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Molecular Cloning and Characterization of a Novel Putative Carboxylesterase, Present in Human Intestine and Liver

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A full-length cDNA coding for a putative intestinal carboxylesterase (iCE) was isolated from a human small intestine cDNA library. The cDNA has an open reading frame of 559 amino acids with up to 65 % homology to other carboxylesterases of different mammalian species. The deduced amino-acid sequence contains many structural features, that are highly conserved among all carboxylesterase isoenzymes, like the serine esterase active site, an ER-retention signal and one Asn-Xxx-Thr site for N-linked carbohydrate addition. Northern blot analysis revealed that the corresponding mRNA is 3.4–3.6 kb in size and is preferentially expressed in human intestine with a weak signal also in liver. Analysis of cells from the gastrointestinal tract unveiled site-specific, transcriptional regulation of iCE, with higher expression in small intestine and lower expression in colon and rectum. The high expression in small intestine is attributable to a higher expression in jejunum compared to duodenum and ileum.

Carboxylesterases (E.C. 3.1.1.1) are a group of serine esterases that are found in a wide range of tissues and organisms. They hydrolyze many xenobiotics, such as carboxyl esters, thioesters and aromatic amides, and they are involved in the detoxification and activation of ester and amide prodrugs (1,2). Some carboxylesterase isoenzymes (3,4) have been purified and shown to differ in biochemical, immunological, and genetic properties. Therefore, mammalian carboxylesterases are now considered to constitute a family of isoenzymes (5). The physiological role of these enzymes is still elusive, although their involvement in the hydrolysis of several lipids, such as acylcarnitines and palmitoyl-CoA, has been documented (2). Some members of the carboxylesterase/serine esterase multigene family, like the bile salt stimulated lipase (BSSL), or the acid cholesteryl ester hydrolase (ACEH) catalyze the hydrolysis of cholesteryl esters (6). Investigations from several laboratories have implicated a role of the BSSL in mediating cholesterol absorption in the gut (7). Involvement in the esterification of cholesterol within the villus cell has also been demonstrated for BSSL (8,9,10). Another member of the carboxylesterase multigene family has been shown to play a role in human cellular cholesterol homeostasis (11).

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MATERIALS AND METHODS

cDNA cloning and sequencing. A human placental λgt 11 cDNA library (Clontech Laboratories Inc., Palo Alto, CA) was screened using the ^{32}P-labeled human carboxylesterase coding sequence (18) as described previously (22). Positive recombinant phage plaques were purified and phage DNA was isolated using the Qiagen Lambda kit (Qiagen, Chatsworth, CA, USA). DNA inserts were obtained by digestion with *Eco*RI and subcloned into a pUC 18 vector. Nucleotide sequences were determined by the dideoxy chain-termination method (23) with T7 DNA polymerase and fluorescent dye-labeled primer using an automated DNA sequencer (Pharmacia Biotech, Freiburg, Germany).

5′-RACE (rapid amplification of cDNA ends) PCR. In order to identify the 5′end of the carboxylesterase gene, the 5′-RACE-Ready cDNA prepared from human intestine poly(A)$^+$ RNA (Clontech, Palo Alto, CA, USA) was amplified according to the instructions of the manufacturer. A primary PCR reaction was conducted with the provided anchor primer and the gene specific primer iCE1 (5′ GCTGAGGTACAGGCAGTCCTCAGAC 3′) complemen-tary to nucleotide positions +358 to +389 with respect to the ATG codon. An aliquot of the initial PCR reaction served as template in a secondary PCR reaction (nested PCR) with the anchor primer iCE2 (5′ GCTAAGAAACTCTGACTCCACTGCG 3′) matching bases +300 to +324 with respect to the ATG codon, resulting in a 402 bp amplification product. An additional 5′-RACE PCR was performed using primer iCE3 (5′

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CCCTCACACCACTCCAAGATTCAGG 3′) resulting in a 337 bp PCR product. Both PCR products were analyzed on a 1.4 % agarose gel, blotted onto nylon membranes and hybridized with primer iCE4 (5′ GCGGTGGCCTGTGGGCTTCTGC 3′) (data not shown). The verified DNA fragments were cloned in pUC18 and sequenced.

