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COMMENTARY

FUNCTIONAL CONSEQUENCES OF LIPID-MEDIATED PROTEIN–MEMBRANE INTERACTIONS

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The dynamics of protein–membrane interactions have been the focus of intense investigation. Recently, two novel mechanisms for the tight association of proteins with the plasma membrane have been uncovered. These mechanisms involve the covalent attachment of proteins to lipid moieties that are embedded in the bilayer. In one case, lipids such as myristate, palmitate or isoprenoids are covalently attached to the protein, conferring sufficient hydrophobicity to facilitate its interaction with the bilayer. In the second case, the C-terminal amino acid of the protein is attached to an inositol phospholipid via a unique oligosaccharide moiety. The resulting glycosyl-phosphatidylinositol (glycosyl-PI) anchor produces sufficient hydrophobicity to induce attachment to the lipid bilayer. These two broad mechanisms of protein–lipid interaction have evolved into a diverse number of forms that allow for the subtle modulation of protein–membrane affinity. Salient features of these structures are illustrated in Fig. 1. In this commentary, we compare the structural features of these two mechanisms of protein–membrane attachment, and discuss some of their potential regulatory roles.

COVALENT ATTACHMENT OF LIPIDS TO INTRACELLULAR PROTEINS

Fatty acylation or isoprenylation of a number of intracellular proteins is required for optimal biological activity. The field has been reviewed recently [2–4], and our intention here is to highlight some of the recent advances.

Protein N-myristoylation

In all cases described, N-myristoylation of proteins occurs via an amide linkage to the α-amino group of an N-terminal glycine [2–4]. N-Myristoylation occurs cotranslationally or very soon after the completion of polypeptide synthesis, as demonstrated by the incorporation of myristate into nascent peptides [5], and blockage of this process by protein synthesis inhibitors [6]. The precise function of protein-bound myristic acid is not fully understood. In some cases it is required to target proteins to the plasma membrane [2, 4], but myristoylated proteins are also found in the cytosol, endoplasmic reticulum and nucleus [7, 8]. As is the case for glycosyl-PI-anchored proteins (see below), the diversity of proteins known to be myristoylated indicates a number of possibilities regarding the functional role of this modification. The list of myristoylated proteins includes several known to be involved in intracellular signalling pathways, such as the tyrosine kinases $p60^{src}$ [8] and $p56^{lck}$ [9], the catalytic subunit of cAMP-dependent protein kinase [10], the regulatory subunit of protein phosphatase 2B [11], a prominent protein kinase C substrate [12] and the G-proteins [13, 14]. A number of viral proteins are also myristoylated. In most cases, this modification is required for membrane attachment and for an intact replicating cycle [2–4].

(1) *Enzymology*. The acylation reaction is catalyzed by a myristoyl CoA: protein *N*-myristoyl transferase (NMT) which does not utilize fatty acids with chain lengths greater than 14 carbons [2]. An NMT has been purified and cloned from *Saccharomyces cerevisiae* [15, 16]. It exhibits a high degree of selectivity for the sequence of its substrate peptide. A loose consensus sequence required for effective myristoylation has been elucidated with the following rules: (i) an amino-terminal Gly is required; (ii) a neutral amino acid at position 2 is required. Aromatic or bulky residues at this position yield poor substrates, while charged residues and Pro are not allowed; (iii) the requirements for positions 3 and 4 are less stringent, although peptides with uncharged residues at position 3 yield higher affinity; (iv) position 5 is critical in NMT–ligand interactions, with Ser being highly favored. A charged residue at this position renders a peptide inactive as an NMT substrate, as does Pro at either position 5 or 6; and (v) residues beyond the first six amino acids play a role in substrate recognition by NMT, although the precise structural requirements in this region remain to be elucidated. The consensus data described above are supported by the sequences of known myristolylated proteins [2].

(2) *Regulation*. There are a number of potential points of regulation of N-myristoylation. Since acylation occurs cotranslationally or very soon after the completion of polypeptide synthesis [5], and since all cells examined contain active NMT, the transcription and translation of the candidate protein

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may indirectly regulate its N-myristoylation. N-Myristoylation occurs exclusively on an N-terminal glycine residue. Since the initiator methionine must first be removed by an *N*-methionine amino peptidase to expose the acceptor glycine, this peptidase could potentially regulate N-myristoylation. Moreover, the activity or subcellular location of the NMT might be regulated. Thus, certain proteins bearing a myristoylation consensus sequence might be translated on ribosomes that are not in the proximity of the NMT. In this context it is interesting to note that a subset of G-proteins contains the N-terminal glycine but is not myristoylated [17, 18].

Although all known N-myristoylated proteins that have been sequenced bear a glycine after the initiator methionine, it is possible that proteins could be acylated at a cryptic site exposed by proteolytic cleavage.

It is possible that certain proteins may undergo multiple cycles of myristoylation followed by deacylation. However, in all cases investigated thus far, it appears that myristic acid remains stably bound to the protein until it is degraded. Whether enzymatic demyristoylation of proteins occurs has not yet been rigorously investigated.

(3) *Role of N-myristoylation in protein function.* Although myristic acid confers membrane binding capacity on some proteins, the detection of a number of myristoylated proteins in the cytosol suggests that the fatty acid has other roles as well. Because their functional diversity makes it difficult to generalize, we discuss here the properties of selected myristoylated proteins.

