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Fatty Acylated Proteins as Components of Intracellular Signaling Pathways†

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An increasing number of proteins of diverse origin are being found to undergo covalent modification by the addition of long-chain, saturated fatty acids. The two most common fatty acids found associated with proteins are myristate, linked through an amide bond to N-terminal glycine, and palmitate, which is usually attached to cysteine residues via a thioester bond. These two fatty acids differ in chain length by only two carbon atoms, but the enzymes catalyzing their transfer to proteins, as well as the subcellular distributions of the proteins to which they are attached, exhibit striking differences. Although clues as to the function of covalent fatty acids on proteins are available in only a few cases, the specificity with which these modifications occur suggests that amide- and ester-linked fatty acids confer distinct properties on the proteins to which they are attached. An intriguing observation has been that many fatty acylated proteins participate in intracellular growth factor signaling pathways in which numerous intermolecular interactions must occur. In addition, a number of such proteins are associated with the cytoplasmic face of the plasma membrane, where the increased hydrophobicity provided by a long-chain fatty acid might be expected to facilitate interaction with the lipid bilayer and/or other membrane proteins. Indeed, studies to date indicate that covalent fatty acids, in most cases, are necessary for optimum biological activity of the parent protein. In this paper, we will briefly review the enzymology of cellular protein fatty acylation and will focus on current knowledge concerning those fatty acylated proteins believed to function in growth factor signaling pathways.

Enzymology of Protein Fatty Acylation

The 16-carbon saturated fatty acid palmitate is attached to proteins posttranslationally, usually through labile thioester bonds that can be readily cleaved by hydroxylamine or potassium hydroxide (Olson et al., 1985; Magee & Courtneidge, 1985; McIlhinney et al., 1985). Two distinct classes of cellular proteins contain covalent palmitate, suggesting the existence of multiple palmitoyltransferases capable of catalyzing the esterification of palmitate to proteins.

The majority of palmitoylated proteins appear to be synthesized on free polysomes and transported to the plasma membrane posttranslationally. Identification of fatty acylated proteins by metabolic labeling of tissue culture cells with ^{3}H-labeled fatty acids revealed that the majority of these proteins are localized to the inner face of the plasma membrane, where they are resistant to extraction by agents known to release classical peripheral membrane proteins (Wilcox & Olson, 1987). The absence of palmitoylated proteins in the soluble fraction indicates that the enzymes responsible for ester-linked fatty acylation of nonsecretory proteins reside in or near the plasma membrane. Many palmitoylated proteins can be acylated several hours after inhibition of protein synthesis (Olson & Spizz, 1986). This may reflect relatively slow transport of the proteins to the intracellular site of palmitoylation, reversible fatty acylation, or both.

A minor subset of cellular palmitoylated proteins are transmembrane glycoproteins and, in general, appear to acquire covalent palmitate a short time after synthesis, probably in the endoplasmic reticulum or Golgi apparatus. Included in this class are the insulin and β_2-adrenergic receptors (see below), as well as the α subunit of the voltage-sensitive sodium channel (Schmidt & Catterall, 1987). Palmitoylation of the transferrin receptor has also been demonstrated, but this apparently takes place at the plasma membrane and is therefore likely to be catalyzed by an enzyme distinct from that responsible for acylation of glycoproteins early in the secretory pathway (Omary & Trowbridge, 1981).

In a few cases, ester-linked fatty acids have been found to undergo turnover, indicating that reversible fatty acylation may provide an additional mechanism by which certain activities of a given protein may be modulated (Magee et al., 1987; Jing & Trowbridge, 1987; Staufenbiel, 1987). We have recently identified a palmitoylated protein in BC_3H1 cells that is deacylated following serum or growth factor stimulation, pro-

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viding direct evidence for modulation of fatty acylation in response to external stimuli (James & Olson, 1989a). Thus, it will be important to identify the enzymes involved in protein deacylation and to define their mode of regulation. In this regard, a fatty acyl esterase has been described in microsomal membrane preparations that removes palmitate from proteins (Berger & Schmidt, 1986).

The enzymes catalyzing ester-linked fatty acylation are somewhat relaxed with regard to fatty acyl CoA substrates, in that they transfer palmitoyl-CoA preferentially but recognize fatty acyl CoA's of shorter and longer chain lengths as well (Berger & Schmidt, 1984; Olson et al., 1985). A question that arises is whether this apparent preference for palmitate reflects true enzyme specificity or rather is a result of the high abundance of palmitate in cells compared to fatty acids of different chain lengths. The relaxed specificity of these enzymes for fatty acid substrates is in contrast to the strict specificity of *N*-myristoyltransferase (NMT) for myristoyl-CoA (see below) and may reflect in ester-linked acyl proteins the need for a general hydrophobic domain, as opposed to the apparent absolute requirement for myristate in proteins acylated by NMT. Thus far, no palmitoyltransferases have been purified or cloned.

The rare 14-carbon fatty acid myristate is covalently attached via a stable amide linkage to the α-amino group of N-terminal glycine residues, constituting the other major type of protein fatty acylation [for reviews, see Olson (1988) and Schultz et al. (1988)]. Although the somewhat leaky nature of enzymes involved in ester-linked fatty acylation of proteins allows a low level of posttranslational incorporation of myristate by certain proteins, acylation of classical myristoylated proteins occurs cotranslationally on nascent polypeptide chains and is thus completely abolished by inhibitors of protein synthesis (Wilcox et al., 1987). In contrast to the near exclusive localization of palmitoylated proteins to the plasma membrane, myristoylated proteins are found in a number of subcellular compartments, including the cytosol, plasma membrane, endoplasmic reticulum, and nucleus [see Olson and James (1989)]. The existence of soluble myristoylated proteins, together with the specific targeting of myristoylated proteins to distinct locations within the cell, suggests that the myristoyl moiety serves as more than just a hydrophobic membrane anchor. Rather, it appears that this relatively rare modification is necessary for correct orientation of the protein within appropriate membrane structures and/or interaction with critical substrates.

N-Myristoyltransferase (NMT), the enzyme responsible for myristoylation of proteins, has been purified and cloned from *Saccharomyces cerevisiae* and its substrate specificity thoroughly characterized (Towler & Glaser, 1986; Towler et al., 1987a,b, 1988a). The properties of this enzyme have been reviewed elsewhere and will not described in detail here (Towler et al., 1988b; Olson, 1988). The enzyme exhibits remarkable substrate specificity for fatty acyl donor and protein acceptor: only N-terminal glycine can be acylated, and myristoyl-CoA is highly favored as fatty acyl donor. Using myristoylation of synthetic peptides as an assay, Towler and co-workers have defined additional amino acids near the N-terminus which influence the ability of a protein to serve as a substrate for NMT. These studies have resulted in the formation of a loose consensus sequence for myristoylation that has been found in all proteins known to be myristoylated in vivo.