Northern blot analysis. Ten micrograms each of total RNA from the human mucosa of different intestinal tissues and other sources (CLONTECH, Palo Alto, CA, USA) were denaturated in formamide and formaldehyde at 65 °C for 5 min and electrophoresed in an agarose gel containing formaldehyde. RNA was then blotted onto Hybond Nylon membranes (Amersham, Buckinghamshire, England), and hybridized with a full-length cDNA probe labeled with α^{32}P-dCTP using a random priming kit (Amersham). The membranes were washed in 2 × SSC-0.1% SDS, then in 0.1 × SSC-0.1% SDS at 65 °C for 15 min each and visualized by autoradiography with Kodak XR5 film at −80 °C with an intensifying screen.

RESULTS AND DISCUSSION

Library screening. A λgt11 cDNA library, constructed from human small intestine, was screened by plaque hybridization using a 520-bp *Pst*I fragment from a human carboxylesterase cDNA (9), isolated from human liver. From 1×10^6 plaques tested, 18 positive clones were isolated and the insert recovered by PCR amplification. The largest cDNA inserts were subcloned into pUC18 and sequenced with T7 DNA polymerase and fluorescent dye-labeled primer using an automated DNA sequencer (ALF, Pharmacia).

Sequencing of full-length cDNA. In order to obtain the full-length cDNA sequence of the iCE mRNA, a 5′-RACE-Marathon-Ready cDNA (Clontech, Palo Alto), prepared from human small intestine poly $(A)^+$ RNA, was amplified by 5′-RACE according to the instructions of the manufacturer (Clontech). A 402-bp long 5′-RACE PCR product, that overlapped the cDNA by 112 bp, was obtained using a nested PCR reaction with 2 iCE specific primers (iCE1, iCE2) from the 5′end of the iCE cDNA. The origin of the PCR product was verified through positive hybridization with an oligonucleotide (iCE4) preceding the primers used for RACE-PCR. Cloning and se-

