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A single activity carboxyl methylates both farnesyl and geranylgeranyl cysteine residues

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Members of the Ras superfamily of small GTP-binding proteins, γ-subunits of heterotrimeric G proteins and nuclear lamin B are subject to a series of post-translational modifications that produce prenylcysteine methylester groups at their carboxyl termini. The thioether-linked polyisoprenoid substituent can be either farnesyl (C_{15}) or geranylgeranyl (C_{20}). Small molecule prenylcysteine derivatives with either the C_{15} or C_{20} modification, such as *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine (AFC), *S-trans,trans*-farnesylthiopropionate (FTP), as well as the corresponding geranylgeranyl derivatives (AGGC and GGTP) are substrates for the carboxyl methyltransferase. *Saccharomyces cerevisiae ste14* mutants that lack RAS and a-factor carboxyl methyltransferase activity are also unable to methylate farnesyl and geranylgeranylcysteine derivatives. Moreover, C_{20}-substituted cysteine analogs directly compete for carboxyl methylation with the C_{15}-substituted cysteine analogs and vice versa. Finally, AGGC is even more effective than AFC as an inhibitor of Ras carboxyl methylation, despite the fact that Ras is methylated at a farnesylcysteine rather than a geranylgeranylcysteine residue.

Protein carboxyl methylation; GTP-binding protein; Transforming protein; Membrane attachment; Prenylcysteine

1. INTRODUCTION

A variety of important regulatory proteins in eukaryotic cells are originally translated with a cysteine 4 amino acids from the C-terminus [1]. Because the 2 amino acids that follow the cysteine in these proteins are generally aliphatic, this structural motif has previously been referred to as a "CAAX tail", where C represents a cysteine, A is an aliphatic residue and X is the C-terminal amino acid [2]. In both yeast and mammalian cells, 2 distinct proteins prenyltransferase activities have been identified that modify CAAX-tail proteins. One catalyzes the transfer of the C_{20} polyisoprenoid, geranylgeranyl, from geranylgeranyl pyrophosphate to the CAAX-tail cysteine [3,4]. The other catalyzes the corresponding reaction with the C_{15} polyisoprenoid, farnesyl [5–9]. Because of the differential specificities of the prenyltransferases [3,10], CAAX-tail proteins that end in a methionine or serine such as nuclear lamin B [11], Ras [1,12–15] and the γ-subunit of transducin [16] are modified with the C_{15} substituent; while proteins with CAAX tails that end in a leucine, such as G25K [17], Rap1A [14], Rap1B [18] and Gγ's from neural tissue [19,20] are modified by the C_{20} group. The initial processing event, addition of either the C_{15} or C_{20} isoprenoid, is followed by proteolytic cleavage of the 3 carboxyl-terminal amino acids with subsequent methylation of the exposed prenylcysteine carboxylate [12,21].

The pathways that result in different prenyl substituents could provide a means for differential regulation of individual proteins through interaction with target proteins that recognize the C-termini, such as membrane receptors, methyltransferases and methylesterases [22]. To examine the role of the C-termini in target recognition, we have synthesized a variety of small molecule analogs.

Previously, we have reported that the carboxyl methyltransferase activities that modify C_{15}-substituted cysteines at the C-termini of proteins such as nuclear lamin B, Ras and the γ-subunit of transducin, also catalyze the carboxyl methylation of small molecular weight C_{15}-substituted cysteine analogs such as *N*-acetyl-*S*-farnesylcysteine, AFC [22,23]. AFC methyltransferase activities are ubiquitous to all eukaryotic cell types [22,23]. *Saccharomyces cerevisiae ste14* mutants that are deficient in RAS and a-factor methyltransferase activity [24,25] also lack the ability to methylate AFC [22]. Moreover, AFC inhibits the methylation of CAAX-tail proteins [22,26,27].

