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Fatty acylation of proteins

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Contents

I. Introduction

Many proteins are modified during or after their synthesis. Some of these modifications have been known for many years, as for instance glycosylation, phosphorylation and proteolytic cleavage of precursor polypeptides. These examples have been extensively covered in a number of review articles and in biochemical textbooks over the years.

Another type of protein modification discovered more recently is the covalent attachment of lipid molecules like phospholipid, diacylglycerol and various species of long chain fatty acids. Such binding of lipid molecules is expected to change the physical properties of the respective entity quite dramatically, because largely hydrophilic residues are converted into very hydrophobic

Abbreviations: VSV, vesicular stomatitis virus; SFV, Semliki Forest Virus; ER, endoplasmic reticulum; PAT, protein fatty acyltransferase; NMT, *N*-myristoyltransferase.

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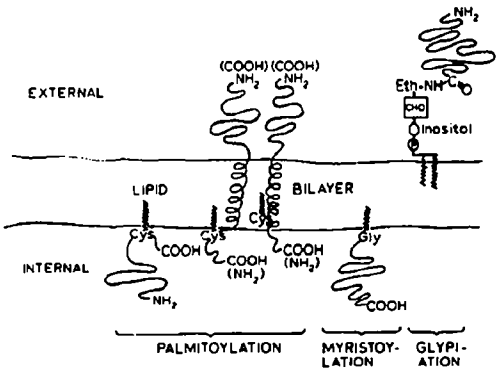

Fig. 1. Schematic diagram of hydrophobic modifications of proteins: palmitoylation, myristoylation and glypiation. Cys, cysteine; Gly, glycine; CHO, carbohydrate; Eth-NH_2, ethanolamine. Threonine has been identified as the palmitoylation site in one case only, with bovine brain myelin proteolipid apoprotein [90]. All other palmitate linkage sites chemically identified are cysteine residues (compare Table II).

ones. This will, of course influence the interactions between such modified proteins and other molecules present in their vicinity, be it other proteins, lipids or even nucleic acid. Likewise, intermolecular processes may be influenced, such as oligomerisation of polypeptides during biosynthesis or the co-operativity between subunits of a multimeric protein.

The present review aims at summarizing developments in the field of hydrophobic modifications of proteins since their discovery. Special emphasis will be placed on the acylation of viral and eukaryotic polypeptides with long chain fatty acids. This modification yields what could be regarded as a new class of proteins, which by analogy with glyco- or phosphoproteins, I suggest by termed 'acylproteins'. Two types of acylprotein are presently distinguished in the literature, those which contain exclusively the fourteen carbon myristic acid (tetradecanoic acid) in an amide-linkage, and those which are predominantly modified with palmitic-, stearic- and oleic acid (hexa- and octadecanoic acids) in ester- or thioester-type linkages. The various biochemical and cell biological aspects of palmitoylation and myristoylation will be discussed.

Another hydrophobic modification of proteins, the covalent attachment of a glycolipid tail to some membrane proteins (glypiation), will not be described here. Current reviews cover this area, which the reader is referred to [1–4]. Fig. 1 presents a schematic representation of the above-mentioned hydrophobic modifications of proteins.

II. Ester-type palmitoylation

Even before the term palmitoylation became widely used in the literature, a few protein species had been previously identified that contained fatty acids as part of their covalent structure. These were the proteolipid apoprotein isolated from the myelin membrane, which is also termed lipophilin [5,6], the proteolipid component of preparations of the sarcoplasmic Ca^{2+}/Mg^{2+}-ATPase [7] and the bacterial lipoproteins [8,9]. The fatty acid species in these above-mentioned proteins more or less reflected the acyl patterns of the lipids of the membrane from which they had been isolated. In all these cases palmitic-, stearic- and oleic acid were the predominant acyl species. Although these proteins, due to their solubility in organic solvents, had initially been termed proteolipids, they can be classified as true acylproteins because of their covalently bound fatty acid residues. It should be mentioned that some other proteolipid species have been described. Despite their lack of any covalently bound fatty acid, these are soluble in organic solvents, but insoluble in water [10].

