Dietary Free and Esterified Cholesterol Absorption in Cholesterol Esterase (Bile Salt-stimulated Lipase) Gene-targeted Mice*

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The involvement of pancreatic cholesterol esterase (bile salt-stimulated lipase) in cholesterol absorption through the intestine has been controversial. We have addressed this issue by using homologous recombination in embryonic stem cells to produce mice lacking a functional cholesterol esterase gene. Cholesterol esterase knockout mice and their wild type counterparts were fed a bolus dose of [3H]cholesterol and a trace amount of [3-13C]cholesterol by gavage. The ratio of the two radiolabels excreted in the feces over a 24-h period was found to be similar in the control and cholesterol esterase-null mice. Similar results were observed when the radiolabeled sterols were supplied in an emulsion with phospholipid and triolein or in lipid vesicles with phosphatidylcholine. Cholesterol absorption results were similar between the control and cholesterol esterase-null mice regardless of whether the animals were fed a low fat diet or a high fat/high cholesterol diet. The rate of [3H]cholesterol appearance in the serum of the gene-targeted mice paralleled that observed in control animals. In contrast to these results, when experiments were performed with [3H]cholesteryl oleate instead of [3H]cholesterol, a higher amount of the 3H radiolabel was found excreted in feces and dramatically less of the radiolabel was detected in the serum of the cholesterol esterase-null mice in comparison with that detected in control animals. Serum cholesterol levels were not significantly different between control and cholesterol esterase-null mice fed either control or an atherogenic diet. These results indicate that cholesterol esterase is responsible for mediating intestinal absorption of choleseryl esters but does not play a primary role in free cholesterol absorption.

Cholesterol esterase, also called bile salt-stimulated lipase or carboxyl ester lipase (abbreviated as CEL), is a lipolytic enzyme capable of hydrolyzing triacylglycerol, phospholipid, lysophospholipid, and cholesteryl esters. The enzyme is synthesized in the acinar cells of the pancreas and is stored in zymogen granules. Cholesterol esterase is released into the intestinal lumen upon food ingestion and constitutes 1–2% of total protein in pancreatic juice (1).

While the high concentration of CEL in pancreatic juice suggests that it may play a role in mediating nutrient absorption, the precise physiologic function of the enzyme remains controversial. Early studies with isolated intestinal cells suggested a role for CEL in dietary cholesterol absorption (2). However, subsequent studies yielded contradictory results. For example, using pancreatic diverted rats, Watt and Simmonds (3) showed normal absorption and esterification of cholesterol. In contrast, using the same experimental system, Gallo et al. (4) showed an 80% reduction in cholesterol absorption, which could be restored by infusion of pancreatic juice containing CEL but not by juice depleted of the enzyme.

Cholesterol absorption has also been studied using a variety of inhibitors. Bennett Clark and Tercyak (5) demonstrated a reduction in cholesterol transmucosal transport in rats with inhibited acyl CoA:cholesterol acyltransferase and normal pancreatic function, which suggested that acyl CoA:cholesterol acyltransferase, and not CEL, was responsible for this process. However, using similar inhibitors, Gallo et al. (6) showed no inhibition of cholesterol absorption, which again suggested the involvement of CEL. In later studies, CEL inhibitors, such as the phenoxyphenyl carbamates WAY-121,751 and WAY-121,898, were shown to be effective inhibitors of cholesterol absorption in normal and cholesterol-fed rats and dogs (7). Thus, whole animal studies have not consistently shown the importance of CEL in cholesterol absorption.

The possible role of CEL in mediating intestinal absorption of cholesterol has also been investigated in vitro without resolution. Bhat and Brockman (8) showed that incubation of rat intestinal sacs with cholesterol-containing micelles in the presence of CEL resulted in a 3–5-fold enhancement of intracellular cholesterol and cholesteryl ester accumulation compared with intestinal sacs incubated in the absence of the enzyme. More recently, Lange and colleagues, using Caco-2 cells as a model for intestinal epithelium, showed that CEL addition was necessary for the transfer of exogenous cholesterol to a “physiologically important pool” that could be esterified and assembled into lipoproteins (9). In contrast to these results, our laboratory could not demonstrate CEL-mediated uptake of unesterified cholesterol by Caco-2 cells (10). Our in vitro data were confirmed and extended in a recent publication by Fisher and colleagues (11). Both laboratories reported that the enzyme was only effective in facilitating cellular uptake of esterified cholesterol.

