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MODIFICATION OF EUKARYOTIC SIGNALING PROTEINS BY C-TERMINAL METHYLATION REACTIONS

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Abstract—Eukaryotic polypeptides that are initially synthesized with the C-terminal sequence –Cys–Xaa–Xaa–Xaa, including a variety of signal-transducing proteins, such as small G-proteins, large G-proteins and cGMP phosphodiesterases, can be targeted for a series of sequential post-translational modifications. This processing pathway includes the isoprenylation of the cysteine residue with a farnesyl or geranylgeranyl moiety, followed by proteolysis of the three terminal residues and α-carboxyl methyl esterification of the cysteine residue. The potential reversibility of the last step suggests that it may be involved in modulating the function of these proteins. Firstly, methylation may play a role in the activation of cellular peptides or proteins. Secondly, this modification may aid in the membrane attachment of cytosolic precursor proteins. Thirdly, methylation may protect the polypeptide from C-terminal proteolytic degradation once the three terminal amino acid residues are removed. Finally, reversible methylation may directly regulate the function of its target proteins. Therapeutically, inhibitors of C-terminal isoprenylcysteine methylation or demethylation reactions may prove to be useful pharmacological tools as anti-cancer and anti-inflammatory agents.

Keywords—Signal transduction, isoprene, -CXXX, protease, isoprenyltransferase, methyltransferase, C-terminal methylation.

CONTENTS

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Abbreviations—AFC, *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine; AGGC, *N*-acetyl-*all-trans*-geranylgeranyl-L-cysteine; EGF, epidermal growth factor; LPS, lipopolysaccharide; MTA, 5′-methylthioadenosine; NGF, nerve growth factor.

1. INTRODUCTION

Cellular proteins can undergo a variety of post-translational covalent modifications that can be both functionally and structurally important. Perhaps the most widely characterized of these modifications is the phosphorylation of proteins involving the protein kinase-catalyzed transfer of inorganic phosphate from ATP to the side chain hydroxyl groups of serine, threonine or tyrosine residues of acceptor proteins (Fig. 1) (Kemp and Pearson, 1990). These modifications are readily reversible by the action of specific protein phosphatases in the cell (Cohen and Cohen, 1989). The activation and deactivation of protein functions by cycles of phosphorylation and dephosphorylation are highly regulated and crucial to a large number of cellular processes. A potentially parallel type of protein covalent modification involves the methyltransferase-catalyzed transfer of a methyl group from *S*-adenosyl-L-methionine to carboxyl groups on acceptor proteins and their removal by methylesterases (Springer *et al.*, 1979). Here, the methyltransferase can be considered analogous to the protein kinase and the methylesterase analogous to the protein phosphatase.

However, it now appears that protein carboxyl methylation reactions may play more complex roles in cellular physiology. Methylation is more energetically costly than phosphorylation, requiring about 12 ATP equivalents per reaction (Atkinson, 1977; Stock and Simms, 1988). This high metabolic energy cost may limit the widespread use of protein methylation in a simple regulatory mechanism analogous to phosphorylation, reserving this modification for specialized instances where the phosphorylation/dephosphorylation system would not represent an appropriate or effective mechanism.

Protein carboxyl methyltransferases have been found in a wide variety of organisms and there now appears to be at least four distinct classes of these enzymes. Two of these types do not appear to be involved in regulatory reactions in higher cells. The first type, the Class I L-glutamyl methyltransferase, has been found only in chemotactic bacteria. Here, a cytosolic protein methyltransferase catalyzes the formation of L-glutamyl γ-methyl esters on specific membrane-associated chemoreceptors involved in chemotaxis (Clarke, 1985; Stock and Simms, 1988). An L-glutamyl γ-methylesterase has also been identified in these cells that can enzymatically hydrolyze the methyl ester moiety. This esterase can also deamidate specific glutamine residue on these methyl-accepting proteins to form glutamate residues that can then be methylated. Together, these two reactions function to regulate the bacterial response to chemoattractants and chemorepellents

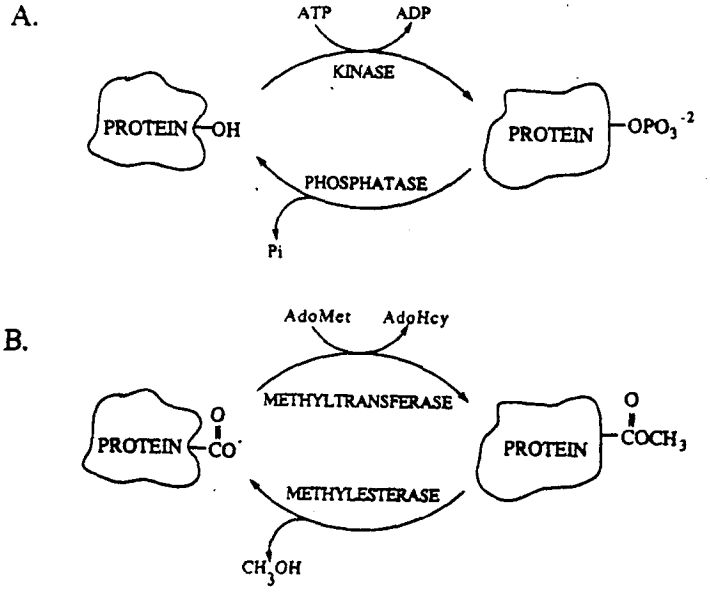

FIG. 1. Comparison of reversible phosphorylation and methylation reactions. A: The phosphorylation of hydroxyl groups on acceptor polypeptides is catalyzed by protein kinases that utilize one ATP equivalent per phosphorylation event. This covalent modification can be reversed by the action of protein phosphatases. B: Methylation of protein carboxyl groups is catalyzed by methyltransferases utilizing *S*-adenosylmethionine (AdoMet) as the methyl donor leaving as a by-product *S*-adenosylhomocysteine (AdoHcy). This covalent modification can be reversed by cellular methylesterases.

much as protein phosphorylation and dephosphorylation reactions regulate other cellular processes (Dunten and Koshland, 1991). To date, no evidence has been reported identifying such an L-glutamyl methyltransferase/esterase system in eukaryotic cells.

The second type of enzyme, the Class II D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase is also localized to the cytosol. This enzyme does not appear to recognize normal residues, but instead catalyzes the methyl esterification of deamidated, isomerized and racemized aspartyl and asparaginyl residues on a wide variety of polypeptide substrates in many prokaryotic (Li and Clarke, 1992) and in most eukaryotic cells (Clarke, 1985; Aswad and Johnson, 1987). Unlike L-glutamyl methylation, this methyl esterification does not appear to have any regulatory function and no specific esterases have been identified that can catalyze the removal of the methyl ester. An attractive hypothesis for the *in vivo* role of this enzyme is that it is involved in the repair or degradation of spontaneously damaged cellular proteins and polypeptides (McFadden and Clarke, 1982). After methylation of the carboxyl group of the damaged residue, spontaneous intramolecular rearrangements can occur via succinimide intermediates to ultimately return the residue to its normal form (Johnson *et al.*, 1987a,b; McFadden and Clarke, 1987).