Here we address the specificity and characteristics of the methyltransferase activities that modify C-terminal C_{20} substituted cysteines. Our results indicate that the

Abbreviations: AFC, *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine; FTA, *S-trans,trans*-farnesylthioacetic acid; FTP, *S-trans,trans*-farnesyl-3-thiopropionic acid; AGGC, *N*-acetyl-*S*-all-*trans*-geranylgeranyl-L-cysteine; GGTA, *S*-all-*trans*-geranylgeranylthioacetic acid; GGTP, *S*-all-*trans*-geranylgeranyl-3-thiopropionic acid; AGC, *N*-acetyl-*S-trans*-geranyl-L-cysteine; AdoMet, *S*-adenosyl-L-methionine; DTT, dithiothreitol.

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STE14 activity in yeast and a single activity in mammalian cells can catalyze the caboxyl methylation of both C_{15}-substituted analogs, such as AFC and C_{20}-substituted analogs, such as *N*-acetyl-*S*-all-*trans*-geranylgeranylcysteine (AGGC). This raises the possibility that the carboxyl methylation of both classes of CAAX-tailed proteins may be catalyzed by the same methyltransferase.

2. MATERIALS AND METHODS

2.1. *Materials*

Except as noted, reagents were purchased from Aldrich Chemical Co. (Milwaukee). *N*-Acetyl-L-cysteine and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis). All-*trans*-geranylgeraniol was a kind gift from R. Coates or from Kuraray Co. (Tokyo). *S*-Adenosyl-L-methionine was purchased from Boehringer Mannheim (Indianapolis). *S*-Adenosyl-[^{3}H-methyl]L-methionine and [^{3}H] farnesylpyrophosphate were purchased from DuPont-NEN (Boston). Unlabeled farnesylpyrophosphate was a kind gift from J. Backer. HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh).

2.2. S-*Prenyl-Compounds*

AFC, FTA, FTP, AGGC, GGTA and GGTP and their corresponding methyl esters were prepared through incubation of either *trans,trans*-farnesyl bromide or all-*trans*-geranylgeranyl bromide with cysteine, mercaptoacetic acid or 3-mecaptopropionic acid or methyl ester in a manner analogous to our previously reported method for the synthesis of AFC and AFC methyl ester [23]. All-*trans*-geranylgeranyl bromide was prepared from all-*trans*-geranylgeraniol by an adaptation of a previously reported method [28]. All products were purified by reverse-phase HPLC over a semi-preparative C_8 column (20 × 250 mm, 120 A, 15 μ spherical particle size, A-243-15, YMC, Morris Plains, NJ) with 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile as elution buffers. Appropriate fractions were pooled, dried, then characterized by NMR and mass spectroscopy.

2.3. *In vitro reconstitution of p21$^{Ki\text{-}ras\text{-}2B}$ post translational processing system*

Recombinant human p21$^{Ki\text{-}ras\text{-}2B}$ was expressed in *Escherichia coli* strain BL21[DE](pLysS) transformed with pT7K-Ras-Gly. The p21$^{Ki\text{-}ras\text{-}2B}$ was recovered from inclusion bodies by low speed centrifugation of sonicated cell extracts. The pellet fraction was dissolved in 6 M guanidine-HCl then dialyzed against 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 50 μM GTP. After gel filtration (7.5 × 300 mm, Ultropac column TSK G2000SW, LKB, Bromma, Sweden), p21$^{Ki\text{-}ras\text{-}2B}$ was judged to be greater than 90% pure by SDS-PAGE.

Partially purified farnesyltransferase from rat brain was prepared by Mono Q ion exchange chromatography of rat brain cytosol [5]. Unmodified p21$^{Ki\text{-}ras\text{-}2B}$ was farnesylated by incubation in the presence of this enzyme plus farnesylpyrophosphate. The farnesylated p21$^{Ki\text{-}ras\text{-}2B}$ was further processed by addition of rat brain membranes that cleave the 3 amino acids after the farnesylated cysteine. In the presence of [^{3}H]AdoMet, the membranes also catalyze [^{3}H]methylation of the resultant farnesylcysteine α-carboxyl group.

