Mammalian Prenylcysteine Carboxyl Methyltransferase Is in the Endoplasmic Reticulum*

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Prenylcysteine carboxyl methyltransferase (pcCMT) is the third of three enzymes that posttranslationally modify C-terminal CAAX motifs and thereby target CAAX proteins to the plasma membrane. Here we report the molecular characterization and subcellular localization of the first mammalian (human myeloid) pcCMT. The deduced amino acid sequence of mammalian pcCMT predicts a multiple membrane-spanning protein with homologies to the yeast pcCMT, STE14, and the mammalian band 3 anion transporter. The human gene complemented a ste14 mutant. pcCMT mRNAs were ubiquitously expressed in human tissues. An anti-pcCMT antiserum detected a 33-kDa protein in myeloid cell membranes. Ectopically expressed recombinant pcCMT had enzymatic activity identical to that observed in neutrophil membranes. Mammalian pcCMT was not expressed at the plasma membrane but rather restricted to the endoplasmic reticulum. Thus, the final enzyme in the sequence that modifies CAAX motifs is located in membranes topologically removed from the CAAX protein target membrane.

A number of signaling molecules, including Ras and G proteins, are targeted to the inner leaflet of the plasma membrane by a sequence of posttranslational modifications of a C-terminal CAAX motif that include prenylation, proteolysis, and carboxyl methylation (1). In some cases palmitoylation of an upstream cysteine is also required (2). These modifications render otherwise hydrophilic proteins hydrophobic, promoting association with membranes. The relative contributions of prenylation, proteolysis, and carboxyl methylation to membrane targeting are not well understood. Whereas neutralization of the negative charge on the α-carboxyl group by methyl esterification adds to overall hydrophobicity, particularly for farnesylated proteins, this modification contributes little to the affinity of geranylgeranylated proteins for membranes (3). Although processed CAAX proteins can associate with phospholipid vesicles in vitro (4), it is not known whether membrane proteins participate in prenylcyte membrane association in vivo. The Saccharomyces cerevisiae mating pheromone, α-factor, is a CAAX-processed polypeptide, and both its secretion via the Ste6p transporter (5) and its engagement of the Ste3p G protein-linked receptor (6) are dependent on prenylcyte carboxyl methylation, suggesting a role for this modification in protein-protein interactions. A cycle of prenylcyte carboxyl methylation is associated with neutrophil activation (7), and inhibitors of this enzyme block signal transduction in neutrophils (7), macrophages (8), and platelets (9), suggesting that, like bacterial chemotaxis (10), some eukaryotic processes may be regulated by reversible carboxyl methylation.

Because prenylcyte carboxyl methylation cannot be abolished by mutation of the substrate without also eliminating prenylation, elucidation of the role of carboxyl methylation will require characterization and disruption of the methyltransferase. Until recently, the only prenylcyte carboxyl methyltransferase (pcCMT) characterized at the molecular level was the STE14 gene product of S. cerevisiae (5). Homologs in Schizosaccharomyces pombe and Xenopus laevis have now been reported (11). Here we report the molecular cloning and preliminary characterization of the first mammalian pcCMT. In addition, using pcCMT tagged with green fluorescent protein (GFP) we show that pcCMT is expressed in the endoplasmic reticulum (ER) but excluded from the plasma membrane that is the target of many CAAX proteins, including Ras.

** Experimental Procedures

Molecular Cloning—A text-based search of the expressed sequence tag (EST) data base of the National Center for Biotechnology Information identified a 426-bp partial cDNA from murine placenta (mh77d6.r1) that has amino acid homology to the STE14 gene product of S. cerevisiae. Primers based on this sequence (forward: 5’-CGCCGACTCAAACCGGCTGCTGCTGCTACTCTCTA-3’; reverse: 5’-CGTTGACTCCAGGCTGTGATCCAGGAA-3’) were used to amplify by reverse transcriptase-PCR a 218-bp homologous cDNA fragment from HL60 cells. This fragment was then 32P-labeled and used to screen a unidirectional, size-fractionated HL60 cDNA library constructed in the λZAP system (provided by Dr. Philip Murphy, NIAID). Hybridizations were performed at 58 °C overnight. Of ~105 recombinant plaques screened, a single doubly positive clone was identified and proved positive on secondary screen. This clone contained a 3.6-kb cDNA insert that was sequenced in both directions.