(a) *Proto-oncogenic tyrosine kinases.* Proto-oncogenes encode a class of regulatory proteins whose mutation or aberrant expression may result in oncogenic transformation. The *src* gene family consists of a number of closely related tyrosine protein kinases of which a subset including *src, yes, syn/fyn,* and *lck* has been shown to be myristoylated [2]. Myristoylation of $p60^{v\text{-}src}$ is essential for association of the protein with the plasma membrane and for expression of transforming potential [19, 20]. Since non-myristoylated $p60^{v\text{-}src}$ retains its tyrosine kinase activity [19, 20], it is probable that myristoylation functions to target the enzyme to the plasma membrane where it exerts its effect by phosphorylating relevant membrane-bound substrates. To test this hypothesis Hanafusa and coworkers constructed chimeric proteins by fusing the first 14 amino acids of $p60^{src}$, which contains the myristoylation site, to either the *fps* tyrosine kinase or to α-globin, both non-myristoylated, soluble proteins. In each case the fusion protein was myristoylated and associated with the membrane [21]. However, while myristic acid is necessary for membrane attachment of $p60^{src}$, it is not sufficient. A transformation-defective mutant of $p60^{src}$ has been described that does not associate with the membrane despite its myristoylation. This and other data suggest that the myristic acid does not promote membrane attachment merely by acting as a nonspecific hydrophobic anchor, but rather by promoting the association of the protein with a membrane-bound "receptor" for myristoylated $p60^{src}$.

$p56^{lck}$, a myristoylated member of the *src* family of proto-oncogenes, has been shown recently to associate with the CD4 and the CD8 T-lymphocyte surface glycoproteins [22, 23]. These surface antigens are expressed in a mutually exclusive manner on mature T-lymphocytes. CD4 + T-cells possess a helper phenotype in that they activate B-lymphocytes by producing lymphokines [24], and CD8 + T-cells fulfill cytotoxic functions by killing virally infected or abnormal host cells. The data suggest that the major histocompatibility molecules on antigen-presenting cells interact with CD4 on the T-cell. This activates *lck*, resulting in the phosphorylation of the T-cell receptor and subsequent signalling to the nucleus [25]. The myristic acid moiety of *lck* is necessary (but not sufficient) for its attachment to CD4 [26, 27]. In this sense CD4 represents the first myristoyl–protein receptor to be defined.

(b) *GTP-binding proteins.* Heterotrimeric guanine nucleotide-binding proteins (G-proteins) transduce signals across cell membranes by coupling receptors for hormones and sensory stimuli to effector enzymes and ion channels [for review see Ref. 28]. The G-protein complex consists of a large family of α subunits and a more restricted number of β and γ subunits [28]. The β and γ subunits are always tightly associated as a complex and are capable of binding to membrane phospholipids [28]. The α subunit binds to the $\beta\gamma$ complex in the unactivated state and is released from the complex receptor upon activation. The free α subunit generally transduces the signal to the effector molecule. Several of the α subunits (α_{i1}, α_{i2}, α_{i3}, α_o, α_t, α_z) have been shown to be myristoylated [13, 14, 17, 18]. While the fatty acid is not required for the association between the α and $\beta\gamma$ subunits, it is required for continued association of the protein with the plasma membrane after dissociation from $\beta\gamma$. The α_s subunits are not myristoylated although they bear an N-terminal glycine. This may be due to the presence of an asparagine at positions 4 and 5 which makes them unfavorable substrates for the NMT [17, 18].

(c) *MARCKS protein.* The myristoylated, alanine-rich C kinase substrate (MARCKS) is a major specific protein kinase C (PKC) substrate which is also known as the 87K substrate in neuronal cells [29–31], the 80K substrate in fibroblasts [32, 33] and the 68K substrate in macrophages [12]. The protein is phosphorylated rapidly during neurosecretion, growth factor-dependent mitogenesis in fibroblasts, and macrophage activation. MARCKS was first shown to be myristoylated in macrophages and neuronal cells [12], a finding that was later confirmed in a number of cell lines. Cloning of cDNAs for the genes encoding bovine brain MARCKS [34], chicken brain MARCKS [35], and murine macrophage MARCKS* revealed 32 kD, 28 kD and 30 kD proteins respectively. The N-terminal myristoylation domain and the phosphorylation domain are conserved between the three species, but the remainder of the molecule exhibits considerable divergence. The phosphorylation domain is an

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amphipathic helix and in the unphosphorylated state is capable of binding calmodulin [36].

The association of the MARCKS protein with the plasma membrane depends upon its myristoylation [37]. Interestingly, this attachment process is regulated by the phosphorylation state of the protein.* Phosphorylation displaces the myristoylated protein from the plasma membrane *in vivo* [12] and *in vitro* [38], and dephosphorylation is accompanied by the reassociation of the myristoylated protein with the membrane.* This cycle of membrane attachment and detachment, directed by myristic acid and regulated by the phosphorylation state of the protein, represents a novel mechanism by which a protein can shuttle to and from the membrane. It is quite possible that the membrane attachment of other myristoylated protein kinase C substrates such as $p60^{src}$ and $p56^{lck}$ may be regulated in a similar manner. The observation that phosphorylated $p56^{lck}$ has lower affinity for CD4 than its nonphosphorylated counterpart is consistent with this proposal [39]. The role of protein phosphorylation in modulating the association of MARCKS with the membrane suggests that the protein binds to a specific membrane receptor, rather than a nonspecific association with the lipid bilayer.

