COMMENTARY

Lipid modification of proteins and its relevance to protein targeting

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Introduction

Great advances in our knowledge of how proteins reach their final destinations in cells have been achieved in recent years; in particular the classical pathway of secretion *via* the endoplasmic reticulum and Golgi complex is now becoming understood in molecular detail (Rothman and Orci, 1990). However, a number of paradoxical examples of proteins which are apparently secreted, but not *via* this pathway, have been reported (March *et al.* 1985; Burgess and Maciag, 1989). The diversity of ways in which eukaryotic cells can target proteins is now emerging. A novel secretory pathway involves plasma membrane 'pumps' related to the products of the multi-drug resistance (MDR) genes (Featherstone, 1990). Amongst the molecules secreted by this pathway are a number of small lipopeptide mating factors. Recent progress in our understanding of lipid modifications of proteins has identified a relationship between these lipopeptides and the products of the *ras* oncogenes and several other important proteins (Hancock *et al.* 1989). It is the purpose of this commentary to illustrate how different lipid modifications are used by eukaryotic cells to create flexibility and specificity in protein targeting. Interest in this field has been amply demonstrated at two recent international meetings (*ras* Proteins: Function and Evolution, Cold Spring Harbour Lab., May 9–13, 1990; ASBMB/ASI Meeting, New Orleans, June 4–7, 1990).

Four classes of covalent lipid modification have been identified in eukaryotic systems over the last decade (Schmidt, 1989; Thomas *et al.* 1990; Hancock *et al.* 1989) and their general structures are shown in Fig. 1.

Apical polarity of GPI-anchored proteins

The GPI-anchor structure was first characterised from *Trypanosoma* Variant Surface Glycoprotein (VSG) and mammalian Thy-1 antigen, and has now been observed attached to many cell surface proteins of eukaryotic cells, from yeast to man (reviewed in Ferguson and Williams, 1988). The use of bacterial phosphatidylinositol-specific phospholipases C (PI–PLC) to release GPI-anchored proteins pioneered by Low has provided a simple though not infallible test for this kind of linkage (Low, 1989). Several structures have now been determined in detail, largely due to the efforts of Ferguson and his colleagues, and a general structure has emerged (Fig. 1.1). The C-terminal residue of the protein moiety is amide-linked to ethanolamine which is in turn phosphodiester-linked to a tri-mannosyl glucosaminyl core glycan. This is attached to phosphatidylinositol (PI) *via* the 6 position of the inositol and inserted in the outer leaflet of the lipid bilayer of the plasma membrane, which is apparently the exclusive location of this class of proteins. Several possibilities for variation exist. The R_1 and R_2 substituents on the glycerol of the PI moiety can be either alkyl or acyl, with a range of chain lengths. The inositol ring can be substituted with palmitate, thus rendering the structure resistant to PI-PLC. The glycan can carry extra sugars, usually αGal, βGalNAc or αMan, and/or ethanolamine phosphate. Thus a wide diversity of structures with different physical, biochemical and immunological properties can be generated.

The biosynthesis of GPI-anchored proteins is complex. They are translocated into the endoplasmic reticulum (ER) using a typical signal sequence. A weakly hydrophobic C-terminal extension is believed transiently to anchor the protein to the ER membrane until it is proteolytically removed and replaced with a preformed GPI-anchor (Ferguson and Williams, 1988; Doering *et al.* 1990). During transport of the protein to the cell surface, the anchor can be modified in its glycan moiety and can also undergo remodelling of the R_1 and R_2 substituents (Masterson *et al.* 1990). Recent reports have investigated the distribution of GPI-anchored proteins in polarised epithelial cells and have yielded the interesting observation that they are exclusively found in the apical membrane of these cells (Lisanti *et al.* 1988). Conventionally anchored membrane proteins with transmembrane peptide segments, however, are found on both apical and basolateral surfaces. Conversion of a conventional protein into a GPI-anchored protein by recombinant DNA-mediated swapping of C-terminal sequences results in exclusive targeting of the chimaera to the apical membrane, showing that the information resides in the GPI anchor itself (Lisanti *et al.* 1989). The mechanism of this selective transport is unclear at present. There could be a specific recognition event ('receptor') which sorts GPI-anchored proteins intracellularly and directs them into an apical route. Alternatively the GPI-anchor may confer self-aggregative properties on these proteins, causing them to patch and be segregated by a physical mechanism. Glycolipids, which are also highly concentrated in the outer leaflet of the apical membrane, have been shown to have such aggregative properties and may even co-cluster with the GPI-anchored proteins. Whatever the mechanisms, the presence of a GPI-anchor allows the cell another level of control over the distribution of membrane proteins. Whether GPI-anchors have other functions has

