Isoprenylcysteine Carboxyl Methyltransferase Deficiency in Mice*

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After isoprenylation, Ras and other CAAX proteins undergo endoproteolytic processing by Rce1 and methylation of the isoprenylcysteine by Icmt (isoprenylcysteine carboxyl methyltransferase). We reported previously that Rce1-deficient mice died during late gestation or soon after birth. We hypothesized that Icmt deficiency might cause a milder phenotype, in part because of reports suggesting the existence of more than one activity for methylating isoprenylated proteins. To address this hypothesis and also to address the issue of other methyltransferase activities, we generated Icmt-deficient mice. Contrary to our expectation, Icmt deficiency caused a more severe phenotype than Rce1 deficiency, with virtually all of the knockout embryos (Icmt−/−) dying by mid-gestation. An analysis of chimeric mice produced from Icmt−/− embryonic stem cells showed that the Icmt−/− cells retained the capacity to contribute to some tissues (e.g. skeletal muscle) but not to others (e.g. brain). Lysates from Icmt−/− embryos lacked the ability to methylate either recombinant K-Ras or small molecule substrates (e.g. N-acetyl-S-geranylgeranyl-l-cysteine). In addition, Icmt−/− cells lacked the ability to methylate Rab proteins. Thus, Icmt appears to be the only enzyme participating in the carboxyl methylation of isoprenylated proteins.

After isoprenylation, Ras and other proteins that terminate with a CAAX sequence undergo two additional C-terminal modifications (1). First, the last three amino acids of the protein (i.e. the -AX) are released by an endoprotease associated with the endoplasmic reticulum (1–3). Second, the carboxyl group of the newly exposed isoprenylcysteine is methylated (4, 5). These post-isoprenylation processing steps may help target CAAX proteins to membrane surfaces within cells (1). The endoprotease and methyltransferase steps have attracted interest because they offer a potential means for modulating the activity of CAAX proteins, many of which participate in cell signaling (1). Several groups have hypothesized that inhibiting the endoprotease or the methyltransferase might retard the growth of tumors caused by mutation-activated Ras proteins (1, 2, 6, 7). At this point, however, testing such hypotheses appears to be a few years away. No specific high affinity inhibitors suitable for animal testing have been developed, either for the endoprotease or for the methyltransferase. Just as importantly, neither the spectrum of substrates nor the physiologic importance of the two processing steps has been explored fully. This is particularly the case for the methyltransferase.

To define the physiologic relevance of the post-isoprenylation processing steps, our laboratory generated and characterized mice lacking the endoprotease Rce1 (8). Membranes from Rce1-deficient embryos and cells were completely unable to carry out the endoproteolytic processing of Ras and a host of other CAAX proteins. Surprisingly, the consequences of knocking out Rce1 in the mouse were relatively mild. Although most of the Rce1 knockout mice died before birth, the embryos remained viable until late in gestation, and as late as embryonic day 18.5 many were normal in size, appeared healthy, and had no obvious histologic abnormalities. A few of the Rce1 knockout mice were born and lived for a few weeks.

A methyltransferase for mammalian CAAX proteins, isoprenylcysteine carboxyl methyltransferase (Icmt), has been identified recently (5) and shown to be located in the endoplasmic reticulum (5, 9). We used gene-targeting techniques to produce a mouse embryonic stem (ES) cell line lacking both Icmt alleles and documented that membranes from those cells lacked the ability to methylate recombinant K-Ras (10). It is important to note, however, that existing reports have raised the possibility that certain isoprenylated proteins might be methylated by other enzymatic activities, at least in some cell types. For example, Giner and Rando (11) concluded that there were distinct methyltransferase activities for the two classes of carboxyl-methylated isoprenylated proteins, the CAAX proteins and the CXC Rab proteins (11). That conclusion was based on a variety of reciprocal inhibition studies with different methyltransferase substrates and inhibitors.

