Novel Methyltransferase Activity Modifying the Carboxy Terminal Bis(geranylgeranyl)-Cys-Ala-Cys Structure of Small GTP-Binding Proteins†

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ABSTRACT: Proteins containing CX_3, CXC, and CC (where C is cysteine and X is undefined) undergo posttranslational isoprenylation at their cysteine residues. In the case of proteins which terminate in CX_3, proteolytic removal of X_3 is followed by the carboxymethylation of the isoprenylated cysteine residue. CXC proteins also undergo C-terminal methylation. The present study addresses the question of whether this methylation is catalyzed by a different isoprenylated protein methyltransferase than that previously described for CX_3 proteins. The *S*-adenosylmethionine (AdoMet) dependent methylation of a small peptide—*N*-acetyl-*S*-geranylgeranyl-L-cysteinyl-L-alanyl-*S*-geranylgeranyl-L-cysteine (Ac(GG)CysAla(GG)-Cys)—was investigated using membranes from a variety of bovine tissues as sources of enzyme. Ac-(GG)CysAla(GG)Cys was a substrate for methylation, while Ac(GG)Cys(GG)Cys was not. Reciprocal inhibition studies on the methylation reactions of the CXC peptide and of *N*-acetyl-*S*-farnesyl-L-cysteine (AFC), a previously described methyltransferase substrate, suggested that these reactions are catalyzed by distinct enzymatic activities. Farnesylthioacetic acid (FTA), a potent competitive inhibitor of the methylation of AFC, did not inhibit the methylation of the CXC peptide. Moreover the K_I values for *S*-adenosylhomocysteine and *S*-adenosylethionine inhibition differed for the two enzymatic activities. These data indicate that more than one AdoMet-dependent methyltransferase is involved in the carboxymethylation of isoprenylated proteins.

The carboxymethylation of isoprenylated proteins is an enzymatic process of possible regulatory significance, since this reaction is the only reversible one in the isoprenylation pathway (Tan & Rando, 1991). Many physiologically important proteins, including the heterotrimeric G-proteins (e.g., transducin), the ras-like small G-proteins, and the nuclear lamins, are modified by isoprenylation and methylation (Clarke, 1992). Thus, proteins terminating in a CX_3 motif undergo S-isoprenylation at cysteine by either (*all-trans*)-farnesyl ($C_{15}H_{25}$) or (*all-trans*)-geranylgeranyl ($C_{20}H_{33}$) pyrophosphate, followed by proteolytic trimming of the X_3 peptide and methylation of the newly exposed isoprenylcysteine carboxyl terminal residue.

The AdoMet[1]-dependent carboxymethylation reaction in this sequence has been shown to be reversible (Pérez-Sala et al., 1992), and a specific methylesterase for isoprenylated protein methyl esters has been identified (Tan & Rando, 1991), suggesting the possibility that methylation may represent a point of physiological control. Although methylation of the nuclear lamins is thought to be linked to the dissolution and reconstitution of the nuclear envelope (Chelsky et al., 1987), the overall function and importance of isoprenylated protein methylation is unclear and is currently under active investigation. However, if isoprenylated protein methyltransferases are to be of any essential physiological significance, specific methyltransferase for different isoprenylated proteins would be expected to exist.

In addition to proteins terminating in CX_3, another group of small G-proteins related to ras, bearing CC and CXC carboxyl-terminal motifs, are also isoprenylated (Takai et al., 1992). Such proteins have been shown to be modified by the geranylgeranyl group (Khosravi-Far et al., 1991; Farnsworth et al., 1991). The C-terminal CXC motif of several proteins has been shown to be methylated (Farnsworth et al., 1991; Newman et al., 1992; Li & Stahl, 1993). The situation of doubly geranylgeranylated CC terminating proteins with respect to methylation remains unclear, but it appears that these proteins are not carboxymethylated (Wei et al., 1992).

It had previously been shown that the enzyme responsible for C-terminal methylation of the farnesylated ($C_{15}H_{25}$) γ-subunit of transducin does not distinguish between the farnesylated and geranylgeranylated small molecule substrates AFC (Scheme 1) and AGGC (Pérez-Sala et al., 1992). However, processing of the CC and CXC proteins diverges from processing of CX_3 proteins in so far as a specific geranylgeranyltransferase is responsible for their isoprenylation (Moores et al., 1991; Horiuchi et al., (1991). Does a specific methyltransferase for this class of isoprenylated proteins also exist?

In this study, we describe experiments with small molecules representing the minimal C-terminal sequence of CC and CXC proteins (Scheme 1). The results show that the

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[1] Abbreviations: AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; AdoMet, *S*-adenosyl-L-methionine; AdoEth, *S*-adenosylethionine; AdoHcy, *S*-adenosyl-L-homocysteine; ROS, rod outer segment; RPE, retinal pigmented epithelium; CAC peptide or Ac(GG)CysAla(GG)Cys, *N*-acetyl-*S*-geranylgeranyl-L-cysteinyl-L-alanyl-*S*-geranylgeranyl-L-cysteine; CC peptide or Ac(GG)-Cys(GG)Cys, *N*-acetyl-*S*-geranylgeranyl-L-cysteinyl-*S*-geranylgeranyl-L-cysteine; FTA, *S*-farnesylthioacetic acid.

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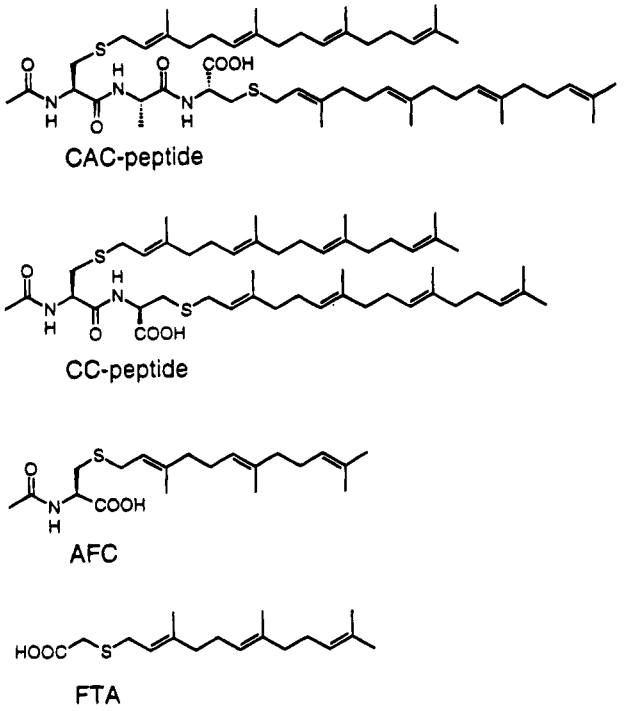
Scheme 1: Structures of Isoprenylated Analogs



CXC sequence represents a substrate for a new *S*-adenosylmethionine-dependent methyltransferase. On the other hand, CC-containing peptides are not carboxymethylated.

