Cloning and sequencing of rat liver carboxylesterase ES-4 (microsomal palmitoyl-CoA hydrolase)

Mariette ROBBI*, Emile VAN SCHAFTINGEN and Henri BEAUFAY

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL 75.39, Avenue Hippocrate, 75 B-1200 Brussels, Belgium

A cDNA which encodes a carboxylesterase of 561 amino acid residues including a cleavable signal peptide is described. The enzyme expressed in COS cells migrates during PAGE (SDS-, and non-denaturing) as a single prominent band in the region of liver ES-4. It ends in the C-terminal cell-retention signal -HNEL, which, in COS cells overexpressing the enzyme, appears to be slightly less efficient than the signals -HTEL and -HVEL of ES-3 and ES-10 respectively. Glycosylation is not essential for intracellular retention, but leads to a higher activity. As do many carboxylesterases, the enzyme expressed in COS cells hydrolyses *o*-nitrophenyl acetate and α-naphthyl acetate. It also hydrolyses acetanilide, although less efficiently than ES-3, and, distinctively, palmitoyl-CoA. In addition to the four canonical Cys residues of the carboxylesterases, it contains a fifth, unpaired Cys^{336}, which apparently is not essential for the catalytic properties. Indeed, treatment with iodoacetamide or substitution of Cys^{336} by Phe does not markedly alter the activity of the enzyme on the various substrates. The predicted structure of ES-4 is highly homologous to that of two other recently cloned esterases which also end in -HNEL [Yan, Yang, Brady and Parkinson (1994) J. Biol. Chem. **269**, 29688–29696; Yan, Yang and Parkinson (1995) Arch. Biochem. Biophys. **317**, 222–234]. Together, these isoenzymes probably account for the closely spaced bands observed in the region of ES-4 in non-denaturing PAGE.

INTRODUCTION

Rat liver microsomes contain a number of carboxylesterases which are encoded by different genes [1]. These enzymes are best resolved by isoelectric focusing and, therefore, they were initially designated by their pI value. They are glycoproteins of the high-mannose-containing type, made of a single polypeptide chain (approx. 60 kDa), except for the ES-10 isoenzyme (also designated pI 6.0 or pI 6.1 esterase [2,3], RH1 [4], or carboxylesterase A [5]) which is a homotrimer [2,3]; most occur in slightly different molecular forms and show a set of closely spaced bands in non-denaturing PAGE [2,3]. Carboxylesterases are active on a variety of small aliphatic and aromatic esters, but can be distinguished from one another on the basis of their action on drugs [6] and lipids [7]. They are believed to be involved in detoxication, or activation of xenobiotic compounds.

Starting from rat liver, testis and kidney cDNA libraries, several carboxylesterases have been cloned and sequenced [8–14]. In our laboratory, a major goal was to investigate the mechanism by which a number of these enzymes are retained in the lumen of the endoplasmic reticulum. We have reported the cDNA sequence of the microsomal enzyme ES-10 [10] and identified the cDNA encoding ES-2 (the major carboxylesterase in the rat blood plasma [15,16]), a cDNA which was originally cloned by Long et al. [8] and Takagi et al. [9] and presumed to encode a microsomal enzyme. Manipulations of the C-terminal end of these cDNAs and expression of these messages in COS cells have demonstrated that the consensus motif for retention of carboxylesterases in the animal cells lies in the C-terminal sequence -HXEL [15,17]. This conclusion has been corroborated in other laboratories [18,19] and in our studies on the microsomal isoenzyme ES-3 [11] (also designated pI 5.6 or 5.5 esterase [2,3], RL2 [4], or egasyn [20]).

The present work has been undertaken to identify and characterize a carboxylesterase ending in -HNEL [18], which had been recognized during the course of our earlier cloning procedures. The cDNA has been cloned from a rat liver cDNA library, sequenced and transfected in COS cells. The product was identified as ES-4 (also designated pI 6.2/6.4 or pI 6.4 esterase [2,3], RL1 [4], or microsomal long-chain acyl-CoA hydrolase [21]).