In all known cases, myristoylation of cellular protein occurs on penultimate glycine residues, thus requiring prior removal of the initiating methionine. Although NMT does not possess an intrinsic aminopeptidase activity (Towler et al., 1987b), an aminopeptidase is known to catalyze removal of amino-terminal methionine residues after elongation of the first 30–40 amino acids (Palmiter, 1977). NMT activity is found in both the crude membrane and soluble fractions (Towler & Glaser, 1986), suggesting that it may be a peripheral membrane protein. Alternatively, it may associate with ribosomes as part of an amino-terminal processing complex, as is the case with some *N*-acetyltransferases (Pestana & Pitot, 1975), thus facilitating stoichiometric myristoylation of substrate proteins. Indeed, the fact that purified myristoylated proteins appear to be quantitatively blocked at their N-termini suggests that myristoylation occurs stoichiometrically. The cotranslational nature of myristoylation also indicates that NMT must interact with the elongating polypeptide soon after initiation of protein synthesis (Wilcox et al., 1987).

The extreme specificity exhibited by NMT for fatty acyl CoA donor, coupled with the fact that myristate comprises only 1–3% of total fatty acids in eukaryotic cells (Khandwala & Casper, 1971), suggests that the myristoyl moiety provides a structural feature that is critical for the normal function of the parent protein and that fatty acids other than myristate cannot duplicate this feature. Indeed, incorporation of a myristate analogue (11-oxymyristic acid) of reduced hydrophobicity into cellular proteins resulted in altered membrane association of $p60^{v\text{-}src}$ and a related protein from BC_3H1 cells (Heuckeroth & Gordon, 1989). This finding is of particular interest in light of the recent identification of a $p60^{src}$ receptor in the plasma membrane which binds only the myristoylated form of the protein (Resh, 1989). Taken together, these data strongly suggest a specific role for myristoylation in the sorting of myristoyl proteins within cells and demonstrate that the precise structural features of myristate may be required for this function.

Fatty Acylated Proteins in Growth Factor Signaling Pathways

Studies involving metabolic labeling of tissue culture cells with ^3H-labeled fatty acids have revealed numerous proteins acylated by either palmitate or myristate (Olson et al., 1985; Magee & Courtneidge, 1985; McIlhinney et al., 1985). Of the small number that have been identified, many (but not all) are known to be critical components of growth factor signaling pathways, and in at least some cases, this modification has been found to be essential for efficient functioning of the relevant pathway. To date, proteins that function as cell surface receptors, tyrosine and serine/threonine kinases, their substrates, a phosphatase, G-proteins, and Ca^{2+}-binding proteins are known to be fatty acylated (for references, see below). It thus seems likely that additional fatty acylated proteins will be identified which perform key regulatory functions in growth control. In the following sections, we will discuss evidence, much of it very recent, concerning the functions of fatty acylation in the establishment of intracellular signaling pathways.

RAS and RAS-Related Proteins. The mammalian ras gene products, as well as their yeast homologues, bind guanine nucleotides and exhibit intrinsic GTPase activity. Point mutations in mammalian RAS proteins that abolish GTPase activity induce cellular transformation, presumably due to persistent activation of one or more growth factor signaling pathways [see Barbacid (1987) for review]. RAS proteins incur a number of posttranslational modifications (now known to include C-terminal proteolysis of three amino acids, carboxyl methylation, polyisoprenylation, and palmitoylation), which

ultimately result in their binding to the inner face of the plasma membrane (Tamanoi et al., 1988; Clarke et al., 1988; Gutierrez et al., 1989; Hancock et al., 1989). Proteolytic mapping experiments have shown that RAS is not deeply embedded in the bilayer, despite its tight association with the membrane (Grand et al., 1987).

Palmitoylation of RAS was first demonstrated by Buss and Sefton (1986), and it was postulated to provide a mechanism for targeting the protein to the membrane. A C-terminal sequence of four amino acids (C-A-A-X, where C = cysteine, A = aliphatic R group, and X = any amino acid) is conserved among virtually every member of the RAS and RAS-related family of proteins. Initial mutational analyses revealed that these four C-terminal amino acids are required for membrane localization, palmitoylation, and transformation (Willumsen et al., 1984a,b). These studies suggested that the cysteine residue in this conserved sequence served as the acceptor amino acid for acylation. However, more recent work has demonstrated that this C-terminal cysteine actually acquires a polyisoprenoid moiety in the cytosol (Hancock et al., 1989). Palmitoylation of RAS proteins occurs at a cysteine residue located two to six amino acids upstream of the C-terminal cysteine (the exact position varies between different members of the family). The earlier finding, that deletion of the C-terminal cysteine residue resulted in a nonacylated protein, is now explained by the fact that this residue is polyisoprenylated in the cytosol, which apparently facilitates interaction of RAS proteins with the membrane. Palmitoylation is thus dependent upon prior isoprenylation. It is not known for certain whether the dependence of palmitoylation on isoprenylation reflects a recognition event by the palmitoyltransferase or is due to the specific localization of the palmitoyltransferase to the plasma membrane. Isoprenylated but nonpalmitoylated mutants of RAS interact weakly with the plasma membrane and exhibit reduced biological activity. Palmitoylation increases both membrane affinity and biological activity, indicating that the palmitate moiety is required for optimal functioning of RAS proteins, perhaps by mediating appropriate interactions between RAS and other proteins in the membrane.

Additional data that suggest a critical functional role for palmitoylation in regulating the activity of RAS proteins come from a study that demonstrated rapid turnover of the fatty acid moiety associated with $p21^{N\text{-}ras}$ (Magee et al., 1987). In T15 cells overexpressing the human N-ras gene, fatty acid turnover on $p21^{N\text{-}ras}$ occurred with a half-life of $\sim$20 min, whereas the half-life of the protein itself was $\sim$1 day. This implies that a rapid cycle of acylation/deacylation takes place during normal functioning of RAS proteins. Whether fatty acid turnover occurs with similar kinetics, if at all, on transforming RAS mutants has not been reported.

Further attempts to define the role of fatty acylation in RAS function have employed chimeric proteins possessing a myristoylation signal at their N-termini. Membrane binding and transforming potential of activated H-ras proteins, lacking the C-terminal cysteine residue required for proper processing and membrane localization, was restored by fusion of either 15 amino-terminal amino acids of $p60^{src}$ or 11 amino acids of the Gag protein of Rasheed leukemia virus, both of which direct myristoylation of the fusion protein (Buss et al., 1989). These results indicated that activated RAS proteins are capable of transmitting an oncogenic signal regardless of the lipid moiety that anchors them to the plasma membrane and suggest that palmitoylation and myristoylation may be interchangeable for RAS function. However, it was found that normal cellular RAS proteins also acquired transforming potential upon addition of a myristoylation signal. These data can be interpreted to indicate that reversible fatty acylation (i.e., palmitoylation) serves to modulate the avidity of membrane binding or coupling between RAS proteins and key effector proteins, whereas myristoylated forms of these proteins remain tightly associated with the membrane and thus are unable to be uncoupled from other regulatory proteins. It will be important to determine if cellular RAS proteins possessing a myristoylation signal still become palmitoylated at their C-termini and, if so, whether their ability to become deacylated has been altered.

