Effects of Farnesylcysteine Analogs on Protein Carboxyl Methylation and Signal Transduction*

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Several proteins associated with signal transduction in eukaryotes are carboxyl methylated at COOH-terminal S-farnesylcysteine residues. These include members of the Ras superfamily and γ-subunits of heterotrimeric G-proteins. The enzymes that catalyze the carboxyl methylation reaction also methylate small molecules such as N-acetyl-S-trans,farnesyl-L-cysteine (AFC). AFC inhibits carboxyl methylation of p21WAF1 and related proteins both in vitro and in vivo. Saturating concentrations of AFC cause a >80% inhibition of chemotactic responses of mouse peritoneal macrophages. Our results suggest that carboxyl methylation may play a role in the regulation of receptor-mediated signal transduction processes in eukaryotic cells.

In bacteria, the activities of membrane receptor-transducer proteins are regulated by carboxyl methylation at glutamate residues (Springer et al., 1979; Ninfa et al., 1991; Stock et al., 1991). Recently, several important classes of signal transduction proteins in eukaryotes have been shown to be carboxyl methylated. These include members of the Ras superfamily of guanine nucleotide-binding proteins (Clarke et al., 1988; Gutierrez et al., 1988; Stimmel et al., 1990), the γ-subunits of heterotrimeric G-proteins (Fukada et al., 1990; Fung et al., 1990; Yamane et al., 1990), the a-subunit of cGMP phosphodiesterase from retinal rods (Ong et al., 1989), and a class of fungal mating pheromones (Sakagami et al., 1981; Ishibashi et al., 1984; Anderegg et al., 1988). This raises the possibility that carboxyl methylation may play a central role in the regulation of stimulus-response coupling in eukaryotic cells.

Eukaryotic signal transduction proteins are carboxyl methylated at modified cysteine residues that are produced by a series of post-translational modification events (Clarke et al., 1988; Hancock et al., 1989). The nascent proteins have a cysteine residue 4 amino acids from the COOH terminus within a characteristic sequence that has been termed a CAAX tail (Barbacid, 1987). A polysignoprotein group is coupled to this cysteine through a thioether bond, the 3 residues that follow the cysteine are proteolytically cleaved, and the resultant COOH-terminal carboxyl group is methyl esterified.

The polyisoprenoid linked to p21WAF1 (Casey et al., 1989; Buss et al., 1991), Saccharomyces cerevisiae RAS2 protein (Stimmel et al., 1990), the γ-subunit of retinal transducin (Fukada et al., 1990), and S. cerevisiae a-factor (Anderegg et al., 1988) is a 15-carbon farnesyl group.

Here we report the effects of a specific inhibitor of the COOH-terminal S-farnesylcysteine methyltransferase. Evidence is presented that the enzyme that methylates Ras will also methylate small molecule analogs of the modified COOH-terminal amino acid, S-farnesylcysteine. One of these analogs, N-acetyl-S-trans,farnesyl-L-cysteine (AFC), has a high affinity for the methyltransferase (KM = 20 μM) and functions as a specific inhibitor of the COOH-terminal S-farnesylcysteine methyltransferase both in cell-free extracts and in living cells. The availability of a specific inhibitor for this type of protein carboxyl methylation in eukaryotic cells has allowed us to begin to directly assess the physiological significance of this reaction. Our results indicate that in vertebrate cells, as in bacteria, protein carboxyl methylation plays a role in receptor-mediated signal transduction processes.

MATERIALS AND METHODS

S-Substituted Cysteine Compounds—S-Substituted cysteine compounds were prepared from the chloride or bromide of the desired substituent in a manner analogous to our previously reported syntheses of S-farnesylcysteine derivatives (Volker et al., 1990). The products were characterized by NMR, mass spectrometry, and HPLC.

Cell Lines and Strains—Two rat embryo fibroblast cell lines co-transfected with plasmids that contain activated p21WAF1 (Gly-12 to Val-12 substitution) and murine p53 that contains a linker insertion mutation at amino acid position 215 (KH215.A2.T23 and MSV.KH215.T22-4.1-4) were employed. The genotypes of the S. cerevisiae cells used were: strain MS46, MATa urd-52 ade2-101; strain MSV.KH215.T22-4.1-4 used. The Bacillus subtilis cells were used: strain MS46, MATa urd-52 ade2-101 leu2-3; strain MS10, MATa urd-52 ade2-101 leu2-3; strain MS46, MATa leu2-3 ade5-4. The Echerichia coli cells used were: strain K12, strain S22, strain S85 (CSC3004(CR34)), thi-1 thr-1 leuB6 lacY1 tonA21 supE44, and W3110 strain S85 (trpC 9830).