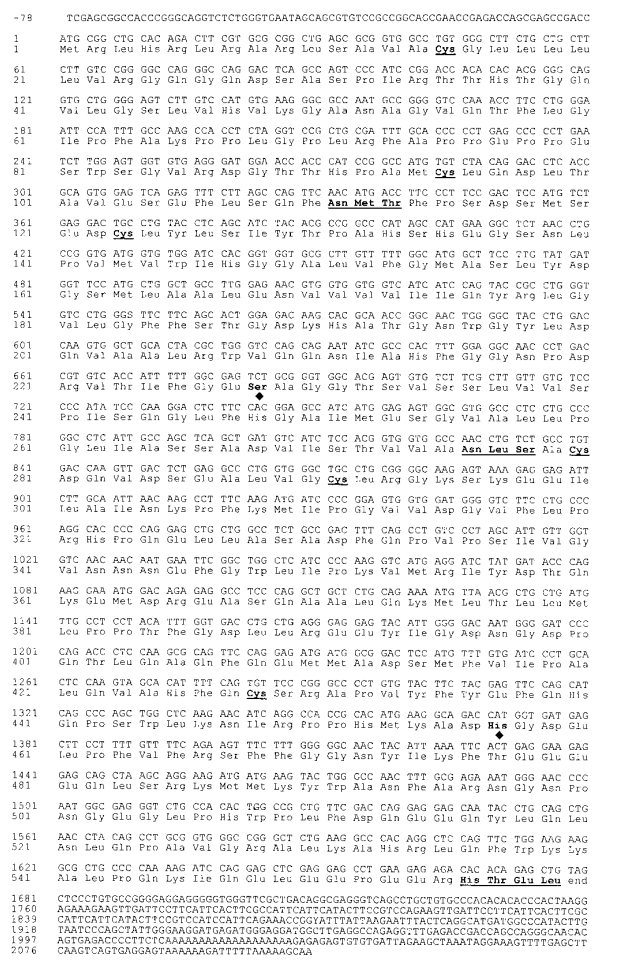

FIG. 1. The human intestine cDNA sequence (iCE) and the deduced amino acid sequence are shown. Translation starts at position 1 and terminates at position 1677. A putative signal peptide is indicated by broken underscore. Serine and histidine residues presumed to be part of the active site are labeled (♦). Potential glycosylation sites, cysteine residues and the C-terminal retention signal are indicated by boldface underscoring.

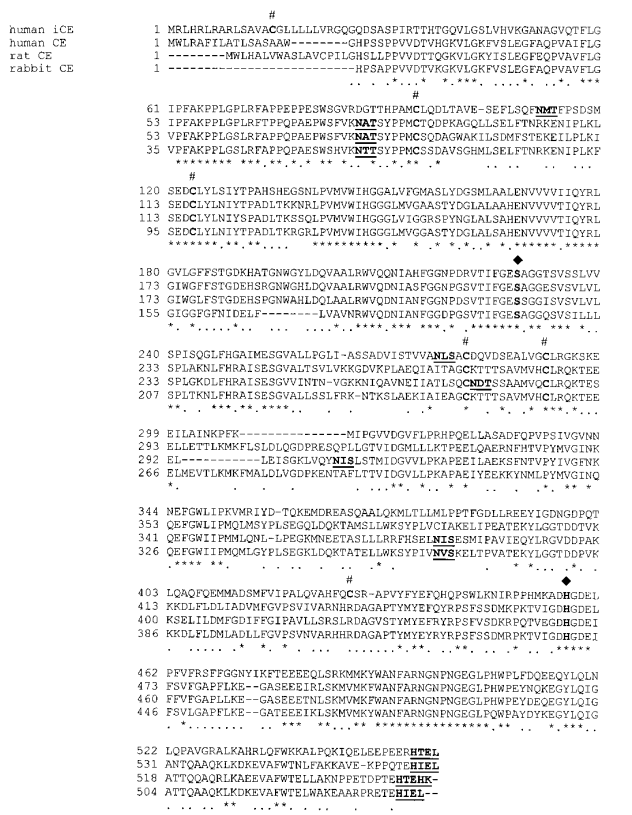

FIG. 2. Amino acid sequence comparison between the human iCE, rat E1 (17), rabbit CE (16) and human carboxylesterases (15,18). Alignment was carried out using MULTALIGN from the HUSAR package (DKF2, Heidelberg). An asterisk indicates identity of all carboxylesterases; a dot shows homology in three members of the comparison. Potential glycosylation sites and the C-terminal retention signal are indicated by boldface underscoring. Serine and histidine residues presumed to be part of the active site are labeled (♦). Five cysteine residues are indicated (#).