Much knowledge regarding the processing and function of proteins in the RAS family has come from studies in yeast. RAS1 and RAS2 proteins from *S. cerevisiae* are structurally similar to mammalian RAS proteins (Powers et al., 1984). These proteins bind GTP, possess intrinsic GTPase activity, and have been shown to modulate the activity of yeast adenylate cyclase (Broek et al., 1985; Toda et al., 1985). At least one RAS protein is required in yeast, as mutants defective in both *ras* genes are incapable of vegetative growth, whereas haploid cells lacking only one grow normally (Kataoka et al., 1984; Tatchell et al., 1984). Consistent with the situation in mammalian systems, RAS1 and RAS2 proteins are palmitoylated posttranslationally (Fujiyama & Tamanoi, 1986). Mutational analysis of RAS2 has also shown that fatty acylation is required for membrane association and complementation of $RAS1^-$ mutants (Deschenes & Broach, 1987). Three allelic yeast mutants (supH, ste16, and dpr1) have been identified which are required for RAS function as well as for production of the mating hormone α-factor (Powers et al., 1986; Fujiyama et al., 1987). It was originally proposed that this allele, termed RAM (RAS protein and α-factor maturation function), might encode an acyltransferase (Powers et al., 1986). At that time it was believed that yeast α-factor precursors were also palmitoylated. The predicted C-terminal sequence of both α-factor polypeptide precursors consists of Cys-Val-Ile-Ala (Betz et al., 1987), matching the sequence previously believed to be a consensus for palmitoylation of ras proteins. However, the recent finding by Hancock et al. that the C-A-A-X sequence directs polyisoprenylation rather than palmitoylation, combined with another recent report that yeast α-factor precursors are indeed isoprenylated at their C-termini (Anderegg et al., 1988), strongly suggests that the RAM gene actually encodes an enzyme involved in isoprenylation. Although the precise order of events involved in C-terminal modification of RAS proteins is not known, it has been proposed that polyisoprenylation occurs first and creates a recognition signal for a carboxypeptidase which removes the three C-terminal amino acids (Hancock et al., 1989). The newly generated α-carboxyl group of the C-terminal cysteine would then be available for methylation. The fact that isoprenoid chains could not be detected on newly synthesized $pro\text{-}p21^{ras}$ suggests that the conversion from pro-p21 to processed c-p21 (involving isoprenylation, C-terminal proteolysis, and carboxyl methylation) occurs very rapidly and may be mediated by a single enzyme or multienzyme complex. Reconstitution experiments utilizing in vitro translated RAS and RAM gene products should aid in defining the catalytic activity encoded by the RAM gene. If the enzymes responsible for polyisoprenylation and C-terminal proteolysis/carboxyl methylation are encoded by distinct loci, it should be possible to isolate yeast mutants that are polyisoprenylation positive but defective in one or both of the latter.

Additional yeast and mammalian proteins have been identified which share significant homology with mammalian RAS

Table I: RAS and Related Proteins

protein	C-terminal sequence	polyisoprenylated[a]	palmitoylated	refs
H-ras	ESGPGCMSCKCVLS	+	+	Sefton et al., 1982
N-ras	DGTQGCMGLPCVVM	+	+	Magee et al., 1987
K(A)-ras	KTPGCVKIKKCVIM	+	+	Shimizu et al., 1983; Capon et al., 1983; Buss & Sefton, 1986
K(B)-ras	KKKKKKSKTKCVIM	+	-	Shimizu et al., 1983; Capon et al., 1983; Buss & Sefton, 1986
Krev-1	VEKKKPKKKSCLLL	+	-	Kitayama et al., 1989
Ras1	NARKEYSGGCCIIC	+	+	Powers et al., 1984; Fujiyama & Tamano, 1986
Ras2	EASKSGSGGCCIIS	+	+	Powers et al., 1984; Fujiyama & Tamano, 1986
α-factor	IIKGVFWAPACVIA	+	+	Betz et al., 1987; Anderegg et al., 1988
YPT1	KGNVNLKGGCC	-	+	Molenaar et al., 1988; Gallwitz et al., 1983
rhoA	QARRGKKKSGCLVL	+	-	Hall, 1989
rhoB	YGSQNGCINCCKVL	+	+	Hall, 1989
rap-1a	VEKKKPKKKSCLLL	+	-	Hall, 1989
rap-2	PDKDDPCCSACNIQ	+	+	Hall, 1989

[a] Either demonstrated or predicted, on the basis of studies by Hancock et al. (1989).

Table II: GTP-Binding Proteins

protein	N-terminal sequence	C-terminal sequence	predicted substrate for NMT	myristoylated	pertussis toxin sensitive[a]	refs
$G_{i\alpha}$	MGCTLSA	CGLF	+	+	+	Buss et al., 1987; Nukada et al., 1986; Bray et al., 1987
$G_{o\alpha}$	MGCTLSA	CGLY	+	+	+	Van Meurs et al., 1987; Itoh et al., 1986
$G_{s\alpha}$	MGCLGNS	YELL	-	-	-	Robishaw et al., 1986; Yatsunami & Khorana, 1985
$G_{t\alpha}$[b]	MGAGASA	GCLF	+	-	-	Lochrie et al., 1985; Tanabe et al., 1985
$G_{x\alpha}$	MGCRQSS	IGLC	o[c]	unknown	-	Matsuoka et al., 1988
G_{olf}	MGCLGNS	YELL	-	unknown	-	Jones & Reed, 1989
$G_{t\gamma}$	MPVINIE	CVIS	-	-	+	Hurley et al., 1984
G_{γ}	MASNNIA	CAIL	-	-	+	Gautam et al., 1989

[a] Either known or predicted, on the basis of the presence of a cysteine near the carboxy terminus. [b] Two $G_{t\alpha}$ proteins have been characterized. However, their amino acid sequences are identical within the first six residues. [c] o indicates "consensus" sequence for myristoylation.

proteins. These include yeast and mammalian YPT1 (Gallwitz et al., 1983) and Krev-1 (a suppressor of ras transformation) (Kitayama et al., 1989) and members of the rho, rab, and rap gene families [for review, see Hall (1989)]. Yeast YPT1, which is required for cell viability, lacks a C-terminal consensus for polyisoprenylation but does possess two C-terminal cysteine residues and has been shown to be palmitoylated (Molenaar et al., 1988). Although the protein is found in both the membrane and soluble fractions, palmitoylated YPT1 is localized to the membrane, indicating that palmitoylation occurs immediately prior to, or concomitant with, membrane association. Mutants lacking either one of the C-terminal cysteines were functionally indistinguishable from wild-type proteins. Deletion of both C-terminal cysteine residues, however, yielded a nonpalmitoylated mutant that was biologically inactive and found exclusively in the soluble fraction, suggesting that palmitoylation does indeed regulate the subcellular distribution of YPT1 (Molenaar et al., 1988).