In an attempt to resolve this controversy, we have used the approach of gene targeting in embryonic stem (ES) cells to produce mice lacking in CEL. The CEL(−/−) mice provide a unique in vivo model to assess the physiological function of the bile salt-stimulated cholesterol esterase.

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‡Abbreviations used are: CEL, cholesterol esterase (carboxyl ester lipase or bile salt-stimulated lipase); ES cells, embryonic stem cells; kb, kilobase pair; bp, base pair; neo, neomycin resistance gene; FC, free cholesterol; CE, cholesteryl ester; PC, phosphatidylcholine; TG, triglyceride.
Cholesterol Esterase Knockout Mice

Experimental Procedures

Cloning of the Mouse Cholesterol Esterase Gene and Production of the Targeting Vector—A strain 129 mouse genomic library made in λ-DASH phage vector was obtained from Dr. Thomas Doetschman at our institution and used to isolate the mouse CEL gene. The 2-kb full-length rat cholesterol esterase cDNA (12) was used as the probe for screening the library. A positive clone that also hybridized with probes corresponded to both the 5′-flanking region and the 3′-flanking region of the rat CEL gene was selected for further characterization. Restriction mapping, Southern hybridization with various cholesterol esterase cDNA fragments, and partial nucleotide sequencing were performed to determine the intron and exon locations of the mouse CEL gene.

A 4.7-kb Sad DNA fragment, encoding sequences from 540 bp upstream of exon 1 to intron 7 of the mouse cholesterol esterase gene, was subcloned into a similarly-digested PTZ18U plasmid. A 1.7-kb frag-ment containing a thymidine kinase promoter-driven neomycin resistance gene (neoR) was isolated from SspI/HindI-digested pcDNA3neo (Stratagene) and subcloned into the unique BalI site in exon 4 of the 4.7-kb Sad clone (Fig. 1). A plasmid containing neoR inserted in the same orientation as the CEL gene was selected for the gene-targeting experiment. After CsCl purification, the targeting vector was digested with Sad, and the Sad DNA fragment containing the disrupted CEL gene sequence was purified by agarose gel electrophoresis.

Targeted Disruption of the Cholesterol Esterase Gene in ES Cells—Gene targeting experiments were performed using the R1 ES cell line derived from the 129 mouse strain by Nagy et al. (13). Cells were grown and passaged as described (14). When ES cells were grown at high densities, the medium was supplemented with isoenzyme inhibitory factor T9 (8). On the 3rd or 4th day of the expansion and freezing method, originally described by Zilversmit and Hughes (27) and Smithies (22). Primer concentrations were each 2.5 μM.

Gene targeting experiments were performed using the R1 ES cell line (Stratagene) and subcloned into the unique SacI site in exon 4 of the CEL gene was selected for the gene-targeting experiment. After CsCl purification, the targeting vector was digested with Sad, and the Sad DNA fragment containing the disrupted CEL gene sequence was purified by agarose gel electrophoresis.

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PCR products were amplified from mouse genomic DNA with the following primers: 5′-TTTCCCTTACTGCTTTCAG-3′, GGTATCCGCTGCTCTTACAGTC-3′, and the downstream CEL primer, 5′-TCACTATTCCCGCTTACAGTC-3′, amplify a 244-bp fragment from the wild type exon 4 but do not amplify the targeted allele because of the insertion of neoR between their cognate sequences. Identical conditions were used for both primer sets in two separate reactions. A positive result was obtained with both primer sets indicating both wild-type and positive results with both sets indicated a heterozygote, and a positive result with the neoR primers only was scored as a homozygous knockout.