3. RESULTS

3.1. *Prenylcysteine methyltransferase activities in rat tissues*

We have previously demonstrated that membrane fractions from eukaryotes catalyze the carboxyl methylation of AFC [22,23]. The corresponding geranylgeranyl derivative, AGGC, is also methylated by eukaryotic membrane fractions. In both rat brains and liver, similar subcellular distributions of AFC and AGGC methyltransferase activities were observed (Table I).

Table I

Subcellular localization of *S*-prenylcysteine methyltransferase activity in rat tissues

Fraction	Initial rate of formation of AFC methyl ester (pmol/mg/min)	Initial rate of formation of AGGC methyl ester (pmol/mg/min)
Brain		
Total	8.4	8.5
Nuclear/E.R.	12	12
Mitochondrial	6.3	6.5
Microsomal	18	16
Soluble	0.37	0.45
Liver		
Total	0.58	0.60
Nuclear/E.R.	1.0	1.0
Mitochondrial	1.4	1.4
Microsomal	2.8	2.4
Soluble	0.02	0.03

Subcellular fractions, prepared as previously described [20], were incubated with [^{3}H]AdoMet (10 μM; 8000 cpm/pmol) and AFC (100 μM) or AGGC (75 μM) in 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.9, in a total volume of 175 μl. After 10, 20 and 30 min, 50 μl aliquots were removed from each reaction mixture and the levels of [^{3}H]AFC methyl ester or [^{3}H]AGGC methyl ester determined by our previously reported heptane extraction method [22]. The level of protein in each incubation mixture was adjusted to be 75 μg.

Table II

S-prenylcysteine methyltransferase activity in *Saccharomyces cerevisiae*

Strain	Fraction	Initial rate of formation of AFC methyl ester (pmol/mg/min)	Initial rate of formation of AGGC methyl ester (pmol/mg/min)
MS46 (*MAT*α)	Membrane	1.1	1.2
	Soluble	<0.001	<0.001
MS10 (*MAT*a)	Membrane	1.2	1.1
	Soluble	<0.001	<0.001
H629-2d (*ste14 MAT*a)	Membrane	<0.001	<0.001
	Soluble	<0.001	<0.001

Subcellular fractions, prepared as previously described [20], were incubated with [^{3}H]AdoMet (10 μM; 8000 cpm/pmol) and AFC (100 μM) or AGGC (100 μM) in 50 mM Tris-HCl, 1 mM DTT, pH 7.9, in a total volume of 175 μl. After 10, 20 and 30 min, 50 μl aliquots were removed from each reaction mixture and the levels of [^{3}H]AFC methyl ester or [^{3}H]AGGC methyl ester determined by our previously reported heptane extraction method [22]. The level of protein in each incubation mixtures was adjusted to be 75 μg.