Hancock et al. demonstrated that a C-terminal C-A-A-X sequence is sufficient to direct polyisoprenylation and weak membrane binding of a heterologous protein. However, only proteins that also contain a cysteine residue a short distance upstream of the C-terminus are predicted to be palmitoylated. Those proteins lacking a site for palmitoylation, however, contain a polybasic domain, consisting of at least five positively charged basic amino acids immediately upstream of the C-A-A-X motif, which might be expected to facilitate interaction with negatively charged polar head groups on the surface of a lipid bilayer. As noted in this study, it is interesting that the ras, rho, and rap families are each predicted to encode both palmitoylated and nonpalmitoylated proteins (see Table I). Thus, the confinement of these proteins to the plasma membrane indicates that mechanisms in addition to fatty acylation participate in determining their subcellular localization and suggests that specific protein–protein interactions, perhaps in the form of RAS "receptors", are also involved. It has not been reported whether binding of fully processed RAS proteins to the membrane can be duplicated in artificial liposomes or if protein components are also required. Determination of the precise functional role for palmitoylation of ras and ras-related proteins will likely await the identification of effector proteins whose interaction with ras is required for normal signaling.

Additional GTP-Binding Proteins. Heterotrimeric G proteins, composed of α, β, and γ subunits, participate in transmembrane signaling by coupling the intracellular domains of cell surface receptors with appropriate effector proteins. G_s and G_i are involved in stimulation and inhibition, respectively, of adenylate cyclase [see Gilman (1987) for review]. The function of G_o, another member of the G protein family (Sternwies & Robishaw, 1984; Neer et al., 1984), is unknown, but a role for this complex in regulation of calcium channels has been proposed (Hescheler et al., 1987). Transducin (G_t) is localized to disc membranes of retinal rod outer segments and activates cGMP phosphodiesterase in response to photosignal transduction [see Stryer et al. (1981)]. The GTP-binding and GTPase activities of G proteins reside in the α subunit, whereas the β/γ subunits are thought to help anchor the complex in the plasma membrane.

cDNA clones encoding at least nine different α subunits have now been isolated from various sources (see Table II). The deduced amino acid sequence of each of these contains glycine as the penultimate residue, and additional residues within the first six amino acids of each protein are compatible with the minimal sequence requirements established by Towler and co-workers for recognition by NMT in vitro. However, Buss et al. have reported that only the α subunits of G_i and G_o are myristoylated, whereas covalent myristate was not detected on $G_{s\alpha}$ or $G_{t\alpha}$ (Buss et al., 1987). Closer inspection of the N-terminal amino acid sequence of $G_{s\alpha}$ revealed glycine and asparagine at positions 4 and 5, respectively. In their in vitro studies of NMT, Towler and co-workers found that placing asparagine in position 5 of a known myristoylated

peptide (corresponding to the N-terminus of the catalytic subunit of cAMP-dependent protein kinase, see below) yielded a substrate with an ~10-fold higher K_m compared to that of the parental peptide. Similarly, glycine in position 4 of the same parental peptide resulted in a 9-fold increase in K_m (Towler et al., 1988). While these data may explain the absence of myristate on $G_{s\alpha}$, similar peculiarities within the N-terminal sequence of $G_{t\alpha}$ were not found, and it is currently unclear why myristoylation of this protein was not detected. Perhaps the in vitro substrate specificities of NMT are subject to additional constraints in an in vivo setting, which might result in myristoylation of only a subset of proteins possessing an N-terminal myristoylation "consensus" sequence. Alternatively, the absence of myristate on $G_{t\alpha}$ may reflect failure to remove the initiating methionine. An additional G protein α subunit, designated $G_{x\alpha}$, has recently been identified which has significant amino acid similarity with $G_{i\alpha}$ and $G_{s\alpha}$ (including a glycine residue at position 2), but the possibility that this protein might be myristoylated was not examined (Matsuoka et al., 1988). Given the apparent exception, in the case of $G_{t\alpha}$, to the consensus sequence for N-terminal myristoylation, it is not possible to predict with certainty whether $G_{x\alpha}$ might be myristoylated. Finally, an olfactory neuron specific G protein α subunit, termed G_{olf} has been characterized which can activate adenylate cyclase in a heterologous system (Jones & Reed, 1989). The first six amino acids of the deduced sequence of G_{olf} are identical with those found in $G_{s\alpha}$, and therefore, by inference, one would not expect this protein to be myristoylated. This remains to be demonstrated, however, as sequences beyond the first six amino acids may contribute to a protein's ability to be recognized by NMT. Thus, as for the ras family, the G protein gene family codes for both acylated and nonacylated proteins.

It is interesting, in light of the recent findings concerning targeting of ras proteins to the plasma membrane, that the predicted amino acid sequences of several members of the G protein family terminate with the C-A-A-X motif, which was shown to be sufficient to direct the isoprenylation of a heterologous protein (Hancock et al., 1989). This C-terminal sequence is found in all pertussis toxin sensitive G protein α subunits (see Table II), as well as in both G protein γ subunits characterized to date. Indeed, the cysteine residue of this sequence in $G_{t\alpha}$ has been identified as the ADP-ribose acceptor site (West et al., 1985). Pertussis toxin insensitive α subunits lack this cysteine residue. It will be interesting to determine if any members of this family are isoprenylated and, if so, how this might affect their availability as a substrate for pertussis toxin.

The functional consequences of myristoylation of $G_{i\alpha}$ and $G_{o\alpha}$ are currently unknown. It has been suggested that interaction of G proteins with the plasma membrane might involve formation of a β–γ subunit "anchor" to which α subunits bind (Gilman, 1987). The possibility of γ subunits being isoprenylated, as mentioned above, would support their proposed role as part of an anchor. Myristoylation of certain α subunits might be required for their interactions with β–γ complexes, or it might facilitate their coupling with receptors and effector proteins, thus allowing efficient utilization of the relevant signaling pathway. Mutational analyses of $G_{i\alpha}$ and $G_{o\alpha}$ should result in the definition of specific, myristoylation-dependent protein–protein interactions in which these two proteins are involved.