In contrast to the enzymes described above, two newly discovered classes of methyltransferases may have functional roles in the modulation of higher cell function. The third type of enzyme, the Class III isoprenylcysteine protein carboxyl methyltransferase, catalyzes the transfer of a methyl group to the α-carboxyl group of a C-terminal cysteine residue that has been modified with a farnesyl or geranylgeranyl moiety via a thioether linkage. Unlike the other classes of protein carboxyl methyltransferases that are cytosolic, the C-terminal isoprenylcysteine methyltransferase is membrane-associated in all eukaryotic cells examined to date (Hrycyna and Clarke, 1990; Stephenson and Clarke, 1990; Hrycyna *et al.*, 1991; Volker *et al.*, 1991a; Perez-Sala *et al.*, 1992; Stephenson and Clarke, 1992). Interestingly, bacterial cells do not appear to possess this activity (Hrycyna *et al.*, 1991). Finally, a fourth class of enzyme has recently been identified in the cytosolic fraction of bovine brain and other eukaryotic cells that also catalyzes the methyl esterification of a C-terminal amino acid residue. In this case, the enzyme recognizes C-terminal leucine residues on 36-kDa cytosolic polypeptides (Xie and Clarke, 1993). Interestingly, the substrate for this reaction is itself a protein phosphatase, suggesting a link between phosphorylation and methylation systems (H. Xie and S. Clarke, submitted).

This review will, thus, be centered on the role of post-translational C-terminal modification reactions, especially with regard to cell signaling interactions. The focus will be on the type III methylation system and the two other related post-translational modifications, isoprenylation and proteolysis, that lead to the maturation of candidate polypeptide precursors. We will also discuss biological systems where indirect evidence for a role for carboxyl methylation has been obtained but where the exact nature of the chemistry and enzymology have not been fully defined. It is possible that some of these systems may involve L-glutamyl or other novel types of protein carboxyl methyltransferases that will enlarge the eukaryotic cell's repertoire for reversible protein modification.

2. OVERVIEW OF -CXXX PROCESSING STEPS

In 1988, it was first suggested that eukaryotic proteins and polypeptides initially synthesized with the C-terminal sequence -CXXX, where X represents a variety of amino acid residues, can be post-translationally modified by lipidation and methylation reactions (Fig. 2) (Clarke *et al.*, 1988). The resulting structures were first observed in the peptidyl mating factors from the jelly fungi *Tremella mesenterica* and *T. brasiliensis* and contain C-terminal cysteine residues that are modified by both an *S*-isoprenyl group linked via a thioether linkage and α-methyl esterification (Sakagami *et al.*, 1981; Ishibashi *et al.*, 1984). It is now known that a large number of proteins in both yeast and mammalian systems are, in fact, modified by three sequential reactions; isoprenylation of the cysteine residue is followed by proteolytic cleavage of the three C-terminal amino acids and ultimately the newly exposed lipidated cysteine residue is methylated (Hrycyna and Clarke, 1990; Stephenson and Clarke, 1990). Examples of the proteins and polypeptides known to undergo these modifications include the *Saccharomyces cerevisiae* a-mating factor (Betz *et al.*, 1987; Anderegg

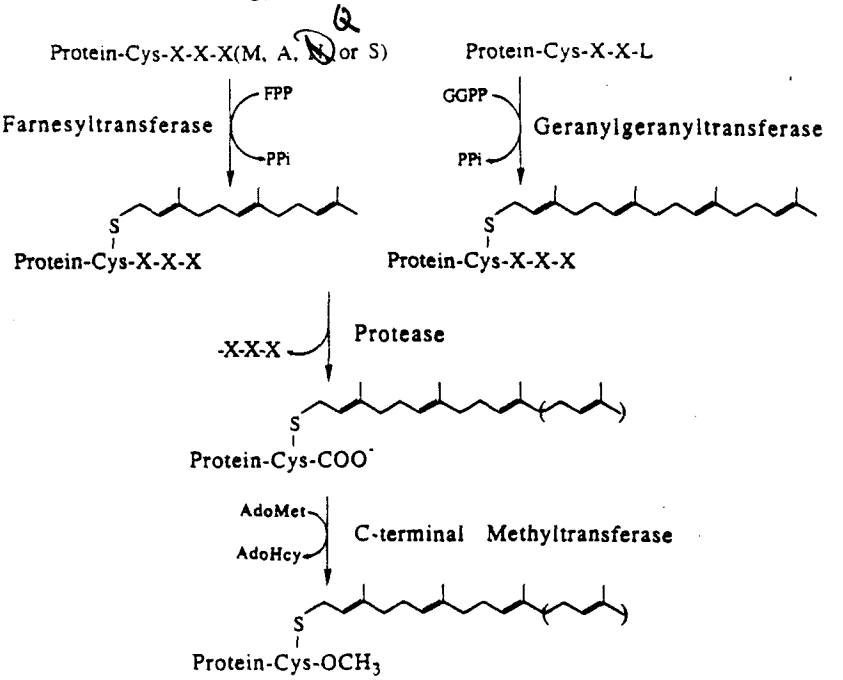

FIG. 2. Post-translational modifications of -CXXX-containing polypeptides. Candidate substrates initially synthesized with the C-terminal sequence -CXXX can undergo a series of post-translational reactions that modify their C-termini. When the final amino acid residue (X) is either Met (M), Ala (A), Gln (Q) or Ser (S), a soluble farnesyltransferase catalyzes the transfer of a farnesyl group from farnesyl pyrophosphate (FPP) to the cysteine residue (Cys) of the acceptor polypeptide. Alternatively, if the C-terminal amino acid residue is Leu (L), a geranylgeranyltransferase can modify the polypeptide by catalyzing the transfer of a geranylgeranyl moiety from geranylgeranylpyrophosphate (GGPP) to the cysteine residue. The three terminal amino acids of these lipidated substrates are then cleaved by a proteolytic activity and finally, the newly exposed isoprenylcysteine residue is methylated by a membrane-associated C-terminal methyltransferase.

et al., 1988; Hrycyna *et al.*, 1991) and RAS proteins (Deschenes *et al.*, 1989; Fujiyama and Tamanoi, 1990; Hrycyna *et al.*, 1991) and the mammalian RAS proteins (Clarke *et al.*, 1988; Casey *et al.*, 1989; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Schafer *et al.*, 1989), large heterotrimeric G-proteins (Fukada *et al.*, 1990; Fung *et al.*, 1990; Lai *et al.*, 1990; Yamane *et al.*, 1990), several ras-related small G-proteins (Kawata *et al.*, 1990; Buss *et al.*, 1991; Farnsworth *et al.*, 1991; Yamane *et al.*, 1991), bovine and rat cGMP phosphodiesterase α- and β-subunits (Ong *et al.*, 1989; Catty and Deterre, 1991; Anant *et al.*, 1992) and nuclear lamins (Chelsky *et al.*, 1987; Wolda and Glomset, 1988; Chelsky *et al.*, 1989; Farnsworth *et al.*, 1989; Holtz *et al.*, 1989; Vorburger *et al.*, 1989). It has also become evident that both C_{15} farnesyl and C_{20} geranylgeranyl isoprene moieties are used to modify these polypeptides *in vivo*. In fact, it appears that the C-terminal amino acid of the -CXXX motif specifies the nature of the isoprene group. Polypeptides that end with a leucine residue are geranylgeranylated and those ending with methionine, serine, alanine or glutamine residues are targeted for farnesylation (Fig. 2) (Kawata *et al.*, 1990; Kinsella *et al.*, 1991b; Moores *et al.*, 1991; Reiss *et al.*, 1991b).