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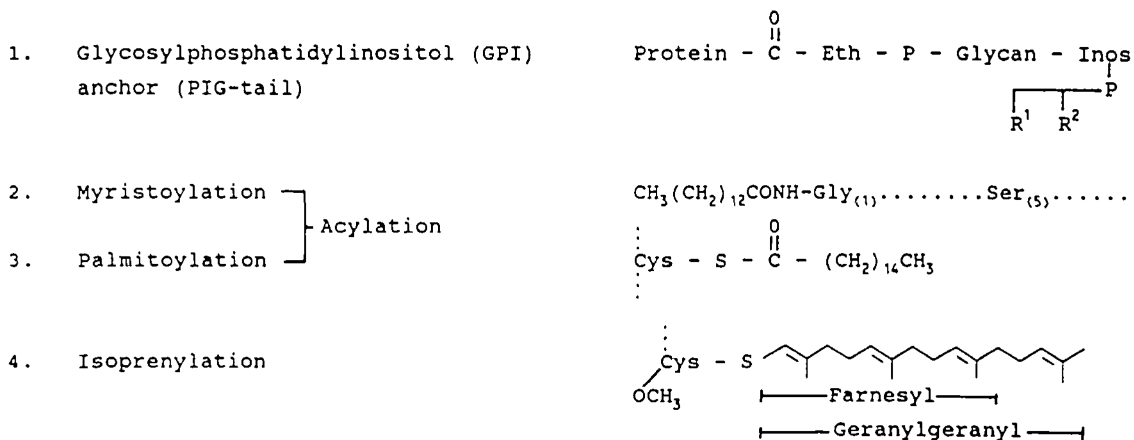

Eth, ethanolamine; P, phosphate; Inos, inositol; R_1, R_2, alkyl/acyl groups

Fig. 1. Structure of covalent lipid modifications of eukaryotic proteins.

been a matter of some debate (Thomas *et al.* 1990). However, the widely held idea that these proteins have a higher lateral mobility in the membrane does not seem to stand up to extensive measurements on a wide range of these proteins (M. Edidin, personal communication).

Myristoylation

In the early 1980s two types of covalent modification of proteins with long chain fatty acids ('acylation') were reported (reviewed by Schmidt, 1989). In one of these types the rare 14-carbon saturated fatty acid myristate is amide linked to N-terminal glycine residues of many cellular polypeptides (Fig. 1.2). Somewhat surprisingly many of these proteins were found to be primarily cytosolic, implying that simple hydrophobic interaction of the acyl chain with lipid bilayers was not the sole function of the modification (Magee and Courtneidge, 1985). The primary structural requirements for myristoylation are now well defined (see consensus sequence in Fig. 1.2) and an *N*-myristoyl transferase has been purified from yeast and mammalian sources and its properties studied (Towler *et al.* 1988). The enzyme is soluble and catalyses co-translational myristoylation of suitable substrates, after removal of the initiator Met, using myristoyl-coenzyme A as a co-substrate. Studies with fatty acid analogues have shown that the acyl chain length is crucial for utilisation by the enzyme while the hydrophobicity is not (Heuckeroth *et al.* 1988). Indeed, analogues with one methylene group replaced by O or S atoms, thus reducing their hydrophobicity, can be incorporated into a subset of myristoylated proteins *in vivo*. This results in a partial redistribution of some of the membrane-bound myristoylated proteins into the soluble fraction. Interference of this type with the membrane targeting of some myristoylated proteins could be of pharmacological use in the treatment of human disease. Not only do these analogues disrupt targeting of transforming pp60^{src} protein (see below) but they also interfere with the replication of HIV by virtue of preventing membrane localisation and processing of the viral *gag* structural protein (Bryant *et al.* 1989). They may also have effects on the HIV *nef* protein which has also been shown to be myristoylated (Guy *et al.* 1987).