Cholesterol Esterase Knockout Mice—The single-dose, dual-isotope feeding method, originally described by Zilversmit and Hughes (27) and validated for measuring cholesterol absorption in mouse by Duedal et al. (28), was used to determine cholesterol absorption efficiency in control and CEL gene-targeted mice. Test meal was prepared either as lipid emulsion or as sonicated vesicles. Emulsified substrate was prepared by sonication in a Bransonic-32 bath sonicator. When cholesteryl ester absorption was being studied, the mix also included 0.2 mg of unlabeled cholesteryl oleate. For some of the cholesterol esterase studies, the emulsion consisted of 50 μl of [1H]cholesteryl oleate, 1 μl of [125I]cholesteryl oleate, 5 μl of cholesterol, and either 5 or 35 μl of phosphatidylcholine in 1 ml of water for 5 min of a Heidolph mixer. The cholesterol absorption media was analyzed on 10% SDS-polyacrylamide gel (23). The electrophoresed samples were either stained with Coomassie Blue or transferred to nitrocellulose (24) for immunoblotting with affinity-purified rabbit anti-rat cholesterol esterase and [3H]-labeled antibody (Amersham Corp.) as described (25). CEL activity was determined as described (26) using 0.1 μM of the above supernatant, 15 mm bile salt, and 10 μM cholesteryl [14C]oleate (30 μCi/μmol).

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FIG. 1. Diagram of the mouse cholesterol esterase gene and targeting construct. Panel A, partial restriction map and exon/intron arrangement of the mouse CEL gene. Exons are indicated by boxes. Panel B, construct used for targeting. nea<sup>0</sup> (shaded box) was inserted into the Ball site in exon 4 of the 4.7-kb SacI fragment. Panel C, PstI/SalI 5' probe used for screening ES colonies and mice for homologous recombination. Panel D, BglII/Sall 3' probe used for screening ES colonies and mice for homologous recombination. Restriction enzymes indicated are as follows: Ball (B), EcoRI (E), HindIII (H), NcoI (N), SphI (P), SacI (S), and Xbal (X). Arrows indicate the approximate positions of the primers used for polymerase chain reaction analysis of mice.

...tests using SigmaStat software from Jandel Corporation.

Sera for Cholesterol Analysis—Blood was collected from euthanized animals by severing the inferior vena cava. Samples were allowed to clot on ice, and serum was collected after centrifugation. Total cholesterol levels were determined using commercially available enzymatic kits from Sigma.

RESULTS

Targeting of the Cholesterol Esterase Gene in Mouse Embryonic Stem Cells—Restriction mapping and Southern blot analysis revealed that the mouse cholesterol esterase gene is highly homologous to the rat CEL gene and contains 11 exons interrupted by 10 introns (30). A 4.7-kb SacI fragment that contains exon 1 to intron 7 was used to construct the targeting DNA as described under "Experimental Procedures" and schematically outlined in Fig. 1B. The insertion of nea<sup>0</sup> into exon 4 of the CEL gene disrupts the CEL coding sequence immediately preceding the active site domains of cholesterol esterase (25, 30). Additionally, any truncated polypeptide derived from the disrupted CEL gene could not be secreted by the pancreas due to the absence of the exon 11 domain, which is important for this function (31). Thus, homologous recombination of the endogenous CEL gene with this targeting construct will provide an animal with a CEL-null phenotype.

The electroporation of 5.5 × 10<sup>5</sup> mouse ES cells with the targeting DNA resulted in approximately 4,800 G418-resistant colonies. One-fourth of the colonies were picked and expanded individually in 24-well dishes. A total of 268 colonies were selected for Southern blot analysis to screen for homologous recombination between the targeting DNA and the resident CEL gene. For the initial screening, ES colony DNA was digested with EcoRI and hybridized with an −1100-bp PstI/SalI DNA fragment corresponding to genomic sequence 5' from the targeting DNA (Fig. 1C). As shown in Fig. 2A, the wild-type allele gives rise to a fragment >30 kb in length, while a correctly targeted gene yields a fragment 6.5 kb in length due to the insertion of two EcoRI sites present in the thymidine kinase promoter of nea<sup>0</sup> (Fig. 1B). To confirm that the putative targeting events had taken place as planned, additional aliquots of the ES colony DNA were digested with Xbal and hybridized with an −1100-bp BglII/SalI DNA fragment corresponding to sequences 3' from the targeting DNA (Fig. 1D). Fig. 2B shows that the wild-type allele yields a 7.2-kb fragment, while the correctly targeted allele yields a 9.0-kb fragment due to the insertion of 1.75 kb of DNA corresponding to the selectable marker cassette. Of the 268 colonies screened, 11 were positive in both tests. The overall targeting efficiency was 4.4%.