MATERIALS AND METHODS

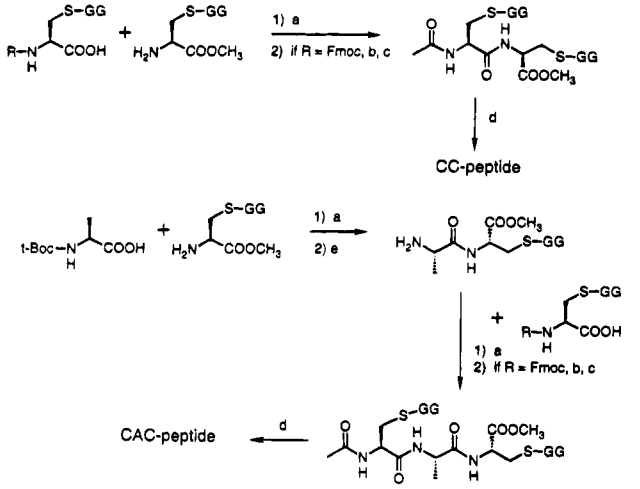
Materials

S-Adenosyl-L-[*methyl*-^{3}H]methionine (10 Ci/mmol) was purchased from Du Pont-New England Nuclear. *S*-Adenosyl-L-methionine, *S*-adenosyl-L-ethionine, *S*-adenosyl-L-homocysteine, and sinefungin were purchased from Sigma Chemical Co. Geranylgeraniol was purchased from TCI America. AFC and FTA were prepared as previously reported (Tan et al., 1991). ROS membranes were prepared as previously described and stored frozen until use (Pérez-Sala et al., 1992). Membranes from other bovine tissues were prepared by homogenizing 1 g of fresh bovine tissue (10 eye cups were used for the RPE membranes) in 5 mL of phosphate buffer in a ground glass homogenizer. The supernatants after two low-speed centrifugations (600*g*) were centrifuged to pellet the membranes (100000*g*). The pellets were resuspended in 5 mL of buffer and stored at −80 °C.

Methods

Methyltransferase Assay. Substrates and inhibitors were added as DMSO solutions to a mixture containing ROS membranes suspended in buffer (200 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$) and [^{3}H-*methyl*]-*S*-adenosylmethionine in 6.7 mM H_2SO_4/ethanol 9:1 at 4 °C. The final incubation mixture consisted of 50 μL containing 44 μL of suspended ROS membranes, 5 μL of *S*-adenosylmethionine solution, and 1 μL of substrate solution; final protein concentration = 3 mg/mL, DMSO = 2%. After 30 min at 37 °C, the assays were cooled to 0 °C and extracted with 250 μL of ethyl acetate. The layers were separated by centrifugation, and the organic extracts were analyzed by normal-phase HPLC (hexane/ethanol 24:1) with on-line radioactivity detection as previously described (Pérez-Sala et al., 1992). All points were determined in triplicate. Data analysis was carried out using the Enzyme Kinetics program (Trinity Software).

Scheme 2: Synthesis of Geranylgeranylated Peptides[a]



[a] Reagents: (a) EDAC, HOBT, DMF; (b) Et_2NH; (c) Ac_2O; (d) Na_2CO_3; (e) concentrated HCl/MeOH. R = acetyl or Fmoc (fluorenylmethoxycarbonyl); GG = geranylgeranyl; t-Boc = *tert*-butoxycarbonyl.

Synthesis of Isoprenylated Peptides

(*all-trans*)-Geranylgeraniol (2.5 g) in 25 mL of dry Et_2O was converted to (*all-trans*)-geranylgeranyl bromide by the addition of 1 mL of PBr_3 at 0 °C. After 30 min, the reaction mixture was poured into 50 mL of an ice-cold saturated solution of NH_4Cl and extracted with hexane. The organic layer was filtered through a pad of celite in hexane/Et_2O (9:1) and concentrated by rotary evaporation without heating. The crude geranylgeranyl bromide was mixed with 1 equiv of cysteine methyl ester or acetylcysteine in dry DMF and allowed to react in the presence of 1.1 equiv i-$PrEt_2N$. The resulting products were purified by silica chromatography [(*S*-geranylgeranyl)cysteine methyl ester, 0.1% TEA in hexane/EtOAc (1:1); (*S*-geranylgeranyl)acetylcysteine, 0.3% HOAc in hexane/acetone (2:1)]. Synthesis of the isoprenylated peptides was carried out using standard solution methods for peptides coupling as shown in Scheme 2 (Bodansky & Bodansky, 1984). Thus, to prepare *N*-acetyl-(*S*-geranylgeranyl)cysteinyl-(*S*-geranylgeranyl)cysteine methyl ester (CC peptide methyl ester), 3 mmol each of (*S*-geranylgeranyl)cysteine methyl ester and (*S*-geranylgeranyl)acetylcysteine was treated with 3 mmol each of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) hydrochloride and 1-hydroxybenzotriazole (HOBT) at room temperature in 5 mL of DMF for 16 h, followed by extraction with diluted hydrochloric acid and EtOAc and purification by silica gel chromatography (hexane/EtOAc (2:1)). The methyl esters could be converted to the free acids by mild basic hydrolysis (10% Na_2CO_3/acetonitrile (1:1), room temperature, vigorous stirring, 16 h). Flexibility was introduced to the synthesis through the use of N-terminal fluorenylmethoxycarbonyl (Fmoc) derivatives. These could be converted to the desired *N*-acetyl compound by treatment with diethylamine/ethyl acetate (1:4) (room temperature, 1 h), followed by acetylation with acetic anhydride (room temperature, 16 h).