MATERIALS AND METHODS

Expression studies in COS cells

The E-HNEL insert was subcloned in the *Eco*RI site of pcDL-SRα296 (kindly provided by DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA, U.S.A.) for expression in COS cells under the control of SRα which is composed of the simian virus 40 early promoter and the R-U5 segment of the human T-cell leukaemia virus type-1 long terminal repeat [22]. The ES-10 [10] and the ES-3 [11] cDNAs were similarly subcloned in pcDL-SRα296. On the day before transfection, the COS cells were seeded at a density of 1.2×10^6 cells per 10-cm tissue-culture dish. The cells were cultured and transfected as earlier described [17], except that 20 μg of each recombinant DNA was used per dish, including in co-transfections. Radiolabelling, immunoprecipitation, endo-β-*N*-acetylglucosaminidase H (endo-H) treatment and analysis by PAGE were performed as described previously [10,17].

Construction of the ES-4 mutant in which Cys^{336} is substituted by Phe

The cDNA inserted in plasmid pcDL-SRα296 was mutated by PCR amplification using divergent oligonucleotides and Pwo (*Pyrococcus woesei*) DNA polymerase (Boehringer) (M. Veiga da Cunha, S. Courtois, A. Michel, E. Gosselain and E. van

Abbreviation used: endo-H, endo-β-*N*-acetylglucosaminidase H.

* To whom correspondence should be addressed.