Much interest was generated when it was shown that the transforming protein pp60^{src} of Rous sarcoma virus was myristoylated, and that this modification was essential for membrane binding and transforming activity (Kamps *et al.* 1986). This protein is found primarily in the plasma membrane, and in particular in association with adhesion plaques. The addition of myristate alone would seem inadequate to generate such specificity and this is supported by the localisation of other myristoylated proteins to the cytosol or to other intracellular membranes (Magee and Courtneidge, 1985). Several groups have thus embarked on a search for a specific myristoyl-*src* 'receptor' and indeed plasma membranes do appear to harbour saturable binding sites for myristoyl-*src* but not for the non-myristoylated protein. Recent data indicate that a $32\times10^{-3} M_r$ receptor protein can be purified after cross-linking to a synthetic iodinated myristoyl-*src* peptide (Resh and Ling, 1990). Further studies will be required to confirm these observations, but the intriguing possibility exists that specificity may be generated by a synergistic recognition of myristate and nearby peptide sequences. The myristate itself may interact directly with another protein rather than with lipid, as has been shown for VP4 of polio virus (Chow *et al.* 1987).

Palmitoylation

The other type of protein fatty acylation involves the thioester linkage of primarily palmitic acid (C16:0) to the side chains of cysteine residues, which can apparently reside at any point in the primary structure of the protein (Fig. 1.3). The enzymology of this process is poorly understood owing to the difficulty of purifying labile, membrane-bound palmitoyl transferases. Palmitoyl-CoA is the best acyl donor but there is little evidence for any specific sequence requirements in the protein substrate. It has been noted, however, that palmitoylation sites usually occur near the transmembrane region of membrane-spanning proteins, on the cytoplasmic side (Schmidt, 1989). In addition to many transmembrane proteins, a number of otherwise hydrophilic proteins associated with the cytoplasmic face of cellular membranes are palmi-

toylated, e.g. $p21^{ras}$, GAP-43, ankyrin (Magee *et al.* 1987; Staufenbiel, 1987; Skene and Virag, 1989).

The finding of an oncogene product, $p21^{ras}$, which was palmitoylated has sparked much interest in this area. The *ras* proteins are primarily plasma membrane-bound. Interestingly the palmitoylation of *ras* proteins has been found to turn over rapidly *in vivo* under normal growth conditions (Magee *et al.* 1987). Several other palmitoylated proteins show significant rates of acyl group turnover, e.g. transferrin receptor (Omary and Trowbridge, 1981), ankyrin (Staufenbiel, 1987) and GAP-43 (Skene and Virag, 1989), which is in stark contrast to myristoylation that is apparently stable and irreversible. This turnover may be regulatory since both acylation and deacylation appear to be enzymatic (L. Gutierrez, J. Childs and A. I. Magee, unpublished observations) and under the control of serum factors (L. Gutierrez, J. de Bony and A. I. Magee, unpublished observations). In addition, the localisation of the enzymatic activities is different: the deacylating activity co-fractionates with plasma membrane while the acyl transferase activity fractionates with Golgi markers (L. Gutierrez and A. I. Magee, unpublished observations). This latter observation suggests that the *ras* acyl transferase may be related to, or even identical with, the enzyme responsible for palmitoylating transmembrane proteins; however, alternative explanations are possible. If correct, these data imply that palmitoylated *ras* proteins may cycle between the Golgi complex and the plasma membrane, perhaps being carried passively by membrane flow. The presumed mode of action of *ras* proteins, as growth signal transducers at the plasma membrane, should be considered in the light of this possible dynamic relocation.