Isolation of Peritoneal Macrophages—Adult BALB/C mice were injected intraperitoneally with 1 ml of Brewer's thioglycolate medium (Fisher Scientific Co.). Peritoneal exudate cells (PEC's) were collected 5 days later by peritoneal lavage using Hank's buffered saline solution (GIBCO) containing 5 units of heparin/ml. The cells were stored for nonspecific esterase activity using α-naphthyl acetate as substrate (Kaplow, 1981) or with Wright-Giemsa stain (Camoo Quik Stain II, American Scientific Products). Cell suspensions that had no more than 2% contamination of neutrophils were pooled together. Cell suspensions were washed twice in Hank's buffered saline solution (Fisher Scientific Co.). Peritoneal exudate cells (PEC's) were collected 5 days later by peritoneal lavage using Hank's buffered saline solution (GIBCO) containing 5 units of heparin/ml. The cells were stained for nonspecific esterase activity using α-naphthyl acetate as substrate (Kaplow, 1981) or with Wright-Giemsa stain (Camoo Quik Stain II, American Scientific Products). Cell suspensions that had no more than 2% contamination of neutrophils were pooled together. Cell suspensions were washed twice in Hank's buffered saline solution (Fisher Scientific Co.). Peritoneal exudate cells (PEC's) were collected 5 days later by peritoneal lavage using Hank's buffered saline solution (GIBCO) containing 5 units of heparin/ml. The cells were stained for nonspecific esterase activity using α-naphthyl acetate as substrate (Kaplow, 1981) or with Wright-Giemsa stain (Camoo Quik Stain II, American Scientific Products). Cell suspensions that had no more than 2% contamination of neutrophils were pooled together. Cell suspensions were washed twice in Hank's buffered saline solution (Fisher Scientific Co.).
tion, then resuspended to a concentration of $3 \times 10^6$ cells/ml in RPMI 1640 (GIBCO), pH 7.2, with 10% heat-inactivated fetal bovine serum (GIBCO), with or without AFC, AGC, or phorbol 12-myristate 13-acetate, as described.

Tissue Extracts—Extracts from mouse tissues were prepared at 4°C by homogenization with a Teflon-glass homogenizer in 5 ml/g wet weight, tissue of 100 mm Tris-Cl, 1 mM EDTA, 1 mM DTT, pH 7.9 (buffer A). Bovine brain extracts were prepared from whole brains that were homogenized in a blender with 3 ml of buffer A/g, wet weight, tissue, followed by filtration through cheesecloth. Extracts of S. cerevisiae and E. coli were prepared similarly, except for the cell disruption procedure. Bacteria were lysed by sonication in buffer A, and yeast cells were broken by vortexing with glass beads in buffer A with 0.1 mM phenylmethylsulfonyl fluoride (buffer B). Rat tissue extracts were prepared with a Teflon-glass homogenizer in 9 volumes of 320 mm sucrose, 7.5 mm Tris-Cl, 1 mM CaCl$_2$, 1 mM DTT, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (buffer C).

Rat tissue extracts were centrifuged for 10 min at 8000 × g. The pellets were washed once in buffer C, then resuspended in 320 mm sucrose, 7.5 mm Tris-Cl, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (buffer D), to yield the crude nuclear fractions. The postnuclear supernatants and washes were combined, an additional 1 mM EDTA added, then centrifuged for 20 min at 16,000 × g. The pellets were washed once in buffer D, then resuspended in buffer D to yield the crude mitochondrial fractions. The postmitochondrial supernatants and washes were combined and centrifuged for 200 min at 100,000 × g. The supernatants containing soluble fractions. The high speed pellets were washed, then resuspended in buffer D to produce the crude microsomal fractions. All resuspensions were performed using a Teflon-glass homogenizer.