The cDNA sequence of carboxylesterase ES-4 is registered in the EMBL data library under the accession number X81825.

```
   1                                                                      ctgtcc    6

   7  atgcaag atg tgc ctc agc ttc ctg atc ctg gtg tcc cta gca acc tgt gtg gtt tat gga    67
 -18          met cys leu ser phe leu ile leu val ser leu ala thr cys val val tyr gly    -1
          → 1-HNEL
  68  aac ccc tct tca cca ccc gtg gtg gac acc acg aaa ggc aaa gtc ctg ggg aag tac gtc   127
   1  asn pro ser ser pro pro val val asp thr thr lys gly lys val leu gly lys tyr val    20

 128  agc tta gaa gga gtc aca cag tct gta gct gtc ttc ctg gga gtc cct ttt gcc aag ccc   187
  21  ser leu glu gly val thr gln ser val ala val phe leu gly val pro phe ala lys pro    40

 188  cct ctg gga tct ctg agg ttt gct cca cca cag cct gca gag ccc tgg agc ttc gtg aag   247
  41  pro leu gly ser leu arg phe ala pro pro gln pro ala glu pro trp ser phe val lys    60

 248  aac acc acc acc tat cca cct atg tgc tct caa gat gca gca aaa ggg cag agg atg aat   307
  61  asn thr thr thr tyr pro pro met cys ser gln asp ala ala lys gly gln arg met asn    80
                                      ***
 308  gat ctc cta acc aac aga aag gag aaa atc cat ctc gag ttt tct gaa gat tgt ctc tac   367
  81  asp leu leu thr asn arg lys glu lys ile his leu glu phe ser glu asp cys leu tyr   100
                                                                      ***
 368  ctg aat att tac act cct gca gac ttt aca aag aat agc agg ctg cca gtg atg gtg tgg   427
 101  leu asn ile tyr thr pro ala asp phe thr lys asn ser arg leu pro val met val trp   120

 428  atc cat gga ggt gga atg aca ctg ggc ggg gca tca acc tat gat ggc cgg gtc ctc tct   487
 121  ile his gly gly gly met thr leu gly gly ala ser thr tyr asp gly arg val leu ser   140

 488  gcc tat gaa aac gtg gtg gta gtg gcc att cag tat cgc ctg ggc atc tgg gga ttc ttc   547
 141  ala tyr glu asn val val val val ala ile gln tyr arg leu gly ile trp gly phe phe   160

 548  agc aca ggg gat gaa cac agc agg gga aac tgg ggt cat ttg gac caa gtg gct gcg ctg   607
 161  ser thr gly asp glu his ser arg gly asn trp gly his leu asp gln val ala ala leu   180

 608  cac tgg gtc cag gac aac att gcc aac ttt ggg ggt gac cca ggc tct gtg acc atc ttt   667
 181  his trp val gln asp asn ile ala asn phe gly gly asp pro gly ser val thr ile phe   200

 668  gga gag tca gca gga ggt ttc agt gtc tct gtt ctt gtg ttg tcc cca ctg acc aag aac   727
 201  gly glu ser ala gly gly phe ser val ser val leu val leu ser pro leu thr lys asn   220
              ###
 728  ctc ttc cac agg gcc att tct gag agt ggg gtg gtc ttc ctt cct gga ttg tta acc aag   787
 221  leu phe his arg ala ile ser glu ser gly val val phe leu pro gly leu leu thr lys   240

 788  gat gtt aga cca gcc gct aag caa att gct gat atg gct gga tgt gaa acc acc aca tct   847
 241  asp val arg pro ala ala lys gln ile ala asp met ala gly cys glu thr thr thr ser   260
                                                          ***
 848  gcc atc att gtt cac tgc ctg cgt caa aag aca gaa gag gag ctc tta gag atc atg aag   907
 261  ala ile ile val his cys leu arg gln lys thr glu glu glu leu leu glu ile met lys   280
                          ***
 908  aaa atg aat ctg att aaa ctc agt tca caa agg gat aac aaa gag agc tac cac ttt ttg   967
 281  lys met asn leu ile lys leu ser ser gln arg asp asn lys glu ser tyr his phe leu   300

 968  tca act gtg gtt gac aat gta gtg ctg ccg aag gac cca aaa gag atc ctg gct gag aag  1027
 301  ser thr val val asp asn val val leu pro lys asp pro lys glu ile leu ala glu lys   320
                                2-HNEL ←
1028  aac ttc aac acc gtg ccc tac att gtg gga atc aac aag caa gaa tgt ggc tgg ctt ctg  1087
 321  asn phe asn thr val pro tyr ile val gly ile asn lys gln glu cys gly trp leu leu   340
                                                              ***
1088  cca aca atg atg gga ttt gta cca gct gat gta gaa ttg gac aag aag atg gcc att acg  1147
 341  pro thr met met gly phe val pro ala asp val glu leu asp lys lys met ala ile thr   360

1148  ctc ctg gag aaa ttt gct tcc cta tat ggt ata cca gag gat att att cca gtt gcc att  1207
 361  leu leu glu lys phe ala ser leu tyr gly ile pro glu asp ile ile pro val ala ile   380

1208  gag aag tac aga aaa ggt agt gat gac tcc atc aag atc aga gat gga atc ctt gcc ttt  1267
 381  glu lys tyr arg lys gly ser asp asp ser ile lys ile arg asp gly ile leu ala phe   400

1268  att ggg gat gtg tca ttt tct atc cca tca gtg atg gtg tcc cgt gac cac aga gat gct  1327
 401  ile gly asp val ser phe ser ile pro ser val met val ser arg asp his arg asp ala   420

1328  gga gct ccc acc tac atg tat gag tat caa tac tac ccg agc ttc tca tca ccc caa aga  1387
 421  gly ala pro thr tyr met tyr glu tyr gln tyr tyr pro ser phe ser ser pro gln arg   440

1388  ccc aag cat gta gta gga gac cat gca gat gat ctc tac tct gtc ttt ggt gcc cca att  1447
 441  pro lys his val val gly asp his ala asp asp leu tyr ser val phe gly ala pro ile   460
                                  ###
1448  tta aga gat ggt gcc tca gaa gag gag atc aag ctc agc aag atg gtg atg aaa ttt tgg  1507
 461  leu arg asp gly ala ser glu glu glu ile lys leu ser lys met val met lys phe trp   480

1508  gcc aac ttt gct cgg aat ggg aac cct aat ggc cga ggg cta cct cat tgg cca cag tat  1567
 481  ala asn phe ala arg asn gly asn pro asn gly arg gly leu pro his trp pro gln tyr   500

1568  gac cag aaa gaa gaa tat ctg cag att ggt gca acc acc cag caa tcg cag aga ctg aaa  1627
 501  asp gln lys glu glu tyr leu gln ile gly ala thr thr gln gln ser gln arg leu lys   520

1628  gca gag gaa gtg gct ttt tgg aca cag tta ctg gct aag aga caa cct cag cca cac cac  1687
 521  ala glu glu val ala phe trp thr gln leu leu ala lys arg gln pro gln pro his his   540

1688  aac gag ctg tga atgcaagtctctgtcatcttcagaacaagcaagccaagatatagttcttccactaaagatgta  1762
 541  asn glu leu ---                                                                   543

1763  tgtaaatggaagatggatctggaggatcctgaagaattttgtcaaagagacagggagaacccaggaaagagaaatattt  1841

1842  gtacttatggcaccaatttagagaataaatgacatttttacggtcaaaaaaaaaaaaaaa                       1901
```

