Topology of Mammalian Isoprenylcysteine Carboxyl Methyltransferase Determined in Live Cells with a Fluorescent Probe

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Isoprenylcysteine carboxyl methyltransferase (Icmt) is a highly conserved enzyme that methyl esterifies the α carboxyl group of prenylated proteins including Ras and related GTPases. Methyl esterification neutralizes the negative charge of the prenylcysteine and thereby increases membrane affinity. Icmt is an integral membrane protein restricted to the endoplasmic reticulum (ER). The Saccharomyces cerevisiae ortholog, Ste14p, traverses the ER membrane six times. We used a novel fluorescent reporter to map the topology of human Icmt in living cells. Our results indicate that Icmt traverses the ER membrane eight times, with both N and C termini disposed toward the cytosol and with a helix-turn-helix structure comprising transmembrane (TM) segments 7 and 8. Several conserved amino acids that map to cytoplasmic portions of the enzyme are critical for full enzymatic activity. Mammalian Icmt has an N-terminal extension consisting of two TM segments not found in Ste14p and therefore likely to be regulatory. Icmt is a target for anticancer drug discovery, and these data may facilitate efforts to develop small-molecule inhibitors.

Two classes of proteins are targeted to cellular membranes by posttranslational modifications that include polyisoprenylation (7). The first are proteins that end with a CAAX sequence, in which an invariant cysteine lies three residues from the final amino acid of the primary translation product and “A” denotes an amino acid that is usually, but not always, aliphatic. CAAX proteins are prenylated by either farnesyltransferase or geranylgeranyltransferase I (10). Once prenylated, CAAX proteins are modified by Ras-converting enzyme I (Rce1), a protease that removes the AAX amino acids, rendering the prenylcysteine the new C terminus. The second class of prenylated proteins is Rab proteins that end with a CC or CXC sequence and are prenylated by geranylgeranyltransferase II (5). Prenylated CAAX and CXC Rab proteins are substrates for a highly conserved enzyme, isoprenylcysteine carboxyl methyltransferase (Icmt), that methyl esterifies the α carboxyl group of the prenylcysteine (21, 27).

Prenylation allows otherwise hydrophilic proteins to associate with cellular membranes, presumably by the insertion of the prenyl lipid into the phospholipid bilayer. Such an insertion would bring the α carboxyl group of the prenylcysteine into proximity with the negatively charged head groups that are predominant among the phospholipids of the cytosolic leaflet of the plasma membrane. Methyl esterification removes the negative charge on the cysteine and thereby eliminates the electrostatic repulsion that would otherwise occur. Thus, carboxyl methylation of prenylproteins is believed to be a mechanism to increase their affinity for the plasma membrane. This model has been supported by in vitro studies of protein binding to liposomes (11) and studies of membrane targeting of green fluorescent protein (GFP)-tagged proteins in living cells (13, 14). In bacteria, carboxyl methylation of glutamate side chains regulates protein-protein interactions (22), and in some contexts, carboxyl methylation of prenylcysteine residues may serve a similar function (17, 23).

Mammalian genomes encode only one member of the Icmt class of methyltransferases, and Icmt lacks homology to any other protein methyltransferase (8). The fact that Icmt is highly conserved from yeast to humans suggests that its function is critical. Indeed, a deletion of the Icmt locus in mice by homologous recombination results in embryonic lethality (3, 12).

The best-studied substrates for Icmt are Ras and related GTPases. Ras is the proto-oncogene mutated more often in human cancer than any other. Accordingly, much effort has gone into developing agents that interfere with Ras function. The most successful approach to anti-Ras drug development has been to target the CAAX processing enzymes. Farnesyltransferase inhibitors are in the late stages of clinical development, although their efficacy has been somewhat disappointing (20). Icmt is also considered to be a target for anti-Ras drug discovery. Pharmacological studies (25, 26), in vitro studies of Icmt null cells (2), and analysis of tumors in animals that are conditionally deficient in Icmt (24) have recently provided a proof of principle for the idea of inhibiting Icmt to treat Ras-dependent cancer. The development of Icmt inhibitors will require detailed knowledge of the structure and function of the enzyme.