We have investigated the subcellular location of MARCKS in macrophages by immunofluouresence and immunoelectron microscopy. The protein colocalizes with protein kinase C, vinculin and talin in punctate structures at the substrate adherent surface of macrophage filopodia. PKC-dependent phosphorylation displaces MARCKS from the punctate structures (and from the membrane). This event is accompanied by major cytoskeletal rearrangement and filopodial retraction. While the function of MARCKS is not yet clear, it seems likely that the protein regulates the actin cytoskeleton at points of attachment of the cell to the substratum.

(d) *Cyclic AMP activated protein kinase-catalytic subunit.* While the myristic acid moiety is clearly required for membrane attachment of *src*, *gag*, G-proteins and MARCKS, its role in the biology of the catalytic subunit of protein kinase A remains obscure. This protein is one of the subset of myristoylated proteins found in the cytosol [40]. Mutation of the N-terminal glycine of the catalytic subunit to alanine prevents myristoylation, but does not alter the specific activity of the kinase or its capacity to associate with its regulatory subunits [41]. Furthermore, the non-myristoylated C subunit remained capable of inducing dramatic changes in the cytoskeleton, restored steroidogenesis in the mitochondria of defective adrenocortical cells, and induced the transcription of genes. Since these events occur in defined subcellular compartments, it appears unlikely that myristoylation is required for targeting in these cases. It is possible, however, that myristoylation may induce the association of the C subunit with its substrates thereby increasing the sensitivity of cAMP, or that myristoylation is required for a subset of functions such as the phosphorylation of integral membrane proteins.

(e) *Viral proteins.* A number of proteins important for virus assembly and structure have been shown to be myristoylated and, in general, acylation is required for their function. Thus, preventing myristoylation by mutation of the N-terminal Gly to Ala of the *gag* proteins of Moloney murine leukemia virus, Mason–Pfizer monkey virus, and HIV inhibits the binding of the protein to the membrane and subsequent virus particle formation and budding [42–44]. The absolute requirement of myristoylation for the life-cycle of viruses such as HIV has suggested that the NMT may be a potential target for therapeutic intervention. In this regard it is interesting that sulfur- and oxygen-substituted analogs of myristic acid, which are incorporated into proteins but which are much less hydrophobic than myristate, inhibit HIV and Moloney murine leukemia virus replication without associated cell toxicity [45].

(4) *Is there a membrane "receptor" for myristoylated proteins?* Much circumstantial evidence has accumulated suggesting that myristoylated proteins associate with the membrane by binding to "receptor" molecules. For example, myristoylation is required for the association of $p56^{lck}$ with CD4 and with CD8 [46, 47]. In addition, both $p60^{src}$ and the MARCKS protein, which require myristoylation for effective membrane attachment, are targeted to focal adhesions at the plasma membrane, suggesting that specific protein components are involved in membrane attachment. Phosphorylation displaces myristoylated MARCKS from the membrane, while dephosphorylation is accompanied by its reassociation with the membrane.* These data suggest a membrane receptor for dephosphorylated, myristoylated MARCKS.

Direct evidence for a "myristoyl-*src*" receptor was obtained recently in experiments which demonstrated specific and saturable binding of $p60^{v\text{-}src}$ to plasma membranes *in vitro* [46, 47]. Binding was saturable, depended on myristoylation, and was sensitive to heat and trypsin. In addition, binding of *src* was inhibited competitively by a myristoylated peptide corresponding to the first 11 amino acids of $p60^{v\text{-}src}$ but not by the non-myristoylated peptide or by myristoylated peptides derived from the sequences of other myristoylated proteins.

Palmitoylation of proteins

Many membrane-associated proteins have been shown to be modified with the 16-carbon fatty acid, palmitic acid. The palmitate is typically linked to cysteine residues via thioester bonds [3, 4, 15]. In contrast to myristoylation, palmitoylation occurs posttranslationally. Moreover, this fatty acid has been shown to turn over many times during the life of the polypeptide. Since palmitoylation occurs both in the endoplasmic reticulum and the plasma membrane, it is possible that more than one type of palmitoyl transferase exists. This enzyme(s) is less specific for the fatty acid than the NMT, suggesting that the abundance of palmitoylated proteins may reflect the predominance of this fatty acid in the cell.

Since ester-linked fatty acids have been shown to turn over more rapidly than the protein, it is likely that deacylating enzymes must also exist. Indeed, a

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fatty acyl esterase capable of removing palmitic acid from proteins has been identified in microsomal membranes [48]. In addition, James and Olson [49] have described recently a palmitoylated protein in BC_3H1 cells that is deacylated when the cells are stimulated with growth factors or serum.

A wide variety of cellular and viral proteins has been shown to covalently bind fatty acid via ester linkages [reviewed in Refs. 2–4]. Since a full description of these proteins is beyond the scope of this review, we will briefly describe two examples from which some general properties can be derived.