Figure 1 Nucleotide sequence of the E-HNEL cDNA and the deduced amino acid sequence

Schaftingen, unpublished work). The primers used (mutations in bold) were the following : 5′-pCTT GTT GAT TCC CAC AAT GTA GG-3′ and 5′-CAA GAA T**TC** GGC TGG CTT CTG-3′. The PCR-amplified DNA was purified by phenol extraction and circularized by ligation before transformation of the bacteria. The mutated construct was identified by its additional *Eco*RI site and the length of the resulting *Eco*RI restriction fragments.

Biochemical assays

The transfected COS cells were disrupted in ice-cold water just before measurement of the protein content according to the method of Bradford [23] with BSA as standard. Cell lysis was completed in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.2 % Triton X-100, 12.5 μg/ml soybean trypsin inhibitor and 125 kallikrein-inhibitory units/ml aprotinin. After 30 min on ice and centrifugation, esterase activities were assayed: (i) at 420 nm with *o*-nitrophenyl acetate (0–4.5 mM) in 20 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.1 % Triton X-100 at 26 °C [24]; (ii) at 412 nm with palmitoyl-CoA (0–30 μM) in 30 mM Hepes buffer, pH 7.5, containing 1 mM EDTA, 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid) at 37 °C [25]; or (iii) at 558 nm with acetanilide (0–40 mM) in 0.1 M Tris/HCl buffer, pH 8.6, at 37 °C [26]. Sensitivity of the enzymes to 1 mM bis-*p*-nitrophenyl phosphate was tested after preincubation at pH 7.4 for 30 min at 37 °C.

RESULTS AND DISCUSSION

Cloning of a cDNA encoding a carboxylesterase ending in HNEL

Using as a probe a 1.2 kbp restriction fragment at the 3′ end of ES-3 cDNA [11], we have selected cDNA clones representative of liver carboxylesterases from a λZAP^{R} II cDNA library. The 90 positive clones were further analysed by PCR to identify those encoding an isoenzyme ending in -HNEL [18]. The primers were a 18-mer antisense oligonucleotide bridging the stop codon (Figure 1) and 20-mer oligonucleotides complementary to the vector-specific T3 and T7 promoters. The longest positive clone, 1-HNEL (Figure 1), contained an insert of 1831 bp and ended in a 15 bp poly(A^{+}) tail. Overlapping clones extending further to the 5′ end of the cDNA were searched for among the remaining clones with a similar PCR strategy, using this time a 22-mer antisense oligonucleotide close to the 5′ end of 1-HNEL as gene-specific primer. This approach yielded clone 2-HNEL (Figure 1): its 1053 bp insert consists of 13 bp in the 5′ non-coding region and 1040 bp in the coding region, of which 983 bp overlap with 1-HNEL. The identity between the two clones is complete between nucleotides 158 and 1053 whereas nine substitutions are present between nucleotides 71 and 157 (results not shown), resulting in the replacement of Phe^{25} and Pro^{28} by Val^{25} and Ser^{28}. The identity of these two clones over a large portion of their sequence suggests that they are derived from a single gene by an alternative splicing event.