II-A. Identification of palmitoylated proteins of viral and cellular origin

At the time unaware of the above-mentioned acylated proteolipids, Schmidt and Schlesinger [11,12] observed that typical membrane glycoproteins can be labeled with tritiated long chain fatty acids. Although viral membrane glycoproteins were utilized initially, the same authors reported that fatty acylation also occurred with proteins of non-infected cells [13]. The first cellular membrane glycoprotein to be identified as fatty acylation was the transferrin receptor [14]. Many other species of acylproteins of viral or cellular origin with covalently bound fatty acids were subsequently identified (Table I).

In most of the proteins listed in Table I acylation was detected after labeling the protein with [^{3}H]palmitic acid in the appropriate system followed by identification via immunoprecipitation, PAGE-analysis, chromatographic procedures, or combinations thereof. Although not examined in all acylproteins listed, in nearly all cases in which labeled fatty acids were analysed, palmitic acid was found to represent the major species bound to the acylprotein with stearic- and oleic acid comprising the balance of total radioactive fatty acid recovered from the protein under study. The same results have also been reported from analysis of acyl chains released from non-labeled acylproteins purified in large scale (for references refer to Table I). It should be noted that proteins modified by a glycolipid will incorporate ^{3}H-labeled fatty acids during metabolic labeling, just like the acylproteins. In order to differentiate such glypiated proteins from acylproteins, labeling with [^{3}H]ethanolamine or [^{3}H]inositol should be applied. Only if such label is incorporated, is the protein most likely glypiated [3,4].

TABLE I

Palmitoylated proteins of eukaryotic and viral origin

Protein species	Origin of protein	Ref.
Cellular acylproteins		
Transferrin receptor	human leukemic T-cells	14
Acetyolcholine receptor [a]	muscle cell line	15
Insulin receptor [a]	human lymphocytes (IM-9), Hep G2	16,17
Insulin like growth factor I - receptor	Hep G2, MDCK	18
IgE receptor	leukemic T-cells	19
Rhodopsin	bovine retina	20
Interphotoreceptor	bovine retina	21
Na^+ channel, α-subunit	rat neurons	22
Interleukin-2 receptor	human T-cell line (MT-1)	23
Sialo-gp 2,3 and gp3Pr	erythroblasts	24
HLA B7, DR	lymphoblastoid cell line	25
HLA-D/invariant chain p41	various human lymphoma cells p41 transfected cell	26 27
Ia alpha and beta	mouse spleen cells	28
Galactosyltransferase	Hela cells	29
Golgi mannosidase II	mouse 3T3 and other cells	30
Cardiac 51 kDa protein	mouse	31
Mucus glycoproteins	sublingual salivary glands	32
Mucus glycoproteins	gastric mucosal cells	33
TGF-alpha precursors	TGF-gene transfected cells	34
Butyrophilin	milk fat globule membrane (goat)	35
Xanthine oxidase	milk fat globule membrane (goat)	35
Lipophilin	myelin membrane (bovine)	5,6
DM-20	myelin membrane (rat)	36
PO-protein	myelin of peripheral nerve (rat)	37
Ca^{2+}-ATPase	sarcoplasmic reticulum	7,38
Fibronectin	human fibroblasts	39
Ankyrin	human erythrocytes	40
Band 4.1 protein	human erythrocytes	40
Vinculin [a]	chicken embryo fibroblasts	41,42
Lens membrane proteins	intact occular lens (rat)	43
Ligatin	ileal enterocytes (rat)	44
ras-proteins	various human cell lines	45
Apolipoproteins (AI,E)	human hepatoma cells (Hep-G2)	46
Apolipoprotein B	human LDL	47
Folate binding protein [a]	human KB-cells	48
Developmental gps	sea urchin embryo	49
RAS 1 and RAS 2	yeast cells	50
YPT1	yeast cells	51
Alpha factor	yeast cells	52,53
Membrane glycoproteins	yeast cells	54
Actin (sub population)	slime mold	55,56
Microtubule-binding protein	*Trypanosoma brucei*	57
Light harvesting protein [a]	duckweed plant	58
Rib 1,5-P_2-carboxylase [a]	duckweed plant	58
Six proteins (major 22 kDa)	Tetrahymena mimbres	59
Surfactant protein (SAP35)	alveolar epithelial cells	60
Human tissue factor	human lung fibroblast	61
CD9 surface gp	human platelets	62