(1) *Transmembrane glycoproteins*. These include β_2-adrenergic and insulin receptors, and the α subunit of the voltage-sensitive sodium channel. These proteins are acylated soon after their synthesis, probably in the endoplasmic reticulum or the Golgi [50–52]. The transferrin receptor has been shown to be reversibly palmitoylated at the plasma membrane [53]. Although the functions of these modifications are unknown, it is possible that palmitoylation facilitates protein–protein interactions, thereby modulating signal transduction pathways. Consistent with this, mutation of Cys^{341} results in a non-palmitoylated form of the β_2 adrenergic receptor that is markedly impaired in its ability to mediate agonist stimulation of adenylyl cyclase [50].

(2) *The* ras *family of GTP-binding proteins*. The N-, H-, and K-$p21^{ras}$ proteins are members of a large family of low molecular weight (20–26 kD) guanine nucleotide binding proteins which were originally characterized as transforming oncogenes [54]. The function of *ras* proteins depends upon the binding of GTP and localization at the plasma membrane [55]. Plasma membrane attachment of *ras* proteins is directed by a complex sequence of events which ultimately results in the covalent attachment of two lipid moieties. The initial steps of polyisoprenylation, C-terminal proteolysis, and carboxymethylation are common to all *ras* proteins and to related proteins such as the fungal mating factors which bear the CAAX motif [56–59]. This motif contains a cysteine, two aliphatic amino acids (most often hydrophobic amino acids such a leucine, isoleucine or valine) followed by any amino acid. Early posttranslational processing of *ras* (and fungal mating factors) includes the proteolytic cleavage of the AAX, carboxymethylation of the now C-terminal cysteine (Cys^{186}), and its farnesylation via a thioether linkage to its sulfur atom [58]. This processing is irreversible and probably occurs in the cytoplasm. The farnesyl moiety confers low avidity membrane binding on the protein and may localize *ras* proteins to the membrane/cytosol interface, where they can be acted upon by a membrane bound acyl transferase [58]. This acyl transferase catalyzes the thioesterification of palmitic acid to *ras* proteins bearing a second cyteine residue in the vicinity. For p21-H-*ras* this occurs on Cys^{181} and Cys^{184} and for p21N-*ras* on Cys^{181}. p21-k-^{ras} does not contain a cysteine residue immediately upstream from Cys^{186} and is therefore not palmitoylated [58]. Palmitoylation increases the avidity of the protein for the membrane, and since the palmitic acid on p21-N-*ras* is known to turn over rapidly, this may indirectly regulate its biological activity by controlling its access to membrane.

Since the farnesyl moiety is absolutely required for *ras* activity and since it is derived from mevalonate, it is possible that pharmacologic agents which interfere with mevalonate synthesis may be useful antitumor agents. In this regard, it is interesting that mevinolin prevents the correct processing and membrane localization of *ras*.

COVALENT ATTACHMENT OF LIPIDS TO EXTRACELLULAR PROTEINS

Distribution and structure of glycosyl-PI-anchored proteins

The anchorage of proteins to membranes via glycosyl-PI has been detected in a variety of cell types. The distribution and identification of these proteins have been reviewed extensively [60, 61]. Over forty proteins of considerable functional and evolutionary diversity are attached to biological membranes by this mechanism. This list includes a number of hydrolytic enzymes, adhesion molecules, coat proteins, mammalian antigens and additional proteins, many of unknown function. The only feature common to these proteins is their location on the extracellular face of the plasma membrane or (in three cases) on the luminal surface of intracellular organelles.

Compositional and structural analyses of the glycosyl-PI anchors of several proteins have revealed certain conserved and variant features. Most of the structural information on glycosyl-PI has emerged from studies on the variant surface glycoprotein (VSG) of *Trypanosoma brucei* [62], acetylcholinesterase [63] and the Thy-1 antigen [64]. These studies have revealed a basic structure in which the C-terminal amino acid of the protein is linked to an ethanolamine through an amide bond. The ethanolamine, in turn, is attached via a phosphodiester to an oligosaccharide consisting of a trihexose backbone linked to glucosamine. The presence of this nonacetylated glucosamine is one of the unique features of this molecule, possibly serving as an important recognition site in the biosynthesis and metabolism of glycosyl-PI. The glucosamine is glycosidically linked to the 6-OH position of the inositol ring of PI. This linkage allows for the solubilization of the anchored protein by nitrous acid deamination (causing hydrolysis of the glucosamine–inositol bond) and by bacterial PI-specific phospholipases C (PLCs).

A number of variations in this basic core structure have been observed, perhaps contributing to alterations in functional roles. The presence of varied oligosaccharide side chains for most of the anchors of known structure has been demonstrated. In addition, the Thy-1 anchor appears to possess an additional phosphoethanolamine. Another variation in structure concerns the fatty acid composition of the glycerolipid. In general, the inositol phospholipid moiety of most glycosyl-PI anchors does not resemble the predominant, mature form of PI (1-stearyl, 2-arachidonyl) in most cells, but is more likely to contain saturated fatty acid, or a fatty alkyl group in the 2-position [60, 61, 65]. Another interesting

modification is the addition of an extra fatty acid ester linked to one of the hydroxyls of the inositol moiety. The resulting acylated glycosyl-PI is resistant to cleavage by the PI- and glycosyl-PI specific PLCs [65].