The two clones were recombined at their unique *Xho*I restriction site to produce the composite cDNA E-HNEL: nucleotides 1–361 derive from clone 2-HNEL and nucleotides 362–1901 from 1-HNEL. This cDNA most likely corresponds to a full-length 2-HNEL-type cDNA and encodes a protein which differs from that encoded by 1-HNEL by only 2 amino acid residues.

As shown in Figure 1, translation initiates at the second ATG, which opens a reading frame of 1686 bases and is preceded by a sequence that is more compatible with the context of an initiator codon than the sequence around the first, out of frame ATG [27]. The encoded polypeptide begins with a cleavable signal sequence of 18 residues [28]. The predicted isoelectric point and molecular mass of the processed protein, as calculated by PC Gene (Intelli Genetics, Inc., Mountain View, CA, U.S.A.), are 6.29 and 60.4 kDa respectively. The sequence contains the Ser and His residues that are part of the catalytic centre and has also a single N-glycosylation site, the one present in most carboxylesterases. The N-terminal Asn-Xaa-Ser motif contains a proline residue and is therefore not a glycosylation site [29] contrary to previous suggestions [5,12,14]. Remarkably, the sequence contains five cysteine residues whereas most carboxylesterases contain only four, except carboxylesterase C which has six [14]; the four highly conserved Cys residues are thought to form two intrachain disulphide bridges [30]. As ES-4 is a monomeric protein [2,3], the polypeptide necessarily contains a free, unbridged thiol group.

Comparison with other sequences

The liver cDNA that we have cloned is highly homologous to those of kidney carboxylesterase B [12] and liver carboxylesterase C [14], both from rat. At the nucleotide level, the identity with the sequence reported here is of 98.3 % and 96.8 % respectively. Remarkably, the non-translated 3′ regions of the two liver clones show only one difference in 190 bp, and were therefore closer to each other than the corresponding coding sequences (59 differences for approx. 1630 bp). In contrast, the kidney carboxylesterase B, when compared with the two liver enzymes, showed more divergence in its 3′ untranslated region than in the coding sequence. These data suggest that a recombination event between two different genes may have occurred recently, or that the transcript of a single gene may be spliced differently in liver and kidney.

Figure 2 shows the comparison of the amino acid sequence of the three carboxylesterases mentioned above and of the peptides derived by Alexson et al. [31] from a microsomal acyl-CoA thioesterase. The sequence reported here shows 98.2 % and 93.6 % amino acid sequence identity with carboxylesterases B [12] and C [14] respectively. The N-terminal amino acid sequence predicted for the processed chain is identical to that established by Edman degradation for purified kidney palmitoyl-CoA-hydrolysing carboxylesterase [32] and rat liver microsomal carboxylesterase B [5] over 27–30 amino acid residues. The three complete sequences shown in Figure 2 match the sequence of peptides 1, 4, 5 and 7 (45 residues in total) but similarly differ from peptides 2 (five residues out of 19), 3 (five residues out of 13) and 6 (one residue out of 15). Because of these discrepancies, it was of interest to characterize further the enzyme encoded by our cDNA clone.

The carboxylesterase C cDNA [14] was also identified in our rat liver λZAP^{R} II cDNA library and partially sequenced. It seems thus that at least three different rat carboxylesterases ending in -HNEL (ours, and carboxylesterases B and C [12,14]) are expressed in rat liver; they are barely distinguishable by their predicted pI values (6.29, 6.29 and 6.17) and calculated molecular masses (60.4, 60.5 and 60.6 kDa), and most likely account for the