N-Fluorenylmethoxycarbonyl-(S-geranylgeranyl)cysteinylalanyl-(S-geranylgeranyl)cysteine Methyl Ester. ^{1}H-NMR (500 MHz, δ, $CDCl_3$): 7.765 (2H, d, J = 7.8 Hz, aromatic), 7.594 (2H, br m, aromatic), 7.401 (2H, t, J = 7.4 Hz, aromatic), 7.315 (2H, t, J = 7.8 Hz, aromatic), 6.829 (1H, br m, NH), 6.779 (1H, d, J = 6.8 Hz, NH), 5.647 (1H, br m, NH), 5.256 (1H, t, J = 7.4 Hz, vinyl H), 5.180 (1H, t, J = 7.4 Hz, vinyl H), 5.02−5.12 (6 H, m, vinyl H's), 4.720 (1H, m, α-CH), 4.541 (1H, m, α-CH), 4.421 (2H, br d, CH_2O), 4.294 (1H, br m, α-CH), 4.228 (1H, t, J = 6.9 Hz, fluorenyl-9-CH), 3.747 (3H, s, OMe), 3.05−3.25 (4H, m, allylic CH_2S's), 2.78−2.98 (4H, m, cysteine CH_2S's), 2.00−2.12 (16 H, m, allylic CH_2CH_2's), 1.91−1.99 (8 H, m, allylic CH_2CH_2's), 1.674 (9H, s, Me), 1.657 (3H, s, Me's), 1.594 (6H, s, Me), 1.586 (6H, s, Me's, 1.575 (6H, s, Me's), 1.439 (3H, d, J = 6.8 Hz, alanine Me).

N-Acetyl-(S-geranylgeranyl)cysteinylalanyl-(S-geranylgeranyl)cysteine Methyl Ester. The compound was purified by normal-phase TLC (ethyl acetate). HPLC RT = 15.7 min (AFC HPLC RT = 11.1 min) (hexane/ethanol (24:1), 1.5 mL/min). ^{1}H-NMR (500 MHz, δ, $CDCl_3$): 6.849 (1H, d, J = 7.8 Hz, NH), 6.788 (1H, d, J = 7.8 Hz, NH), 6.349 (1H, d, J = 7.8 Hz, NH), 5.266 (1H, t, J = 7.4 Hz, vinyl H), 5.186 (1H, t, J = 7.8 Hz, vinyl H), 5.07−5.12 (6 H, m, vinyl H's), 4.734 (1H, ddd, J = 7.8, 7.8, 4.8 Hz, α-CH), 4.49−4.56 (2H, ddd, J = Hz, α-CH), 3.748 (3H, s, OMe), 3.236 (2H, d, J = 7.8 Hz, allylic CH_2S), 3.193 (1H, dd, J = 12.7, 8.8 Hz, allylic CH_2S), 3.098 (1H, dd, J = 12.7, 6.8 Hz, allylic CH_2S), 2.953 (1H, dd, J = 13.7, 4.9 Hz, cysteine CH_2S), 2.918 (1H, dd, J = 13.7, 4.9 Hz, cysteine CH_2S), 2.827 (1H, dd, J = 13.7, 6.8 Hz, cysteine CH_2S), 2.785 (1H, dd, J = 13.7, 7.8 Hz, cysteine CH_2S), 2.02−2.12 (16 H, m, allylic CH_2CH_2's), 2.040 (3H, s, CH_3CO), 1.95−1.99 (8 H, m, allylic CH_2CH_2's), 1.687 (3H, s, Me), 1.675 (6H, s, Me's), 1.665 (3H, s, Me), 1.595 (18 H, s, Me's), 1.434 (3H, d, J = 6.8 Hz, alanine Me).

N-Acetyl-(S-geranylgeranyl)cysteinylalanyl-(S-geranylgeranyl)cysteine. The compound was purified by normal-phase TLC (ethyl acetate/acetone/water/acetic acid (180:90:15:1)) and reverse-phase TLC (methanol/water (9:1)). ^{1}H-NMR (500 MHz, δ, acetone-d_6): 7.642 (1H, d, J = 7.8 Hz, NH), 7.573 (1H, d, J = 7.8 Hz, NH), 7.347 (1H, d, J = 7.9 Hz, NH), 5.22−5.28 (2H, m, vinyl H's), 5.08−5.17 (6 H, m, vinyl H's), 4.635 (1H, ddd, J = 7.9, 7.9, 4.9 Hz, α-CH), 4.635 (1H, ddd, J = 7.4, 7.4, 7.4 Hz, α-CH), 4.502 (1H, ddd, J = 6.8, 6.8, 13.6 Hz, α-CH), 3.16−3.30 (4H, m, allylic CH_2S's); 2.994 (1H, dd, J = 13.6, 4.8 Hz, cysteine CH_2S), 2.71−2.91 (3H, m, cysteine CH_2S's), 1.96−2.16 (27H, m, allylic CH_2CH_2's and CH_3CO), 1.704 (6H, s, Me's), 1.655 (6H, s, Me's), 1.619 (6H, s, Me's), 1.604 (6H, s, Me's), 1.592 (6H, s, Me's), 1.337 (3H, d, J = 6.9 Hz, alanine Me).

N-Fluorenylmethoxycarbonyl-(S-geranylgeranyl)cysteinyl-(S-geranylgeranyl)cysteine Methyl Ester. ^{1}H-NMR (500 MHz, δ, $CDCl_3$): 7.760 (2H, d, J = 6.8 Hz, aromatic), 7.596 (2H, d, J = 7.8 Hz, aromatic), 7.395 (2H, t, J = 7.4 Hz, aromatic), 7.311 (2H, t, J = 6.4 Hz, aromatic), 7.098 (1H, br m, NH), 5.678 (1H, br m, NH), 5.262 (1H, t, J = 7.4 Hz, vinyl H), 5.178 (1H, t, J = 7.4 Hz, vinyl H), 5.06−5.14 (6 H, m, vinyl H's), 4.762 (1H, m, α-CH), 4.407 (2H, d, J = 6.8 Hz, CH_2O), 4.38 (1H, br m, α-CH), 4.242 (1H, t, J = 7.4 Hz, fluorenyl-9-CH), 3.747 (3H, s, OMe), 3.18−3.28 (2H, br m, allylic CH_2S), 3.176 (1H, dd, J = 13.2, 8.4 Hz, allylic CH_2S), 3.104 (1H, dd, J = 12.7, 7.8 Hz, allylic CH_2S), 2.80−3.00 (4H, m, cysteine CH_2S's), 2.01−2.11 (16 H, m, allylic CH_2CH_2's), 1.94−1.99 (8 H, m, allylic CH_2CH_2's), 1.674 (9H, s, Me's), 1.643 (3H, s, Me), 1.594 (3H, s, Me), 1.586 (9H, s, Me's), 1.565 (6H, s, Me's).