The goal of the current study was to generate Icmt knockout mice, both to define the physiologic consequences of Icmt deficiency and to further define biochemical roles of Icmt. Based in part on the report of an additional methyltransferase activity (11), our a priori prediction was that Icmt-deficient mice might...
be affected less severely than the Rce1-deficient mice and might even be viable. We also predicted that Icmt-deficient cells might retain the capacity to methylate the CXC-containing Rab proteins. As outlined in this report, both of those expectations were dashed by our experimental results.

MATERIALS AND METHODS

Generation of Icmt-deficient Mice—A sequence replacement gene-targeting vector designed to replace exon 1 of the mouse Icmt gene with a neomycin-resistance gene (10) was electroporated into 129/SvJae ES cells, and targeted cells (Icmt−/−) were identified on Southern blots with 5′-flanking probe (10). Two clones, each with a single gene integration, were used to produce Icmt+/− mice. Timed matings of Icmt+/− mice were performed to assess the viability of homozygotes (Icmt−/−) at different stages of development. The genotype of each embryo was determined by Southern blot analysis (10). Icmt−/− fibroblasts were produced from mouse embryos as previously described (8).

Production of Chimeric Mice from Icmt−/− ES Cells—To assess the contribution of Icmt-deficient ES cells to different tissues, two independent lines of Icmt−/− ES cells (10) were injected into C57BL/6 blastocysts. Ten male chimeric mice were obtained; all were 35–75% chimeras as judged by coat color. At 8 weeks of age, the mice were sacrificed, and genomic DNA was purified from multiple tissues and analyzed by Southern blot with a 32P-labeled Icmt probe. The ratio of Icmt−/− to Icmt+ bands in each tissue was determined by phosphorimager.

Measurement of Icmt Activity in Embryo Lysates—Embryos were harvested and immediately placed in ice-cold buffer A (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 1 mM EDTA, 100 mM NaCl), supplemented with a protease inhibitor mixture (Complete Mini, Roche Molecular Biochemicals). The embryos were homogenized with a Polytron and then centrifuged at 500 × g for 5 min to remove debris. The protein concentration of the homogenate was determined with a Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). To measure Icmt activity, lysates (40–100 μg) were incubated with 10 μM S-adenosyl-L-[methyl-14C]methionine (55 Ci/mmol, Amersham Pharmacia Biotech) and 50 μM of either N-acetyl-S-geranylgerany1-L-cysteine (AGGC, Biomol) or N-acetyl-S-farnesyl-L-cysteine (AFC, Biomol). Recombinant farnesyl-K-Ras (7) and recombinant geranylgeranyl-Rab proteins (described below) were also tested as methyl-accepting substrates. The total volume for the methylation reaction was 50 μl. After a 30-min incubation at 37 °C, the methylation reaction was stopped by adding 50 μl of 1.0 M NaOH containing 0.1% SDS. Most of the reaction mixture (90 μl) was spotted onto a plasticized 2 × 8-cm filter paper wedge in the neck of a 20-ml scintillation vial containing 5 ml of scintillation fluid (ScintiSafe Econo 1, Fisher). The vials were capped and incubated at room temperature for 5 h to allow the [14C]methanol (formed by base hydrolysis of methyl esters) to diffuse into the scintillation fluid (4). The filter papers were then removed, and the vials were counted for radioactivity. Methyltransferase activity (pmol/mg total cell protein/min) was calculated after subtracting the background level of methylation in control reactions (lysates and S-adenosyl-L-[methyl-14C]methionine but no methylated substrates). For the Rab methylation assays, control reactions also contained Rab1A, a CC Rab protein that does not undergo carboxyl methylation (12).

Expression and Purification of Recombinant Rab Proteins—Recombinant Rab proteins, Rab escort protein (REP), and Rab geranylgeranyltransferase (RabGGTase) were purified as previously described (13–15). Briefly, bovine Rab3B, murine Rab3D, and human Rab6 were expressed as N-terminal histidine-tagged fusion proteins in Escherichia coli with pET11b (Novagen, Madison, WI) as the expression vector for Rab3B and Rab6 and pSET (Invitrogen, Carlsbad, CA) for Rab3D. The proteins were purified by affinity chromatography using a Ni2+-Sepharose resin. RabGGTase was expressed in Sf9 insect cells by coinfection with baculoviruses coding for both the α- and β-subunits and then purified by cation exchange chromatography followed by gel filtration chromatography. REP was also expressed in Sf9 cells as a C-terminal histidine-tagged fusion protein and was purified by Ni2+-Sepharose affinity chromatography.