Nucleotides (upper lines) and amino acid residues (lower lines) are numbered in the right- and left-hand margins. Marks show the signal sequence cleavage site (arrow), the single potential glycosylation site (underlined), the Ser and His residues of the active site (#), the Cys residues (*), the C-terminal endoplasmic reticulum retention signal (double underlined), the in-frame stop codon TGA (- - -), the polyadenylation signal AATAAA (stippled). The positions of the primers used to screen the cDNA library, the beginning of clone 1-HNEL and the end of clone 2-HNEL are indicated by arrrows above the nucleotide sequence.

```
1  MCLSFLILVS LATCVVYGNP SSPPVVDTTK GKVLGKYVSL EGVTQSVAVF      32
2  ......F... .......... .......... .......... ..........
3  ...RS.F... ......C... ........M. .......A.. ..........
4                      .. ........

1  LGVPFAKPPL GSLRFAPPQP AEPWSFVKNT TTYPPMCSQD AAKGQRMNDL      82
2  .........P .......... .......... .......... ..........
3  .......... .......... .......... .......... .T........
4                                .. .S........ .VG..VL

1  LTNRKEKIHL EFSEDCLYLN IYTPADFTKN SRLPVMVWIH GGGMTLGGAS     132
2  .......... .......... .......... .......... ..........
3  .......V.. Q......... .........D ..M....... ...L.Q....
4       .N.P. Q--.....

1  TYDGRVLSAY ENVVVVAIQY RLGIWGFFST GDEHSRGNWG HLDQVAALHW     182
2  .......... .......... .......... .......... ..........
3  ....Q..... .......... .......... .......... ..........
4

                         *
1  VQDNIANFGG DPGSVTIFGE SAGGFSVSVL VLSPLTKNLF HRAISESGVV     232
2  .......... ..A....... .......... .......... ..........
3  .......... .......... .......... .....S...Y ..........
4

1  FLPGLLTKDV RPAAKQIADM AGCETTTSAI IVHCLRQKTE EELLEIMKKM     282
2  ..T....... .......... .......... .......... ..........
3  LITE.F.... .......... ...K...... .......... .......E..
4

1  NLIKLSSQRD NKESYHFLST VVDNVVLPKD PKEILAEKNF NTVPYIVGIN     332
2  .........I T......... .......... .......... .N........
3  .......... T......... .I.D...... .......... ..........
4                                              .. .........

1  KQECGWLLPT MMGFVPADVE LDKKMAITLL EKFASLYGIP EDIIPVAIEK     382
2  .......... .......... .......... .......... ..........
3  .......... ..R...P..K .......M.. .....I.... ..........
4                                              .. ..........

1  YRKGSDDSIK IRDGILAFIG DVSFSIPSVM VSRDHRDAGA PTYMYEYQYY     432
2  .......... .......... ....FY.... .......... ..........
3  .......P.. .......... ..L.C..... .......... ...V......
4                                        Q...  ..........

                   *
1  PSFSSPQRPK HVVGDHADDL YSVFGAPILR DGASEEEIKL SKMVMKFWAN     482
2  .......... .......... .......... .......... ..........
3  .......... D........V .......... .......... ..........
4  .

1  FARNGNPNGR GLPHWPQYDQ KEEYLQIGAT TQQSQRLKAE EVAFWTQLLA     532
2  ........A. .......... .......... .......... ..........
3  ........A. .......... .......... .......... ..........
4                                        .... ..........

1  KRQPQPHHNE L                                            543
2  ..E....... .
3  .......... .
4
```

Figure 2 Alignment of the predicted amino acid sequences for three rat carboxylesterases ending in -HNEL

Line 1, sequence reported in this paper; line 2, carboxylesterase B [12]; line 3, carboxylesterase C [14]; line 4, tryptic peptides of long-chain acyl-CoA thioesterase [31]. Amino acid residues are numbered in the right-hand margin starting from the N-terminal end of the mature protein; dots indicate the residues common with the first sequence; dashes indicate gaps. The serine and histidine residues of the active site are indicated with an asterisk and the cysteine residues are indicated with a dot. The C-terminal retention signal and the N-glycosylation site are underlined, and the cleaved signal sequence is italicized.

two prominent bands commonly observed in the region of pI 6.2–6.4 enzymes after histoenzymological staining of gels [2,3]. This heterogeneity is not due to a difference in the degree of glycosylation, since this type of esterase appears to be fully glycosylated in hepatocytes [33].