N-Acetyl-(S-geranylgeranyl)cysteinyl-(S-geranylgeranyl)cysteine Methyl Ester. The compound was purified by normal-phase TLC (hexane/ethyl acetate (2:1)). HPLC RT = 3 min (hexane/ethanol (24:1), 1.5 mL/min). ^{1}H-NMR (500 MHz, δ, $CDCl_3$): 7.144 (1H, d, J = 7.8 Hz, NH), 6.371 (1H, d, J = 6.9 Hz, NH), 5.262 (1H, t, J = 7.3 Hz, vinyl H), 5.186 (1H, t, J = 7.8 Hz, vinyl H), 5.06−5.12 (6 H, m, vinyl H's), 4.736 (1H, ddd, J = 6.8, 6.8, 5.9 Hz, α-CH), 4.571 (1H, ddd, J = 7.8, 5.9, 6.8 Hz, α-CH), 3.752 (3H, s, OMe), 3.265 (1H, dd, J = 13.8, 7.8 Hz, allylic CH_2S), 3.238 (1H, dd, J = 13.8, 6.8 Hz, allylic CH_2S), 3.183 (1H, dd, J = 12.7, 7.8 Hz, allylic CH_2S), 3.110 (1H, dd, J = 12.7, 7.8 Hz, allylic CH_2S), 2.955 (1H, dd, J = 14.7, 4.9 Hz, cysteine CH_2S), 2.913 (1H, dd, J = 14.6, 5.8 Hz, cysteine CH_2S), 2.851 (1H, dd, J = 13.7, 6.8 Hz, cysteine CH_2S), 2.768 (1H, dd, J = 13.7, 7.8 Hz, cysteine CH_2S), 2.01−2.11 (16 H, m, allylic CH_2CH_2's), 2.033 (3H, s, CH_3CO), 1.94−1.99 (8 H, m, allylic CH_2CH_2's), 1.681 (3H, s, Me), 1.669 (6H, s, Me's), 1.651 (3H, s, Me), 1.589 (18 H, s, Me's).

N-Acetyl-(S-geranylgeranyl)cysteinyl-(S-geranylgeranyl)cysteine. The compound was purified by normal-phase TLC (0.3% acetic acid in ethyl acetate). ^{1}H-NMR (500 MHz, δ, $CDCl_3$): 7.282 (1H, d, J = 7.2 Hz, NH), 6.430 (1H, d, J = 7.2 Hz, NH), 5.250 (1H, t, J = 7.2 Hz, vinyl H), 5.204 (1H, t, J = 7.6 Hz, vinyl H), 5.06−5.12 (6 H, m, vinyl H's), 4.681 (1H, ddd, J = 6.8, 6.8, 5.5 Hz, α-CH), 4.623 (1H, ddd, J = 7.0, 7.0, 6.5 Hz, α-CH), 3.15−3.26 (4H, m, allylic CH_2S), 3.011 (1H, dd, J = 14.3, 5.4 Hz, cysteine CH_2S), 2.88−2.94 (2H, m, cysteine CH_2S), 2.807 (1H, dd, J = 13.9, 6.8 Hz, cysteine CH_2S), 2.01−2.11 (16 H, m, allylic CH_2CH_2's), 2.060 (3H, s, CH_3CO), 1.94−1.99 (8 H, m, allylic CH_2CH_2's), 1.677 (9H, s, Me), 1.660 (3H, s, Me's), 1.595 (18H, s, Me).

RESULTS

The C-terminus of smg-25A/rab3A has been clearly shown to be modified by two geranylgeranyl groups and a methyl ester (Farnsworth et al., 1991). We therefore picked the C-terminal sequence of this protein for our investigations of G-protein methyltransferases. N-Acetylcysteinylalanylcysteine was synthesized in a doubly geranylgeranylated form (Ac(GG)CysAla(GG)Cys, CAC peptide) (Scheme 1). In addition, a peptide representing the minimal C-terminal sequence of a CC-terminal G-protein, doubly geranylgeranylated acetylcysteinylcysteine (Ac(GG)Cys(GG)Cys, CC peptide) (Scheme 1), was prepared, although double geranylgeranylation has not been demonstrated unequivocally in CC-terminal proteins, and no reports have detected their methylation.

With these compounds in hand, a variety of membranes from different bovine tissues were surveyed for their ability to methylate the prenylated peptides. Membranes from brain, heart, intestine, kidney, liver, lung, retina, retinal pigmented epithelium, and testes were all shown to be capable of catalyzing the enzymatic methylation of both AFC and the CAC peptide. The overall levels of activity were found to vary among these preparations (Table 1). None of these membrane preparations catalyzed the methylation of the CC peptide.

Table 1: Methylation of AFC−CAC Mixtures (125 μM Each) by Bovine Membranes

membrane source	AFC[a]	CAC[a]	CC[a]
brain	0.4	0.7	0
heart	0.2	0.6	0
intestine	1.1	0.8	0
kidney	1.6	2.3	0
liver	0.5	2.1	0
lung	1.5	1.9	0
retina	2.7	3.5	0
ROS	3.3	5.9	0
RPE	2.3	4.9	0
testes	1.1	1.3	0

[a] In units of pmol min^{-1} mg^{-1}.