Several possibilities have been proposed for the physiological roles of these modifications. Since they give these otherwise hydrophilic polypeptides a greater hydrophobic character, lipidation and methylation may help guide the polypeptides to their functional sites on membrane surfaces (Clarke *et al.*, 1988; Wolda and Glomset, 1988; Deschenes *et al.*, 1989; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Holtz *et al.*, 1989; Ong *et al.*, 1989; Vorburger *et al.*, 1989; Fung *et al.*, 1990; Clarke, 1992; Schafer and Rine, 1992). It has also been suggested that methylation of the C-terminus may protect precursor polypeptides from further proteolytic digestion once the three terminal amino acids have been removed (Hrycyna and Clarke, 1992). Additionally, these modifications may serve as recognition signals for specific receptor proteins localized to the plasma membrane or to other intracellular membranes or may activate cellular proteins or peptides (Clarke, 1992). Since many of the proteins targeted for this type of post-translational processing are thought to have signal

transduction roles, these modifications may somehow be involved in modulating their activities *in vivo* (Clarke *et al.*, 1988; Deschenes *et al.*, 1989; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Ong *et al.*, 1989; Fung *et al.*, 1990; Clarke, 1992; Schafer and Rine, 1992) or are perhaps involved in the regulation of the cell cycle (Chelsky *et al.*, 1987, 1989). As more is learned about C-terminal protein carboxyl methylation, it may turn out that it plays a distinct role in the individual functions of a variety of cellular proteins.

3. FARNESYLTRANSFERASES AND GERANYLGERANYLTRANSFERASES

Two distinct isoprenyltransferases have been identified that can catalyze the isoprenylation of -CXXX-containing proteins and polypeptides in yeast and mammalian systems. In yeast, a farnesyltransferase comprised of two heterologous subunits, the *RAM1* (Powers *et al.*, 1986; Fujiyama *et al.*, 1987; Goodman *et al.*, 1988) and *RAM2* gene products (Goodman *et al.*, 1990; He *et al.*, 1991; Moores *et al.*, 1991), appears to be responsible for the lipidation of -CXXX-containing polypeptides, such as the RAS proteins (Goodman *et al.*, 1990), the *STE18* gene product (Finegold *et al.*, 1990) and the **a**-factor mating pheromone (Anderegg *et al.*, 1988; Marcus *et al.*, 1991), with a C_{15} farnesyl group. In mammalian systems, the corresponding subunits α and β of the -CXXX farnesyltransferase have also been extensively characterized and appear to be similar to their yeast homologues (Chen *et al.*, 1991a,b; Reiss *et al.*, 1991a,b; Pompliano *et al.*, 1992; Reiss *et al.*, 1992).

The second enzyme is a geranylgeranyltransferase that catalyzes the addition of a C_{20} geranylgeranyl moiety to acceptor proteins and polypeptides. This -CXXX geranylgeranyltransferase, or GGT-I, is comprised of two subunits encoded by the *CDC43/CAL1* gene (Johnson *et al.*, 1991; Ohya *et al.*, 1991) and the *RAM2* gene in *S. cerevisiae* (Finegold *et al.*, 1991; Mayer *et al.*, 1992; Gomez *et al.*, 1993). A similar enzyme has also been characterized in mammalian cells (Joly *et al.*, 1991; Moores *et al.*, 1991; Seabra *et al.*, 1991; Yokoyama *et al.*, 1991; Yoshida *et al.*, 1991).

Another multisubunit geranylgeranyltransferase in both yeast and mammalian cells, GGT-II, does not recognize the -CXXX motif but instead lipidates polypeptides terminating in the sequence -CC or -CXC, where X is any amino acid residue. In mammalian cells, this enzyme is also termed Rab geranylgeranyltransferase because several of the Rab GTP-binding proteins are substrates for the enzyme (Moores *et al.*, 1991; Seabra *et al.*, 1992a,b, 1993). In yeast, it has been proposed that a variety of low molecular weight GTP-binding proteins, such as YPT1 and SEC4, may be substrates for this enzyme (Moores *et al.*, 1991; Rossi *et al.*, 1991).

Several reviews of protein prenylation have recently appeared, including those of Casey (1992), Clarke (1992), Cox and Der (1992), Glomset *et al.* (1992), Maltese (1990), Schafer and Rine (1992) and Sinensky and Lutz (1992).

4. C-TERMINAL PROTEASES

Less is known about the protease activity that removes the three C-terminal amino acid residues from isoprenylated polypeptide precursors in both yeast and other eukaryotic cell types. Indirect evidence for the proteolytic event was initially obtained from the isolation of α-carboxyl-methylated isoprenylcysteine derivatives from proteins whose cDNAs encode for an additional three C-terminal amino acid residues (Ong *et al.*, 1989; Ota and Clarke, 1989; Stimmel *et al.*, 1990; Yamane *et al.*, 1990, 1991). In 1989, Gutierrez *et al.* demonstrated the removal of the three terminal amino acids from the mammalian p21ras protein directly by introducing a unique tryptophan residue into the protein precursor at each of the three C-terminal positions. The loss of the residue was monitored during biosynthetic processing *in vivo*. Similarly, Fujiyama and Tamanoi (1990) demonstrated loss of the three C-terminal residues from the *S. cerevisiae* RAS2 protein during its post-translational biosynthetic processing. Finally, a membrane-associated proteolytic activity in canine microsomes has been identified that reportedly increases the membrane binding of farnesylated p21H-ras 2-fold over the nonproteolyzed species (Hancock *et al.*, 1991).

Enzyme activities from mammalian systems responsible for the cleavage of the three C-terminal amino acids have recently been identified and subjected to biochemical characterization. Ashby

et al. (1992) identified a membrane-associated endoproteolytic activity in rat liver specific for a farnesylated peptide substrate that releases the terminal three amino acids as a tripeptide. A microsomal enzymatic activity from calf liver has also been identified that can specifically cleave a farnesylated tetrapeptide between the isoprenylated cysteine residue and the adjacent residue, as well as the related farnesylated tri- and dipeptides (Ma *et al.*, 1992; Ma and Rando, 1992). More recently, a distinct activity from brain microsomal membranes capable of sequentially removing the three terminal amino acids from a synthetic heptapeptide representing the C-terminus of the mouse N-ras protein was identified (Akopyan *et al.*, 1992). This activity was characterized as a thiol-dependent serine carboxypeptidase that has a higher affinity for the farnesylated peptide substrate than the nonfarnesylated analog. Interestingly, none of the proteolytic activities capable of cleaving the three terminal amino acid residues from farnesylated precursors identified so far in mammalian systems are soluble.

Proteolytic activities in the yeast *S. cerevisiae* capable of recognizing synthetic isoprenylated -CXXX peptide substrates have also been characterized. At least three distinct activities in yeast have been identified using the synthetic peptide substrate *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM *in vitro*, a membrane-associated enzyme and two soluble activities. One of these soluble activities has been shown to be the vacuolar protease carboxypeptidase Y (Hrycyna and Clarke, 1992). The membrane-associated enzyme is similar in its inhibitor specificity to an activity identified by Ashby *et al.* (1992) that is capable of catalyzing the removal of the three terminal amino acids as a tripeptide from a farnesylated peptide substrate *in vitro*. The partially purified second soluble activity, also identified by Ashby *et al.* (1992), was characterized as a 110-kDa enzyme that initially appeared to be a metallocarboxypeptidase that could cleave both farnesylated and nonfarnesylated -CXXX-containing peptides (Hrycyna and Clarke, 1992). However, most recently, we have purified the soluble activity to apparent homogeneity and determined that this enzyme represents a novel metalloendopeptidase capable of preferentially cleaving substrates on the C-terminal side of hydrophobic amino acid residues (Hrycyna and Clarke, 1993). The purified enzyme, consisting of a single 68-kDa polypeptide as determined by sodium dodecyl sulfate polyacrylamide-gel electrophoresis, appears to be encoded by the *YCL57w* gene (Hrycyna and Clarke, 1993), previously identified as an open reading frame on chromosome III in *S. cerevisiae* (Oliver *et al.*, 1992).