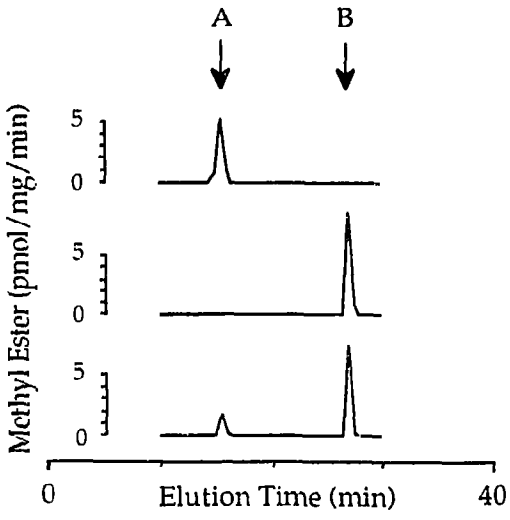

Fig. 1. In vitro carboxyl methylation of AFC and AGGC in rat brain. An 800 × g membrane fraction from rat brain (16 μg protein) was incubated at 37°C with [^{3}H]AdoMet (10 μM; 3800 cpm/pmol) and AFC or AGGC in 100 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.9, in a total volume of 50 μl. Formation of [^{3}H]AFC methyl ester and [^{3}H]AGGC methyl ester was determined in a manner analogous to our previously reported method [22]. Briefly, after 25 min of incubation, each reaction was vortexed together with 250 μl of ice-cold heptane. A 200 μl aliquot of the heptane layer was removed, dried in a vacuum centrifuge (Savant SVC-100H), resuspended in 500 μl acetonitrile, then subjected to reverse-phase HPLC over a C_{18} column (Vydac 201TP54). The column was eluted at 1.5 ml/min for 5 min with 50% solvent B followed by a linear gradient from 50% solvent A to 100% solvent B over 50 min. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in water and solvent B was 0.1% (v/v) TFA in acetonitrile. Fractions were collected every 30 s, mixed with 5 ml scintillation fluid (ICN EcoLite+) and analyzed by liquid scintillation spectrometry. Arrows indicate the elution times of synthetically prepared AFC methyl ester (A) and AGGC methyl ester (B). (Top trace) 50 μM AFC; (center trace) 50 μM AGGC; (lower trace) 50 μM AFC + 50 μM AGGC.

3.2. *Prenylcysteine methyltransferase activities in* Saccharomyces cerevisiae

Mature *RAS* and *MAT*a-type mating pheromones (a-factor) from *S. cerevisiae* each have a C-terminal *S*-farnesylcysteine methyl ester [1,29]. A *ste14* mutant strain lacks the activity requires to methylate both RAS and a-factor [24,25]. This mutant also lacks both AFC and AGGC methyltransferase activity (Table II). The *STE14* gene product is membrane-associated [25], as are the AFC and AGGC methyltransferase activities (Table II).

3.3. *AFC and AGGC compete for carboxyl methyltion*

A reverse-phase HPLC assay was used to determine the extent of carboxyl methylation of AFC and AGGC (Fig. 1). Incubation of AFC (50 μM) with rat brain membrane extract resulted in the formation of 11 pmol/mg/min of AFC methyl ester under the conditions described in the figure legend. When AGGC (50 μM) was incubated with the extract, 11 pmol of AGGC methyl ester was also produced. The amount of AFC methyl ester formed was reduced by 80% and the amount of AGGC methyl ester was reduced by 20% when AFC and AGGC were present together in the incubation mixture. These results are consistent with a direct competition of AFC and AGGC for the same methyltransferase activity where AGGC has a higher affinity for the enzyme.

Table III

Kinetics of prenylcysteine carboxyl methylation

Substrate	K_m(μM)	K_i of FTA (μM)	K_i of GGTA (μM)
AFC	25	3	2
FTP	20	5	5
AGGC	7	3	3
GGTP	5	3	3

An 800 × g membrane fraction from rat brain (48 μg protein) was incubated at 37°C with [^{3}H]AdoMet (10 μM; 3800 cpm/pmol) together with AFC, FTP, AGGC or GGTP (0, 9, 23 or 90 μM for C_{15} substrates; 0, 6, 15 or 60 μM for C_{20} substrates) and FTA or GGTA (0, 2, 9, 23 or 45 μM) in 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.9, in a total volume of 175 μl. After 10, 20 and 30 min, 50 μl aliquots were removed from each reaction mixture and the levels of [^{3}H]methyl esters of AFC, FTP, AGGC or GGTP determined by our previously reported heptane extraction method [23]. Initial rates of formation of [^{3}H]methyl ester as a function of inhibitor concentration were determined at each concentration of substrate, then plotted as double reciprocal plots vs. substrate concentration. The apparent K_m's for the substrates, as determined from the double reciprocal plots, were then plotted as a function of inhibitor concentration.