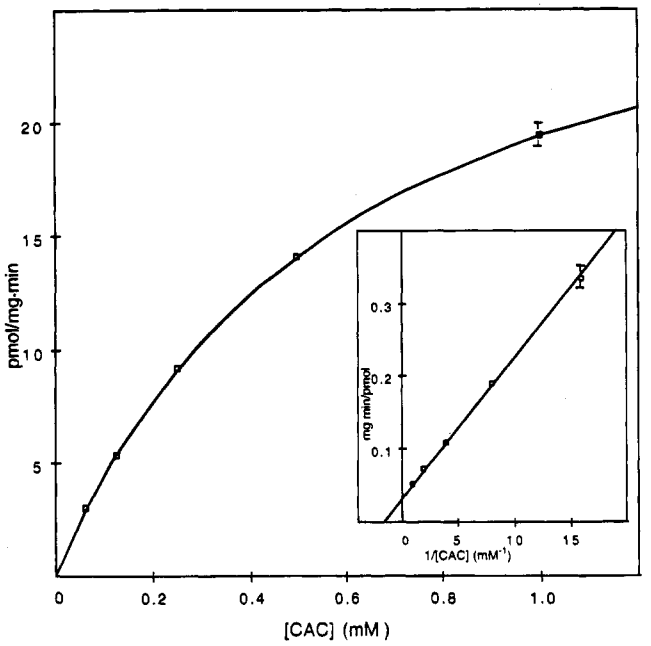


FIGURE 1: Enzymatic methylation of the CAC peptide. Plot of specific activity vs concentration of the CAC peptide (AdoMet = 9.28 μM). The inset shows the same data plotted as 1/specific activity vs 1/concentration of CAC peptide.

Table 2: Kinetic Data for AFC and CAC Peptide Methyltransferases

substrate	K_M (μM)	V_{max} (pmol min^{-1} mg^{-1})
	AFC Methyltransferase	
AFC	56.5 ± 5.8[a,b]	9.87 ± 0.54[b]
AdoMet	3.60 ± 0.46[c]	9.20 ± 0.94[c]
	CAC Peptide Methyltransferase	
CAC peptide	594 ± 60[a,b]	30.9 ± 1.8[b]
AdoMet	3.92 μM ± 0.47[d]	21.1 ± 2.1[d]

[a] The K_M of the prenylated substrate is linearly dependent on the membrane concentration. These values are those obtained under the conditions described in the Methods section. [b] Measured at 9.28 μM AdoMet. [c] Measured at 125 μM AFC. [d] Measured at 500 μM CAC peptide.

The kinetic constants for CAC peptide methylation were determined for the rod outer segment (ROS) membrane-associated methyltransferase (Figure 1; Table 2). The K_M value for the CAC peptide is an order of magnitude higher than that of AFC, and the V_{max} value for its methylation is roughly 3-fold as great.

Experiments were carried out to determine if the methyltransferase acting on the CAC peptide is unique or is the same as the methyltransferase previously described for the

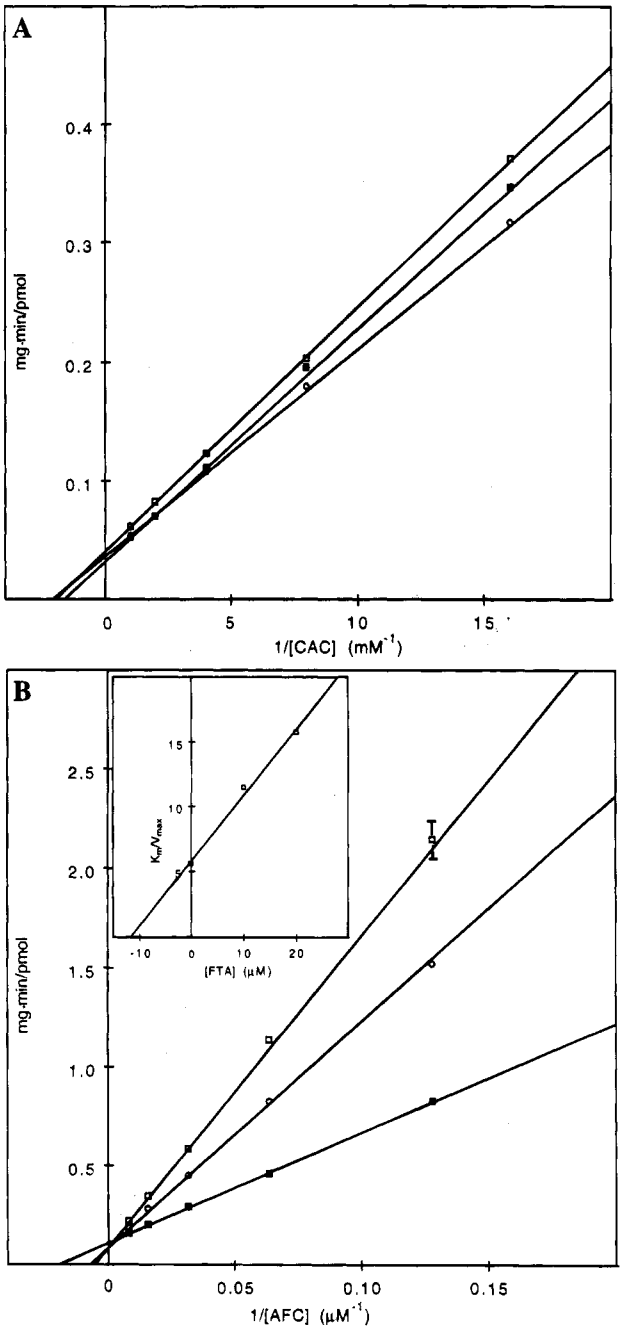


FIGURE 2: FTA inhibition of AFC and CAC peptide methylation. (A) FTA inhibition of CAC peptide methylation (AdoMet = 9.28 μM). Plot of 1/specific activity vs 1/concentration of CAC peptide. Solid squares = 0 μM FTA, open circles = 10 μM FTA, open squares = 20 μM FTA. (B) FTA inhibition of AFC methylation (AdoMet = 9.28 μM). Plot of 1/specific activity vs 1/concentration of AFC. Solid squares = 0 μM FTA, open circles = 10 μM FTA open squares = 20 μM FTA. The inset shows a replot of the slopes of lines vs concentration of FTA.

methylation of AFC and the transducin γ-subunit (Pérez-Sala et al., 1991; Tan et al., 1991). We had previously shown that FTA is a potent competitive inhibitor of AFC methylation (Tan et al., 1991). However, FTA, which under the conditions of the assay had a K_I of 11.2 ± 1.3 μM as measured with AFC as the substrate, showed no clear inhibition of CAC peptide methylation (Figure 2). In cross-inhibition experiments (Figure 3), AFC showed no clear inhibition of CAC peptide methylation, nor did the geranylgeranylated analog AGGC. Therefore, the methyltrans-