However, this soluble enzyme may not represent the physiologically relevant species in the post-translational processing of cytosolic isoprenylated polypeptide precursors in *S. cerevisiae*. The enzyme activity is not specific for farnesylated -CXXX peptide substrates (Hrycyna and Clarke, 1992; Hrycyna and Clarke, 1993) and subcellular localization studies suggest that the soluble enzyme is not present in the cytosolic fraction but instead may be sequestered in a subcellular compartment (Hrycyna and Clarke, 1993). Thus, the membrane-associated proteolytic activity specific for farnesylated -CXXX substrates identified by both our laboratory and by Ashby *et al.* (1992) may be the physiologically relevant enzyme species, although importantly, no mutants defective in this activity have been isolated to date.

5. METHYL ESTERIFICATION OF C-TERMINAL ISOPRENYLCYSTEINE RESIDUES

5.1. IDENTIFICATION AND CHARACTERIZATION OF C-TERMINAL METHYLTRANSFERASES

In 1989, the first identification of an activity capable of methylating a C-terminal residue was described (Ota and Clarke, 1989). These studies showed that an activity in bovine retinal membranes could methylate C-terminal cysteine residues in an *S*-adenosylmethionine-dependent reaction. Since this time, methylation has been definitively shown to occur on the α-carboxyl group of a C-terminal isoprenylated cysteine residue and the enzymes involved in this reaction have been well characterized in mammalian systems as well as in the yeast *S. cerevisiae* (Hrycyna and Clarke, 1990; Stephenson and Clarke, 1990; Volker *et al.*, 1991a; Perez-Sala *et al.*, 1992; Stephenson and Clarke, 1992).

We have shown that a membrane-associated C-terminal methyltransferase, the product of the *STE14* gene in *S. cerevisiae*, can catalyze the methylation of both farnesylated and geranylgeranylated synthetic peptide substrates *in vitro* and is present in both **a** and α cell types (Hrycyna and Clarke, 1990; Hrycyna *et al.*, 1991). This methyltransferase is responsible for the *in vivo* methylation

of several cellular polypeptides, including RAS1, RAS2 and the peptide mating pheromone a-factor (Hrycyna *et al.*, 1991). Presumably, this enzyme is responsible for the C-terminal methyl esterification of a variety of -CXXX-containing yeast proteins, but it remains to be determined if geranylgeranylated polypeptides are methylated by this enzyme *in vivo*. It is also unclear whether yeast polypeptides terminating with -CC or -CXC are methylated at their C-terminus and, if so, if the *STE14* gene product is responsible.

An enzyme possessing similar characteristics to the *STE14* methyltransferase has been described in mammalian systems (Stephenson and Clarke, 1990; Volker *et al.*, 1991a; Perez-Sala *et al.*, 1992; Stephenson and Clarke, 1992). This methyltransferase is thought to be responsible for the methyl esterification of a variety of polypeptides, including the ras proteins (Clarke *et al.*, 1988; Casey *et al.*, 1989; Hancock *et al.*, 1989), several ras-related small G-proteins (Kawata *et al.*, 1990; Buss *et al.*, 1991; Farnsworth *et al.*, 1991; Yamane *et al.*, 1991), heterotrimeric large G-proteins (Fukada *et al.*, 1990; Fung *et al.*, 1990; Lai *et al.*, 1990; Yamane *et al.*, 1990), the nuclear lamin proteins (Kitten and Nigg, 1991) and the bovine and rat cGMP phosphodiesterase α- and β-subunits (Ong *et al.*, 1989; Catty and Deterre, 1991; Anant *et al.*, 1992). Like the yeast enzyme, this methyltransferase has also been shown to recognize both farnesylated and geranylgeranylated synthetic peptide substrates *in vitro*, though it remains to be seen whether it functions similarly *in vivo* (Stephenson and Clarke, 1990; Tan *et al.*, 1991; Volker *et al.*, 1991a; Perez-Sala *et al.*, 1992). Furthermore, it appears that this methyltransferase requires only an isoprenylated thiopropionate moiety and does not need any peptide sequence for substrate recognition (Tan *et al.*, 1991; Gilbert *et al.*, 1992).

The subcellular localization of the C-terminal methyltransferase has not been determined definitively. Interestingly, biochemical studies have shown that both the yeast and mammalian enzymes have been found associated with the membrane fraction (Hrycyna and Clarke, 1990; Stephenson and Clarke, 1990, 1992). The deduced amino acid sequence of the yeast *STE14* gene corroborated this biochemical data. The sequence reveals a 239 amino acid residue polypeptide that appears to contain multiple membrane spanning domains that are indicative of integral membrane proteins.* In light of this evidence, these enzymes represent the only membrane-associated protein carboxyl methyltransferases described to date. It will be interesting to determine whether methylation is carried out at the plasma membrane or on the cytoplasmic face of an intracellular membrane such as the endoplasmic reticulum. In fact, recent data suggests that the bulk of the mammalian enzyme activity may be localized to the endoplasmic reticulum (Stephenson and Clarke, 1992). One possible and attractive hypothesis is that these three reactions involved in the post-translational maturation of CXXX-terminating proteins are carried out by a higher order complex comprised of the isoprenyltransferase and methylation machinery and the proteolytic enzyme(s). Importantly, however, the isoprenyltransferase has been found in the soluble fraction (Goodman *et al.*, 1990; Schafer *et al.*, 1990; Reiss *et al.*, 1992; Seabra *et al.*, 1992a), though a weak association with a cellular membrane or membrane proteins might not be readily detected.

5.2. Possible Functions of C-terminal Methylation

5.2.1. *Biological Activity of Proteins and Polypeptides*

One possible function of methylation is to activate certain cellular polypeptides by structural modification. An example of this type of activation is observed with the mating pheromone a-factor from *S. cerevisiae*, an *in vivo* substrate for the STE14 methyltransferase (Hrycyna *et al.*, 1991). The structure of the mature peptide contains a C-terminal farnesylcysteine methyl ester (Anderegg *et al.*, 1988). Exogenously added a-factor lacking this modification results in a severe decrease in the biological activity of the pheromone (Marcus *et al.*, 1991). Thus, the methyl ester on a-factor appears to be necessary either for binding of a-factor to its receptor (STE3) on the surface of MATα cells (Hagen *et al.*, 1986; Kurjan, 1992) or for the subsequent activation step that leads to G1 arrest and mating. The a-factor methyl ester also appears to be a crucial recognition determinant for the

*Sapperstein, S., Berkower, C. and Michaelis, S. (1989) DNA sequence analysis of *STE14*, a gene required for biogenesis of the *Saccharomyces cerevisiae* a-factor mating pheromone. In: *Abstracts Cold Spring Harbor Yeast Meeting, Cold Spring Harbor, NY, August 12–18, 1989*, p. 139, Cold Spring Harbor Press, New York.

a-factor transporter, STE6, since a-factor export is completely blocked in a *ste14* mutant (S. Michaelis, personal communication; Hrycyna *et al.*, 1991). The absolute dependence of a-factor export on the presence of the STE14 methyltransferase again reinforces the idea that the methyl ester may be crucial for facilitating protein–protein interactions, although the basis for this effect remains to be determined.