Many other members of the *ras* superfamily of low molecular weight GTP-binding proteins have potential palmitoylation sites, and indeed palmitoylation of one of them (YPT1) at C-terminal cysteine residues has been reported (Molenaar *et al.* 1988). Many of these proteins are now recognised to be involved in directing membrane traffic, and to undergo cyclic association with, and dissociation from, intracellular membranes (Walworth *et al.* 1989). It is thus tempting to speculate that regulated acyl group turnover could contribute to this phenomenon.

Isoprenylation

During recent studies of the palmitoylation of *ras* proteins it was found that they also undergo a complex triplet of modifications at the C terminus, dependent on the primary sequence motif CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid) (Gutierrez *et al.* 1989; Hancock *et al.* 1989). The first event is addition of a fifteen-carbon, isoprenoid lipid farnesol, to the cysteine residue (Casey *et al.* 1989). This is followed by proteolytic removal of the AAX, and carboxyl-methylation of the resulting α-carboxyl group to give a highly modified C terminus (Fig. 1.4). Interestingly, these modifications are identical to those occurring on the fungal mating factors mentioned earlier, although they clearly do not result in secretion of *ras* proteins. In the case of *ras* proteins, CAAX modification is usually followed by, and required for, palmitoylation of nearby upstream cysteine residues. The CAAX-dependent modifications, unlike palmitoylation, are irreversible.

Many other cellular proteins contain CAAX motifs and are modified similarly. However, it has recently emerged that the most protein-bound isoprenoid is the twenty-carbon geranylgeranyl (Farnsworth *et al.* 1990; Rilling *et al.* 1990). Slight differences in the sequence of the CAAX motif seem to enable the cell to select which isoprenoid to attach. Indeed, recent studies on purified farnesyl transferase show that its activity is highly dependent on CAAX sequence (Reiss *et al.* 1990). It seems likely that a combination of different isoprenoid moieties and amino acid sequences contained in the carrier protein can direct that protein to distinct intracellular membrane destinations. In one case, that of nuclear lamins, this mechanism is used to target the protein to the inside of the nuclear membrane, using a combination of CAAX modification (farnesylation) and a nuclear targeting signal (Holtz *et al.* 1989). Having done its job, the isoprenylated C terminus of lamin A is then proteolytically removed (Vorburger *et al.* 1989).

The *ras* superfamily boasts many CAAX-containing members, but in addition several others terminate in CC or CXC motifs. Evidence exists for the YPT1 CC motif being palmitoylated (see above), but data is now starting to emerge suggesting that these alternative C-terminal sequences may be subject to isoprenylation and possibly other modifications. The possibilities for directing differential localisation by mixing and matching these signals are legion. Also the ability to interfere with isoprenoid modification using drugs which block isoprenoid synthesis (mevinolin, compactin) provides an experimental approach to understanding their function. Mevinolin has been shown to block secretion and processing of fungal mating factors, demonstrating the importance of correct modification for interaction with this novel secretory pathway (Miyakawa *et al.* 1985).

Progress is now being made on the enzymology of CAAX modification. A soluble farnesyl transferase has been purified and characterised (Reiss *et al.* 1990) and is being cloned, and a membrane-bound methyltransferase has been detected (Stephenson and Clarke, 1990). Presumably families of enzymes exist which recognise variant C-termini and process them differently.

Conclusions

Four types of lipid modification of eukaryotic proteins have now been recognised (Fig. 1) and others may be yet undiscovered. In total cell lysates from a variety of cells, between ten and fifty proteins of each type can be detected by metabolic labelling and one-dimensional SDS–PAGE analysis (A. I. Magee, unpublished observations). One can thus make a crude estimate that perhaps 10–50% of all cell proteins may carry some kind of lipid modification. It would not be surprising therefore if every aspect of cell metabolism was influenced by lipid-modified proteins. At this early stage it is clear that lipid modifications can dramatically affect protein localisation in cells, perhaps in a regulatory way. The further analysis of these pathways will undoubtedly aid our understanding of cellular function.

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