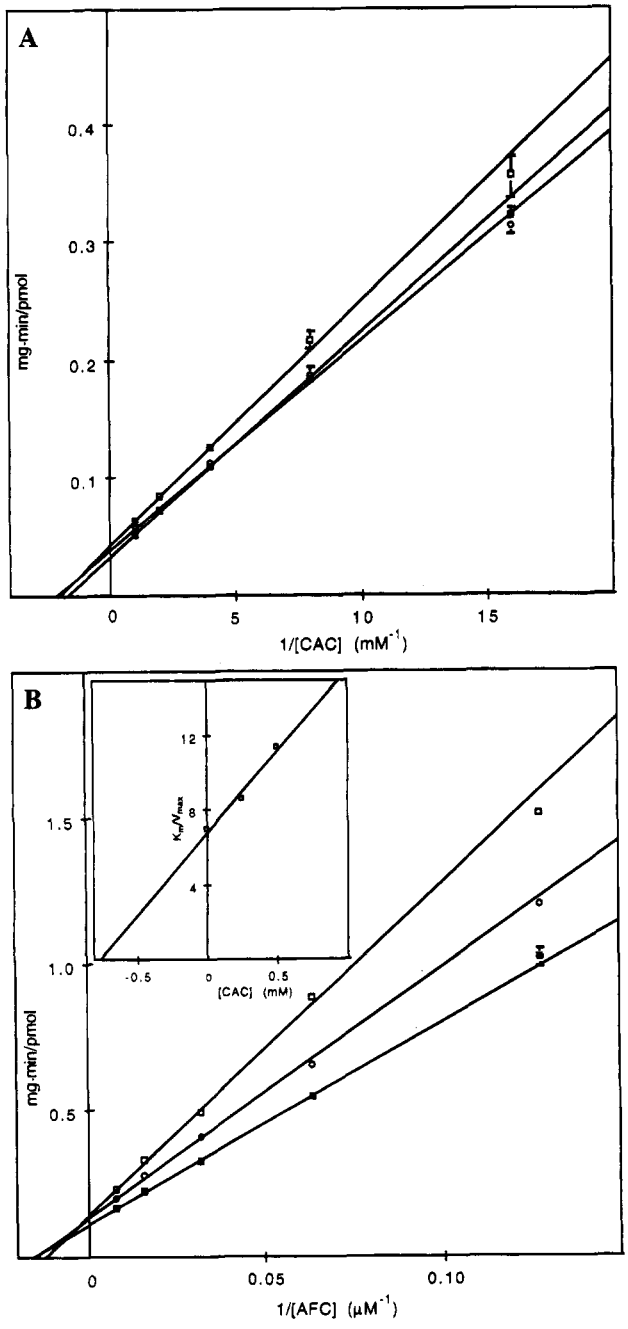


FIGURE 3: Cross-inhibition of AFC and CAC peptide methylation. (A) AFC inhibition of CAC peptide methylation (AdoMet = 9.28 μM). Plot of 1/specific activity vs 1/concentration of CAC peptide. Solid squares = 0 μM AFC, open circles = 62.5 μM AFC, open squares = 125 μM AFC. (B) CAC peptide inhibition of AFC methylation (AdoMet = 9.28 μM). Plot of 1/specific activity vs 1/concentration of AFC. Solid squares = 0 mM CAC peptide, open circles = 0.25 mM CAC peptide, open squares = 0.5 mM CAC peptide. The inset shows a replot of the slopes of lines vs concentration of CAC peptide.

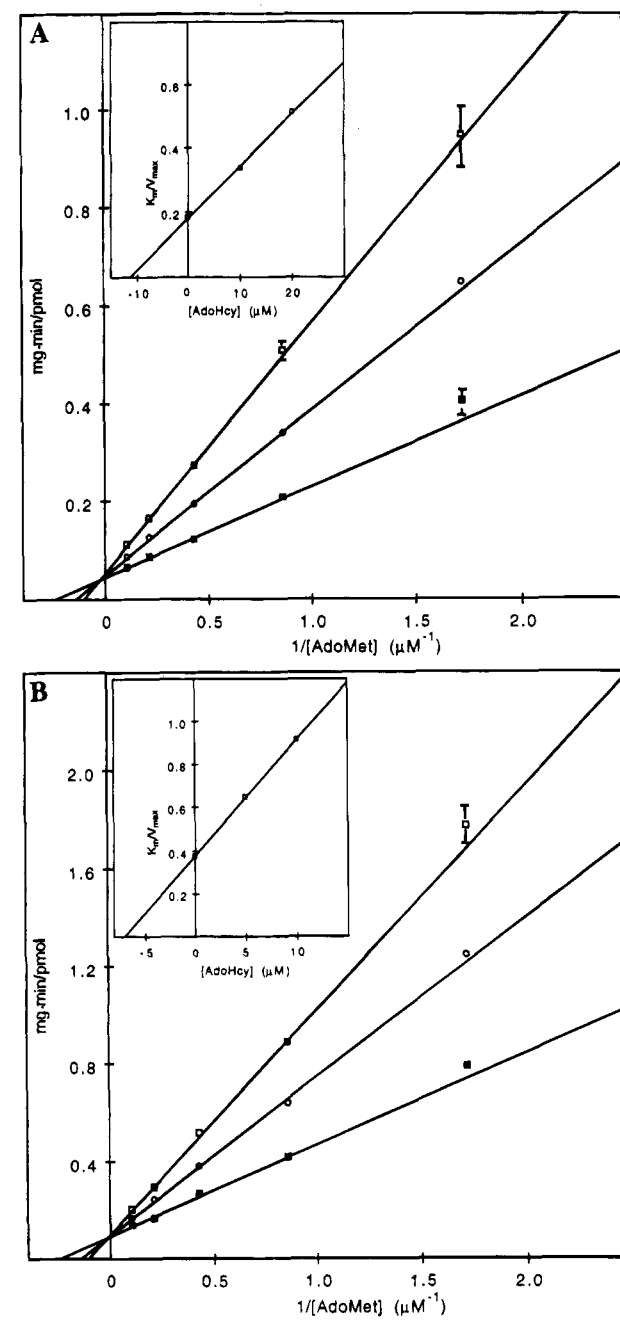


FIGURE 4: AdoHcy inhibition of AFC and CAC peptide methylation. (A) AdoHcy inhibition of CAC peptide methylation (CAC = 500 μM). Plot of 1/specific activity vs 1/concentration of AdoMet. Solid squares = 0 μM AdoHcy, open circles = 10 μM AdoHcy, open squares = 20 μM AdoHcy. The inset shows a replot of the slopes of lines vs concentration of AdoHcy. (B) AdoHcy inhibition of AFC methylation (AFC = 125 μM). Plot of 1/specific activity vs 1/concentration of AdoMet. Solid squares = 0 μM AdoHcy, open circles = 5 μM AdoHcy, open squares = 10 μM AdoHcy. The inset shows a replot of the slopes of lines vs concentration of AdoHcy.

ferases responsible for the methylation of the CAC peptide and AFC are different.