Bovine brain fractions were prepared in the same way, except buffer B was used throughout, with 0.1 mM phenylmethylsulfonyl fluoride (buffer B). Rat tissue extracts were centrifuged for 10 min at 800 × g. The pellets were washed once in buffer C, then resuspended in 320 mm sucrose, 7.5 mm Tris-Cl, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (buffer B). Rat tissue extracts were centrifuged for 90 min at 100,000 × g with 0.1 mM phenylmethylsulfonyl fluoride (buffer B). Rat tissue extracts were centrifuged for 20 min at 16,000 × g. The pellets were washed once in buffer D, then resuspended in buffer D to yield the crude mitochondrial fractions. The postmitochondrial supernatants and washes were combined and centrifuged for 200 min at 100,000 × g. The supernatants containing soluble fractions. The high speed pellets were washed, then resuspended in buffer D to produce the crude microsomal fractions. All resuspensions were performed using a Teflon-glass homogenizer.

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. To determine the concentration of bovine serum albumin, the extinction coefficient at 280 nm for a 1 mg/ml solution was taken to be 0.66 (Tanford and Roberts, 1952).

Protein Carboxyl Methylation Assay—To determine the level of carboxyl methylated proteins from dried SDS-PAGE gels, lanes were cut into 3-mm slices that were each placed into 1.5-ml open-topped vials and 1 ml of a cell disruption mixture. Bacteria were lysed by sonication in buffer A, and yeast cells were broken by vortexing with glass beads in buffer A with 0.1 mM phenylmethylsulfonyl fluoride (buffer B). Rat tissue extracts were prepared with a Teflon-glass homogenizer in 9 volumes of 320 mm sucrose, 7.5 mm Tris-Cl, 1 mM CaCl$_2$, 1 mM DTT, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (buffer C).

Rat tissue extracts were centrifuged for 10 min at 8000 × g. The pellets were washed once in buffer C, then resuspended in 320 mm sucrose, 7.5 mm Tris-Cl, 1 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 (buffer D), to yield the crude nuclear fractions. The postnuclear supernatants and washes were combined, an additional 1 mM EDTA added, then centrifuged for 20 min at 16,000 × g. The pellets were washed once in buffer D, then resuspended in buffer D to yield the crude mitochondrial fractions. The postmitochondrial supernatants and washes were combined and centrifuged for 200 min at 100,000 × g. The supernatants containing soluble fractions. The high speed pellets were washed, then resuspended in buffer D to produce the crude microsomal fractions. All resuspensions were performed using a Teflon-glass homogenizer.

Bovine brain fractions were prepared in the same way, except buffer A was used in each step. With yeast, buffer B was used throughout, and the 16,000 × g fractionation step was omitted.

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. To determine the concentration of bovine serum albumin, the extinction coefficient at 280 nm for a 1 mg/ml solution was taken to be 0.66 (Tanford and Roberts, 1952).

**RESULTS**

**S-Farnesylcysteine Carboxyl Methyltransferase Activity**—Cell-free extracts from a wide variety of eukaryotic sources catalyze the transfer of methyl groups from S-adenosyl-L-methionine (AdoMet) to AFC. By following the radioactivity from [3H]AdoMet we were able to identify by reverse phase HPLC the product of the methylation reaction as the α-carboxyl methyl ester derivative of AFC (Volker et al., 1990).

Significant methyltransferase activity was detected in all eukaryotic cells and tissues that were examined, including S. cerevisiae (Table I). The highest levels were found in mammalian brain and the lowest in blood. No methyltransferase activity was detected in E. coli.

An analysis of the subcellular distribution of AFC methyltransferase activity in brain, liver, and heart indicates that the enzyme is tightly associated with membranes (Table II). Although significant levels were detected in all membrane fractions, the highest specific activity was found in the crude microsomal fractions. The total activity was associated primarily with the material that sedimented with the crude nuclear fractions. This correlates with the location of nuclear lamin B, a protein that is reversibly methylated at a COOH-terminal S-farnesylcysteine residue in a cell cycle-dependent manner (Chelsky et al., 1987).

Mutant strains of S. cerevisiae with defects in STE14 are deficient in the ability to methylate α-factor and other peptides with a COOH-terminal S-farnesylcysteine (Marr et al., 1991; Hrycyna and Clarke, 1990). A ste14 mutant was also found to be totally deficient in AFC methylation (Table III).

In yeast, as in mammalian tissues, S-farnesylcysteine methyltransferase activity is tightly associated with membrane fractions (Table III).