A glycophospholipid with structural similarity to the glycosyl-PI anchor has been described in a number of cell types. This lipid shares the core structure of PI-glucosamine, linked to additional monosaccharides, but lacks ethanolamine, and is not attached to protein. Numerous studies in tissue culture cells have demonstrated that this lipid is hydrolyzed by a PLC mechanism in response to insulin and related hormones. The resulting hydrolytic products, diacylglycerol and an inositol phosphate glycan, are thought to mediate some of the actions of insulin in fat, liver and muscle cells [66, 67].

Protein–glycosyl-PI attachment

The anchoring of proteins to glycosyl-PI appears to occur by the attachment of the proteins to a preformed lipid precursor. This attachment requires the removal of a relatively short, C-terminal hydrophobic domain. The formation of this linkage appears to occur cotranslationally or posttranslationally in the endoplasmic reticulum (ER), as judged by sensitivity to cleavage by PI-PLC or reactivity with antibodies that recognize the glycan moiety of the anchor [68–71]. The rapid kinetics of anchor addition suggest that cleavage of the hydrophobic C-terminal peptide and glycosyl-PI attachment may be catalyzed by the same enzyme (transpeptidase or transamidase) and that a presynthesized glycosyl-PI moiety is added *en bloc*.

The protein signals that direct attachment to glycosyl-PI appear to be localized to C-terminal hydrophobic regions. Initial studies comparing the amino acid sequence predicted from cDNAs of Thy-1 or trypanosomal VSG with the actual peptide sequence demonstrated the loss of the predicted C-terminal peptide prior to replacement with the glycosyl-PI moiety [72, 73]. Several glycosyl-PI-anchored proteins are alternatively expressed with transmembrane hydrophobic peptide regions (e.g. N-CAM [74], FcγRIII [75, 76], and LFA-3 [77]), or with sequences that produce secreted proteins (e.g. DAF [78], Qa-2 [79], and N-CAM [80]). These variations are derived from differences in the C-terminal regions, generated either by alternate splicing of a single gene or transcription of alternative genes.

Recent studies have revealed several structural features of this carboxy terminal sequence which are involved in glycosyl-PI attachment. Sequence analysis of a number of proteins has suggested that a relatively short hydrophobic domain of 20–30 amino acids is contained within this COOH terminal region. Glycosyl-PI attachment occurs 8–12 residues to the NH_2 terminus of this hydrophobic domain, in a region containing short side chain amino acids. A number of studies employing progressive deletion mutants of the glycosyl-PI-anchored enzyme placental alkaline phosphatase have indicated that the length of this hydrophobic domain may play an important role. Reduction of the hydrophobic domain to 17 amino acids resulted in a mutant that was still glycosyl-PI anchored, while a deletion mutant that contained only a 13 amino acid hydrophobic domain was secreted, rather than glycosyl-PI anchored [81, 82]. Although the precise sequence of the hydrophobic domain that is replaced by the glycosyl-PI anchor may not be highly constrained, in one case, the MHC class I protein Qa_2, a single charged amino acid (Asp) within the hydrophobic domain was demonstrated to be essential for glycosyl-PI attachment [83].

Further studies have defined two different regions within the C-terminal glycosyl-PI attachment "signal" of DAF: a 17 amino acid C-terminal hydrophobic region and the 20 amino acids located just upstream. Removal of the 17 amino acid C-terminal hydrophobic segment of decay accelerating factor prevented glycosyl-PI anchoring, although the addition of this hydrophobic domain to a secreted protein (human growth hormone) did not confer glycosyl-PI anchoring, indicating that this hydrophobic segment is necessary, but not sufficient to direct lipid attachment [84]. Thus, additional information (cleavage and attachment sites) must reside within the adjacent 20 upstream amino acids. Moreover, it is likely that the signal nature of the 17 amino acid C-terminal hydrophobic region depends upon overall hydrophobicity, since replacement with a random hydrophobic sequence or by the N-terminal ER signal sequence of human growth hormone still allowed glycosyl-PI attachment [85]. Similar deletion or chimeric gene experiments employing glycosyl-PI-anchored placental alkaline phosphatase, Qa-2 and Thy-1 have also localized the signal to C-terminal hydrophobic regions [81, 83, 86].

The amino acid that is amide-linked to the glycosyl-PI anchor is highly constrained. A survey of a number of glycosyl-PI-anchored proteins reveals that Ser, Cys, Asp, Asn, Gly and Ala are found at this position, and that hydrophobic or bulky side chain amino acids are excluded. Recently, studies have indicated that a single amino acid at the glycosyl-PI attachment site may determine which membrane anchor is to be utilized. A human IgG Fc receptor expressed on granulocytes (FcγRIII-1) is glycosyl-PI linked, while a protein with >95% identity, expressed in macrophages and NK cells, utilizes a transmembrane amino acid domain (FCγRIII-2). Substitution of a Ser in FcγRIII-1 for a Phe in III-2 at the glycosyl-PI attachment site reverses the membrane anchors for these proteins [87–89]. Mutational analyses of the hydrophobic transmembrane domain and cytoplasmic domain have revealed that the amino acid at the glycosyl-PI attachment site is dominant over other structural features. These studies suggest that the hydrophobicity of the transmembrane domain, as well as the length of the cytoplasmic sequence may influence the efficiency of glycosyl-PI anchoring.