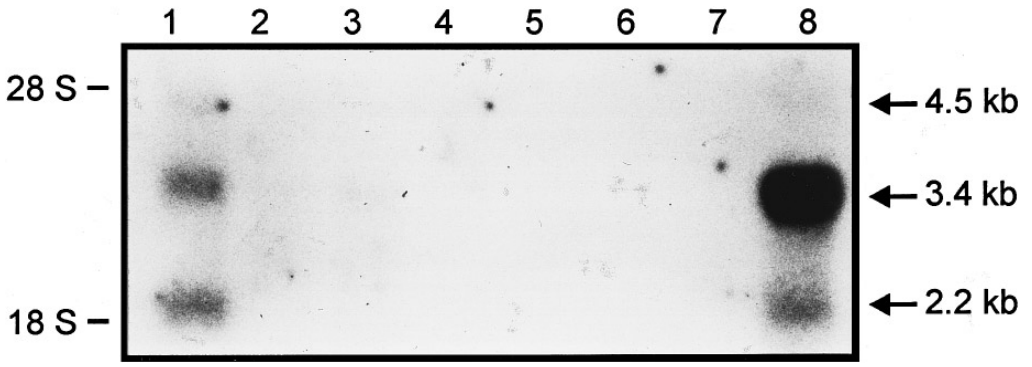

FIG. 3. Northern blot analysis of human liver (1), lung (2), peritoneal cells (3), synovial cells (4), Caco-2 cells (5), monocytes (6), monocytes (cultured for 7 days) (7) and small intestine(8) RNA with cDNA of human iCE. Total RNA (10 μg) was electrophoresed on agarose gels, transferred to nylon membranes and hybridized to the ^{32}P-labeled full-length iCE cDNA probe.

quencing revealed that transcription starts 78 nucleotides upstream of the presumed ATG start codon. This transcription start point was also verified by an independent 5′-RACE-PCR with oligonucleotides derived from sequences downstream of iCE1 and iCE2.

The nucleotide and deduced aminoacid sequence of iCE is shown in Fig. 1. The cDNA is 2154 base pairs (bp) long with an open reading frame of 1677 bp encoding a polypeptide of 559 amino acid residues. The putative start codon is preceded by a 78 bp long 5′-UTR. The nucleotide sequence surrounding the first ATG codon is in agreement with the Kozak consensus sequence (12) for functional initiation codons often found in eucaryotic mRNAs (11). The cDNA contains 6 Cys residues with one of them being part of the presumed signal peptide. Cysteines are known to be conserved in carboxylesterases and presumably form disulfide bonds. In addition, two Asn residues at position 111 and 276 in the N-X-S/T motif, which is known to be a putative N-glycosylation site (12), are present. The Ser at 228 in the G-X-S-X-G motif, which is common to the active site of the serine esterase family (13), is believed to be part of the active site of the iCE (14). A tetrapeptide at the C-terminus (His-Thr-Glu-Leu) very likely represents the ER-retention signal.

Amino acid sequence comparison. Shibata et al. (15) classified carboxylesterases into 3 groups, according to the C-terminal tetrapeptide and the presence or absence of a peptide sequence encoded by exon 8 of the human carboxylesterase gene. Fig. 2 shows an alignment of the amino acid sequence of the intestinal carboxylesterase with those of three carboxylesterases that belong to 3 distinct groups. Rabbit CE (16) for group I, rat E1 (17) for group II and human carboxylesterases (15,18) for group III. The protein sequence of the human iCE showed 62.9 %, 65.6 % and 65.1 % homology to the group I, II and III carboxylesterases, respectively. The serine 228 in the pentapeptide G-X-S-X-G and the histidine 457 that constitute the active site, are conserved in iCE. In addition, the four cysteines involved in disulfide bonds in carboxyl-esterases are also conserved in iCE. The C-termini of the human intestinal, liver and rabbit carboxylesterases contain the identical H-X-E-L tetrapeptide, a signal for protein retention at the luminal side of endoplasmatic reticulum (19, 20). The C-terminal protein sequences of group II enzymes have conserved H-T-E-H-X amino acid residues which are believed to be relevant for secretion (19, 21). All of the carboxylesterases have N-X-T/S sites for N-linked carbohydrate addition.

Tissue distribution of iCE mRNA. In order to determine the tissue distribution of iCE mRNA expression, Northern blot analysis was performed at a selected set of RNAs. Equal amounts of total RNA isolated from human intestine (mucosa), lung, liver, synovial cells, peritoneal cells, Caco-2 cells, monocytes and monocytes cultured for 7 days, were separated by gel electrophoresis, blotted and hybridized with a ^{32}P-labeled iCE cDNA probe. As seen in Fig. 3, the iCE mRNA is moderately expressed in liver and highly expressed in intestine. Three mRNA species of different size (3.4 kb, 4.5 kb and 2.2 kb) were identified. Whether these forms originate because of the use of different transcription start points, or are the result of alternative splicing, is still elusive. It is also conceivable that, because of the relatively high homology among the carboxylesterases, the hybridization signals may reflect cross-hybridization with so far uncharacterized isoenzymes. Overexpression and purification of the iCE will enable the characterization of functional specificities as well as the analysis of tissue distribution using antibodies raised against the protein, in comparison to other carboxylesterases.