Under the conditions used, AFC methyl ester was produced at a constant rate for 25 min. The rate increased proportionately with protein level. The activity in both mouse and bovine

**TABLE I**

| Source                  | Methyltransferase activity | % of total | mg | pmol/mg/min |
|-------------------------|---------------------------|------------|----|-------------|
| Mouse tissue homogenates | Brain                     | 8.4        | 100| 7.9         |
|                         | Testis                    | 2.7        | 100| 11          |
|                         | Thigh muscle              | 1.1        | 100| 6.3         |
|                         | Thymus                    | 0.68       | 100| 16          |
|                         | Heart                     | 0.31       | 100| 18          |
|                         | Small intestine           | 0.28       | 100| 18          |
|                         | Spleen                    | 0.16       | 100| 20          |
|                         | Lung                      | 0.022      | 100| 16          |
|                         | Kidney                    | 0.020      | 100| 0.20        |
|                         | Liver                     | 0.012      | 100| 0.20        |
|                         | Blood                     | 0.002      | 100| 0.20        |
| Cell homogenates        | S. cerevisiae MATα        | 0.52       |    |             |
|                         | S. cerevisiae MATα        | 0.48       |    |             |
|                         | E. coli K12               | <0.001     |    |             |
|                         | E. coli W3110             | <0.001     |    |             |

**TABLE II**

| Source | Fraction   | Methylation specific activity | Total protein | Overall activity | % of total | pmol/mg/min |
|--------|------------|-------------------------------|---------------|-----------------|------------|-------------|
| Rat    | Brain      | Total                          | 7.9           | 140             | 100        |
|        | Crude nucleus |                               | 11            | 86              | 86         |
|        | Crude mitochondrial |                          | 6.3          | 18              | 10         |
|        | Crude microsomal |                              | 18           | 2.0            | 3          |
|        | Soluble     |                               | 0.20         | 39              | 1          |
| Rat    | Liver      | Total                          | 0.78         | 4300            | 100        |
|        | Crude nucleus |                               | 1.4          | 1700            | 71         |
|        | Crude mitochondrial |                          | 1.6          | 460             | 22         |
|        | Crude microsomal |                              | 2.7          | 74              | 6          |
|        | Soluble     |                               | 0.040        | 2000            | 2          |
| Rat    | Heart      | Total                          | 0.096        | 28              | 100        |
|        | Crude nucleus |                               | 0.12         | 18              | 80         |
|        | Crude mitochondrial |                          | 0.33         | 1.2             | 15         |
|        | Crude microsomal |                              | 0.50         | 0.23            | 4          |
|        | Soluble     |                               | 0.004        | 8.3             | 1          |
TABLE III
S-Farnesylcysteine methyltransferase activity in S. cerevisiae

| Strain Fraction | Methyltransferase specific activity pmol/mg/min |
|-----------------|---------------------------------------------|
| MS46 (MATα)     | Membrane 1.1  Soluble <0.001 |
| MS10 (MATα)     | Membrane 1.2  Soluble <0.001 |
| HR129-2a (ste14 MATα) | Membrane <0.001  Soluble <0.001 |

Inhibitor of Ras Methylation

Carboxyl methylation of the 70, 45, and some of the 18-26 kDa molecular mass proteins is inhibited by AFC (Fig. 2). Thus, AFC inhibits the methyltransferase activities that modify proteins that are known to be modified at COOH-terminal S-farnesylcysteine residues. In contrast, methylation of the 36-kDa species is not affected by AFC. We have identified a cytosolic methyltransferase activity that catalyzes the carboxyl methylation of the 36-kDa protein, and this activity is clearly distinct from the membrane-associated S-farnesylcysteine methyltransferase.

Thus, AFC provides a useful means to distinguish at least two major classes of eukaryotic protein carboxyl methyltransferases. The precise nature of the methylated residues in the 36-kDa protein remain to be established.

Inhibition of p21<sup>ras</sup> Carboxyl Methylation in a Reconstituted Post-translational Modification System—We examined the methylation of Ras in a reconstituted post-translational modification system. Unmodified p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> was overproduced in E. coli and purified. This protein is an efficient substrate of the farnesyltransferase that has recently been characterized by Hess et al. (1990), and we have identified a membrane-associated protease in rat brain that cleaves the COOH-terminal 3 amino acids from farnesylated p21<sup>His<sub>5</sub>-<sup>ras</sup></sup>.