Expression studies in COS cells

The E-HNEL cDNA was transiently expressed in COS cells to characterize further the encoded product. After non-denaturing

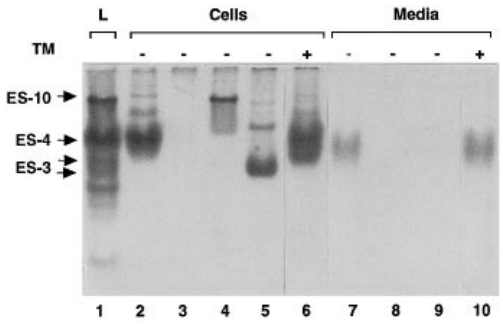

Figure 3 Expression of different esterase isoenzymes in COS cells

The cells were transfected with recombinant cDNA encoding ES-4 (E-HNEL cDNA; lanes 2, 6, 7 and 10), antisense E-HNEL cDNA (lane 3), ES-10 (lanes 4 and 8), or ES-3 cDNA (lanes 5 and 9). After 48 h in culture they were reincubated overnight in the absence (−) or in the presence (+) of tunicamycin (TM). The products extracted from the cells with Triton X-100 were resolved by non-denaturing PAGE (lanes 2–6). Proportional samples of the culture media (lanes 7–10) were similarly analysed after dialysis, lyophilization and rehydration in the presence of Triton X-100. Lane L was loaded with an extract from rat liver microsomes. The gels were histoenzymologically stained by incubation with α-naphthyl acetate.

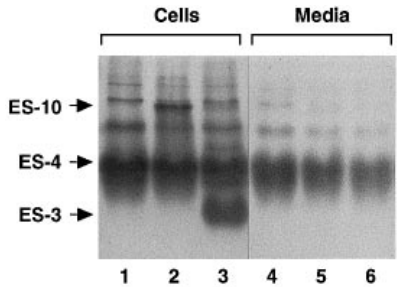

Figure 4 Co-expression of several esterase isoenzymes in COS cells

The cells were transfected with the recombinant cDNA encoding ES-4 alone (lanes 1 and 4), or in combination with the cDNA encoding ES-10 (lanes 2 and 5) or ES-3 (lanes 3 and 6). The experimental protocol was as described in Figure 3, except that reincubation with tunicamycin was omitted.

PAGE (Figure 3) the esterase activity identified by histoenzymological staining with α-naphthyl acetate showed as a single prominent band in the region of ES-4 from liver microsomes (Figure 3, lane 2 versus lane 1) and was distinct from the esterase activities of independent transfections with ES-10 (lane 4) and ES-3 (lane 5) cDNAs. Besides the prominent band, lanes 2 and 5 each showed slower-migrating components which most likely represent the enzyme artifactually associated with other cellular proteins, as commonly observed in crude detergent extracts; in lane 4 the streak under the ES-10 band is presumably due to progressive dissociation of the trimeric form [17]. Esterase activity remained undetected in the COS cells transfected with antisense E-HNEL cDNA (Figure 3, lane 3). These results identify the E-HNEL cDNA as encoding the ES-4 isoenzyme.

Although the bulk of recombinant ES-4 was retained in the cells, a minor part was consistently found in the culture medium (Figure 3, compare lane 2 with lane 7). In this respect ES-4 differed from ES-10 and ES-3, which could not be detected in the medium (Figure 3, lanes 8 and 9 respectively). COS cells were also co-transfected with the cDNAs of ES-4 and ES-3, or with the cDNAs of ES-4 and ES-10 (Figure 4). In each case a small fraction of ES-4 was present in the culture medium, while ES-10

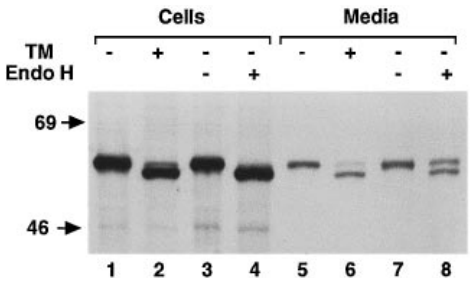

Figure 5 Fluorogram of the E-HNEL cDNA product expressed in COS cells

Cells were labelled overnight with 0.4 mCi of PRO-MIX in the absence (−) or in the presence (+) of tunicamycin (TM). The products were precipitated with an antiserum to ES-4 from the Triton X-100 extracts of cells (lanes 1–4) and from the culture media (lanes 5–8). Some immunoprecipitated products were incubated in the absence (−) or the presence (+) of endo-H before analysis by SDS/PAGE and fluorography. Arrows on the left indicate the positions of BSA (69 kDa) and ovalbumin (46 kDa).