In order to elucidate the physiological functions of iCE , Northern blot analysis using RNAs from different intestinal tissues was performed (Fig. 4). The expression of iCE mRNA in healthy tissues of the small intestine, colon and rectum revealed high expression in

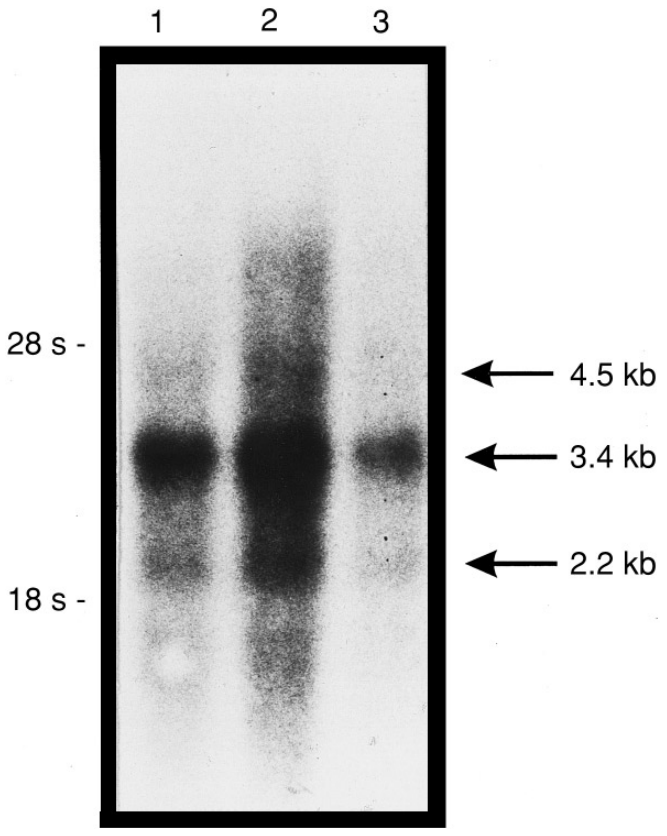

FIG. 4. Northern blot analysis of different gastrointestinal tissues. Each lane was loaded with 10 μg of total RNA isolated from enterocytes of the mucosa of a healthy individual (duodenum, lane 1; jejunum, lane 2; ileum, lane 3). The RNA was extracted with guanidinium-thiocyanate buffer and purified by CsCl centrifugation, separated on a denaturing gel, transferred to Gene Screen plus membranes (New England Nuclear, Boston, MA 02118) and probed with a ^{32}P-labeled full length iCE cDNA.

small intestine and low expression in rectum (data not shown). The higher expression in small intestine is mainly attributable to a higher expression in the jejunal region (Fig. 4, lane 2), compared to a moderate expression in duodenum (Fig. 4, lane 1) and low expression in ileum (Fig. 4, lane 3). These data possibly indicate that iCE may be important for xenobiotic control and detoxification of the intestinal mucosa. Whether the iCE expression pattern responds to certain gastrointestinal inflammatory diseases, in particular of the colon (Ulcerative Colitis and Crohnś disease), thus indicating an involvement in the xenobiotic control and detoxification of the intestinal mucosa, is currently under investigation.

We have cloned and characterized a new member of the carboxylesterase family that is expressed in human intestine and liver. Overexpression and purification of this novel protein, followed by the characterization of functional specificities and analysis of the tissue distribution of iCE using antibodies, will contribute to understand its specific role in the large family of carboxylesterases.

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