Northern Blotting—Total RNA (40 μg) from HL60 cells grown with or without 1.25% MeSO for 5 days was fractionated on a 1.2% denaturing
formaldehyde-agarose gel, transferred to a nylon filter, and hybridized with [32P]dATP-labeled full-length HL60 pcCMT cDNA in Express Hybridization solution (CLONTECH, Palo Alto, CA) at 68 °C. A human multiple-tissue Northern blot (CLONTECH) was hybridized with the same probe according to the manufacturer’s instructions. The same hybridization solution (CLONTECH, Palo Alto, CA) at 68 °C. A human multiple-tissue Northern blot (CLONTECH) was hybridized with the same probe according to the manufacturer’s instructions.

**RESULTS AND DISCUSSION**

Repeated efforts to characterize mammalian pcCMT by STE14 homology cloning and by enzyme purification and microsequencing were unsuccessful. However, we identified a partial murine cDNA (GenBank accession no. AA022888) in

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**FIG. 1. Deduced amino acid sequence of human pcCMT and homology to related genes.** *S. cerevisiae* STE14 (5) and *S. pombe* and *X. laevis* mam4 (11) have prenylcysteine carboxyl methyltransferase activity in vitro. Murine EST nh77d06.r1 and two *C. elegans* genes were identified by searching the NCBI GenBank. Amino acid identities between human pcCMT and other gene products are indicated by shading. Hydrophobic stretches of human pcCMT that may represent membrane-spanning domains are indicated with

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**Antiserum—**A peptide corresponding to deduced amino acids 185–201 of HL60 pcCMT (FNHVVQNEKSDTHTLV) was synthesized, linked to keyhole limpet hemocyanin, and used to immunize rabbits. The resulting antiserum was used for immunoblotting of neutrophil light membranes prepared by nitrogen cavitation and discontinuous sucrose density centrifugation as described (12), for immunoprecipitation of lysates of HL60 cells metabolically labeled withL-[35S]methionine, and for sucrose density sedimentation (12), for immunoprecipitation of lysates.
the methyl donor S homologous to the degree of divergence in the N-terminal third of the molecule, brane-spanning protein. Comparison of human pcCMT with all ing the hypothesis that human pcCMT is a multiple mem-

teration that active pcCMT cannot be extracted from membranes but can be partially reconstituted in phospho-

somes derived from nitrogen cavitation of human neutrophils (1–4). Hydropathy analysis revealed six hydrophobic sequences homologous sequence. Using this fragment an HL60 cDNA employed to amplify from human myeloid HL60 cells a 218-bp primers based on this sequence, reverse transcription-PCR was the EST data base of the National Center for Biotechnology

The first 65 amino acids of human pcCMT, with detergents but can be partially reconstituted in phospho-

shpes (20) were not apparent, arguing against an evolution-

ary link.

To determine whether the HL60 Ste14p homolog has prenylcyesteine carboxyl methyltransferase activity, the cDNA was subcloned into a mammalian expression vector and transiently overexpressed in COS-1 cells. Membranes prepared from these cells were used as a source of recombinant enzyme. Farnesylated Ras GTPases require detergent for extraction from membranes. Therefore geranylgeranylated neutrophil cytosolic Rho GTPases that remain soluble by virtue of association with an accessory protein, guanine nucleotide dissociation inhibitor (GDI), were utilized as endogenous substrates in an in vitro assay (Fig. 2). Untransfected COS-1 cell membranes had little pcMT activity toward Rho proteins compared with that of membranes derived from human neutrophils (Fig. 2a). Transfection with the HL60 cDNA conferred pcMT activity toward Rho GTPases on COS-1 membranes, and this activity was blocked by the competitive pcMT inhibitor, AFC, with an ED50 (10 μM) identical to that for endogenous neutrophil pc-