Preparation of Germinalynoglycanlated REP-Rab Complexes—Geranylgeranylated Rab/REP complexes (REP/RabGG) were formed in vitro by incubating Rab proteins with RabGGTase, and geranylgeranylated 32P-labeled GPP (GPPG) in the presence of limiting amounts of REP (16). Rab protein (10 μg) was incubated with 2.5 μM REP1, 0.7 μM RabGGTase, and 20 μmol unlabeled GPPG (Sigma) in a 50 mM sodium HEPES buffer, pH 7.2, containing 5 mM MgCl2, 1 mM dithiothreitol, and 0.05 mM protein-grade Nonidet P-40 (Calbiochem). The 50 μl reaction was incubated at 37 °C for 45 min. The complexes were stored at 4 °C with 1 mg/ml of bovine serum albumin as a carrier protein. To determine the efficiency of geranylgeranylation, parallel reactions were performed in the presence of [3H]GPPG (1–15 Ci/mmol, PerkinElmer Life Sciences), and the incorporated [3H]GPP was measured by scintillation after filtration through glass fiber filters (15).

Northern Blot Analysis—A 282-base pair 32P-labeled Icmt cDNA probe (spanning exons 1–3 of the Icmt gene) was hybridized to a mouse multiple-tissue poly(A)+ RNA blot (CLONTECH, Palo Alto, CA); hybridization and washing were performed as described previously (7). The blot was exposed to x-ray film for 72 h at ~80 °C.

Quantification of Substrate Accumulation in Icmt−/− cells—To assess the level of methylation substrates within cells, whole-cell lysates (Icmt+/+, Icmt−/−, and Icmt−/+ embryos) were incubated with S-adenosyl-L-[methyl-14C]methionine and recombinant Ste14p (10 μg of membrane protein from Sf9 cells that overexpress yeast STE14) (7). The amount of base-labile methylation was quantified as described above.

RESULTS

Biochemical Analysis of Icmt-deficient Embryos—Icmt+/+ mice were produced from two independent lines of ES cells. Genotyping of 21-day-old offspring from Icmt+/− intercrosses revealed that about two-thirds (58 of 82) were heterozygotes, and the remainder were wild-type. Genotyping of embryos revealed that Icmt−−/+ embryos constituted 25% of the litter until embryonic day 10.5 (Fig. 1B). By E11.5, there were only 1 Icmt−−/+ embryos among 329 recovered at different time points. C, Icmt−−/+; Icmt−+; and Icmt−−− embryos at E11.5.

FIG. 1. Death of Icmt−−/+ embryos at mid-gestation. A, Southern blot of the genomic DNA from the yolk sacs of Icmt+/+, Icmt−−/+, and Icmt−−− embryos. Genomic DNA was digested with BamHI. The Southern blot was hybridized with a 5′ probe (10). The BamHI fragment in the wild-type allele is 5.0 kb, whereas it is 6.8 kb in the targeted allele. B, percentages of Icmt+/+, Icmt−−/+ ( ), and Icmt−−− ( ) embryos surviving at different time points. C, Icmt−−/+; Icmt−+; and Icmt−−− embryos at E11.5.
methyltransferase substrates in lysates from Icmt−/− embryos (i.e. an accumulation of cellular proteins that could be methylated by the yeast ortholog of Icmt, Ste14p, Fig. 3C).