3.4. *Kinetics of prenylcysteine carboxyl methylation*

A variety of small molecule analogs of C-terminal *S*-prenylcysteine residues were synthesized to assay the substrate specificities of the methyltransferase activities. Inhibition of AFC methylation is a useful screen for compounds that inhibit the C-terminal *S*-farnesylcysteine carboxyl methyltransferase activity [23]. By this method we found that several of the analogs compete with AFC for the *S*-farnesylcysteine methyltransferase. Two of the compounds, FTA and GGTA, were excellent inhibitors, but were not substrates. This property enabled us to use our previously reported heptane-extraction method [23] to determine kinetic parameters of the carboxyl methylation reaction (Fig. 2). The method is based on our observations that when an aqueous incubation mixture of [^{3}H]AdoMet, AFC and enzyme source is extracted with *n*-heptane, any [^{3}H]AFC methyl ester produced is quantitatively transferred into the heptane layer, whereas most other [^{3}H]-containing species in the mixture remain in the aqueous layer. This method is equally useful for AGGC, FTP and GGTP, since their methyl esters are also quantitatively transferred into heptane.

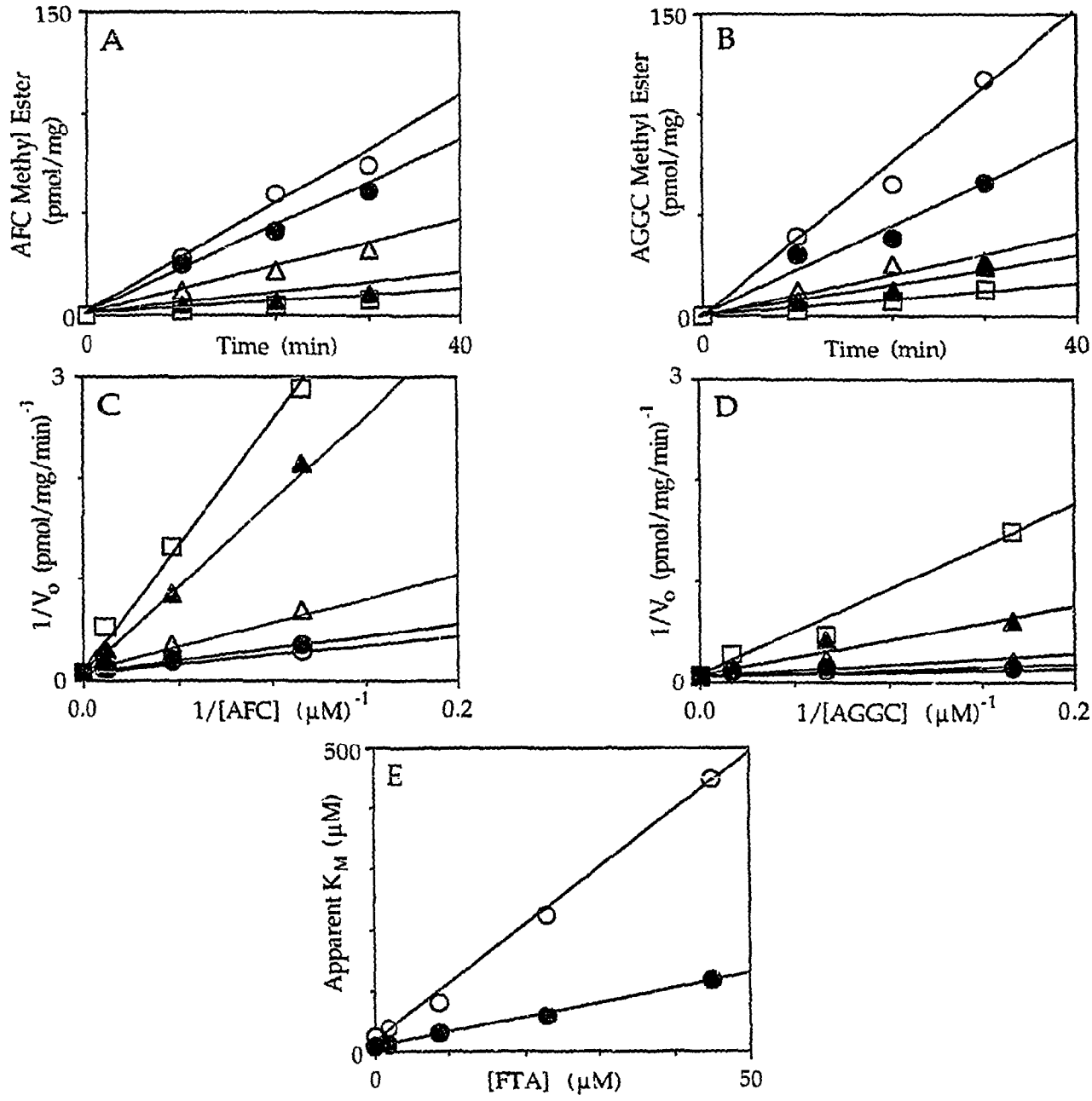

Fig. 2. Effect of FTA on the rates of formation of AFC methyl ester and AGGC methyl ester. An 800 × *g* membrane fraction from rat brain (48 μg protein) was incubated at 37°C with [^{3}H]AdoMet (10 μM; 3800 cpm/pmol) with AFC (0, 9, 23 or 90 μM) or AGGC (0, 6, 15 or 60 μM) and FTA (0, 2, 9, 23 or 45 μM) in 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.9, in a total volume of 175 μl. After 10, 20 and 30 min, 50 μl aliquots were removed from each reaction mixture and the levels of [^{3}H]AFC methyl ester and [^{3}H]AGGC methyl ester determined by our previously reported heptane extraction method [22]. (A) Incubation of AFC (9 μM) with