5.2.2. *Increasing Hydrophobicity and Membrane Attachment*

A frequently suggested explanation for the role of C-terminal methylation is that the modification increases the hydrophobicity of an otherwise hydrophilic polypeptide by eliminating the negative charge of the C-terminal α-carboxyl group. Since most of these candidate -CXXX precursor proteins, when mature, are associated with a specific cellular membrane, the addition of the methyl group might be working in concert with the isoprene modification to aid in the membrane attachment of these polypeptides (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Schafer and Rine, 1992; Sinensky and Lutz, 1992). It is conceivable that these hydrophobic modifications may help to anchor the proteins into the lipid bilayer of target membranes by insertion of the isoprene and/or methyl moieties, but is also clear that these modifications alone cannot facilitate complete membrane-association. In most cases, an additional factor is necessary for efficient membrane-association. For example, an upstream palmitoylation site on mammalian N-ras and Ha-ras has been implicated in the efficient membrane-association of these proteins (Hancock *et al.*, 1989, 1990). Also, the mammalian p21K-ras(B) protein requires an upstream polylysine sequence in addition to the modifications made to the C-terminal -CXXX sequence (Hancock *et al.*, 1990). Importantly, p21K-ras(B) methylation significantly increased membrane-association of the protein (Hancock *et al.*, 1991). Methylation has also been shown to aid in the efficient membrane localization of the yeast RAS2 protein (Hrycyna *et al.*, 1991). From these results and from the examination of the membrane-association of other small G proteins (Yamane and Fung, 1989; Yamane *et al.*, 1991) and the nuclear lamins (Holtz *et al.*, 1989), it is clear that multiple signals are required for membrane localization, but the modifications of the C-terminal -CXXX motif, including methylation, play an important role in the membrane attachment of a variety of cellular proteins.

Alternatively, instead of being inserted into target membranes, these modifications may be associating with specific 'receptor' proteins or other cellular factors (Clarke, 1992). An example of this type of association is the interaction between lipidated and methylated a-factor mating pheromone and the G-protein coupled receptor STE3 on α cells in *S. cerevisiae* (Hagen *et al.*, 1986; Kurjan, 1992). It has been previously observed that both nonlipidated and unmethylated *S. cerevisiae* a-factor derivatives are significantly less biologically active than the fully modified pheromone, suggesting that each of these modifications are crucial for receptor recognition, but it remains to be seen how the receptor is recognizing these modifications (Anderegg *et al.*, 1988; Marcus *et al.*, 1991). Interestingly, other nonpeptide isoprenoid pheromones, such as the insect juvenile hormones (Gilbert *et al.*, 1980) and the immediate precursor to the insect juvenile hormone III, methyl farnesoate (Landau *et al.*, 1989), are also carboxyl methylated and may interact with receptor proteins that play a role in the control of development in insects and crustaceans. These reports, taken together with the results discussed above, suggest that the methyl group may serve a dual function in the cell. Methylation may initially help to guide proteins to a membrane by eliminating the negative charge at the C-terminus of the polypeptide, but ultimately the role of the modification, perhaps in conjunction with isoprenylation, is to interact with a specific protein at a specific subcellular site. It is interesting to note, however, that these -CXXX-containing proteins are all modified similarly at their C-termini but are targeted to different subcellular membranes. This observation suggests that the situation may be more complex and that other upstream amino acid sequences in the protein may be required for correct subcellular localization.

5.2.3. *Protection from Proteases*

The possibility has been raised that C-terminal methyl esterification of the newly exposed cysteine residue generated after the cleavage of the three terminal amino acid residues may help to protect the maturing polypeptide from further proteolytic digestion at the C-terminus (Hrycyna and

Clarke, 1992). By this model, a competition between the methyltransferase and the protease for C-terminal isoprenylated cysteine residues may be established *in vivo* representing a new mechanism for regulating the levels of modified proteins in the cell. Previous studies in our laboratory on the processing of the RAS2 protein in *S. cerevisiae* strains lacking STE14 methyltransferase activity (Hrycyna *et al.*, 1991) showed an apparent accumulation of a soluble form migrating on sodium dodecyl sulfate-gel electrophoresis at the position where the nonisoprenylated precursor p41 is normally found to migrate (Fujiyama *et al.*, 1987). Since these cells, though deficient in methyltransferase activity, do contain active farnesyltransferase activity, it was unclear why the loss of the methylation reaction should dramatically slow the isoprenylation reaction. We suggest that isoprenylated proteins may, in fact, form normally in the absence of the methyltransferase but then may be digested further by the proteolytic enzyme. This cleavage reaction could remove the farnesylated cysteine residue and perhaps other amino acid residues resulting in the reversion of the polypeptide to a form indistinguishable on sodium dodecyl sulfate-gel electrophoresis from the unmodified precursor protein. Presumably, the loss of several amino acids would have little to no effect on the migration of the protein in comparison to cleavage of the isoprene group. This hypothesis may explain the apparent persistence of the precursor species. In support of this explanation, preliminary *in vitro* experiments in *S. cerevisiae* suggested that neither the soluble nor the membrane-associated proteolytic activity was able to hydrolyze the methyl ester from the substrate *N*-acetyl-*S*-farnesylcysteine-[^{3}H]methyl ester (Hrycyna and Clarke, 1992). Importantly, the localization of the protease responsible for the cleavage event is a factor in the feasibility of this model. Although no evidence has been presented as to the biochemical localization of the membrane-associated enzyme, it is thought that the active site must necessarily reside on either the plasma membrane or on the cytoplasmic face of an intracellular membrane in order to be physiologically relevant in the processing of cytoplasmic precursor polypeptides (Ashby *et al.*, 1992; Hrycyna and Clarke, 1992). Interestingly, although preliminary localization results suggest that the soluble enzyme may reside in a subcellular compartment within the cell (see Section 4), it is unclear whether this activity can function in the cytoplasm to some extent *in vivo*.

5.2.4. *Regulation of Protein Function*

It is well established that the receptor-mediated chemotactic response of bacteria to stimulants is regulated by reversible methylation reactions involving the Class I L-glutamyl methyltransferase and the esterase specific for glutamine and methylated glutamate residues (Clarke, 1985; Stock and Simms, 1988). These observations led to a great interest in determining whether methylation/demethylation regulatory systems exist in mammalian systems as well. One suggestion has been made that these types of reactions may play an important role in the assembly and disassembly of nuclear lamin B during the cell cycle in mammalian cells (Chelsky *et al.*, 1987, 1989).

The first evidence for a link between receptor-mediated chemotaxis and reversible carboxyl methylation in mammalian cells was presented by O'Dea *et al.* (1978). A rapid but transient increase in protein carboxyl methylation in rabbit neutrophils was observed upon exposure to the chemotactic peptide fMet–Leu–Phe. In 1980, this phenomenon was extensively re-evaluated and although a similar effect was observed, it was found to be greatly variable (Venkatasubramanian *et al.*, 1980). It was later determined that the formylpeptide chemoattractant receptor in mammalian HL-60 cells is directly coupled to a large heterotrimeric guanine nucleotide-binding protein (large G-protein) that when activated helps transduce the signal through the cell (Polakis *et al.*, 1988). Subsequently, it was found that the γ-subunit of large G-proteins from rabbit and bovine brain are, in fact, α-carboxyl methylated at their C-termini (Backlund *et al.*, 1990; Fung *et al.*, 1990; Yamane *et al.*, 1990). These pieces of information taken together suggested that the γ-subunit of the G-protein coupled to the chemoreceptor may also be modified similarly and that the methylation of the large G-protein involved in this and other large G-protein-mediated signal transduction pathways may play a key role in regulating cellular function. Unfortunately, this hypothesis has not yet been tested experimentally.

In addition to the possibility that regulated methylation reactions are targeting the γ-subunit of the large G-protein coupled to the receptor itself, it has been suggested that fMet–Leu–Phe treatment may also be affecting the methylation state of various cellular small guanine

nucleotide-binding proteins (small G-proteins). In human neutrophils exposed to fMet–Leu–Phe, a transient increase in carboxyl methylation of 20- to 24-kDa polypeptides was observed (Philips *et al.*, 1993). These polypeptides are thought to be related to the Ras family of GTP-binding proteins. Although not previously addressed, it is also possible that in addition to these small G-proteins the γ-subunit of the large G-protein coupled to the fMet–Leu–Phe receptor may be affected as well as other cellular polypeptides. Clearly, further work is needed to identify the methylated proteins involved in the chemotactic response to the peptide fMet–Leu–Phe.