An earlier study (11) concluded that distinct S-adenosylmethionine-dependent methyltransferase activities were responsible for the methylation of the CAAX and CXC groups of isoprenylated proteins. That result would predict that lysates from Icmt−/− embryos would retain the ability to methylate CXC Rab proteins. This was not the case. Lysates from Icmt−/− cells were incapable of methylating three different CXC Rab proteins, although those proteins were readily methylated by lysates from Icmt+/+ cells (Fig. 4). The importance of Rab methylation by Icmt remains obscure, but one obvious possibility is that it is important for membrane targeting. In preliminary experiments, we have used cell fractionation experiments and the expression of GFP-Rab fusions to assess the localization of Rab6 (a Golgi CXC Rab protein) in Icmt−/− and Icmt+/+ cells but did not observe noticeable differences (data not shown).

**DISCUSSION**

Icmt catalyzes the formation of a carboxyl methyl ester on the isoprenylcysteine of CAAX proteins. The methylation reaction is the last of three sequential CAAX-box modifications and the most subtle, at least from the perspective of the primary structure of the protein. Methylation changes the molecular mass of the protein by a mere 14 daltons versus several hundred for both the isoprenylation and endoprotease steps. We had predicted that Icmt-deficiency might produce a relatively mild phenotype. First, deletion of the methyltransferase gene for both the isoprenylation and endoprotease steps. We had predicted that Icmt-deficiency might produce a relatively mild phenotype. First, deletion of the methyltransferase gene from Icmt−/− embryos yielded a more severe phenotype, with most Icmt−/− embryos dying between E10.5 and E11.5. Importantly, our biochemical studies with embryo lysates did not uncover a residual or redundant Icmt-like activity, and the lysates manifested a striking increase in Ste14p substrates.

The Icmt−/− embryos probably died because Icmt-deficient cells failed to grow and contribute to the formation of various organs. Southern blots of tissues from chimeric mice generated with Icmt−/− cells revealed that Icmt-deficient cells are se-
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verely defective in their capacity to contribute to the formation of certain organs (e.g. liver and brain) although they retained the ability to contribute to the formation of others (e.g. skeletal muscle). We doubt that this finding was spurious, for several reasons. First, similar results were obtained with two lines of Icmt−/− ES cells. Second, there was a reasonably strong inverse correlation between normal levels of Icmt expression and the ability of Icmt−/− ES cells to contribute to the formation of a tissue. Third, the Icmt chimeric mouse experiments were performed in parallel with studies with Zmpste24−/− ES cells, which robustly populated all of the tissues of chimeric mice.

Why were the developmental abnormalities more severe in Icmt−/− embryos than in Rce1−/− embryos? One possibility is simply that Icmt has more substrates than Rce1. Indeed, our experiments revealed for the first time that the CXC Rab proteins (which are not processed by Rce1) are methylated by Icmt. Rab proteins terminating in CXC and CC are geranylgeranylated at both cysteines (12). The CXC Rab proteins, but not the CC Rab proteins, are then carboxyl-methylated (12, 19–23). For example, Rab3a and Rab4, which terminate in Cys-Ala-Cys and Cys-Gly-Cys, respectively, are carboxyl methylated (19, 20), whereas Rab1A and Rab2, which terminate in Cys-Cys, are not (12, 23). Interestingly, replacement of the CXC terminus of Rab3a with CC abolishes methylation, whereas the opposite result is obtained when the CC terminus of Rab1A is replaced with a CXC sequence (12).

Our current studies show that the methylation of the CXC Rab proteins is carried out by Icmt, the same methyltransferase that is responsible for CAAX protein methylation. Thus, Icmt almost certainly has more substrates than Rce1, which has no role in Rab protein processing. If these additional substrates (i.e. the Rab proteins) lie at the root of the more severe developmental defects in Icmt deficiency, one might predict that the methylation of Rab proteins has a significant impact on their function. In the case of Rab6, we did not observe a detectable effect of methylation on the intracellular localization of the protein, but we caution against overinterpreting those results. Those experiments did not assess other potential effects of Rab methylation such as effects on protein function or stability.

A second potential explanation for the more severe developmental problems in the Icmt−/− embryos is that the position-
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