In the converse experiment, AFC methylation was inhibited by the CXC peptide with a K_I of 827 ± 85 μM. However, because of the high K_I value, it could not be determined whether the inhibition was competitive or noncompetitive. The CC peptide, even at concentrations of 1 mM, did not inhibit the methylation of either AFC or the CAC peptide.

Further experiments were carried out to determine if multiple methyltransferases are involved. The K_M for AdoMet is the same for both activities (Table 2). End product inhibition by AdoHcy (Figure 4; Table 3) is competitive for AdoMet in both enzymes, indicating that both enzymes operate with ordered sequential binding (ordered BiBi) (Shi & Rando, 1992). However, the K_I's of end product inhibition by AdoHcy differed for the two, with the K_I for AdoHcy inhibition of CAC peptide methylation being about twice the value measured for the AFC methylation (Table 3). The measured K_I's of a potent inhibitor of

Table 3: Inhibition of AFC and CAC Peptide Methyltransferases by AdoMet Analogs

inhibitor	K_I (μM)[a]
AFC methyltransferase	
AdoHcy	6.58 ± 1.03[b]
sinefungin	0.318 ± 0.044[b]
AdoEth	6.45 ± 0.91[b]
CAC Peptide Methyltransferase	
AdoHcy	11.5 ± 1.0[c]
sinefungin	0.296 ± 0.012[c]
AdoEth	21.8 ± 4.7[c]

[a] All inhibitors were competitive vs AdoMet. [b] Measured at 125 μM AFC. [c] Measured at 500 μM CAC peptide.

AdoMet methyltransferases, sinefungin (Pugh et al., 1978), was equal for both activities (Table 3). However, another analog of AdoMet, *S*-adenosylethionine (AdoEth), inhibited the methylation of AFC three times more potently than the methylation of the CAC peptide (Figure 5; Table 3). These results together with the inability of AFC or FTA to inhibit CAC peptide methylation indicate that separate enzymes are responsible for the methylation of the CAC peptide and AFC.

DISCUSSION

Small molecules have proven to be suitable substrates and inhibitors for enzymes involves in the posttranslational modification of isoprenylated proteins. For example, AFC is an excellent substrate for the isoprenylated protein methyltransferase which methylates proteins terminating with the isoprenylated C (Tan et al., 1991), and S-farnesylated tetrapeptides have proven to be excellent substrates for the isoprenylated protein endoprotease (Ma & Rando, 1992). Furthermore, the isoprenylated protein methyltransferase previously described can methylate both geranylgeranylated and farnesylated substrates (Pérez-Sala et al., 1992). This could be demonstrated by showing that AFC was a competitive inhibitor of AGGC methylation and that its K_I equaled its K_M as a substrate (Pérez-Sala et al., 1992). Cross-inhibition of AGGC methylation by AFC gave the same result. Moreover, FTA and GGTA competitively inhibited the methylation of either AFC or AGGC with the same K_I values (Pérez-Sala et al., 1992). Finally, the enzymatic methylations of AFC and AGGC showed kinetically identical behavior with respect to AdoMet and SAH (Pérez-Sala et al., 1992).

From an enzymological standpoint, it is perhaps not difficult to understand how the same relatively specific enzyme might methylate both AFC and AGGC. The issue that arose here is whether the same enzyme, or any enzyme for that matter, might be able to methylate doubly geranylgeranylated model peptides terminating in CC or CAC and whether this enzymatic activity is distinguishable from the previously described AFC methylating activity.

Small G-proteins terminating in the CC motif have been shown to be geranylgeranylated. Site-specific mutagenesis experiments with rab1b and YPT1 suggest that both cysteine residues are isoprenylated (Molenaar et al., 1988; Moores et al., 1991; Khosravi-Far et al., 1992; Kinsella & Maltese, 1991; Wilson & Maltese, 1993). However, methylation could not be demonstrated in p23rab2, YPT1, or YPT3 (Wei et al., 1992; Newman et al., 1992). In the case of CXC proteins, double geranylgeranylation has been convincingly demonstrated for smg p25A/rab3A (Farnsworth et al., 1991).