FTA (0 μM, ○; 2 μM, ●; 9 μM, △; 23 μM, ▲; 45 μM, □) (B) Incubation of AGGC (6 μM) with FTA (0 μM, ○; 2 μM, ●; 9 μM, △; 23 μM, ▲; 45 μM, □). (C) Initial rates of formation of AFC methyl ester as a function of FTA concentration were determinated at all concentrations of AFC from the slopes of the lines as shown in (A) for 9 μM AFC, then plotted as a double reciprocal plot vs. AFC concentration. (D) The same method described in (C) was employed to construct a double reciprocal plots of AGGC methyl ester formation vs. AGGC concentration. (E) The apparent K_m's of AFC (○) and AGGC (●), as determined from the double reciprocal plots (C) and (D), are plotted as a function of FTA concentration.

Geranylgeranyl analogs, AGGC en GGTP, have a 3–4 fold lower K_m than the corresponding farnesyl derivatives, AFC and FTP (Table III). The K_i's of the competitive inhibitors, FTA and GGTA, do not vary for different classes of substrates, however (Table III). These data are consistent with the conclusion that C_{15} and C_{20} prenylcysteine derivatives are substrates of the same methyltransferase.

3.5. *Inhibition of Ras carboxyl methylation in a reconstituted post-translational modification system*

We examined the methylation of Ras in a reconstituted post-translational modification system. Unmodified human recombinant $p21^{Ki\text{-}ras\text{-}2B}$ was overproduced in *Escherichia coli* and purified. In the experiment reported in Table IV, $p21^{Ki\text{-}ras\text{-}2B}$ was farnesylated, cleaved and used as a substrate for the rat brain methyltransferase. In the presence of 50 μM AFC or AGGC the methylation of $p21^{Ki\text{-}ras\text{-}2B}$ was inhibited as compared to the control lane without any added prenylcysteine analog. *N*-Acetyl-*S-trans*-geranylcysteine (AGC), a structurally similar compound where the isoprenoid is the C_{10} geranyl group, is not an effective inhibitor.