In several proteins, alternative forms of membrane anchoring have been found. Two genes encode FcγRIII: one of these is expressed exclusively in granulocytes, encoding the glycosyl-PI linked form, and the second is expressed in NK cells and macrophages, encoding a transmembrane protein [89]. Regulation of glycosyl-PI attachment for

FcγRIII is transcriptional, determined by the selective expression of these two genes. Transcriptional regulation for these genes must be tightly controlled, since co-expression of these transcripts has not been detected. In contrast, the regulation of glycosyl-PI attachment for N-CAM is post-transcriptional. N-CAM can exist as a glycosyl-PI-anchored or transmembrane-attached protein, with each form exhibiting identical extracellular domains. These differences are due to alternative mRNA splicing through the substitution of a hydrophobic domain for one which is preceded by a glycosyl-PI attachment domain which observes the consensus sequence [74, 80, 90]. These transcripts are differentially expressed, suggesting that regulation of glycosyl-PI attachment for N-CAM occurs posttranscriptionally. In distinction to the cases cited above, in which distinct cells express either a glycosyl-PI-anchored or transmembrane protein, LFA-3 has been shown to be expressed on the surface of the same cell in both forms [77]. A single gene encodes the LFA-3 protein and alternative cDNAs have been described which differ in the length of a putative cytoplasmic domain. However, since both molecules predicted by these cDNAs have identical glycosyl-PI attachment and transmembrane domains, the significance of these two cDNAs in determining the membrane topology of LFA-3 has yet to be determined. The regulation of glycosyl-PI attachment of LFA-3 may be posttranslational. In the case of FcRγIII, glycosyl-PI attachment can be influenced by posttranslational protein interactions. The transmembrane form of that receptor, III-2, requires the co-expression of a second subunit for surface expression. The γ chain of FcεRI and the ζ chain of the CD3/TcR complex both interact with FcγRIII-2, resulting in its surface expression. Interaction of these related molecules with FcγRIII-2 is likely to be dependent upon the transmembrane sequences of these molecules. The glycosyl-PI-attached form of FcγRIII, III-1, can be converted to a transmembrane molecule through its interaction with the γ chain of FcεRI [77]. The precursor form of III-1 does not undergo the transamidation/peptidase cleavage normally involved in glycosyl-PI anchor attachment when co-expressed with the γ chain. While this type of regulation has been seen in transfected cells, it has yet to be observed *in vivo*, in neutrophils which normally express III-1.

Functional roles of the glycosyl-PI anchor

Although the basic structural features of the glycosyl-PI anchor are known, the broad diversity of the anchored proteins makes assignment of a function for the anchor quite difficult. Nevertheless, the unusual properties of this anchor, and the evolution of a complex mechanism to remove a hydrophobic peptide domain for lipid attachment suggest that the utilization of this mechanism of anchoring merely as an inert structural anchor is unlikely. Three possible functional roles for this glycosyl-PI anchor are discussed.

(1) *Protein distribution and targeting.* Most of the glycosyl-PI-anchored proteins identified thus far are located at the cell surface and face the extracellular space. A few glycosyl-PI-anchored proteins exhibit intracellular localizations (i.e. GP-2 in the zymogen granules of the exocrine pancreas [91]). Elongation Factor EF-1α in the endoplasmic reticulum of V79-UF Chinese hamster fibroblasts [92] and a fraction of PH-20 in the acrosome of guinea pig sperm [93]); however, each of these is distributed in the lumen of intracellular vesicles, and not on the cytoplasmic face. Interestingly, two of these proteins (GP-2 and PH-20) undergo regulated secretion, suggesting a potential mechanism for release involving the glycosyl-PI anchor. The observed asymmetric orientation of all glycosyl-PI-anchored proteins is compatible with their incorporation into the lumen of the ER via N-terminal signal sequences with subsequent processing and transfer to glycosyl-PI.

In experiments with COS and MDCK cells [78, 84], attachment of the C-terminal peptide of a glycosyl-PI-anchored protein to a secreted protein resulted in glycosyl-PI addition and targeting to the plasma membrane. These and similar experiments suggested that glycosyl-PI-anchored proteins must require additional signals for intracellular retention and targeting to a pathway for regulated secretion, as has been shown for regulated secretory proteins, such as insulin, growth hormone and pre-pro-somatostatin. Although this observation may seem trivial, since in most cells transport to the cell surface is by default and retention at specific compartments along the secretory pathway is signal-mediated, it is especially relevant to protein transport in polarized cells that contain multiple plasma membrane domains of distinct lipid and protein composition. In a variety of polarized epithelial cell lines (MDCK I and II, LLC-PK_1, Caco-2, and SK-CO15), glycosyl-PI-anchored proteins are selectively enriched in the apical domain, while depleted or absent from the basolateral cell surface [94–96]. This correlation between glycosyl-PI anchoring and apical localization is highly conserved across species (pig, dog and human) and tissue type (renal and intestinal), suggesting that the anchor may act as an apical transport signal to target the attached protein to the apical cell surface. Recombinant transfer of glycosyl-PI-attachment signals to proteins known to exhibit basolateral distribution on polarized cells, such as the viral envelope glycoproteins, HSV gD-1 or VSV G protein or to a regulated secretory protein, such as human growth hormone, resulted in glycosyl-PI anchoring and targeting to the apical membrane. Other polarized cells also exhibit an asymmetric distribution of glycosyl-PI-anchored proteins. For example, neural cell adhesion molecule 120, F3, 5′-nucleotidase and acetylcholinesterase are localized to neuronal outgrowths and neuronal ramifications. The distribution of PH-20 varies with the degree of sperm maturation. This protein is randomly distributed in testicular sperm, localized to the posterior head region in epididymal sperm, and undergoes redistribution to the anterior head region after the acrosome reaction. Paradoxically, PH-20 is "immobile" when randomly distributed, but freely diffusible when localized to the head region (posteriorly or anteriorly). Thus, linkage to glycosyl-PI may convey specific patterns of cell surface localization in different polarized cell types or membrane microdomains.