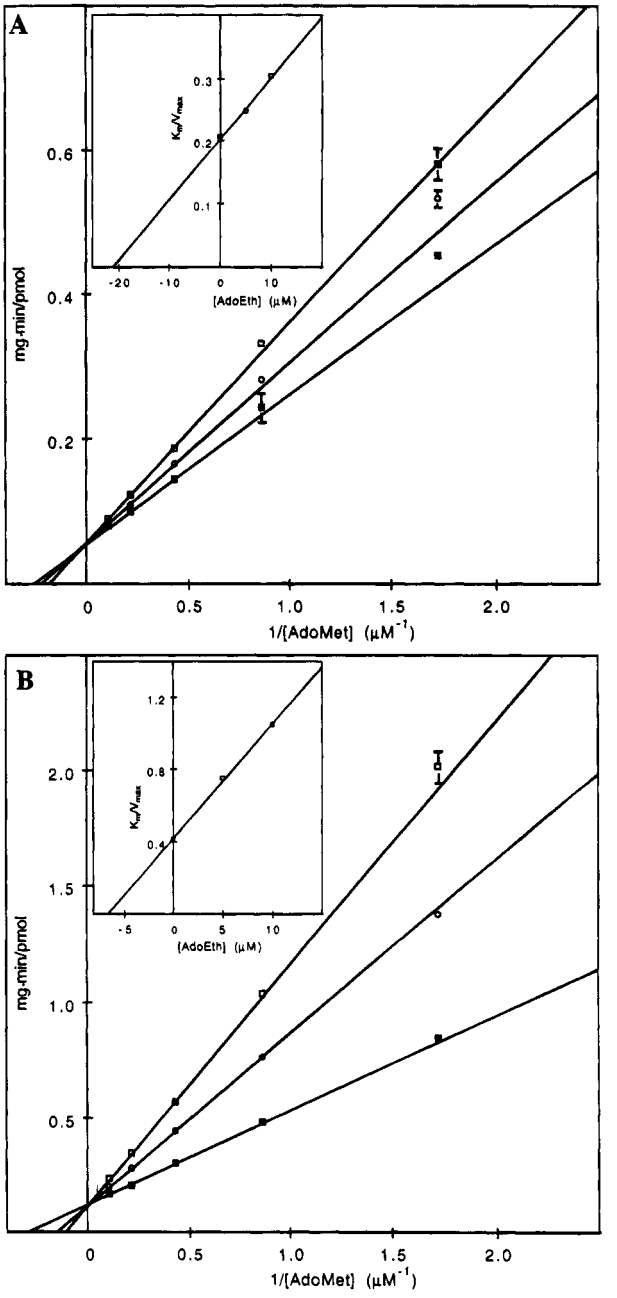


FIGURE 5: AdoEth inhibition of AFC and CAC peptide methylation. (A) AdoEth inhibition of CAC peptide methylation (CAC = 500 μM). Plot of 1/specific activity vs 1/concentration of AdoMet. Solid squares = 0 μM AdoEth, open circles = 5 μM AdoEth, open squares = 10 μM AdoEth. The inset shows a replot of the slopes of lines vs concentration of AdoEth. (B) AdoEth inhibition of AFC methylation (AFC = 125 μM). Plot of 1/specific activity vs 1/concentration of AdoMet. Solid squares = 0 μM AdoEth, open circles = 5 μM AdoEth, open squares = 10 μM AdoEth. The inset shows a replot of the slopes of lines vs concentration of AdoEth.

This same study showed that this protein is C-terminally methylated. This protein bears the Cys-Ala-Cys terminus. Two other proteins appear to undergo the same C-terminal modifications, YPT5 and rab4 (Giannakouros et al., 1993; Li & Stahl, 1993). These proteins terminate in Cys-Ser-Cys and Cys-Gly-Cys, respectively. We chose the Cys-Ala-Cys sequence for our studies, since its C-terminal modification had been best characterized, and the CAC sequence is present in both the bovine and human proteins.

Two model peptides were synthesized as putative methyltransferase substrates; the first (Ac(GG)CysAla(GG)Cys) modeled after the CXC-terminating proteins and the second (Ac(GG)Cys(GG)Cys) modeled after the CC-terminating proteins. We were able to demonstrate that the CAC peptide is methylated by all bovine tissues examined, whereas the CC peptide is not processed by any of these tissues. The negative result with the CC peptide is consistent with the lack of any reported observations in the literature on the existence of methylated proteins terminating with CC. However, our result on the lack of methylation of the CC peptide must be interpreted with caution since this highly abbreviated sequence may be insufficient for enzyme recognition.

Simple cross-inhibition experiments using ROS membranes as a source of methyltransferase were utilized to determine whether or not the methyltransferase which processes the CAC peptide is the same as the one which methylates AFC. Interestingly, both AFC and FTA failed to inhibit the methylation of the CAC peptide competitively. The CAC peptide active methyltransferase was also not inhibited by the geranylgeranyl analog of AFC (AGGC), which rules out the prenyl group as a simple recognition element. These experiments show that a new enzymatic activity is involved in methylating the doubly geranylgeranylated substrate. If the same enzyme was responsible for the methylation of both substrates, then cross-competitive inhibition should have occurred, as found in the AFC/AGGC example discussed above (Pérez-Sala et al., 1992). The K_I of AdoHcy inhibition was also quite different for the two enzymatic activities as was the K_I of AdoEth inhibition. The enzymes seem to be similar in some respects, however, having the same binding order (ordered BiBi) and K_M values for AdoMet.

The fact that the enzyme responsible for the methylation of AFC cannot methylate the CC peptide is not surprising the light of published structure−activity studies (Ma et al., 1994). Bulky substituents adjacent to the carboxyl group of a putative substrate hinder methylation (Ma et al., 1994). For example, neither *N*-benzoyl- nor *N*-pivaloyl-*S*-farnesyl-L-cysteine is a substrate or inhibitor of the enzyme (Ma et al., 1994). Why the CC peptide is not processed by the CAC peptide processing enzyme is unknown since extensive structure−activity studies on this enzyme have not yet been performed. However, the fact that the CAC peptide methyltransferase activity does not methylate the CC peptide or AFC strongly suggests that this enzyme is relatively specific for the isoprenylated CAC peptide moiety.

The CAC peptide weakly inhibited the methylation of AFC by an undetermined mechanism. The structure of the CAC peptide, unlike that of the CC peptide, contains a spacer of one small amino acid between the two sterically bulky amino acids and thus conforms to the steric requirements of the AFC methylating enzyme (Ma et al., 1994). However, if this peptide is a substrate for the AFC methylating enzyme, it must be processed with a very low V_{max} otherwise AFC, AGGC, and FTA should have been able to inhibit the methylation of the CAC peptide significantly.

The finding of a new isoprenylated protein methyltransferase activity lends support to the notion that isoprenylated protein methylation may be of important physiological significance. The literature is ambiguous on this point. Methylation is clearly essential for yeast mating factor **a** function (Anderegg et al., 1988) and may also be important for nuclear lamin function (Chelsky et al., 1987). It has been reported that methylation of ras enhances its ability to interact with membranes (Hancock et al., 1991). Moreover, recent results from this laboratory show that methylation of the γ-subunit of transducin enhances the ability of the transducin heterotrimer to interact with rhodopsin in disk membranes (Parish & Rando, 1994). On the other hand, it has been reported that the state of isoprenylation but not of methylation of the smg-25A/rab3A protein, a protein containing a CXC motif, is essential for its interactions with membranes and its GDI (Musha et al., 1992). However, it has also been reported that, in the very similar YPT5 protein, methylation is required for membrane association (Giannakouros et al., 1993). It is thus clearly possible that methylation may be of physiological importance in some systems but not in others. It would be of some interest to have potent inhibitors of the CAC peptide methyltransferase in order to test whether it has a physiologically significant role to play. The fact that the CAC peptide has a high K_M renders it impractical as an inhibitor of CXC-terminal G-protein methylation. However, structural modifications, including more extensive peptide backbones, may lead to the development of more potent substrates and specific inhibitors of the new isoprenylated protein methyltransferase and these will be useful probes of function.

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