In the experiment reported in Fig. 3, farnesylated and cleaved p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> was used as a substrate for the methyltransferase. In the presence of 100 μM AFC (lane 2), the methylation of p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> was greatly inhibited as compared to the control lane without AFC (lane 1). At the identical concentration, N-acetylated S-trans-geranyl-L-cysteine (AGC, lane 3), which has a 50-fold larger Km (Table IV), is a poor inhibitor of p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> carboxyl methylation. AFC (lane 4), which has a Km nearly as low as AFC (Table IV), also significantly inhibits carboxyl methylation of p21<sup>His<sub>5</sub>-<sup>ras</sup></sup>. Although it is a good inhibitor, AGC is not methylated as well as AFC (Table IV). This feature may prove useful to the design of pharmacologically relevant inhibitors of S-farnesylcysteine methylation. Recently, AFC has also been reported to inhibit the <i>in vitro</i> carboxyl methylation of other CAAX tail proteins, including platelet Rap1 (Huzoor-Akbar et al., 1991) and the γ-subunit of transducin in retinal rod outer segments (Perez-Sala et al., 1991).

Inhibition of p21<sup>ras</sup> Carboxyl Methylation in Transformed Rat Embryo Fibroblasts—AFC is a relatively hydrophobic amino acid derivative that might be expected to move across cell membranes by simple diffusion or through an amino acid or peptide transport system. To explore this possibility we examined the effects of AFC on the methylation of p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> in transformed fibroblasts. Levels of p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> methylation were examined in cells grown for 3 h with [methyl-<sup>3</sup>H]methionine in the presence or absence of AFC. Under these conditions, 100 μM AFC caused a 60-70% decrease in p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> carboxyl methylation (Fig. 4). Mature p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> contains 4 methionine residues (Barbacid, 1987) so that under steady state labeling conditions the incorporation of 1 methyl ester per p21 monomer would give a 1:4 ratio of <sup>3</sup>H-methyl esters to [<sup>3</sup>H]methionine. In the absence of inhibitor, a ratio of 1:6 was obtained after the 3-h incubation. This indicates a stoichiometry of 0.7 methyl groups/molecule. In the presence of AFC, this value was reduced to 0.2-0.3. AFC had no detectable effect on [<sup>3</sup>H]methionine incorporation into p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> protein. Despite its inhibitory effect on carboxyl methylation, AFC had little effect on rates of proliferation of these p21<sup>His<sub>5</sub>-<sup>ras</sup></sup> transformed cells.

Inhibition of Macrophage Chemotaxis with AFC—General methylation inhibitors have been previously shown to block...
Inhibitor of Ras Methylation

### TABLE IV

Specificity of the S-farnesylcysteine methyltransferase activity

The indicated cysteine derivatives were incubated at 37 °C with [³H]AdoMet (10 μM, specific activity: 2500 cpm/pmrol) and mouse brain homogenate (300 mg of protein) in buffer A for various times up to 25 min. Carboxyl methylation of the derivatives was assayed by our previously reported HPLC or heptane extraction methods (Volker et al., 1990). Initial rates were determined at 6 concentrations of each substrate, except for FC and ABC where 2 concentrations were used. ND, not determined.

| Substrates                                      | KM (mM) | Vmax (pmol/mg/min) | Vmax/KM (%AFC) |
|------------------------------------------------|---------|--------------------|----------------|
| N-Acetyl-S-trans,trans-Farnesyl-L-Cysteine (AFC) | 0.02    | 8                  | 100            |
| N-Acetyl-S-trans-Geranyl-L-Cysteine (AGC)        | 1       | 2                  | 0.6            |
| N-Acetyl-S-3,3-Dimethylallyl-L-Cysteine (ADC)    | 5       | 1                  | 0.07           |
| N-Acetyl-S-trans,trans-Farnesyl-D-Cysteine (D-AFC)| 0.04    | 0.5                | 3              |
| S-trans,trans-Farnesyl-L-Cysteine (FC)           | ND      | ND                 | 2              |
| N-Acetyl-S-Benzyl-L-Cysteine (ABC)               | ND      | ND                 | < 0.05         |
macrophage chemotaxis (Snyderman, 1985). To determine the potential role of S-farnesylcysteine carboxyl methylation in this response pathway, we incubated mouse peritoneal macrophages with various concentrations of AFC and measured their response toward endotoxin-activated mouse serum. AFC was found to dramatically reduce the chemotactic response toward endotoxin-activated mouse serum.