Site-specific integration of the targeting DNA at the CEL locus was confirmed by additional Southern blot analysis with both the 5'- and 3'-flanking probes. The addition of the 1.75-kb nea<sup>0</sup> cassette to the endogenous CEL gene resulted in an 8.3-kb HindIII fragment that hybridized with the 5' probe in addition to the 6.5-kb band observed for the controls (Fig. 2A). Using the 3' probe, a 7.5-kb SphI band resulted from the insertion of an SphI site present in the nea<sup>0</sup> gene in targeted clones in addition to the 18-kb SphI band observed for the wild-type allele (Fig. 2B). These hybridization patterns were consistent with those predicted for the site-specific insertion of the nea<sup>0</sup> cassette into exon 4 of the endogenous CEL gene (Fig. 1). A total of eight enzymes, informative with either the 5' or 3' probe, were used to confirm that the gene targeting had occurred as planned. In addition, a nea<sup>0</sup>-specific probe was used to confirm that the targeting DNA had inserted in only one site in the genome (data not shown).

Two of the 11 cell lines with proper CEL gene targeting were used to generate chimeric mice. One cell line yielded only one chimeric mouse, which was female and had only ~5% agouti coat color. However, the second cell line produced 22 chimeric mice (from 119 injected and reimplanted blastocysts), all with extensive agouti coat color. Nineteen of these were male, and 15 of the 19 were able to transmit the modified gene to their offspring. Progeny from these test matings (chimerics × Black Swiss), which carried the modified allele, were bred to generate homozygous knockout animals. Southern blot analysis of the genomic DNA from representative wild type, heterozygous, and homozygous CEL-targeted mice is shown in Fig. 3. Because of an apparent restriction fragment length polymorphism between the ES cells and the outbred mice used in the initial breeding, Xbal was not informative, and NcoI was used as a diagnostic enzyme for the 3' end of the recombination. This enzyme yields a 9.5-kb fragment from the wild-type allele and a 6.5-kb fragment from the targeted allele due to the insertion of the NcoI site in the nea<sup>0</sup> gene (see Fig. 1 for details).

General Characteristics of CEL Gene-targeted Mice—The targeted allele was transmitted by the chimeric males at approximately the expected frequency (40%, 39 CEL+/− mice of 97 agouti pups), indicating no obvious disadvantage to embryos or...
neonates heterozygous at the CEL locus. Furthermore, the crosses between these CEL(+/−) animals yielded progeny with the expected 1:2:1 ratio of wild-type to heterozygous to homozygous knockout genotypes (37:89:40). Both homozygous and heterozygous CEL gene-targeted mice grew normally and appeared to be healthy by inspection. The serum cholesterol levels for CEL(−/−) mice fed control diet (104.1 ± 3.33 mg/dl) or 24 weeks of atherogenic diet (356 mg/dl ± 21.6) were not significantly different (p = 0.2420) from those of their wild type littermates (114.9 ± 6.76 and 399 ± 17.6 mg/dl).

To verify that the gene targeting abolished expression of CEL protein, pancreatic homogenates from control, heterozygous, and homozygous CEL gene-targeted mice were examined for CEL expression using immunoblotting techniques (Fig. 4). The levels of CEL protein in pancreatic extracts of the heterozygous animals were approximately half those of the wild type mice. No CEL protein was detected in homogenates of the CEL(−/−) mice. Furthermore, no CEL-immunoreactive polypeptides of any size were detected, indicating that no fusion protein or truncated protein was being produced as a result of the modified gene. These extracts were also assayed for cholesteryl ester hydrolytic activity. Table I shows that cholesteryl oleate hydrolysis is reduced 98% in the pancreatic extracts of CEL(−/−) animals.