CMT. Carboxyl methylation of Rac2 and RhoA by HL60 pc-

CMT-transfected COS-1 cell membranes was enhanced by CMT. Carboxyl methylation of Rac2 and RhoA by HL60 pc-

membranes (7). Carboxyl methyltransferase activity was quan-

titated in membranes of COS-1 cells transfected with HL60 pcCMT using prenylcysteine analogs as defined substrates (14). Untransfected COS-1 cell membranes had 23 ± 10% (n = 5) of the pcCMT specific activity of neutrophil membranes toward AFC. Transfection of COS-1 cells with HL60 pcCMT resulted in an 18–53-fold increase in specific AFC carboxyl methyltransferase activity (3.0 ± 0.8 to 74.5 ± 6.8 pmol/ mg min, n = 5, p < 0.0005). Carboxyl methylation of N-acetyl-S-all-trans-geranylgeranyl-L-cysteine (AGGC) was increased in parallel with methylation of AFC (18–versus 20-fold increase, n = 2), consistent with previous studies demonstrating that a single activity carboxyl methylated both farnesylated and gera-

VARYing substrates (21). The Michaelis constants of the recombinant enzyme, Km = 7 μM for AFC and 0.6 μM for AGGC, were similar to those for the endogenous neutrophil enzyme (19). Thus, the cDNA described above encodes authentic human myeloid pcCMT.

To determine whether human pcCMT could substitute in vivo for the S. cerevisiae pcCMT, Ste14p, we performed a complementation analysis using mating as a biological read-

out. The HL60 pcCMT cDNA expressed from a plasmid in a Δste14 yeast strain partially restored the mating phenotype (Fig. 3), indicating that a-factor could be carboxyl methylated by human pcCMT. Thus, HL60 pcCMT is a functional human homolog of S. cerevisiae Ste14p.
membranes of mature, peripheral blood neutrophils. Both transcripts were ubiquitously expressed in human tissues (Fig. 4b).

To characterize endogenous pcCMT, a polyclonal antiserum was raised against an internal HL60 pcCMT peptide (amino acids 185–201, Fig. 1). Immunoblots of neutrophil and pcCMT-transfected COS-1 cell membranes using this antiserum revealed a 33-kDa protein (Fig. 4c) corresponding to the predicted size of the protein encoded by the HL60 pcCMT cDNA, confirming that this cDNA, which lacked a termination codon 5′ of the ORF is, in fact, full-length. The same antiserum immunoprecipitated a 33-kDa protein from HL60 cells (Fig. 4c). Although the deduced amino acid sequence of pcCMT reveals a potential N-glycosylation site (Fig. 1), these data argue against glycosylation.

Ras is constitutively carboxy methylated (22) and expressed at the plasma membrane (23), a localization required for its biological activity (24). Like all CAAX proteins, Ras lacks a signal peptide, is synthesized in the cytosol, and modified immediately posttranslationally by a cytosolic prenyltransferase (25). Because prenylcyto
tine carboxyl methylation is catalyzed by an intrinsic membrane protein and represents the last of the three posttranslational modifications of the CAAX cysteine that enhance the affinity of Ras for membranes, the simplest model of plasma membrane targeting predicts pcCMT expression in the target membrane. However, activities associated with the further processing of prenylated proteins, including pcCMT (26), S-isoprenyl-CAAX high affinity binding (27), S-isoprenyl-CAAX protease (28, 29), and palmitoyltransferase (30) activities, have all been reported in micromolar fractions. Furthermore, one of the two S-isoprenyl-CAAX proteases recently identified in yeast has a putative ER retention sequence (31, 32), and a double deletion of these genes led to mislocalization of yeast Ras2p to internal membranes and cytosol (31). Nevertheless, none of these studies excluded expression of prenylcyto
tine-modifying activities from plasma membranes. Indeed, we have reported pcCMT activity in neutrophil subcellular fractions enriched for surface membrane (19).