We have shown that Icmt is an integral membrane protein (18) restricted to the endoplasmic reticulum (ER) (8) that cannot be extracted in the active form by detergent (18). The Saccharomyces cerevisiae ortholog of Icmt, Ste14p, has been characterized as a multiple-membrane-spanning protein with
six transmembrane (TM) segments (19). In this study, we developed a novel method to study the topology of an ER membrane protein and applied the method to human Icmt. We conclude that Icmt passes through the ER membrane eight times with both its N and C termini disposed toward the cytosol. Our topological map allowed us to predict residues involved in catalysis, reveals a conserved helix-turn-helix structure for the final pair of TM segments, and predicts an N-terminal regulatory domain consisting of two TM segments not found in the yeast enzyme.

MATERIALS AND METHODS

Plasmids. pEGFP-N3-Icmt was previously described (8). The Icmt coding region of this plasmid was PCR amplified and inserted into pECPF-C1 and pECFP-N1 vectors (Clontech, Mountain View, CA) to generate cyan fluorescent protein (CFP)-Icmt and Icmt-CPF, CFP-Icmt-Ras binding domain (RBD) and RBD-Icmt-CPF were generated by PCR amplification of the Ras binding domain of cRaf-1 (codons 51 to 131) with appropriate overhangs, which was inserted into the pECPF-C1-Icmt and pECFP-N1-Icmt constructs. Icmt mutations were generated in pEGFP-C1-Icmt using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). CFP-Icmt C-terminal truncation constructs were generated by PCR amplification using pEGFP-C1-Icmt as a template, with EcoRI and BamHI restriction sites encoded into the primers to allow the insertion into pECPF-C1. Oligonucleotides encoding either the RBD (Raf-1 codons 51 to 131) or codons 2 to 18 of bovine rhodopsin (designated gly) were then cloned into the 3′-UTR of both constructs. We previously described KDEL receptor (KDELR)-GFP (6), and this plasmid was used to generate KDELR-RBD by inserting an oligonucleotide encoding the RBD sequence with a 3′ stop codon between the KDELR and GFP coding sequences. pEYFP-C1-Kras12V, directing the expression of yellow fluorescent protein (YFP)-Kras12V, was previously described (4), and YFP-Kras12V185S was generated by site-directed mutagenesis. GFP-vesicular stomatitis virus G protein (VSVG) was a gift from Jennifer Lippincott-Schwartz (NICHD). All plasmids were sequenced prior to use.

Cell culture, transfection, and imaging. COS-1 and HEK293 cells obtained from the American Type Culture Collection were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Cellgro, VA) in 5% CO2 at 37°C. Cells were seeded at 2 × 104 cells in 35-mm plastic plates with a glass coverslip sealing a 1-cm round, central cutout (MatTek, Ashland, MA). Twenty-four hours later, the cells were transiently transfected with 1 to 2 μg of plasmid DNA using SuperFect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Live cells were imaged the next day using an inverted Zeiss 510 laser scanning confocal microscope (63× Plan-Neofluar 1.25-numerical-aperture oil).

Glycosylation assay. COS-1 cells were transfected with CFP-Icmt-gly or the various C-terminal truncation mutants thereof as described above. After 24 h, the cells were lysed with radiolabeled immunoprecipitation assay buffer (20 mM Tris HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), and clarified lysates were immunoprecipitated with rabbit polyclonal anti-GFP antibody (Invitrogen, Carlsbad, CA) conjugated to protein A-agarose beads. Washed immunoprecipitates on the beads were suspended in 50 mM Na-citrate (pH 5.5) with or without 5 μl endoglycosidase H (endo H) (Roche, Indianapolis, IN) overnight at 4°C. Beads were then eluted with SDS sample buffer, and the eluates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis with a mouse monoclonal anti-GFP antibody (Roche, Indianapolis, IN).

