation techniques may lead to some monoacylated proteins being discarded — particularly those using a non-lipid second signal for membrane binding. For instance, the preparation of membranes in the presence of high salt concentrations (121) may result in the shielding of proteins using a polybasic track as the second signal (Figure 2). Finally, a significantly larger proportion of proteins (96% *vs* 76%) were found to be N-acylated in the plasma membrane than in non membrane compartments (125), confirming the high prevalence of N-Myr in this compartment.

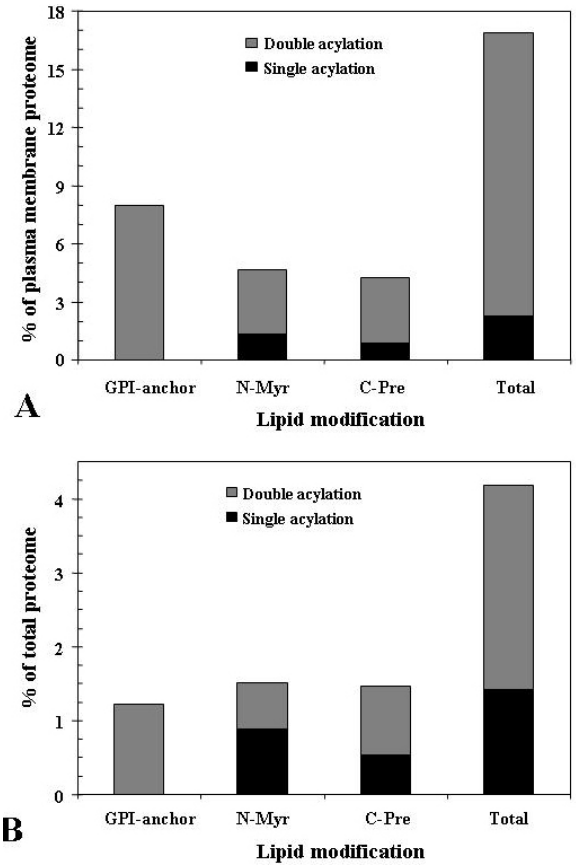


Figure 3. Impact of major lipid modification in the *Arabidopsis thaliana* proteome. The proteome of *A. thaliana* (TAIR v 6.0., 20060709) was scanned for the motifs indicated in Table 1 to identify all protein candidates predicted to undergo modification (C-Pre or N-Myr, NS-Palm). GPI proteins correspond to all those identified in Refs(85, 135). The entries are available upon request.

6. FUTURE DIRECTIONS

Protein lipidation is an expanding field of study, and it is clear that many more types of modification, enzymes and protein substrates remain to be identified in the near future. Studies of protein motifs remain a very straightforward way of identifying modifications in the most common proteins. We now have the means to assess the frequency of modification at proteomic level, as lipidation has been shown to concern more than 2% (animals) to 4% (plants) of all proteins, and more than 17% of membrane proteins. Most of these proteins are present in specialized dynamic microdomains — lipid rafts. There is a clear trend toward the development of software (i) for finer prediction of major lipid modifications, such as C-Pre, N-Myr, S-Palm and GPI-anchors and (ii) identifying minor modifications, such as the addition of sterol or triglyceride. Further experimental data are required for the learning process. We also need to increase the capacity of proteomic studies of posttranslational modifications to analyze hydrophobic peptides, information about these peptides often being lost during liquid chromatography or mass spectrometry (126). Lipid modifications usually may render the peptide so hydrophobic that it is retained on liquid chromatography or oligomerized in the mass spectrometer (127), hindering characterization.

Another interesting feature to be considered in this field is the possible competition between lipidation and other modifications, or even the absence of modification. Two populations of a given protein may occur, each modified in a different way and targeted to different compartments. The relative balance between two modifications may also be regulated by the enzymes involved. Active membrane-targeting signals directing competition between N-acetylation and N-Myr recognition on the one hand and N-acetyl transferase type-C-dependent N-acetylation on the other, have recently been identified as important for membrane, organellar, lysosomal and Golgi trafficking for small GTPases of the ARF family (128-131).

Finally, protein lipidation has been little studied in Archaea and it remains unclear whether such modifications occur in this kingdom and whether any modifications that might occur would be of the bacterial or eukaryotic type. Analysis combining gapped BLAST (132) with other tools (see Ref.(133)) of the major enzyme catalysts (see Table 1) may be a simple way of addressing this question. In this context, the availability of almost five thousands of clusters of orthologs groups (KOGs) in the sequenced genomes of unicellular organisms (http://www.ncbi.nlm.nih.gov/COG/) is most useful (51, 134). Such analysis for instance reveals that prokaryotic diacylglycyl transferases (COG0682) and eukaryotic gpi8 (COG5206), GGT (COG5044) or NMT (COG5092) orthologs are not identified in Archaea (51), suggesting that protein lipid modifications – if any – could be catalyzed by specific enzymes in Archaea.

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Abbreviations: C-Pre, C-terminal prenylation , GGT, geranylgeranyl transferase; , GPI, glycosylphosphatidylinositol; , KOG, cluster of orthologs groups; , NMT, N-myristoyltransferase , N-Myr, N-myristoylation; , PAT, palmitoyl S-transferase , PFT, protein farnesyltransferase; , S-palm, S-palmitoylation; , NS-palm, S-palm on a Cys residue next to an N-Myr-Gly

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