TABLE I (continued)

Protein species	Origin of protein	Ref.
Viral structural acylproteins		
G-protein	rhabdoviruses (VSV)	11
E1,E2	togaviruses (Sindbis, SFV)	11,63
HA, HA_2	avian/human influenza A viruses	63
HA	influenza B virus	[b]
HEF I, HEF II	influenza C virus	[b]
F_0, F_1	Newcastle disease viruses	63
F, HN	mumps virus	64
HN	SV5	65
E2	mouse hepatitis virus	66
E2	bovine coronavirus L9	63
G1, G2	bunya viruses (La Crosse)	67
gp 35	Rous sarcoma virus	68
gp 65	spleen focus forming virus	69
p37 k	vaccinia virus	70
gE	herpes simplex virus (type 1)	71
Viral non-structural acylproteins		
E1b 18 kD	Adenovirus 12	72
E1b 19 kD	Adenovirus 1	73
T-antigen (large)	SV40	74
ras-protein	Harvey murine sarcoma virus	75

[a] The fatty acid linkage in these proteins is partially or totally resistant to treatment with mild alkali or hydroxylamine.

[b] Veit, M., Herrler, G. and Schmidt, M.F.G., unpublished data.

Many of the palmitoylated proteins listed in Table I are glycosylated and represent membrane components with widely diverse biological functions. Among these are polypeptides which span the membrane once or multiple times. Usually the palmitoylated membrane proteins are oriented with their carboxyterminus towards the cytoplasmic side of the membrane or homologously towards the inside of the enveloped virus particles listed. However, reversely oriented proteins may also be palmitoylated, e.g., the hemagglutinin-neuraminidase protein (HN) of mumps virus and of paramyxovirus SV5 [64,65] as well as the transferrin receptor [14]. Some of the membrane proteins listed occur as monomers [20], others as homo-oligomers [11,63] or as hetero-oligomers. An example for the latter is the insulin receptor, in which only the β-subunit is palmitoylated [17,18]. Beside the plasma membrane, internal membranes may also contain proteins with covalently linked fatty acid, e.g., the Golgi-located mannosidase II and galactosyltransferase [29,30] as well as the proteolipid component of sarcoplasmic Ca^{2+}-ATPase [7,38]. Recent more generalized studies of the intracellular location of palmitoylated proteins showed that most of them are membrane bound [76–78]. However, the phenomenon is more complex in a functional sense, since a small group of palmitoylated proteins are secreted by the cells [21,33,34,46,47] and a somewhat larger group of acylproteins comprises components of the cellular cytoskeletal elements [39,40,42,55]. Consid-

ering palmitoylated proteins of viral origin it can be stated that with the exception of two serotypes of vesicular stomatitis virus (VSV) [79] and of Sendai virus [65] (see below) all RNA- and DNA-viruses with a lipid envelope analysed so far contain at least one *structural* protein with covalently bound palmitic acid. These are all located in the viral envelope, and except for p 37 k of vaccinia virus [70], are also glycosylated. It is noteworthy that viral spike proteins with a known low pH-dependent fusogenic activity are palmitoylated (see below).

With viral *non-structural* proteins palmitoylation is not as common as myristoylation (see below). However, cells transformed by the non-enveloped viruses Simian virus 40, adenovirus and with the enveloped Harvey murine sarcoma virus express early proteins which become acylated and are located in the plasma membrane of the infected cells [72–75], where they may play a role in the process of cell transformation (see subsection IV-B).