Icmt activity assay. HEK293 cells were transfected as described above with wild-type or mutant Icmt tagged at the N terminus with CFP. Forty-eight hours after transfection, the cells were scraped into homogenization buffer (10 mM Tris-HCl [pH 7.4], 10 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, and Roche protease inhibitor cocktail) and disrupted by Dounce homogenization (20 strokes). The postnuclear supernatant centrifuged at 3,000 × g was separated into membrane and cytosolic fractions by centrifugation at 150,000 × g for 2 h, and P150 was washed with and resuspended in homogenization buffer (100 μl) and then assayed for total protein (BCA assay; Thermo Scientific, Rockford, IL) and used as a source of Icmt. The expression level of each construct was determined by immunoblotting for GFP quantified with [3H]protein A and PhosphorImager analysis. [3H]adenosyl methionine (30 μCi/ml) was used as the methyl donor, and a biotinylated and farnesylated peptide corresponding to the C terminus of Kras (N-biotinyl-S-farnesyl-L-cysteine) was used as the methyl acceptor (1). Each 50-μl reaction mixture contained 8 μg of membrane protein, 5 μM AdoMet, and various concentrations of BFC. After 20 min at room temperature, the reaction was stopped by the addition of 5 μl of 10% Tween 20 to the mixture and transfer to an ice bath. Ten microliters of a 1:1 slurry of streptavidin-conjugated Sepharose beads in capture buffer (20 mM NaHPO4, 150 mM NaCl [pH 7.4]) was added, and the mixture was rotated overnight at 4°C. The beads were washed three times with capture buffer, brought up in 100 μl of the same buffer, and then added to scintillation fluid. The amount of BFC[3H]methyl ester associated with the beads was determined by scintillation counting using a standard curve generated with [3H]AdoMet. The Vmax of each mutant was calculated from double-reciprocal plots of reaction velocity and BFC concentration and normalized to CFP-Icmt expression as determined by immunoblotting. The Vmax of endogenous Icmt in untransfected HEK293 cells was at least an order of magnitude below that of the overexpressed enzyme, and that activity was subtracted as background.

RESULTS

In order to determine the topology of Icmt in the ER membrane, we devised a novel fluorescence-based, live-cell assay.

FIG. 1. Fluorescent probe for ER membrane protein domains disposed toward the cytosol. (A) The probe consists of constitutively GTP-bound Kras12V that lacks a membrane-targeting sequence (18S) and is tagged with YFP at the N terminus. This probe will bind to an ER protein tagged with RBD only if that domain is disposed toward the cytosol. (B) Fluorescent expression patterns in live COS-1 cells of KDELR-GFP (i), YFP-Kras12V (ii), and YFP-Kras12V185S expressed alone (iii) or coexpressed with KDELR-RBD (iv). Bars represent 10 μm.
FIG. 2. Topology of Icmt deduced in live cells with a fluorescent probe. (A) A Sweet-Eisenberg hydropathy plot of human Icmt generated with MacVector 9.5 is annotated with bars depicting putative TM segments. (B) COS-1 cells expressing YFP-Kras12V185S (red channel) and cotransfected with the indicated form of CFP-tagged Icmt (green channel) were imaged alive 24 h after transfection with a Zeiss 510 LSM microscope. (C) COS-1 cells expressing YFP-Kras12V185S (red channel) and cotransfected with the indicated truncations of Icmt tagged at their N termini with CFP and at their C termini with RBD (green channel) were imaged as described above (B). The amino acids of Icmt included are indicated above the image, and the predicted TM segments included are indicated below the images. Bars in B and C represent 10 μm.
We reasoned that if a fluorescent cytosolic reporter protein were expressed in conjunction with an ER protein for which it has high affinity, and the cognate binding domain of the ER protein was disposed toward the cytoplasm, the pattern of fluorescence would correspond to that of an ER protein. Conversely, if the binding domain of the ER protein were disposed toward the lumen, then the reporter would retain a diffuse, cytosolic pattern. To test this method, we chose constitutively active but non-membrane-targeted Kras12V185S as the cytosolic reporter molecule to be fused with YFP and the RBD of Raf-1 as the module to be fused with the ER protein (Fig. 1A) because of the high affinity that these proteins have for each other (6, 9). The KDELR is restricted to the ER and Golgi apparatus and, when tagged with GFP, gives a pattern of fluorescence that is unambiguous and easily identified as endomembrane (Fig. 1Bi). Whereas YFP-Kras12V decorates the plasma membrane as expected (Fig. 1Bii), YFP-Kras12V185S gives a cytosolic pattern with homogeneous fluorescence in the cytosol and nucleoplasm and negatively imaged organelles (Fig. 1Biii). In contrast, when YFP-Kras12V185S is coexpressed with a KDELR extended at its C terminus with an RBD, the fluorescence pattern is converted to that of the KDELR itself, revealing the nuclear envelope, ER reticulum, and Golgi apparatus (Fig. 1Biv). Thus, YFP-Kras12V185S can report the cytosolic disposition of an RBD when fused to an ER protein and can therefore be used as a live-cell probe for ER protein topology.