We previously identified an activity in eukaryotic cells that methylates the α-carboxy group of the small molecular weight S-farnesylcysteine derivative, AFC (Volker et al., 1990). Here we show that this is the same activity that catalyzes the specificity one would expect for a Ras methyltransferase in that the farnesyl group provides an essential recognition element, and an amide linkage to the α-amino group is preferred over the free amino acid; (b) a S. cerevisiae mutant that is defective in its ability to methylate α-factor and other polypeptides at COOH-terminal S-farnesylcysteine is also defective in AFC methyltransferase activity; and (c) AFC functions as a specific inhibitor of the methylation of proteins such as p21WAF1 that are known to be carboxyl methylated at such as p21WAF1, thus obviating the requirement for diacylglycerol, the second messenger generated by phospholipase C-catalyzed hydrolysis in the chemotactic response pathway (Kikkawa and Nishizuka, 1986; Kaibuchi et al., 1985). Thus, AFC interferes with a step in signaling that precedes protein kinase C activation.

**DISCUSSION**

We previously identified an activity in eukaryotic cells that methylates the α-carboxy group of the small molecular weight S-farnesylcysteine derivative, AFC (Volker et al., 1990). Here we show that this is the same activity that catalyzes the methyl esterification of proteins such as Ras at COOH-terminal S-farnesylcysteine residues. The following lines of evidence support this contention: (a) the methyl acceptor activities of small molecular weight cysteine derivatives fit the specificity one would expect for a Ras methyltransferase in that the farnesyl group provides an essential recognition element, and an amide linkage to the α-amino group is preferred over the free amino acid; (b) a S. cerevisiae mutant that is defective in its ability to methylate α-factor and other polypeptides at COOH-terminal S-farnesylcysteines is also defective in AFC methyltransferase activity; and (c) AFC functions as a specific inhibitor of the methylation of proteins such as p21WAF1 that are known to be carboxyl methylated at COOH-terminal S-farnesylcysteine residues.

Proteins and peptides translated with CAAX tails are subject to a series of post-translational modifications that includes thioether linkage of a farnesyl group to the cysteine,
cleavage of the residues distal to this modified group, and carboxyl methylation of the exposed S-farneslycsteine α-carboxyl (Miyakawa et al., 1985; Clarke et al., 1988; Hancock et al., 1989). A mammalian activity that catalyzes the transfer of a farnesyl group from farnesyl pyrophosphate to the CAAX motif are potent inhibitors of farnesylation, and the inclusion of more upstream amino acids does not dramatically increase inhibition.

An activity in rat liver has been characterized that carboxyl methylates an S-farneslycsteine peptide with an analogous sequence to that which is upstream of the CAAX tail cisteine in p21<sup>ras</sup> proteins has been purified and characterized (Reiss et al., 1990; Schaber et al., 1990; Manne et al., 1990; Reiss et al., 1991). The enzyme also farnesylates a peptide that contains only the last 6 amino acids of unprocessed human p21<sup>ras-2B</sup> (Reiss et al., 1990). Moreover, tetrapeptides that consist solely of the COOH-terminal CAAX motif are potent inhibitors of farnesylation, and the inclusion of more upstream amino acids does not dramatically increase inhibition.

In addition to the S-farneslycsteine methyltransferase, which is membrane-associated, there is a soluble methyltransferase activity that methylates a 36-kDa protein. Throughout a wide range of tissues, methylation is more prevalent at 36 kDa than for species that are modified at S-farneslycsteine residues. We are currently in the process of determining the nature of this modification. Our preliminary results indicate that the carboxyl methyl group is associated with an amino acid that is more hydrophobic than an S-farneslycsteine methyl ester. A detailed analysis of the total pool of prenylated proteins in tissue homogenates indicates that a 20-carbon isoprenoid, geranylgeranyl, is actually a more common cys- teine adduct than the 15-carbon farnesyl group (Farnsworth et al., 1990; Rilling et al., 1990). It seems unlikely, however, that the methylated residue in the 36-kDa protein is a COOH-terminal geranylgeranylcysteine. Preliminary results suggest that the same carboxyl methyltransferase that modifies COOH-terminal farneslycsteine residues also methylates proteins that have COOH-terminal geranylgeranylcysteine residues.² In addition, AFC appears to inhibit the methylation of proteins with a COOH-terminal geranylgeranylcysteine. AFC has been shown to inhibit Rap1 methylation in human platelets (Huzoor-Akbar et al., 1991), and Rap1A expressed in baculovirus-infected insect cells has been shown to be geranylgeranylated (Buss et al., 1991). Moreover, we have observed that AFC inhibits the carboxyl methylation of 20-23-kDa proteins in human neutrophils including one that is immunoprecipitated by polyclonal antisera to Rap1 and Rap2.³ A set of 20-23-kDa proteins in a mouse macrophage