4. DISCUSSION

Several CAAX-tail proteins are post-translationally modified to yield mature proteins with either carboxyl-terminal *S*-farnesylcysteine (C_{15}) or *S*-geranylcysteine (C_{20}) methyl esters. We have previously identified a eukaryotic carboxyl methyltransferase activity that methylates proteins that have a C_{15} group, such as Ras, as well as small molecule analogs of the C-terminus, such as *N*-acetyl-*S-trans,trans*-farnesyl-L-cysteine, AFC [22]. Here we show that small molecule analogs of the carboxyl-terminus of proteins with a C_{20} group, such as *N*-acetyl-*S*-all-*trans*-geranylgeranylcysteine, AGGC, can function as substrates of the same activity. The following lines of evidence support this contention: (i) the subcellular distribution of the C_{15} and C_{20} methyltransferase activities are identical in yeast, as well as in both rat tissues examined; (ii) a disruption in the *Saccharomyces cerevisiae STE14* gene blocks methylation of both C_{15} and C_{20} substrates; (iii) the C_{15} and C_{20} analogs mutually compete for carboxyl methylation; (iv) inhibitors of the C_{20} methyltransferase also inhibit the C_{15} methyltransferase with the same K_i's; and (v) both C_{15} and C_{20} analogs specifically inhibit carboxyl methylation of prenylcysteine proteins, such as Ras.

Our results do not preclude the possibility that there may be additional prenylcysteine methyltransferase activities that have varying specificities for different polyisoprenoid substituents. Future purification of the membrane-associated activities will address this question. Clearly, however, the yeast methyltransferase and the major activities from mammalian cells show little preference as to whether a C_{15} or C_{20} modification is involved. Previous findings with fungal sex factor receptors indicate that this may be a general pattern. A synthetic geranylgeranyl derivative of a *Tremella mesenterica* mating factor, Tremerogen A-10, has a higher affinity for receptor than the corresponding farnesyl derivative which is the native product [30]. Perhaps the primary function in mammalian cells of the longer chain polyisoprenoid is simply to confer a slightly higher affinity for a common set of targets in the membrane.

Table IV

Carboxyl methylation of $p21^{Ki\text{-}ras\text{-}2B}$ in a reconstituted processing system

Prenylcysteine analog Added (50 μM)	Ras methylation (% control)[1]	36 kDa-protein methylation (% control)[2]
AGC	97	97
AFC	68	96
AGGC	50	94

Recombinant human $p21^{Ki\text{-}ras\text{-}2B}$ (7 μg) was incubated at 37°C with partially purified rat brain farnesyltransferase (80 μg) and farnesylpyrophosphate (1 nmol) in 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, pH 7.0, for 1 h in a total volume of 35 μl. A 100 000× *g* rat brain crude microsomal fraction (15 μl, 10 μg protein) that was isolated in 50 mM Tris-HCl, 1 mM DTT, pH 7.9 (Tris/DTT buffer), was added. This was the source of both *S*-farnesylcysteine peptidase and *S*-farnesylcysteine carboxyl methyltransferase activities [22]. After a 15 min incubation at 37°C, aliquots (10.5 μl) from the reaction mixture were added to tubes that contained [^{3}H]AdoMet(80 000 cpm/pmol) and AGC, AFC, AGGC or no inhibitor in Tris/DTT buffer, so that the final volume was 15 μl, the AdoMet concentration was 0.67 μM and the inhibitor concentration was 50 μM. After 25 min at 37°C, the methylation reaction was stopped by the addition of 4 × protein gel loading buffer (5 μl) and heating to 95°C for 5 min. The extracts were separated by SDS-PAGE on an 8-cm-long 12.5% polyacrylamide gel [31]. The gel was dried and the individual lanes were sliced into 3 mm sections. In addition to Ras, a 36 kDa methylated protein from rat brain is observed [22]. The extent of protein methylation was assayed by the methanol vapor diffusion procedure as described previously [32].

[1]Ras methylation control, 0.097 pmol.

[2]36 kDa protein methylation control, 0.084 pmol.

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