We previously demonstrated that Icmt is an integral membrane protein and that its catalytic activity requires membrane association (18). Hydropathy plots of the amino acid sequence of human Icmt strongly suggest a polytopic membrane protein, although the number of TM segments is not obvious (Fig. 2A). There are four regions between amino acids 1 and 110 that meet all criteria for TM helices. Amino acids 132 to 152 are less hydrophobic but correspond to a segment that has been found to be TM in Ste14p (19). Amino acids 154 to 173 are also consistent with a TM segment. Amino acids 214 to 244 are hydrophobic but include 31, rather than the standard 20, amino acids 229 and proline 230 of human Icmt are required for proper topology, consistent with a helix-turn-helix. COS-1 cells expressing YFP-Kras12V185S were cotransfected with either CFP-Icmt-RBD (left) or the same construct in which N239 and P240 were both mutated to leucine (right) and imaged as described in the legend of Fig. 2. The bar represents 10 μm.

FIG. 3. Asparagine 229 and proline 230 of human Icmt are required for proper topology, consistent with a helix-turn-helix. COS-1 cells expressing YFP-Kras12V185S were cotransfected with either CFP-Icmt-RBD (left) or the same construct in which N239 and P240 were both mutated to leucine (right) and imaged as described in the legend of Fig. 2. The bar represents 10 μm.

FIG. 4. Topology mapping of Icmt with a glycosylation reporter. (A) COS-1 cells were transfected with the indicated constructs (GFP, GFP-VSVG, or Icmt truncations tagged at their N termini with CFP and at their C termini with an N-glycosylation reporter consisting of amino acids 2 to 18 of bovine rhodopsin), and after 24 h, each protein was immunoprecipitated with an anti-GFP antibody and analyzed by immunoblotting for CFP/GFP-tagged proteins before or after treatment with endo H as described in Materials and Methods. Asterisks indicate proteins affected by endo H. (B) COS-1 cells were transfected with CFP-Icmt-gly (wild type [WT]) or CFP-IcmtNP229/230LL-gly (NP→LL) and then processed as described above (A).
acids required for TM helices. Thus, it is not clear how many TM segments are encoded in the Icmt gene.

To determine the disposition of the N and C termini of human Icmt, we tagged each end of the protein with RBD and tagged the opposite end with CFP. When YFP-Kras12V185S was coexpressed in COS-1 cells with Icmt tagged only with CFP, the reporter remained cytosolic (Fig. 2B). In contrast, when YFP-Kras12V185S was coexpressed with either RBD-Icmt-CFP or CFP-Icmt-RBD, the reporter was recruited to the ER (Fig. 2B). Thus, both the N and C termini of Icmt are oriented toward the cytosol, and there must be an even number of TM segments.