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³ C. Volker and J. B. Stock, manuscript in preparation.
⁴ M. R. Philips, C. Volker, and J. B. Stock, manuscript in preparation.
Fig. 6. **S-Farnesylcysteine targets in eukaryotes.** Specific signal transduction components are reversibly methylated at COOH-terminal S-farnesylcysteine residues. These proteins may have two distinct classes of regulatory targets, those that interact with the proteins in their methylated state (M-targets) and those that interact with the demethylated proteins (D-targets). Changes in levels of methylation would regulate the relative outputs from these two types of effector systems.

Cell line were reported to be carboxyl methylated by a soluble methyltransferase activity (Backlund and Aksamit, 1988), but recent results indicate that the substrates are soluble, not the methyltransferase. Several distinct functions have been proposed for S-farnesylcysteine carboxyl methylation. In the case of nuclear lamin B, it has been suggested that methylation and demethylation play a role in the regulation of nuclear lamina assembly (Chelsky et al., 1987; Chelsky et al., 1989). In the case of *S. cerevisiae*, a-factor pheromone binding to a membrane receptor of the rhodopsin/β-adrenergic superfamily mediates the activity of a heterotrimeric G-protein (Whiteway et al., 1989). a-Factor is much more active in eliciting a mating response when its COOH-terminal S-farnesylcysteine residue is methylated, and it has been proposed that demethylation provides a mechanism for pheromone inactivation (Anderegg et al., 1988).

The functions for methyl esterification of proteins in the Ras and the G, families remain to be established. These proteins interact with other proteins to direct an array of cellular processes. Activating mutations in ras proto-oncogenes can cause uncontrolled growth in some cell types, while activating mutations in homologous guanine nucleotide-binding proteins, such as Rap1A, can antagonize these effects (Buse et al., 1991). It has been estimated that each mammalian cell may have from 30 to 100 different members of the Ras superfamily, each responding to a different spectrum of regulatory inputs, and interacting with a different group of effector activities (Chardin, 1988). Because of this diversity, the physiological significance of S-farnesylcysteine carboxyl methylation may be quite complex.

The discovery that mammalian S-farnesylcysteine methyltransferase activities are specifically inhibited by AFC provides a means to begin to probe the functions of this modification. Our results indicate that inhibition of p21<sup>140-138</sup>-tsf methylation does not significantly affect growth or transformation of p21<sup>140-138</sup>-transformed mammalian cell lines. These findings are consistent with the observation that a methyltransferase defect in yeast, *STE14*, does not preclude the function of the yeast Ras proteins. This does not mean that carboxyl methylation of S-farnesylcysteine residues is generally without import, however. The chemotactic response of mouse peritoneal macrophages is dramatically inhibited by AFC. The effect of AFC is consistent with a defect in the signal transduction pathway. Heterotrimeric G-proteins are integral players in this pathway, and all G subunits that have been investigated are prenylated and methyl esterified at COOH-terminal cysteines. Besides an effect on G<sub>s</sub>, the possibility should not be discounted that AFC may act through a member of the Ras superfamily with an important role in eukaryotic chemotaxis. Despite a lack of detailed information concerning a specific role for carboxyl methylation in eukaryotic signal transduction one can begin to formulate a general mechanism (Fig. 6). Farnesylation directs signal transduction proteins to regulatory targets in the membrane (Hancock et al., 1989; Casey et al., 1989; Schafer et al., 1989). It seems likely that these membrane targets fall into two classes: those that prefer a demethylated acidic COOH terminus (D-targets), and those that prefer a methylated COOH terminus (M-targets). Increases in the level of methylation of a signal transduction protein would increase its interaction with M-targets and decrease its interaction with D-targets. Decreases in methylation would have an opposing effect. According to this view, the a-factor receptor is an M-target since it specifically interacts with methylated a-factor. On the other hand, the S-farnesylcysteine methyltransferase provides an example of a D-target, since it is a membrane protein that preferentially interacts with demethylated signal transduction proteins. A consideration of this model suggests that another possible locus for the inhibition of signal transduction by AFC could be a direct competition for COOH-termini S-farnesylcysteine receptor sites. In effect, inhibition of the methyltransferase may represent only one example of competition of AFC for an S-farnesylcysteine recognition site in a target protein.

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