Cholesterol Absorption Studies—The role of CEL in mediating absorption of dietary cholesterol and cholesteryl esters was assessed by comparing the amount of radiolabeled sterol excreted in the feces after its infusion into the stomach of normal and homozygous CEL gene-targeted mice. Since a role for CEL in the absorption of cholesteryl esters is not in dispute, the initial study used [3H]cholesteryl oleate in a lipid emulsion with cholesterol, tridecyn, and phospholipid as the substrate. A trace amount of β-[14C]sitosterol was added as a marker to normalize the amount of nonabsorbed sterol recovered in the

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**Table I**

| CEL genotype | Cholesteryl ester hydrolyzed |
|--------------|-----------------------------|
|              | No bile salt | Taurocholate | Deoxycholate |
| ++           | 7.8          | 2240         | 427.6        |
| ++/−         | ND           | 2281         | 421.5        |
| −/−          | 19.4         | 37.5         | 1.5          |
Cholesterol Esterase Knockout Mice

The results showed that absorption of the cholesteryl ester was reduced in the CEL(−/−) mice to ~35% of the wild type value (Table II). The experiment was performed using emulsions with either normal (2:0.2:8:50, FC:CE:PC:TG, w/w/w/w) or low (10:1:80:5) triglyceride content. Similar results were obtained in both tests, indicating that the triglyceride content of the test meal had only a limited effect. The absorption of cholesteryl ester in heterozygous animals was found to be similar to wild type despite the reduced CEL protein level in their pancreas.

In contrast to the cholesteryl ester result, when unesterified [3H]cholesterol was included in the emulsion instead of the cholesteryl oleate, no significant difference was found between wild type and CEL-null mice in absorption of the radiolabeled sterol (Table III). Interestingly, males were found to absorb significantly less (59.6 ± 3.05%) cholesterol than females (72.0 ± 1.61%), but this difference was independent of their CEL genotype. For clarity, results from male and female mice are combined in Table III.

The presence of cholesteryl ester in the core of an emulsion particle has been shown to increase the partition of free cholesterol from the surface to the core (32). Our results show that CEL is necessary for digestion of this core cholesteryl ester. In the absence of CEL, the free cholesterol may remain sequestered and unabsorbed. To test this possibility, animals were fed [3H]cholesterol in an emulsion that contained unlabelled cholesteryl ester along with phospholipid and triglyceride. Table III also shows that the presence of the cholesteryl ester in the core had no effect on the ability of CEL(+/+) mice to absorb the free cholesterol.

Published literature indicates that dietary cholesterol and biliary cholesterol may be absorbed from the intestine by different mechanisms (33). Experiments were undertaken to determine the ability of wild type and CEL gene-targeted mice to absorb unesterified cholesterol presented in a vesicular complex with phospholipids, similar to that present in the biliary tract. [β-14C]Sitosterol was used as a marker of recovery as described above. The results showed that, regardless of the ratio of cholesterol to phospholipid used to prepare the lipid vesicles, there was no significant difference between the ratios of [3H]cholesterol to [β-14C]sitosterol recovered in the feces of wild type versus CEL gene-targeted mice (Table III).

The report that phenoxyphenyl carbamate inhibitors of CEL result in delayed absorption of cholesterol (7) prompted additional experiments to compare the rate at which the radiolabel from cholesterol and cholesteryl esters appears in the serum of control and CEL gene-targeted mice. In these experiments, the amount of [3H] in 15 μl of serum was determined at various times after gastric infusion of a radiolabeled sterol. The infusion of the unesterified [3H]cholesterol resulted in the progressive appearance of the radiolabel in the serum of both wild type and CEL gene-targeted mice with a maximum at ~10 h (Fig. 5) and a slow decline thereafter. No significant difference in the rate of radiolabeled cholesterol appearance in the serum was observed between the two groups of animals in this case. In contrast, when the radiolabel was supplied as emulsified [3H]cholesterol oleate, the serum level of the radiolabel after 12 h was ~8-fold higher in the CEL(+/+) mice than in the CEL-null mice. In fact, very little radiolabel was detected in the serum of the gene-targeted mice (Fig. 5).

To examine the possibility that CEL plays a role in cholesterol absorption when mice are fed a high fat, high cholesterol, atherogenic diet, we studied the absorption of free and esterified cholesterol in wild type and CEL-null mice fed this diet for 6 weeks. As shown in Table IV, cholesteryl oleate absorption was reduced in the CEL-null mice, while free cholesterol was absorbed similarly by the wild type and CEL gene-targeted mice. The percentage of cholesterol absorbed was decreased relative to normal diet in both the free cholesterol and cholesteryl ester experiments due to the high level of cholesterol in the atherogenic diet. In fact the total mass of absorbed cholesterol is increased.