Based on the homology between Icmt and Ste14p, on the deduced topology of the yeast enzyme (19), on our demonstration of an even number of TM segments, and on the N-terminal extension observed in mammalian gene products, we hypothesized that mammalian Icmt spans the ER membrane eight times. To test this model, we generated a series of truncation constructs based on putative TM segments that are tagged with CFP at their N termini and with RBD at their C termini. These were coexpressed in COS-1 cells with YFP-Kras12V185S. Constructs consisting of putative TM1-2, TM1-4, and TM1-6 all recruited YFP-Kras12V185S to the ER (Fig. 2C). In contrast, the constructs consisting of putative TM1-3 and TM1-5 failed to recruit the reporter (Fig. 2C). These results, together with the deduced disposition of the N and C termini, establish the topology of TM1 through TM6 as that of a typical multiple-membrane-spanning protein.

The deduced topology of TM1-6 and the demonstration of both termini of the full-length protein disposed toward the cytosol indicated that, as in Ste14p (19), the final 31-amino-acid stretch of hydrophobic amino acids transverses the ER membrane twice as a helix-turn-helix structure. Asparagine 229 and proline 230, which are midway along the sequence of 31 hydrophobic amino acids, are predicted to allow a helix-turn-helix (16). Although many of the amino acids that form the putative TM segments of Icmt are not conserved, the N229-P230 sequence is highly conserved in Icmt genes from S. cerevisiae to humans (8). To test the hypothesis that the N229-P230 sequence permits a helix-turn-helix structure, we mutated these two amino acids to leucine in CFP-Icmt-RBD. Whereas the RBD at the C terminus of wild-type Icmt recruited YFP-Kras12V185S to the ER very efficiently, relatively little of the probe decorated the ER when expressed with CFP-IcmtNP229/230LL-RBD (Fig. 3). The fact that some YFP-Kras12V185S was recruited to CFP-IcmtNP229/230LL-RBD is consistent with the intermediate results of Romano and Michaelis when the same substitution was applied to Ste14p (19) and suggests that either the dielencic motif allows a helix-turn-helix to form inefficiently in some molecules or that amino acids 214 to 244 fail to efficiently insert into the membrane, leaving a molecule that spans the membrane six times. Regardless of which is the case, the marked difference in the ability to recruit YFP-Kras12V185S between CFP-Icmt-RBD and CFP-IcmtNP229/230LL-RBD demonstrates that N229 and P230 are required to maintain the topology of Icmt and indicates a helix-turn-helix structure similar to that deduced for the final TM segments of Ste14p (19).

To confirm our fluorescence-based, live-cell results using a more conventional method of topology analysis, we used a glycosylation reporter (15). Amino acids 2 to 18 of bovine rhodopsin are highly glycosylated when expressed in the lumen of the ER. We fused this sequence to the C termini of a series of truncation mutants of Icmt tagged at their N termini with CFP, similar to those constructed with the RBD reporter. These were expressed in COS-1 cells, immunoprecipitated with anti-GFP antibodies, and analyzed by SDS-PAGE and immunoblotting with and without prior treatment of the immunoprecipitate with endo H. As a negative control, we expressed GFP alone, which was insensitive to endo H. We expressed VSVG as a positive control and found that endo H increased the electrophoretic mobility of the protein. Using this assay, we observed that whereas constructs with putative TM1-3 and TM1-5 were sensitive to endo H, those consisting of an even number of putative TMs, TM1-2, TM1-4, TM1-6 and TM1-8, were insensitive (Fig. 4A). To confirm the fluorescent results with the CFP-IcmtNP229/230LL-RBD construct (Fig. 3), we constructed an analogous CFP-IcmtNP229/230LL-gly reporter using amino acids 2 to 18 of rhodopsin. Whereas CFP-Icmt-gly ran as one species in SDS-PAGE that was unaffected by endo H, CFP-IcmtNP229/230LL-gly ran as a doublet with a slower-migrating minor species that was endo H sensitive (Fig. 4B), consistent with a shift of the C terminus of the protein into the ER lumen, albeit with low efficiency. Thus, the endo H sensitivity assay was entirely concordant with our fluorescence live-cell assay. Asparagine 127 represents the only endogenous consensus glycosylation site in Icmt. The fact that full-length Icmt is endo H resistant suggests that N127 is not glycosylated, a result concordant with our topology that places this residue on the cytoplasmic face of the enzyme.