(2) *Increased lateral mobility*. An obvious consequence of lipid anchoring is an inherent increase in mobility in the plane of the membrane. Diffusion coefficients on the order of $1–4 \times 10^{-9}$ cm^2/sec are observed for Thy-1, alkaline phosphatase, DAF, and PH-20 [93, 97–99]. These are lower than values determined for freely diffusing lipid probes ($0.5–1 \times 10^{-9}$), but much higher than those for transmembrane glycoproteins ($0.5–6 \times 10^{-10}$). Paradoxically, some glycosyl-PI-anchored proteins possess significant immobile fractions, up to 50% in the case of Thy-1. Certain glycosyl-PI-anchored proteins may require increased lateral mobility for function, as in the case of DAF (for rapid inhibition of the complement cascade) or AChE (for deactivation of acetylcholine at the synapse). Other proteins require a high degree of immobility, such as the trypanosomal VSG (diffusion constant 1×10^{-10}) and PH-20. Interestingly, the low lateral mobility of VSG is not a result of some other factor in the trypanosomal cell surface, since implantation of the protein in the BHK cell membrane did not increase its mobility [100]. The diffusion of PH-20 is dependent on the state of differentiation of guinea pig sperm (see above).

(3) *Regulated release at the cell surface*. One of the unique features of the structure of the glycosyl-PI anchor is the presence of sites for enzymatic modification. Within the glycolipid attachment domain, there are a number of sites than represent potential substrates for glycosyl-PI-specific anchor-degrading enzymes (proteases, glycosidases and/or phospholipases), the actions of which may result in release of the attached protein from the cell surface. Such an enzyme-mediated release mechanism could be regulated or constitutive. Regulated degradation of the glycosyl-PI anchor, perhaps under hormonal control, could potentially provide a unique mechanism for down-regulation of the concentration of the anchored protein at the cell surface or for up-regulation of the protein in the circulation, for its actions at a local or downstream target tissue. Similarly, a constitutive degradation of the glycosyl-PI anchor could provide a mechanism for secretion