The α subunits of G_s and G_t are also subject to cholera toxin catalyzed ADP-ribosylation at an internal arginine residue, resulting in the irreversible activation of adenylate cyclase (Cassel & Selinger, 1977) and inhibition of the light-stimulated GTPase activity of rod outer segments (Abood et al., 1982), respectively. Numerous ADP-ribosylation factors (ARFs), 21-kDa GTP-binding proteins that serve as required cofactors in this reaction, have recently been described. Their ability to function as cofactors for α subunit ADP-ribosylation is dependent upon GTP binding, but they possess no detectable GTPase activity (Schleifer et al., 1982; Kahn & Gilman, 1984, 1986). Although ARF was originally purified from a membrane preparation, immunological studies demonstrated that the majority of at least one ARF is present in the cytosol (Kahn et al., 1988). cDNA clones encoding ARFs have been isolated from bovine, yeast, and chicken (Sewell & Kahn, 1988; Price et al., 1988; Alsip & Konkel, 1986). Kahn et al. first demonstrated the presence of covalent myristate on the N-terminal glycine residue from purified bovine brain ARF, and subsequent cDNA cloning revealed a predicted N-terminal amino acid sequence that is consistent with the protein serving as a substrate for NMT. Vaughan and co-workers have described two soluble ARF-like proteins, sARFI and sARFII, from bovine brain. They isolated a cDNA clone, using oligonucleotides based on the amino acid sequences of peptides derived from sARFII, that is nearly identical with that described by Sewell and Kahn (Price et al., 1988). Although the deduced amino acid sequence of the sARF cDNA does not perfectly match the peptide sequences from purified sARFII, it nevertheless appears to encode an ARF-like protein. Both cDNAs are predicted to encode proteins of 181 amino acids. Inspection of these amino acid sequences revealed that 173/181 positions are identical. However, one of the eight substitutions occurred at position 5: alanine in one versus glutamate in the other. The protein with alanine at position 5 has been shown to be myristoylated. Although possible myristoylation of the other clone (glutamate at position 5) was not examined, peptides with a charged residue at position 5 were not substrates for NMT in vitro (Towler et al., 1987b). Whether these sARFs might also associate with the membrane has not been reported. It is noteworthy that the sARFII peptide sequences used for designing oligonucleotide probes match perfectly with sequences found within the ARF described by Sewell and Kahn. This, combined with the observation that a majority of the ARF described by the latter group is found in the cytosol, suggests that these proteins may be identical. However, the isolation of a closely related but distinct ARF-like cDNA demonstrates that a family of genes encoding these proteins is likely to exist. It will be interesting to determine whether ARF-like proteins are universally myristoylated or, as is the case with the ras and G protein α subunit families, if only a subset are subject to fatty acylation. Selective myristoylation represents a potential mechanism whereby the interactions of various members of a given family of proteins with key components of a signaling pathway might be restricted. Identification of the normal physiological role of ARF proteins in eukaryotic cells will undoubtedly contribute to the understanding of how myristoylation participates in their function. However, with cDNA clones in hand, mutational analyses can now be performed to define the requirements, if any, for myristoylation in the interaction between ARFs and G protein α subunits.

Tyrosine Kinases and Substrates. Nonreceptor tyrosine kinases have been implicated as major participants in a number of cellular processes regulating growth and differentiation, and it is now apparent that a large family of these enzymes exists in mammalian cells (see Table III). Since the discovery that the transforming potential of Rous sarcoma virus is due to a

Table III: Nonreceptor Tyrosine Kinase

protein	N-terminal sequence	predicted substrate for NMT[a]	myristoylated	refs
SRC	MGSSKSK	+	+	Takeya & Hanafusa, 1983; Buss & Sefton, 1985
YES	MGCIKSK	+	+	Sukegawa et al., 1987; Sudel et al., 1988
SYN/FYN	MGCVQCK	+	+	Semba et al., 1986; Kypta et al., 1988; Cheng et al., 1988
FGR	MGCVPCK	+	unknown	Inoue et al., 1987
HCK	MGCMKSK	+	unknown	Ziegler et al., 1987; Quintrell et al., 1987
LCK	MGCVCSN	+	+	Voronova et al., 1984; Marchildon et al., 1984; Marth et al., 1985
LYN	MGCIKSK	+	unknown	Yamanashi et al., 1987
FPS/FES	MGFSSEL	-	-	Groffen et al., 1983
FER	MGFGSDL	-	-	Hao et al., 1989

[a] Sequences of the cellular-derived proteins are given.

protein tyrosine kinase encoded by the *src* gene, several other transforming retroviruses have been shown to encode tyrosine-specific protein kinases. These include *v-fgr* of Gardner–Rasheed feline sarcoma virus, *v-yes* of Y73 avian sarcoma virus, and *v-fps* of Fujinami sarcoma virus [for review, see Hunter and Cooper (1985)]. In addition, normal cellular homologues for each of these, as well as several tyrosine kinase encoding protooncogenes not found associated with retroviruses, have recently been identified (see Table III). Members of this family are characterized by a highly conserved C-terminal catalytic domain, whereas the N-terminal regions diverge significantly. Despite this overall divergence in their N-terminal domains, several members of this family are very similar within the first six amino acids following the initiating methonine. Consistent with this observation, SRC, YES, SYN/FYN, and LCK have been shown to be myristoylated, and additional members are predicted to undergo this modification, on the basis of sequence similarities with the three mentioned above (Table III).

Certain members of this family, such as HCK (Ziegler et al., 1987; Quintrell et al., 1987) and LCK (Marth et al., 1985), exhibit tissue or cell type specific expression, whereas others are found in a wide variety of cells. The simultaneous expression of two or more nonreceptor tyrosine kinases in a single cell type suggests that the functions of these enzymes may not be entirely overlapping. Indeed, the variable N-terminal domains of these proteins have been proposed to confer substrate specificity. However, key substrates involved in transformation may be accessible to multiple SRC-related kinases, as an activated form of $p59^{hck}$, which is expressed primarily in hematopoietic cells, has been shown to transform NIH 3T3 fibroblasts (Ziegler et al., 1989). Inspection of the first six N-terminal amino acids of several members of this family reveals another interesting distinction between them. The N-terminal sequence of the first seven proteins listed in Table III indicates that they are likely to serve as substrates for NMT, and myristoylation of SRC, YES, SYN/FYN, and LCK has indeed been demonstrated. In contrast, the N-terminal sequences of FPS/FES and FER, which are more closely related to each other than to other members of the family, are incompatible with recognition by NMR. Specifically, each contains phenylalanine at position 2 (with glycine at position 1) and glutamate (FPS/FES) or aspartate (FER) at position 5. In vitro studies demonstrated that peptides with aromatic side chains at position 2 or charged residues at position 5 were not myristoylated (Towler et al., 1987a,b). The fact that these two proteins fall into both categories, combined with the observation that 60–90% of $p92^{c\text{-}fps/fes}$ is found in the soluble fraction (Young & Martin, 1984), strongly suggests that they are not myristoylated in vivo. Interestingly, $p140^{gag\text{-}fps}$, the viral transforming counterpart of c-fps/fes, is normally membrane associated, while in a temperature-sensitive mutant defective for transformation it is soluble (Moss et al., 1984). The molecular basis for this change in subcellular location has not been reported. Thus, as is the case for the RAS and G protein families, the nonreceptor tyrosine kinase family is apparently composed of both acylated and nonacylated members.