Our results demonstrate that mammalian Icmt is an integral ER membrane protein containing eight TM segments, with the final two consisting of a helix-turn-helix structure, with both the N and C termini disposed toward the cytosol (Fig. 5A). To test the hypothesis that the highly conserved amino acids of Icmt disposed toward the cytoplasm are involved in catalysis, we mutated 14 such amino acids as well as two nonconserved residues and measured Icmt activities of the mutant proteins (Table 1 and Fig. 5B). Wild-type or mutant Icmt tagged at the N terminus with CFP was expressed in HEK293 cells that were then used to make a membrane fraction that served as a source of enzyme. The Vₘₐₓ of each mutant was calculated from double-reciprocal plots and normalized to CFP-Icmt expression as determined by immunoblotting (Table 1). The substa-
TABLE 1. \( V_{\text{max}} \) of wild-type Icmt and various mutants with substitutions of conserved amino acids

| Enzyme   | No. of membranes | \( V_{\text{max}} \) (pmol BFC h\(^{-1}\) g membrane\(^{-1}\)) | SEM | Fold change from wild type | Topology  |
|----------|------------------|------------------------------------------------------------|-----|---------------------------|-----------|
| F124R    | 3                | 0.32                                                       | 0.21| 0.06                      | CL2       |
| F124A    | 5                | 0.84                                                       | 0.25| 0.16                      | CL2       |
| E107A    | 5                | 1.29                                                       | 0.39| 0.24                      | TM4       |
| E251I    | 6                | 1.38                                                       | 0.50| 0.26                      | C-term CS |
| W241A    | 3                | 1.55                                                       | 0.62| 0.29                      | TM8       |
| R63A     | 4                | 1.61                                                       | 0.12| 0.30                      | CL1       |
| F258A    | 4                | 1.91                                                       | 0.76| 0.36                      | C-term CS |
| P275A    | 4                | 2.07                                                       | 1    | 0.39                      | C-term CS |
| E292A    | 4                | 2.56                                                       | 1.44| 0.48                      | C-term CS |
| D122A    | 4                | 2.69                                                       | 0.92| 0.50                      | CL2       |
| RH290/210AA | 6          | 3.26                                                       | 1.46| 0.61                      | CL3       |
| E146A    | 2                | 3.95                                                       | 1.22| 0.74                      | TM5       |
| S123A    | 3                | 4.11                                                       | 0.23| 0.76                      | CL2       |
| F72A     | 4                | 4.12                                                       | 0.64| 0.77                      | TM3       |
| E142A    | 4                | 4.33                                                       | 0.95| 0.81                      | TM5       |
| R209A    | 3                | 5.25                                                       | 0.42| 0.98                      | CL3       |
| Wild type| 3                | 5.37                                                       | 0.67| 1.00                      | C-term CS |
| Y260A    | 4                | 5.77                                                       | 2.28| 1.07                      | C-term CS |

\(^a\) The source of enzyme was total membranes prepared from HEK293 cells expressing wild-type Icmt or the indicated mutant.

\(^b\) The \( V_{\text{max}} \) was calculated from double-reciprocal plots of the reaction velocity and substrate concentration using 8 \( \mu\)M [3H]AdoMet, and various concentrations of BFC. The units are pmol BFC/[3H]methyl ester produced per hour per \( \mu\)g membrane protein. The relative expression of each mutant was determined by immunoblotting, and the apparent \( V_{\text{max}} \) was normalized relative to the expression of wild-type Icmt. The activity of endogenous Icmt in membranes prepared from cells transfected with empty vector was relatively small and subtracted as background.

\(^c\) Putative location of the mutation as predicted by the topological model shown in Fig. 5. C-term, C terminus; CL, cytoplasmic loop; CS, cytosolic.

The DISCUSSION section follows, discussing the implications of the findings.

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