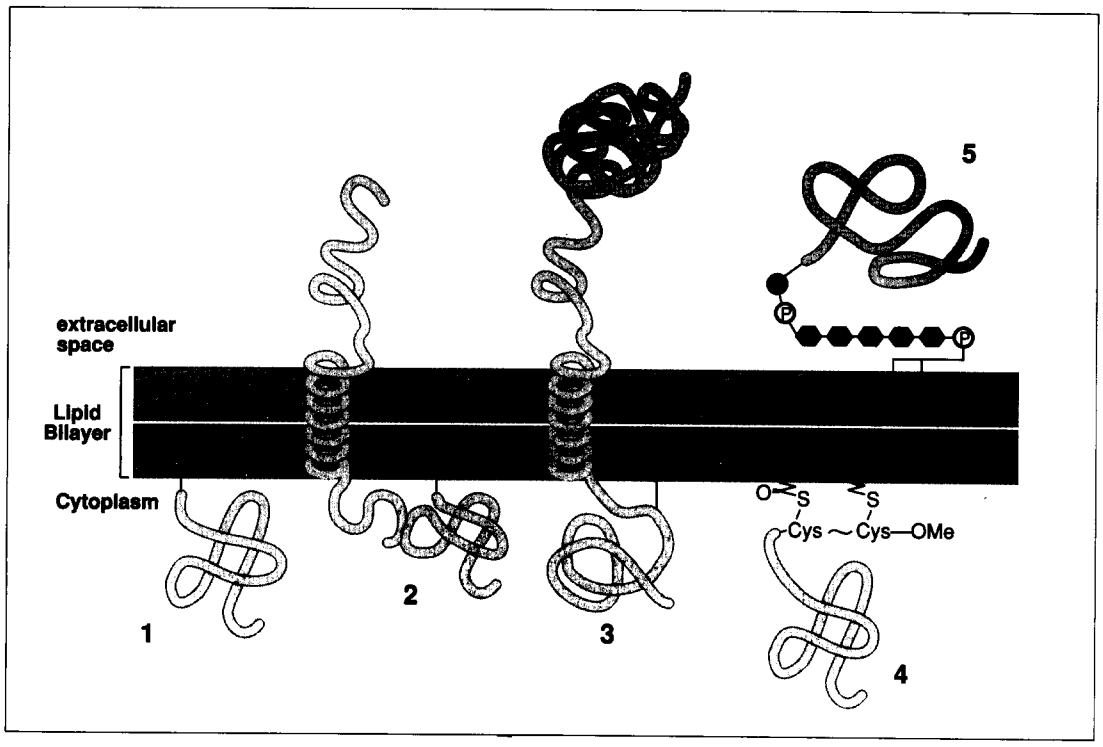

Fig. 1. Lipid–protein interactions at the plasma membrane. Five examples are presented to illustrate mechanisms by which lipid moieties tether proteins to the plasma membrane. (1) The fatty acid moiety of myristoylated or palmitoylated proteins acts as a membrane anchor, allowing direct insertion into the membrane. (2) Covalently attached fatty acid confers a hydrophobic character to the protein which places it in close proximity to a "receptor" protein on the cytoplasmic side of the membrane. The association of $p56^{lck}$ with CD4 may be mediated in this way. (3) A number of integral membrane proteins are thioesterified with palmitic acid. In the case of the β_2-adrenergic receptor, covalently attached palmitic acid appears to be required for effective coupling of the receptor to adenylyl cyclase. (4) The *ras* family of oncoproteins are both isoprenylated and palmitoylated. Isoprenylation initially confers upon the protein an intermediate affinity for the plasma membrane. Palmitoylation then leads to a tighter association with the bilayer. (5) Extracellular proteins such as Thy-1 or alkaline phosphatase are linked in the Golgi to glycosyl-phosphatidylinositol, ultimately resulting in anchoring of the protein to the plasma membrane. (Figure is adapted from Alberts *et al.* [1].)

of proteins such as GP-2 after translocation to the cell surface.

A number of glycosyl-PI-anchored proteins such as alkaline phosphatase, 5′-nucleotidase, DAF, lipoprotein lipase, GP-2, CEA, Qa-2 antigen, Thy-1, FcγRIII-1 from neutrophils and 34 kD placental growth factor have been detected in soluble forms [101]. These soluble forms may result from anchor degradation or differential splicing of mRNAs to produce non-glycosyl-PI-anchored proteins, as documented for N-CAM, Qa-2, and DAF. The detection of free glycosyl-PI molecules that are hydrolyzed in response to hormones suggested the possibility that the glycosyl-PI protein anchor may undergo a similar hormone-sensitive hydrolysis reaction [67]. The acute release of certain glycosyl-PI-anchored proteins from tissue culture cells in response to hormones has been observed, including lipoprotein lipase [102], heparan sulfate proteoglycan [103], 5′-nucleotidase [104], and alkaline phosphatase [105]. Interestingly, the cell surface or circulating concentration of each of these proteins is known to be altered in diabetes [106, 107]. The FcγRIII receptor was released upon neutrophil activation in response to chemotactic peptide or phorbol esters [108]. A survey of the total cell-surface glycosyl-PI-anchored proteins in insulin-sensitive BC_3H1 myocytes indicated that a number of the PLC-releasable proteins were depleted by prior treatment of cells with insulin or serum [109]. Interestingly, exposure to insulin caused the loss of only some of the glycosyl-PI-anchored proteins, while others remained unchanged. These observations suggest the existence of hormone-sensitive and -insensitive "structural" pools of the glycosyl-PI anchor. One intriguing possibility is that structural modifications of the glycosyl-PI moiety play a role in dictating susceptibility to enzymatic degradation. As discussed above, the presence of an ester-linked fatty acid on the inositol ring renders the anchor insensitive to cleavage by PI- or glycosyl-PI-specific PLCs [65].

Although the experiments described above are indirect, they suggest that hormonal treatment results in the activation of an anchor-degrading enzyme(s). However, the nature of this enzyme remains unknown, and could be a specific phospholipase, protease or glycosidase. Glycosyl-PI-specific PLCs with similar properties have been isolated from *Trypanosoma brucei* [110] and mammalian liver [111] and brain (Guochang Z and Saltiel AR, unpublished observations). Both the *T. brucei* and liver enzymes exhibit similar peptide maps, are membrane-associated, calcium independent, and specifically catalyze the hydrolysis of glycosyl-PI, but not other phospholipids [110, 111]. The cDNA for the trypanosomal enzyme has been cloned and sequenced [112]. This predicted sequence revealed no homology to other phospholipases, and gave no indication of a signal sequence or glycosylation sites that would be indicative of an extracellular or transmembrane protein. In addition, immunohistochemical studies indicated an intracellular localization [100]. Thus, it seems unlikely that the mammalian glycosyl-PI-PLC is involved in anchor degradation under normal conditions. However, the possibility remains that other glycosyl-PI-specific phospholipases exist. A glycosyl-PI-specific phospholipase D was found in plasma derived from a number of sources [113–115]. This enzyme specifically hydrolyzes the glycosyl-PI anchor for purified membrane-bound proteins, although it cannot remove cell surface glycosyl-PI-anchored proteins from intact cells.

CONCLUSIONS

Recent investigations into the structural details of membrane proteins have yielded surprising insights into the dynamics of protein–membrane interaction. Although the covalent modification of proteins with lipids has now gained wide acceptance, the functional consequences of these novel interactions are only now emerging. It is interesting to note that the proteins which undergo these modifications exhibit wide diversity of tissue and species distribution, with little or no evolutionary or functional similarity. Although all the fatty acylated or isoprenylated proteins identified thus far are located on the cytoplasmic side of the plasma membrane, there are a number of apparently unrelated consequences of these modifications depending on cell type and protein. Similarly, the list of glycosyl-PI-anchored proteins contains a number of different classes of molecules in a variety of cell types, indicating several possibilities regarding regulation of the properties of these proteins. It is likely that continued investigation in this area will lead to new advances in our understanding of the dynamics of biological membranes.

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