To determine the subcellular localization of pcCMT we constructed a recombinant pcCMT tagged at the C terminus with GFP. In CHO (Fig. 5, a–c), COS-1 (Fig. 5, d–e), and NIH3T3 (not shown) cells, CMT-GFP was visualized in the ER, Golgi, and nuclear membranes but not in the plasma membrane. This assay afforded a sensitivity and resolution that allowed visu-

![Figure 4](image_url)

**Fig. 4. Expression of pcCMT mRNA and protein.** a, total RNA was prepared from HL60 cells that had been grown in DMEM + 10% fetal calf serum without (lane 1) or with (lane 2) 1.25% Me2SO (DMSO) for 5 days to induce granulocytic differentiation. Membranes were hybridized with 32P-labeled full-length pcCMT cDNA (upper panel) and later stripped and rehybridized for β-actin mRNA (lower panel). The results shown are representative of two independent experiments. b, a membrane containing polyadenylated mRNA from the indicated human tissues was hybridized as in a for pcCMT mRNA (upper panel) and subsequently β-actin mRNA (lower panel) according to the manufacturer’s instructions (CLONTECH). c, lysates of HL60 cells metabolically labeled with [35S]methionine/cysteine were immunoprecipitated (lanes 1 and 2) with preimmune (p) or immune (i) serum from a rabbit immunized with a pcCMT peptide. Membranes from human neutrophils (PMN, lanes 3 and 4) and from COS-1 cells transfected with pcCMT (CMT, lanes 5 and 6) or vector alone (VEC, lane 7) were immunoblotted with the same sera. IP, immunoprecipitate; IB, immunoblot.

![Figure 5](image_url)

**Fig. 5. Subcellular localization of human pcCMT.** a–e, CHO cells (a–c) and COS-1 cells (d and e) were transiently transfected with GFP alone (a) or GFP-tagged human pcCMT (b–e) and examined un-
fixed. In c and e the image is intentionally overexposed to reveal the distinct fluorescence of the ER and absence of plasma membrane fluorescence. Arrowheads indicate nuclear envelope fluorescence and asterisks indicate a perinuclear area of intense Golgi fluorescence. f, CHO cells transfected with GFP-tagged pcCMT, fixed, permeabilized, and stained for the ER marker ribophorin I (Texas Red) and viewed by confocal microscopy (0.4 μm optical section) where yellow pseudocolor indicates overlap. g, CHO cells transfected with Myc-tagged human pcCMT, fixed, permeabilized, and stained with anti-Myc antibody 9E10 (Texas Red). h–i, untransfected COS-1 cells stained for endogenous pcCMT with preimmune serum (h) or immune anti-peptide antiserum (i). Bars indicate 10 μm.

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3 M. Philips and T. Oei, unpublished results.
confirm these data by localizing endogenous pcCMT to ER and nuclear membranes in COS-1 cells (Fig. 5, h–i). The absence of Golgi staining for endogenous pcCMT suggests that the Golgi localization of ectopically expressed pcCMT may result from gene overexpression.

These data demonstrate that mammalian pcCMT is an intrinsic membrane protein localized to a compartment topologically removed from the plasma membrane. Similar observations have recently been made in yeast.4 It is curious that proteins such as Ras, synthesized on free ribosomes, prenylated in the cytosol, and destined for the cytoplasmic leaflet of the plasma membrane, are diverted to the ER for processing. Moreover, the ER restriction of pcCMT suggests that an uncharacterized transport pathway must mediate the translocation of fully processed GTPases from internal membranes to the cell surface. Such a pathway could utilize the cytoplasmic surface of secretory vesicles, cytosolic accessory molecules analogous to GDI, or a novel transport system. Investigation of this aspect of Ras biology may open new approaches to the development of pharmacologic inhibitors of oncogenic Ras.

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