Although the precise functions of nonreceptor tyrosine kinases remain unknown, it is now obvious in the case of SRC that the myristoyl moiety it acquires during its synthesis is essential for targeting to the plasma membrane and for specific protein–protein interactions. Initial studies established that SRC is translated and myristoylated in the cytosol and binds to the plasma membrane within 15 min after synthesis (Buss et al., 1984). A number of subsequent mutational analyses demonstrated that nonmyristoylated mutants of SRC, lacking glycine at the penultimate position, are defective in membrane association and transformation, despite having normal tyrosine kinase activities (Cross et al., 1984; Pellman et al., 1985; Kamps et al., 1985; Buss et al., 1986). In addition, the initiating methionine was removed from the N-terminus of these mutant proteins, indicating that lack of myristoylation was not due to nonrecognition by aminopeptidase. Consistent with their lack of membrane association, nonmyristoylated mutants were no longer substrates for protein kinase C (Buss et al., 1986), although their association with the soluble "carrier" proteins p59 and p80 was not affected. Together, these data demonstrated that the covalent modification of SRC with fatty acid plays a crucial role in determining its subcellular location and transforming potential. The basis for the transformation deficiency of these mutants was not immediately clear, as many known substrates of $p60^{v\text{-}src}$ were still phosphorylated by nonmyristoylated mutants (Kamps et al., 1986). However, studies using anti-phosphotyrosine antibodies have resulted in the identification of membrane-associated proteins that are substrates for myristoylated but not nonmyristoylated SRC, suggesting that these proteins participate in the process of transformation (Linder & Burr, 1988; Hamaguchi et al., 1988; Reynolds et al., 1989). Also, it was found that nonmyristoylated mutants of $p60^{v\text{-}src}$ retained their ability to stimulate cell proliferation, suggesting that the SRC substrates regulating proliferation are soluble and distinct from those that participate in transformation (Calothy et al., 1987). Recently, however, overexpression of $p60^{c\text{-}src}$ has been shown to confer enhanced mitogenic responsiveness on 10T1/2 murine fibroblasts in a myristoylation-dependent manner (Luttrell et al., 1988; Wilson et al., 1989). Therefore, while oncogenically activated SRC can induce cell proliferation in the absence of myristoylation, the role of normal $p60^{c\text{-}src}$ in this process is dependent upon membrane association. Interestingly, the hyperresponsiveness of these cells is expressed only in response to epidermal growth factor, as growth in 10% fetal calf serum yielded no increased mitogenic response. These findings not

only provide important clues as to the function of normal SRC in controlling cell growth but also pinpoint a specific growth factor signaling pathway which may be involved in SRC-mediated transformation.

The mutational analyses discussed above demonstrated clearly that membrane association of SRC is dependent upon myristoylation. However, the fact that membrane-bound SRC is localized to the plasma membrane, rather than spread evenly among many intracellular membranes, indicated that the myristoyl moiety must provide a structural feature that confers properties more unique than a nonspecific hydrophobic anchor which might partition into any lipid bilayer. Futhermore, the existence of other myristoylated proteins that reside mainly in the cytosol suggested that myristoylation per se is not sufficient to direct a protein to the plasma membrane and that specific protein–protein interactions are involved as well. Also, the identification of transformation-defective SRC proteins that remain soluble despite being myristoylated provides further evidence that structural properties in addition to myristoylation are required for binding of SRC to the plasma membrane (Buss & Sefton, 1985; Garber et al., 1985). Thus, recent studies that demonstrated the presence of a proteinaceous, plasma membrane localized SRC-specific binding site, which recognizes only the myristoylated form of the protein, generated excitement but little surprise (Resh, 1989; Goddard et al., 1989).

Chimeric proteins, constructed by Hanafusa and co-workers, provided the first hint that the myristoylated N-terminus of SRC contains a recognition signal for targeting to the plasma membrane. They fused the first 14 amino acids of SRC to chimpanzee α-globin, which is normally soluble. The resulting fusion protein was myristoylated, and it fractionated in the crude membrane pellet, demonstrating the ability of the N-terminal region of SRC to target an otherwise soluble protein to the membrane (Pellman et al., 1985). The first evidence of a requirement for membrane components other than the phospholipid bilayer in the binding of SRC was obtained by studying the association of $p60^{v\text{-}src}$ with phospholipid vesicles (Resh, 1988). $p60^{v\text{-}src}$ obtained by detergent extraction of cell membranes was efficiently reconstituted into phospholipid vesicles in a manner dependent upon a myristoylated, N-terminal 10-kDa domain. In contrast, myristoylated SRC molecules obtained from a high-speed supernatant fraction were reconstituted only in the presence of added membrane proteins. Even more convincing evidence for an "SRC receptor" was provided by binding studies using in vitro translated SRC protein and a cellular plasma membrane enriched fraction (Resh, 1989). Binding of newly synthesized $p60^{v\text{-}src}$ occurred on the inner face of the plasma membrane and was saturable, myristoylation dependent, and sensitive to heat and trypsin. Furthermore, binding could be competed by a myristoylated peptide corresponding to the first 11 amino acids of SRC but not by the nonmyristoylated peptide or myristoylated peptides derived from the N-termini of other known myristoylated proteins. Similar results were obtained with an iodinated, 15 amino acid peptide from the N-terminus of SRC and red cell membrane vesicles (Goddard et al., 1989). Again, binding was restricted to the myristoylated SRC peptide and was destroyed by prior protease treatment of vesicles. These results demonstrate the existence of one or more membrane proteins that exhibit the characteristics of a high-affinity receptor, with specificity for a myristoylated SRC "ligand".

The identity of this putative receptor and the nature of its association with the plasma membrane are currently unknown. However, in light of the biological activities of both normal and oncogenic SRC discussed above and their dependence on myristoylation and membrane binding, it seems likely that this receptor represents a key regulatory element in SRC-mediated signaling pathways. The fact that membrane-bound SRC is resistant to salt extraction indicates that the receptor itself also interacts tightly with the membrane, possibly as an integral membrane protein. An intriguing possibility is that the SRC receptor represents the cytoplasmic domain of a transmembrane growth factor receptor, thus providing a direct link between an extracellular stimulus and an intracellular signaling protein, analogous to the coupling of G proteins with β-adrenergic receptors.

An additional question that arises is whether binding of SRC to its receptor can be competed by a SRC peptide with an N-terminal palmitate moiety. Given the extreme specificity for myristoyl-CoA with which NMT has evolved, one would not expect fatty acids of other chain lengths to substitute efficiently for myristate. The in vitro binding assays that have now been developed should allow this question to be answered. An alternative approach to investigating the importance of chain length and hydrophobicity in protein–membrane interactions of myristoylated proteins has recently been reported. An oxygen-substituted analogue of myristate, 11-oxymyristic acid, was synthesized and found to function as a fatty acyl donor for NMT in vitro, albeit at a reduced efficiency that varied among several peptide substrates tested (Heuckeroth & Gordon, 1989). Consistent with the in vitro data, this analogue was incorporated by only a subset of myristoylated proteins from yeast and BC_3H1 cells, a murine myocyte cell line. Incorporation of 11-oxymyristic acid by $p60^{v\text{-}src}$ and a 63-kDa protein from the BC_3H1 cell line resulted in their redistribution from the membrane to the soluble fraction. It appears, therefore, that incorporation of a myristate analogue of similar length but reduced hydrophobicity results in less efficient binding of SRC to its receptor. The ability of specific fatty acid analogues to be selectively incorporated into certain oncogenic proteins and to alter their subcellular distribution raises the possibility that certain analogues might be designed that would interfere with transformation.

We have also observed the 63-kDa protein during our studies of fatty acylated proteins in BC_3H1 cells. Its size, p*I*, and tyrosine phosphorylation suggested that it might represent SRC or a closely related protein. Indeed, cDNA cloning has revealed that this protein is encoded by the *syn/fyn* gene (G. James and E. Olson, unpublished data). The parallel altered distribution of this protein and SRC following incorporation of 11-oxymyristic acid would suggest that additional members of the nonreceptor tyrosine kinase family also possess membrane "receptors" with characteristics similar to those of the SRC receptor. Alternatively, two or more cellular tyrosine kinases may interact with the same receptor. Isolating and characterizing these molecules will be an important area for future studies.