As is apparent from Table I, palmitoylated acylproteins are found in organisms of different levels of complexity. In their evolutionary range of occurrence they reach from the fungi *Dictyostelium* and yeast, across the angiosperma (*Spirodela oligorrhiza*), across the non-vertebrates (sea urchin) up to the vertebrates, mammals and human. As mentioned above, viruses from a variety of hosts as well as prokaryotes contain acylproteins. The latter group will not be dealt with, since it has been covered in a recent review by Wu and Tokunaga [80]. Although the list of palmitoylated proteins is extensive at this point, more acylated species will probably have to be added once the numerous acylproteins detectable after [^{3}H]palmitoylation in vivo of various vertebrate cells have been identified [13,81–83].

II-B. Acyl linkage sites in palmitoylated proteins

From the early studies of myelin proteolipid [5,6] and of the acylproteins present in the viral envelope [11,12,63,84], it is clear that fatty acids are bound by ester-type linkage, since they can be released by mild alkali and hydroxylamine treatment. Stability studies utilizing these agents have since been applied routinely to identify ester-type acylation of most of the viral or cellular palmitoylated proteins listed in Table I. However, direct structural analysis of the palmitate linkage site proved to be a formidable task. Due to their unusual and unpredictable properties, peptides with covalently bound fatty acid moieties derived from palmitoylated proteins were extremely difficult to purify [12,85]. More indirect studies, as for instance limited proteolysis pointed to a location of the fatty acid linkage site close to the membrane spanning segment of acylated membrane proteins [14,63,85–88]. However, despite comparative analysis of the stability of the fatty acid linkage it was initially not even possible, to decide conclusively between an oxygen- and a thioester linkage of the fatty acid to serine or cysteine. For instance, fatty acids linked to Semliki Forest virus (SFV) E1-protein were released with hydroxylamine under the same conditions which cleaved the acetyl-group from *o*-acetyl serine, suggesting an *o*-ester linkage between fatty acid and E1 [89]. However, direct structural analysis has recently revealed a cysteine as the linkage site, and thus a thioester linkage between protein and fatty acid (see below and Ref. 91).

The first direct identification of one of the two palmitoyl linkage sites present in myelin lipoprotein by sequence analysis came from Stoffel and co-workers, who had virtually unlimited supplies of this acylprotein from bovine brain. Their results showed that fatty acid is bound to a threonine residue located in the extracytosolic domain of one of the hydrophilic loops between two of the five membrane segments of lipophilin [90].

A similar protein chemistry approach was also utilized with three other palmitoylated acylproteins, HLA-D associated invariant (il) chain, VSV G-protein and SFV E1-protein. In all three cases palmitic acid was found to be bound to cysteine in thioester linkage. The fatty acylated cysteine of the two viral acylproteins was located on the 'cytoplasmic' (internal) face of the lipid bilayer with SFV-E1 [91] and at the base of the carboxyterminal cytoplasmic tail with the VSV G-protein [92] (compare schematic diagram in Fig. 1). Prior to the biochemical identification of fatty acylation sites, other authors had turned to recombinant DNA technology to locate fatty acids within the primary structure of acylproteins. Replacement of specific cysteine residues suspected to represent the linkage site with serine after site-directed mutagenesis led to a loss of the covalent attachment of fatty acid during in vivo labeling experiments with the mutated *ras*-protein [93,94], the *ras*-like YPT1 from yeast [51], VSV G-protein [95] and the transferrin receptor [97]. The lack of fatty acylation clearly indicated an involvement of the mutated cysteine residues with fatty acid binding. However, it could not be stated with certainty, that those cysteines were the actual linkage sites. Fortunately, the hypothesized Cys residues in ras [98] and the G-protein [92] have since been confirmed as fatty acid linkage sites by direct biochemical methods.

The results of the various analyses of fatty acid attachment sites in different palmitoylated proteins are summarized in Table II. Mainly from comparison of amino acid sequences around suspected acylation sites of the ras family proteins, a consensus sequence for palmitoylation has been proposed recently [99]. However, this so called CAAX-box (C for Cys, A for aliphatic and X for any amino acid) at the C-terminus is by

TABLE II

Cysteine residues as palmitoylation sites and their topography [a]

Palmitoylation sites are marked by asterisks.