The potential involvement of the methylation of large and/or small G-proteins in mammalian chemotaxis has also been inferred from inhibition studies. Saturating amounts of *N*-acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine (AFC), a competitive inhibitor of the C-terminal protein methyltransferase, have been shown to inhibit the chemotactic response in mouse peritoneal macrophages greater than 80% (Volker *et al.*, 1991b). Similarly, inhibition of chemopeptide receptor mediated G-protein activation by AFC was also demonstrated in myeloid differentiated HL-60 granulocytes (Scheer and Gierschik, 1993). It is possible, therefore, that the methylation and demethylation of various G-proteins may be important in the regulation of the mammalian chemotactic response. However, it is unclear how this inhibitor is functioning in the cell. Although it is possible that the compound is affecting the methylation of certain cellular polypeptides, it cannot be ruled out that other nonmethylation reactions may be inhibited (see Section 5.3).

Additionally, C-terminal carboxyl methylation may play a role in regulating a variety of distinct cellular processes that involve other small G-proteins. Interestingly, the methylation of these 23- to 28-kDa polypeptides can be stimulated by the addition of GTP or its nonhydrolyzable analogs (Backlund and Aksamit, 1988). One of these proteins, the G25K small G-protein, has been shown to contain an *S*-(all-*trans*-geranylgeranyl)cysteine methyl ester at its carboxyl terminus (Yamane *et al.*, 1991) and the methyl-esterification reaction has been shown to be stimulated by the addition of GTPγS *in vitro* (Backlund, 1992). Originally, it was thought that all the substrates for methylation, including G25K, were membrane-associated (Backlund and Aksamit, 1988; Yamane and Fung, 1989; Fung *et al.*, 1990). Recently, however, Backlund (1992) identified two distinct pools of G25K in rabbit brain, one soluble and one membrane-associated and determined that the majority of the membrane-associated species appeared to be methylated. Importantly, the soluble G25K species, which appears to be isoprenylated but not methylated, is a good methyl acceptor and associates with the membrane fraction only after being methylated. Interestingly, soluble G25K protein, associated with a soluble 28-kDa polypeptide as a dimeric complex, must be in a guanine nucleotide-bound form in order to be a good methyl accepting substrate (Backlund, 1992). The guanine nucleotide-dependent association of G25K with this soluble factor may play an important role in regulating G25K methylation and subsequent membrane-association and activity.

It is becoming increasingly clear that the rac proteins, a family of ras-related small G-proteins, are involved in the regulation of the inflammatory response in macrophages and neutrophils via the NADPH oxidase system. This system, when activated by exposure to compounds such as fMet–Leu–Phe or phorbol myristate acetate, generates O_2^- and its derivatives in the lumen of the vacuole (created from invagination of the plasma membrane as a result of phagocytosis) increasing the intravacuolar pH and thereby activating neutral proteinases released into this vacuole by the degranulation of cytoplasmic granules (Segal and Abo, 1993). A soluble rac protein species has been identified in both guinea-pig macrophages and human neutrophils (Abo *et al.*, 1991; Knaus *et al.*, 1991). In the guinea-pig system, rac was found complexed to the rhoGDI protein (GDP-dissociation inhibition factor) in the soluble fraction (Abo *et al.*, 1991). A current model based on these and other biochemical findings for the involvement of the rac proteins in the activation of this system has recently been presented by Segal and Abo (1993). In this model, the GDP-bound rac–rhoGDI soluble complex first dissociates and the rac protein associates with the other essential proteins, p67*phox* and p47*phox*, to form an 'activation complex'. At some point this complex exchanges GTP for GDP and associates with a flavocytochrome b in the membrane causing activation of the enzyme activity. The endogenous GTPase activity of the rac protein then causes deactivation and dissociation of the activation complex, leaving the rac protein in the GDP-bound state where it can reassociate with the rhoGDI polypeptide.

The fact that the rac2 protein has been found to be geranylgeranylated (Didsbury *et al.*, 1990; Kinsella *et al.*, 1991a) and methylated (Philips *et al.*, 1993) at the C-terminus coupled to the recent

findings that the rac proteins are somehow involved in the NADPH oxidase system led to an investigation concerning the role of carboxyl methylation of the rac protein (Philips *et al.*, 1993). As was seen for G25K, the prenylated but unmethylated rac2 species is found in the soluble fraction. Upon exposure to GTPγS, rac2 is directed to the membrane where it is subsequently methylated and becomes membrane-bound. On the other hand, the presence of GTPγS does not cause membrane-association of the rhoGDI protein, suggesting that the GDP-GTP exchange causes both the activation of rac and the dissociation of the protein from rhoGDI (Philips *et al.*, 1993). In support of this hypothesis, it has also been shown that GTP-bound substrates are more efficiently carboxyl-methylated than those that are not activated (Philips *et al.*, 1993; Backlund, 1992).

It is possible that this type of regulatory mechanism involving GTP-stimulated carboxyl methylation of target substrates may prove to be generally applicable to a number of other small G-protein systems as well. For example, the rap1 protein from human platelets has been shown to be geranylgeranylated and carboxyl methylated at the C-terminal cysteine residue (Kawata *et al.*, 1990) and this methylation has also been shown to be stimulated by GTPγS (Huzoor-Akbar *et al.*, 1991). Interestingly, the authors stated that methylation of the rap1 protein was only observed when both platelet membranes and cytosol were included in the incubation along with *S*-adenosylmethionine (Huzoor-Akbar *et al.*, 1991). Although the STE14-like methyltransferase is membrane-associated (Hrycyna and Clarke, 1990; Stephenson and Clarke, 1990), no methylation of rap1 would be observed in the membrane fraction if the protein was already fully methylated, as has been suggested for G25K (Backlund, 1992). This argument also suggests that the cytosol may represent a reservoir of nonmethylated precursor protein. Presumably, if the membrane fraction is not added to the incubation, no methylation of the soluble species would be observed. In this system, the addition of GTP may, by some mechanism, stimulate the movement of the soluble rap1 protein to the membrane where it would subsequently be methylated and associate with the membrane fraction. In fact, carboxyl methylation of rap1 from human neutrophils was recently shown to be dependent upon the cytosolic fraction, suggesting that the methyltransferase substrate is in the soluble fraction (Philips *et al.*, 1993) However, no soluble rap1-associated proteins have been identified yet.

From all the studies discussed above, it appears that in eukaryotic cells the levels of C-terminal carboxyl methylation of cellular proteins involved in signal transduction pathways can be regulated by different stimuli and that regulated methylation may play an important role in both large G-protein coupled receptor function and small G-protein function *in vivo*. For these polypeptides, methylation is important for membrane-association, but it remains to be seen whether the moiety simply represents a structural determinant necessary for the proper association with the membrane or with other proteins in the membrane. Once there, it is possible that methylation/demethylation reactions work in concert with GTP hydrolysis to regulate the amount of protein associated with the membrane at a given time. Additionally, it is unclear how many methyltransferase enzymes exist in the cell that can recognize these polypeptides as substrates. So far, only one C-terminal methyltransferase has been described. This enzyme, found in mammalian as well as yeast cells, is relatively nonspecific. It is capable of recognizing any farnesylated or geranylgeranylated C-terminal peptide sequence, suggesting that it does not require upstream polypeptide conformations or sequences for substrate recognition (Stephenson and Clarke, 1990; Hrycyna *et al.*, 1991; Volker *et al.*, 1991a; Perez-Sala *et al.*, 1992; Stephenson and Clarke, 1992). It is possible, however, that in addition to the membrane-associated isoprenylcysteine methyltransferase described, other classes of methyltransferases specific for certain protein substrates may also exist in the cell.