The 36-kDa calpactin I heavy chain (also known as p36 and lipocortin II) is phosphorylated by $p60^{src}$ and exhibits calcium-dependent association with the plasma membrane [for recent review, see Klee (1988)]. This protein has been reported to be myristoylated in a transformation-sensitive manner (Soric & Gordon, 1985). In this study, transformation of chick embryo fibroblasts by $p60^{v\text{-}src}$ resulted in a reduction in the amount of [^{3}H]myristate incorporated by p36 compared to that in nontransformed cells. The radioactivity associated with this protein was identified as authentic myristate, and its resistance to hydroxylamine treatment suggested an amide linkage. However, subsequent molecular cloning of a cDNA

encoding p36 revealed a predicted N-terminal amino acid sequence that cannot serve as a substrate for NMT (Saris et al., 1986). Also, the decreased myristoylation of p36 upon *src* transformation occurred without any apparent reduction in synthesis of the protein, which suggests that p36 is not subject to classical, cotranslational myristoylation. Thus, myristoylation of this protein may be mediated by a unique pathway. It is tempting to speculate that the fatty acyl moiety associated with p36 may participate in its acquisition of a hydrophobic domain during calcium-induced conformational changes that it is known to undergo, thereby facilitating interactions with the plasma membrane.

Serine/Threonine Kinases, Phosphatases, and Substrates. The catalytic subunit of cAMP-dependent protein kinase was among the first proteins demonstrated to contain covalent myristate (Carr et al., 1982), but no information is available concerning any possible role this modification may play in substrate recognition or subcellular localization of the enzyme. The inactive enzyme is a membrane-bound tetrameric complex composed of two regulatory (R) and two catalytic (C) subunits. Elevation of intracellular cAMP levels, provoked by a variety of stimuli, results in the binding of cAMP by an R_2 dimer and the release of two monomeric, active subunits into the soluble fraction [reviewed by Edelman et al. (1987)]. Given the requirement for covalent fatty acids in mediating protein–membrane and protein–protein interactions of other known fatty acylated proteins, it appears likely that myristoylation of the cAMP-dependent protein kinase catalytic subunit plays a significant role in its association with either the regulatory subunits or important substrates. Mutational analyses, similar to those described for SRC, should provide clues as to the role of covalent myristate in the functioning of cAMP-dependent protein kinase.

Another myristoylated protein that has been implicated in serine/threonine kinase signaling pathways in the B subunit of calcineurin (Aitken et al., 1982). Calcineurin is a Ca^{2+}/calmodulin-dependent protein phosphatase composed of two subunits. Subunit A interacts with calmodulin and contains the catalytic site, whereas subunit B binds calcium (Manalan & Klee, 1983; Tonks & Cohen, 1983). Catalytic activity of the A subunit is stimulated by either the B subunit or calmodulin, but only in the presence of calcium (Stewart et al., 1983). As is the case with cAMP-dependent kinase, however, requirements for myristoylation of the B subunit in regulating calcineurin activity have not been reported. The highly selective nature of this modification, combined with its propensity for proteins involved in cellular regulatory pathways, indeed suggests that it provides a necessary feature of calcineurin as well.

A myristoylated protein that has received considerable attention of late is the 67–87-kDa protein kinase C substrate ("80K protein"), which is found in a wide variety of cell types. Although no function has yet been assigned to the 80K protein, its phosphorylation in response to polypeptide growth factors, phorbol esters, and *ras* transformation suggests that it occupies a central position in one or more pathways mediated by protein kinase C (Rozengurt et al., 1983; Rodriguez-Pena & Rozengurt, 1986; Blackshear et al., 1985, 1986; Wolfman & Macara, 1987; Wang et al., 1989). Myristoylation of the 80K protein through an amide linkage was discovered during studies on the response of macrophages to bacterial lipopolysaccharide (LPS) (Aderem et al., 1986, 1988). It was reported that myristoylation of the 80K protein was induced following exposure of macrophages to LPS. The majority of ^{32}P-labeled 80K protein was in the cytosol, whereas the myristoylated protein was almost exclusively associated with the membrane fraction. The discrete subcellular locations of the myristoylated versus the ^{32}P-labeled forms of the protein prompted speculation that LPS-induced myristoylation of the 80K protein may target it to the membrane, where it would be more accessible to active protein kinase C.

We have recently studied myristoylation of the 80K protein in BC_3H1 myocytes in order to determine if it may become fatty acylated through a pathway that is distinct from that utilized by other myristoylated proteins. We found that the 80K protein exhibits the characteristics of a classical myristoylated protein. Its myristoylation occurred cotranslationally via an amide linkage, and there was no evidence for post-translational, stimulus-dependent fatty acylation of a preexisting pool of the protein (James & Olson, 1989b). Furthermore, pulse–chase experiments demonstrated that the 80K protein is not demyristoylated following phorbol dibutyrate stimulation of quiescent cells, despite a 6-fold increase in its level of phosphorylation. The majority of myristate-labeled 80K protein was indeed found in the membrane fraction, but a significant amount (~20%) was soluble, both before and after its phosphorylation by protein kinase C. This finding demonstrated that the overall distribution of this protein between the plasma membrane and cytosol is not influenced by its degree of phosphorylation and that myristoylation is not the sole mechanism by which it associates with the membrane.

Previous studies have reported that immunoreactive 80K is more abundant in the membrane fraction (Albert et al., 1986), whereas the ^{32}P-labeled protein has been found mostly in high-speed supernatants (Patel & Kligman, 1987). Our studies with BC_3H1 myocytes revealed that the intensity of ^{32}P labeling of the 80K protein is approximately equal in the membrane and cytosol, and stimulation with phorbol dibutyrate induced a parallel 6-fold increase in phosphorylation of the protein in each fraction. When compared to the subcellular distribution of the protein under identical conditions, as determined by myristate labeling, it was apparent that cytosolic 80K protein is phosphorylated to a 4-fold higher stoichiometry than the membrane-bound form.

Recent in vitro experiments have shown that phosphorylation of the 80K protein results in its release from membranes (Wang et al., 1989), suggesting that phosphorylation regulates the subcellular distribution of this protein. We found, however, that the in vivo subcellular distribution of the protein is unchanged after a 6-fold increase in its level of phosphorylation. Preliminary experiments indicate that in vitro phosphorylation of the 80K protein by exogenous protein kinase C in isolated membranes from BC_3H1 cells does not result in its solubilization. However, the possibility that soluble factors are also involved in regulating the subcellular distribution of this protein has not been thoroughly investigated in our system. Consistent with this notion, a soluble protein has been identified in bovine brain which stimulates the calcium-dependent phosphorylation of the 80K protein and its release from membranes in vitro (Kligman & Patel, 1986). Taken together, these data suggest that increased phosphorylation of the 80K protein may mediate its cycling between the plasma membrane and cytosol. Thus, phosphorylation at the membrane might result in translocation of the protein to the cytosol, whereas subsequent dephosphorylation in the cytosol would allow it to reassociate with the plasma membrane. If the overall distribution of the 80K protein is maintained in an equilibrium, as our studies suggest, this type of mechanism would not be revealed with intact cells. Indeed, active protein kinase C is known to be associated with the plasma membrane (Halsey et al., 1987; Spach et al., 1986),

suggesting that phosphorylation of the 80K protein could occur there. Alternatively, cytosolic 80K protein may be phosphorylated by the soluble proteolytic fragment of protein kinase C, which is known to be generated following its translocation to the membrane (Tapley & Murray, 1985; Melloni et al., 1986).