Integral membrane proteins		
VSV-G		
NH_2	GLFLVL	RVGIHLC*IKLK ..... COOH
SFV-E1		
NH_2	VVVTC*IGL	RR-COOH
Transferrin-receptor		
HOOC	GYC*ISGSC*	RKPKTVNAK ..... NH_2
Bovine rhodopsin		
NH_2	IYIMM	NKQFRNCMVTTLC*C*G ..... COOH
HLA-D associated invariant chain Ii		
HOOC ..... QYL	YLAG	RSC*K ..... NH_2
Peripheral membrane proteins		
ras-protein		HOOC-SLVC*KC ..... NH_2
YPT1-protein		HOOC-C*C*GGGTN ..... NH_2
(external)	(membrane segment)	(cytoplasmic)

[a] Sequences are from Refs. 92,95,97,101,26,94 and 51 in this order from top to bottom.

definition a rather unprecise recognition sequence. It may be valid for the few palmitoylated ras-type proteins only. When the primary structures of other acylated and non-acylated membrane glycoproteins are compared, due to the wide variability of amino acids around the putative palmitoylation sites, no recognition sequence can be deduced. The only structural theme common to the palmitoylated species is the occurence of at least one cysteine residue usually within about four residues from the putative border between the inner leaflet of the lipid bilayer and the cytoplasm [65,100]. With the VSV G-protein the palmitoylated cysteine residue was located seven residues into the cytoplasm [92]. In a recent report Ovchinnikov and co-workers identified the acylation sites of bovine rhodopsin. The two fatty acylated cysteine residues were located 13 and 14 amino acid residues away from the membrane border into the cytoplasm. These authors suggest that using its fatty acids as anchors, this region of the protein loops back into the lipid bilayer of the photoreceptor membrane [101].

II-C. Biochemistry of palmitoylation

II-C1. Location of palmitoylating activity

From early studies of palmitoylation of viral acylproteins in vivo, it has been apparent that this modification is an event which in the presence of ongoing protein synthesis can be detected by metabolic short pulse labeling [102]. In order to define the intracellular location of palmitoylation, fatty acylation was related to the various stages of oligosaccharide processing, and to the timing of proteolytic processing and intracellular transport. The results of such experiments revealed that palmitoylation of VSV G-protein and Sindbis E1 and E2 occurs shortly after translation and just prior to the acquisition of Endo H resistance [102]. While various trimming inhibitors (swainsonin, deoxynojirimycin, castanospermin) either had no effect on palmitoylation (McDowell, W. and Schmidt, M.F.G., unpublished results) or led to a stimulation [103], the effect of tunicamycin depended on the glycoprotein under study. While tunicamycin had no effect on the acylation of the HLA invariant-chain [26], coronavirus E2 [104,105] and mannosidase II [30], it completely abolished palmitoylation of VSV G-protein [12] and of the sodium channel [22]. Thus, it seems that the influence of tunicamycin on acylation is an indirect one, perhaps by preventing transport of the glycoprotein to the acylation site [106]. This indicates, that glycosylation per se is not required for fatty acylation. Also other experiments pointed to a crucial intracellular location for palmitoylation to operate. Ts-mutants of the G-protein defective in transport between the ER and Golgi could not be acylated at nonpermissive temperatures [12], whereas blocking of transport between the Golgi and the plasma membrane with monensin had no influence on fatty acylation [107]. From pulse-chase experiments with [^{3}H]palmitic acid Dunphy et al. [108] and Quinn et al. [109] reported the *cis*-Golgi to be the intracellular location at which palmitoylation occurs. Subsequently, short pulse labeling in vivo of acylproteins with [^{3}H]palmitic acid was utilized frequently as a marker for the *cis*-Golgi compartment (e.g., Ref. 110). However, more recent data proved that palmitic acid binding begins at an even earlier stage. By cell fractionation after extremely short

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