5.2.4.1. *Methylesterases.* In chemotactic bacteria, the methylation levels of specific chemoreceptor substrates are regulated by the *cheB* methylesterase and not by the *cheR* methyltransferase (Stock and Simms, 1988). The methyltransferase is continuously active, whereas the esterase activity is regulated by reversible phosphorylation reactions. It is interesting to imagine such a system existing in eukaryotic cells and as more is discovered about the role of C-terminal methylation in eukaryotic cells, it becomes imperative to understand how and if this modification is modulated *in vivo* and if esterase activities for specific proteins are present in the cell.

This hypothesis that C-terminal methylation is reversible in eukaryotic cells depends on the

existence of enzymes capable of catalyzing the cleavage of the methyl ester. A possible example of such an activity was described in bovine retinal rod outer segments (Perez-Sala *et al.*, 1991; Tan and Rando, 1992). Enzymatic hydrolysis of the methyl ester from *N*-acetyl-*S*-farnesylcysteine-[^{3}H]methyl ester *in vitro* by a membrane-associated demethylase was observed in the presence of methylation inhibitors, although minimal precautions were taken in these studies to eliminate or inhibit possible contaminating nonspecific proteolytic activities (Perez-Sala *et al.*, 1991). However, in a later study, it was determined that this activity was irreversibly inhibited by the serine esterase inhibitor ebelactone B (Tan and Rando, 1992). It must be noted that although this activity was identified by virtue of its ability to cleave the methyl ester from a small farnesylated substrate, partial loss of methyl esters from methylated transducin was observed in these same bovine retinas. Therefore, it appears that this activity may be capable of recognizing a variety of polypeptides modified with a C-terminal farnesyl cysteine methyl ester structure, but that only the farnesyl cysteine methyl ester structure is necessary for recognition.

Unfortunately, there is little other evidence to support the removal of C-terminal methyl groups in cells. For example, in the yeast *S. cerevisiae*, no nonvacuolar methylesterase activities have been identified using *N*-acetyl-*S*-farnesylcysteine methyl ester as a substrate (Hrycyna and Clarke, 1992). Furthermore, Gutierrez *et al.* (1989) found no evidence for methyl group turnover in mammalian p21H-*ras* over a 2-hr time period. Possibly, however, in *S. cerevisiae*, as well as other eukaryotic cell systems, esterase activities dependent upon protein sequences or conformations upstream from the modified cysteine residue at the C-terminus may exist for specific cellular protein or peptide substrates. From these results, it is also interesting to speculate that the role of methyl ester modification is different for different cellular proteins and polypeptides. In some cases, the modification may be structural and the methyl group may not be turned over at all, as was observed for the ras proteins. Alternatively, the activity of other cellular proteins may be regulated by methylation and demethylation reactions. In any case, further work must be done to resolve these questions.

5.3. Inhibitors as Functional Probes and Pharmacological Agents

The use of inhibitors can allow for a direct assessment of methyltransferase function and for understanding the role of the methyl group in this potentially regulated response. A wealth of information has come from the development of compounds that inhibit *S*-adenosylhomocysteine hydrolase in animal cells (Chiang, 1985; Wolfe and Borchardt, 1991). The inhibition of this enzyme results in the accumulation of *S*-adenosylhomocysteine, a potent inhibitor of almost all *S*-adenosylmethionine-dependent methyltransferases. However, these general inhibitors of methylation reactions potentially may affect a large number of cellular proteins. Therefore, it was of interest to try and develop inhibitors that were specific for proteins modified by the Class III isoprenylcysteine methyltransferase to be used as pharmacological agents. The compounds AFC (Tan *et al.*, 1991; Volker *et al.*, 1991a,b; Perez-Sala *et al.*, 1992) and *N*-acetyl-*all trans*-geranylgeranyl-L-cysteine (AGGC) (Perez-Sala *et al.*, 1992; Philips *et al.*, 1993) have both proven to be effective competitive inhibitors of the C-terminal isoprenyl cysteine membrane-associated methyltransferase. Interestingly, D-AFC is a good inhibitor of methyltransferase activity but is a poor substrate for the enzyme that may prove useful in the design of new pharmaceuticals (Volker *et al.*, 1991b; Gilbert *et al.*, 1992). Various compounds that contain no remnants of peptide or amino acid structure have proven to be good inhibitors but not substrates of this enzyme, making them attractive candidates for use as pharmacological agents. These substances include *S*-(farnesyl-3-thio)butyric acid, *S*-(farnesyl-3-thio)acetic acid, *S*-(farnesyl-3-thio)pyruvic acid and the *cis* and *trans* derivatives of *S*-(farnesyl-3-thio)propionic acid (Tan *et al.*, 1991; Gilbert *et al.*, 1992; Shi and Rando, 1992). *In vitro* experiments using AFC and its derivatives have demonstrated that p21*K-ras 2B* (Volker *et al.*, 1991b), the γ-subunit of transducin (Perez-Sala *et al.*, 1991), the platelet Rap1 protein (Huzoor-Akbar *et al.*, 1991) and the rac2 protein (Philips *et al*, 1993) are effectively not methylated in the presence of inhibitor. However, the possibility does remain that in addition to attenuating methylation levels, the inhibitor may also be affecting other processes, such as the interaction of the isoprenyl groups with membrane-associated receptor proteins or the correct association of polypeptide subunits *in vivo*.

In intact cells, AFC has been shown to inhibit agonist-receptor-induced platelet aggregation as well as aggregation caused by exposure to GTPγS, but does not affect receptor-independent pathways (Huzoor-Akbar *et al.*, 1993). These results are suggestive of a block in the receptor-mediated signal transduction pathway. More specifically, inhibition of platelet aggregation may result directly from inhibiting methylation of the γ-subunit of the large G-protein coupled to the receptor or of distinct small GTP-binding proteins in the cell. Interestingly, unlike other known platelet aggregation inhibitors, AFC does not appear to increase cellular cAMP levels in the cell and may represent a possible novel anticoagulating agent.

As previously mentioned, the addition of AFC to mouse peritoneal macrophages drastically reduced the chemotactic response of these cells, suggesting that methylation may play an important role in receptor-mediated signal transduction (Volker *et al.*, 1991b). AFC did not inhibit chemotaxis when the pathway was turned on with phorbol ester, a known activator of protein kinase C that bypasses the requirement for agonist-receptor binding. AFC was also determined to inhibit G-protein activation in human HL-60 granulocytes (Scheer and Gierschik, 1993). It is clear from these results that the inhibitor is acting at a step in the signal transduction pathway that precedes protein kinase C activation. It is possible then that inhibiting the methylation of the γ-subunit of the large G-protein or of members of the ras family of GTP-binding proteins may be responsible for an attenuated chemotactic response (Volker *et al.*, 1991b). It is also possible that AFC prevents the correct association of the α- and $\beta\gamma$-subunits of the G-protein coupled to the receptor (Fukada *et al.*, 1990) by preventing the methylation of the γ-subunit. Similarly, if the receptor contains a recognition site for methylated isoprenylcysteine residues (Inglese *et al.*, 1992; Pitcher *et al.*, 1992), by inhibiting the methylation of the γ-subunit this signal would be lost. However, Scheer and Gierschik (1993) observed that well-washed membranes, presumably free of the methyl donor *S*-adenosyl-L-methionine, were capable of supporting inhibition by AFC and that *S*-adenosyl-L-homocysteine did not inhibit either basal or fMet–Leu–Phe-stimulated activity, suggesting that AFC itself and not inhibition of the methylation reaction may be responsible for the decreased chemotactic response. These observations led the authors to suggest that AFC might directly interfere with subunit association or may block the 'docking site' for the isoprenylated cysteine residue. Further work needs to be done to resolve these issues, but it is possible that these inhibitors may still prove useful in preventing inflammatory responses *in vivo*, even if their mode of action is not solely to inhibit C-terminal protein methylation.