In vitro phosphorylation of the 80K protein in synaptosomal cytosol has been shown to be inhibited by calmodulin in the presence of calcium (Wu et al., 1982). It should be reiterated here that calmodulin also associates with the protein phosphatase calcineurin A. Thus, the apparent calmodulin-mediated inhibition of 80K phosphorylation may be due to dephosphorylation of the protein. The amino acid sequence of the bovine form of the 80K protein has recently been reported (Stumpo et al., 1989), and the sites for phosphorylation by protein kinase C were found clustered within a 25 amino acid basic domain (Graff et al., 1989). Interestingly, this domain is absolutely conserved between the bovine and chicken proteins, and it was found to have similarity to calmodulin-binding domains of other proteins, suggesting that calmodulin may interact with this region of the protein.

The subcellular distribution of the 80K protein is similar to that observed for SRC (~10% soluble), and in both cases it is known that myristoylation alone is insufficient to confer membrane binding. The fact that membrane-bound 80K protein is resistant to high-salt extraction (Albert et al., 1986) suggests that its association with the membrane is mediated by specific protein–protein interactions. In light of the recent identification of a plasma membrane receptor for SRC, it is tempting to speculate that such receptors might exist for other myristoylated proteins, such as 80K, that partition between the membrane and cytosol. In any event, it is clear that the 80K protein interacts with regulatory proteins in both the plasma membrane and cytosol. It will be important to determine the requirements for myristoylation in these interactions.

Cell Surface Receptors. A recent study has shown that the human β_2-adrenergic receptor is palmitoylated and that the fatty acyl moiety is essential for the functional integrity of the G protein mediated pathway through which it acts (O'Dowd et al., 1989). Mutant receptor molecules in which cysteine 341 was replaced by glycine were no longer palmitoylated and exhibited a dramatic decrease in their ability to activate adenylate cyclase in response to isoproterenol. The palmitoylation site, cysteine 341, lies in the carboxyl-portion, cytoplasmic domain of the molecule, and it was pointed out in this study that this cysteine residue is conserved in every G protein coupled receptor examined, suggesting that palmitoylation may be a universal modification of this receptor family. The β_2-adrenergic receptor has seven putative membrane-spanning domains, thus creating three intracellular and three extracellular loops. Addition of palmitate to cysteine 341, located 12 amino acids from the cytoplasmic surface of the plasma membrane, might be expected to promote binding of this region to the membrane, thus creating a fourth intracellular loop. Reversible fatty acylation of this residue may modulate the formation of a functional G protein binding site. The nicotinic acetylcholine (Olson et al., 1984), insulin, and IGF-1 (Magee & Siddle, 1988) receptors are also palmitoylated, but neither the site for palmitoylation nor the functional consequences of these modification are known.

Other Regulatory Proteins. Transforming growth factor α is a secreted polypeptide growth factor with mitogenic properties, and has been implicated in initiation or maintenance of transformation via an autocrine pathway. TGF-α has been shown to be synthesized as part of a glycosylated and palmitoylated precursor that is transported to the cell surface via the secretory pathway (Bringman et al., 1987). Alternate proteolytic cleavage of the extracellular domain gives rise to multiple TGF-α species, leaving a membrane-associated 12.5-kDa C-terminal fragment containing covalent palmitate, presumably in the cysteine-rich cytoplasmic domain. Tunicamycin had no significant effect on expression or secretion of TGF-α, suggesting that N-glycosylation is not required for transport to the cell surface or proteolytic cleavage. Although the function of covalent palmitate near the C-terminus is unknown, the amino acid sequences of the transmembrane and cytoplasmic segments of the human and rat TGF-α precursors are nearly identical over approximately 60 residues (one conservative substitution), suggesting that these regions play an important biological role. It has been proposed that palmitoylation may serve to slow the movement of the precursor through the endoplasmic reticulum and Golgi, thus allowing for more efficient proteolytic processing. These ideas, however, remain to be tested.

SV40 large T-antigen (T-ag) is a polypeptide that provides various functions required for viral infection and transformation by Simian virus 40 (Butel & Jarvis, 1986). This protein exhibits an unusual subcellular distribution, such that ~95% of intracellular T-ag is found in the nucleus, whereas a small fraction is localized to the plasma membrane. T-ag at the plasma membrane has been subdivided into two classes on the basis of its solubility in the nonionic detergent NP-40 (Klockmann & Deppert, 1983). Approximately one-third of T-ag associated with the plasma membrane was soluble in NP-40, with the remainder tightly bound to the detergent-resistant lamina of the plasma membrane. This detergent-resistant subclass of T-ag was found to contain covalent palmitate, while detergent-soluble and nuclear T-ag were nonacylated. Although it is possible that detergent-soluble T-ag at the plasma membrane may be an artifact of fractionation, selective fatty acylation of only the lamina-associated T-ag suggests that the presence of this subclass is due to specific interactions and that palmitoylation may facilitate tight binding.

Summary

From the studies presented above, it is obvious that fatty acylation is a common modification among proteins involved in cellular regulatory pathways, and in certain cases mutational analyses have demonstrated the importance of covalent fatty acids in the functioning of these proteins. Indeed, certain properties provided by fatty acylation make it an attractive modification for regulatory proteins that might interact with many different substrates, particularly those found at or near the plasma membrane/cytosol interface. In the case of intracellular fatty acylated proteins, the fatty acyl moiety allows tight binding to the plasma membrane without the need for cotranslational insertion through the bilayer. For example, consider the tight, salt-resistant interaction of myristoylated SRC with the membrane, whereas its nonmyristoylated counterpart is completely soluble. Likewise for the RAS proteins, which associate weakly with the membrane in the absence of fatty acylation, while palmitoylation increases their affinity for the plasma membrane and their biological activity. Fatty acylation also permits reversible membrane association in some cases, particularly for several myristoylated proteins, thus conferring plasticity on their interactions with various signaling pathway components. Finally, although this has not been demonstrated, it is conceivable that covalent fatty acid may allow for rapid mobility of proteins within the membrane.

Several questions remain to be answered concerning requirements for fatty acylation by regulatory proteins. The identity of the putative SRC "receptor" will provide important clues as to the pathways in which normal SRC functions, as well as into the process of transformation by oncogenic tyrosine kinases. The possibility that other fatty acylated proteins associate with the plasma membrane in an analogous manner also needs to be investigated. An intriguing observation that can be made from the information presented here is that at least three different families of proteins involved in growth factor signaling pathways encode both acylated and nonacylated members, suggesting that selective fatty acylation may provide a means of determining the specificity of their interactions with other regulatory molecules. Further studies of fatty acylated proteins should yield important information concerning the regulation of intracellular signaling pathways utilized during growth and differentiation.

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