**DISCUSSION**

The results of the current study show that disruption of the CEL gene has no significant effect on the ability of mice to absorb unesterified cholesterol from the gastrointestinal tract. Similar results were observed regardless of the physical characteristics of the substrate or the dietary conditions of the animals. Furthermore, similar results were observed when cholesterol absorption was determined based on the amount of nonabsorbed cholesterol present in the feces or on the appearance of the radiolabeled cholesterol in the serum. These observations demonstrate that CEL is not necessary for cholesterol flux across the intestinal epithelium. In contrast to its effect on unesterified cholesterol absorption this study shows that CEL...
rent results are also in agreement with those of Watt and Simmonds (3), which showed that unesteri-
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fied cholesterol in CEL-null mice, resolves the discrepancy in hydrophobic interactions.

membranes or lipid vesicles of the intestinal epithelium due to that our serum assay was insufficiently sensitive. Alterna-
a minor pathway for absorption of these nutrients exists and base line (Fig. 5). One explanation for this discrepancy is that lesteryl ester-fed, CEL-null mice was not consistent with this

showed no CEL requirement for unesterified cholesterol up-
take by Caco-2 cells reported by Lopez-Candales et al. (9), while intriguing, did not reflect physiologically relevant levels of cho-

is necessary for intestinal absorption of esterified cholesterol. However, since cholesteryl esters constitute only a small frac-
tion of total cholesterol in the diet and are absent from the bile (34), these results strongly suggest that CEL does not play a determining role in the absorption of either dietary or biliary cholesterol.

Although CEL does not appear to be essential for intestinal absorption of unesterified cholesterol, results of this study demonstrate unequivocally that CEL plays a primary role in absorption of cholesteryl esters. These results are consistent with in vitro studies from this laboratory and others that showed a role of CEL in facilitating the uptake of esterified cholesterol but not unesterified cholesterol by intestinal cells (10, 11). Curiously, our fecal sterol experiments indicate a base-line level of cholesteryl ester absorption that is independent of CEL (Table II). However, pancreatic extracts from CEL(−/−) mice lack significant esterolytic activity (Table I). Also, the amount of radiolabel appearing in the serum of cho-

Although our data show that CEL does not participate in free cholesterol absorption, the wide range of absorption values (37–87%) as well as additional studies with inbred strains of mice support the hypothesis that cholesterol absorption is reg-
bulated by at least one gene.2 The recent report by Kirk et al. (37) on the responsiveness of different strains of mice to dietary fat and cholesterol with respect to cholesterol absorption and serum lipid parameters also supports this hypothesis. Candidate genes potentially involved in this process include the cholesterol transfer protein (38), which may be the same as, or closely related to, sterol carrier protein-2 (39, 40), acyl CoA: cholesterol acyltransferase (5, 41), pancreatic lipase (36), and liver fatty acid binding protein (42). Additional experiments are necessary to investigate the physiological role of these and other proteins in cholesterol absorption.

Although CEL does not play a primary role in free cholesterol absorption, its abundance in the intestinal lumen suggests that it may play a different role in the absorption of lipid-based nutrients. This enzyme may complement other lipolytic en-
zymes to increase the efficiency of dietary fat absorption by the small intestine. For example, CEL has been shown to be more efficient than pancreatic lipase in the hydrolysis of long chain

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polyene fatty acids (43, 44). CEL is also capable of hydrolyzing phospholipids and lysophospholipids (45) and thus may play a role in the assimilation of dietary phospholipids. More importantly, a pancreas-derived, vitamin-ester hydrolytic activity has been ascribed to CEL (46, 47), suggesting its importance in the absorption of fat-soluble vitamins, which are primarily esterified in dietary sources. The presence of CEL in the milk of many mammalian species has led to the proposal that the milk CEL is critically important for digestion of milk triglycerides, the major source of energy in infants, before the maturation of the pancreas (48). Finally, in addition to its presence in the gastrointestinal tract, CEL is also found to be present in the milk of many mammalian species, where its level is correlated to that of serum cholesterol (51), where its level is correlated to that of serum cholesterol (52). Thus, CEL may play a role in the modulation of lipoprotein structure and metabolism. The knockout mice described in this report will provide a useful tool to address the role of CEL in various aspects of lipid absorption and metabolism.

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