Another protein implicated in the chemotactic and inflammatory response is the ras-related small G-protein, rac. As discussed in Section 5.2.4, rac is thought to play a regulatory role in the activation of NADPH oxidase in neutrophils and macrophages (Segal and Abo, 1993). The prenylcysteine inhibitors AFC and AGGC were found to inhibit the fMet–Leu–Phe-induced production of superoxide in human neutrophils but were ineffective against phorbol ester induction (Philips *et al.*, 1993). Although the production of this compound is a direct result of activation of the NADPH oxidase system, the inhibitor is probably not directly inhibiting activation of the enzyme because other fMet–Leu–Phe-induced responses in this pathway are observed, such as neutrophil aggregation and cytosolic granule secretion. This data suggests that the mode of action of the inhibitor is at the level of substrate-receptor-mediated coupling of the signal, but that there is a direct link between this response and the carboxyl methylation of a number of ras-related G-proteins.

Although it is apparent that a number of the ras-related proteins in the cell are effectively inhibited by AFC and AGGC, the ras proteins are of immediate interest due to their implicated role in cancer (Casey, 1992; Clarke, 1992; Schafer and Rine, 1992; Sinensky and Lutz, 1992). p21*Ha-ras* methylation has been strongly inhibited by AFC in transformed rat embryo fibroblasts, but, unfortunately, the inhibitor had little effect on the rate of growth or the transforming ability of these cells (Volker *et al.*, 1991b). In a *ste14* mutant of *S. cerevisiae*, which lacks C-terminal isoprenylcysteine methyltransferase activity, it was determined that the lack of methylation had no drastic effects on the activity of the RAS2 protein but did significantly reduce the rate and efficiency of its membrane localization (Hrycyna *et al.*, 1991). Presumably, in both of these systems, even in the absence of methylation enough protein reaches the membrane to elicit the biological response, suggesting that the role of methylation may be to help guide the protein to the membrane. These results suggest that inhibiting the methylation of the ras proteins may not be an effective

inhibitor *in vivo*. Interestingly, though, inhibition of ras farnesylation has a profound effect on the transforming ability of the cells, suggesting that this may prove to be a more effective agent in cancer treatment (Schafer and Rine, 1992).

5.4. POSSIBLE SYSTEMS INVOLVING NOVEL METHYLTRANSFERASE REACTIONS

It is becoming increasingly clear that regulation by methylation and demethylation reactions in eukaryotic cell systems may prove to be a very complex system potentially involving a great number of cellular substrates. Interestingly, though, to date, only one type of C-terminal membrane-associated methyltransferase has been identified that appears to only need an isoprenyl cysteine residue for substrate recognition in both yeast and mammalian systems. From these findings, two possibilities arise to explain how methylation could be involved in the regulation of such a wide variety of polypeptides. First, it is possible that this one methyltransferase actually can methylate all potential cellular substrates. In *S. cerevisiae*, it has been shown that the STE14 methyltransferase is responsible for the C-terminal methylation of at least a-factor and the RAS1 and RAS2 proteins (Hrycyna *et al.*, 1991). If this is the case, then it would be feasible to imagine that regulatory specificity would lie in esterase activities, as is the case for bacterial chemotaxis (see Section 5.4.2.1).

Alternatively, it is possible that other methyltransferase activities are present in the cell that are specific for particular protein or polypeptide substrates. This hypothesis is supported by methyltransferase inhibition studies performed with the inhibitor 5′-methylthioadenosine (MTA). In intact cells, a methyltransferase capable of modifying -CXXX-containing substrates was shown to be inhibited by 3 mM MTA (Chelsky *et al.*, 1989). Interestingly, the nonspecific STE14-like methyltransferase activity described by Stephenson and Clarke (1992) shows no inhibition *in vitro* up to an MTA concentration of 12.8 mM. This finding suggests that a variety of membrane-associated methyltransferases with different substrate specificities may be present in the cell at a number of locations that may be distinguished by their differential sensitivities to MTA (Stephenson and Clarke, 1992). Alternatively, these experiments may all represent the same nonspecific methyltransferase, if in intact cells MTA is converted to an inhibitory compound not present in *in vitro* incubations (Stephenson and Clarke, 1992). If more protein-specific methyltransferases are identified, MTA may prove to be a useful diagnostic tool in differentiating between these and the nonspecific methyltransferase previously described.

5.4.1. *Pre-B Cell System*

In the mouse pre-B lymphocyte cell line (70Z/3), experiments utilizing MTA have offered good preliminary evidence for the involvement of other methyltransferase reactions in the regulation of methylated polypeptide levels. When exposed to the mitogen bacterial lipopolysaccharide (LPS), a pre-B cell signaling pathway is activated. The results of this activation include the mobilization of the transcription factor NF-κB to the nucleus and the subsequent induction of transcription of the κ light-chain gene. Recently, the possibility that the polypeptides involved in this signaling pathway are post-translationally modified by isoprenylation and methylation reactions in response to LPS was explored (Law *et al.*, 1992). Upon LPS treatment, an increase in the amount of carboxyl methylation, possibly representative of either C-terminal, L-glutamate γ-methyl esters or other species, of a number of polypeptides ranging from 106 kDa to 19 kDa was observed. Treatment of 70Z/3 cells with 2 mM MTA or 20 μM mevinolin specifically inhibited κ gene expression as well as the carboxyl methylation of these polypeptides at a step prior to the post-translational activation of NF-κB. The inhibition of the methylation reaction by mevinolin, an inhibitor of the isoprenoid pathway, suggests a link between the methylation and lipidation of these substrates. These results indicate that methylation reactions are potentially involved in NF-κB activation in pre-B cells, but it remains unclear what types of polypeptides are substrates and what types of residues on these substrates are modified. It will also be interesting to determine if other methyltransferase enzymes are responsible for the modification events, since MTA may be a more general methylation inhibitor as well as having a variety of other effects on the cell, such as the inhibition of phosphorylation.

5.4.2. *Pheochromocytoma Cell System*

In rat pheochromocytoma PC-12 cells, the methylation levels of membrane polypeptides in response to nerve growth factor (NGF) or epidermal growth factor (EGF) treatment were examined both in the presence and absence of the methylation inhibitor MTA (Kujubu *et al.*, 1993). Upon treatment with NGF and, to a lesser extent, EGF, the methylation of a variety of proteins was enhanced, including that of polypeptides in the 22–24 kDa range, a molecular mass suggestive of the small G-proteins. It was also determined that MTA can effectively inhibit the signal transduction pathway and methylation of cellular proteins mediated by NGF or EGF stimulation. Although MTA can also act as a kinase inhibitor, these findings suggest that one effect of the compound may be the inhibition of methylation of small G-proteins involved in the signal transduction pathway, such as the ras proteins. Again, the effectiveness of MTA as an inhibitor of the methylation of these polypeptides does not give much information as to the methyltransferase responsible or the type of methylation involved. It is possible that the methylation of these polypeptide substrates may be either at the C-terminus or at an internal site.

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