(Figure 4, lanes 2 and 5) and ES-3 (Figure 4, lanes 3 and 6) could be detected only in the cell extract. This indicates that the ES-4 activity found in the medium was not released from damaged cells, and suggests that the C-terminal end of ES-4 retains the protein in the COS cells less efficiently than those of ES-3 and ES-10. The reason may lie in the replacement of the antepenultimate residue threonine (ES-3) or valine (ES-10) by asparagine, or in the structure of the appending protein [34]. We can, however, not infer from these observations that ES-4 is secreted from rat liver in the blood plasma. The differential retention of these esterases might well be restricted to transfected COS cells overexpressing these enzymes. In earlier studies, on cultured hepatocytes, we did not detect the ES-4 enzyme in the culture medium [33], which suggests that it is tightly retained in the liver cells.

When ES-4 was expressed in the presence of tunicamycin, it showed two bands that were clearly seen in the cell extract (Figure 3, lane 6). The upper band corresponds to the glycosylated form and stains more intensely, although it contains much less protein (see below). A difference in enzyme activity between the glycosylated and the unglycosylated forms of ES-3 was also observed [11], and the unglycosylated form of ES-2 was not active on α-naphthyl acetate [15]. The unglycosylated form of ES-4 was retained in the cells to the same extent as the glycosylated enzyme (Figure 3, lane 10). Thus, glycosylation favours or stabilizes an active conformation of the enzyme, but is not required for its retention in the cell.

These conclusions were confirmed by studies on E-HNEL-transfected COS cells labelled by overnight incubation in the presence of [^{35}S]methionine (Figure 5). The immunoprecipitates obtained with an antiserum to the liver enzyme and developed by SDS/PAGE revealed, as expected [33], a single molecular form which was found predominantly, but not exclusively, in the cells (Figure 5, lanes 1, 3, 5 and 7). A similar distribution was observed in tunicamycin-poisoned cells (Figure 5, lanes 2 and 6), in which case the bulk of the immunoreactive material migrated slightly faster, as expected for the unglycosylated form. The product extracted from the cells was sensitive to endo-H (Figure 5, compare lanes 3 and 4); in contrast a perceptible fraction (approx. 50 %) of the enzyme present in the medium was endo-H resistant (Figure 5, lanes 7 and 8).

Taken together these results strengthen the view that in transfected COS cells (but not in rat liver) a small fraction of ES-4 follows the pathway of secretory proteins. The molecular-mass values estimated for the different forms of ES-4 (60 and 58 kDa) indicate that a single oligosaccharide moiety is added to the polypeptide chain; this is consistent with the very small shift resulting from endo-H treatment of the enzyme synthesized in cultured hepatocytes (see Figure 8 in [33]) and the fact that a single glycosylation site is present in the predicted sequence.

Table 1 Substrate specificity of the carboxylesterases expressed in COS cells

Cell extracts were incubated with the substrates at different concentrations, under the conditions given in the Materials and methods section. Units of enzyme activity refer to 1 mg of cell protein and are defined as the amount (μmol) of product formed per min at the substrate concentration indicated, or at maximal velocity when Michaelis–Menten kinetics is followed, in which case the K_m value is specified.

Substrate	Activity	K_m (mM)
o-Nitrophenyl acetate		
ES-4	7.1 units	0.6
ES-3 ([S] = 4.5 mM)	1.5 units	–
ES-10	0.4 unit	0.14
Acetanilide		
ES-4 ([S] = 40 mM)	20 m-units	–
ES-3	180 m-units	14.5
ES-10	0	
Palmitoyl-CoA		
ES-4 ([S] = 15 μM)	41 m-units	–
ES-3	0	
ES-10	0	

Substrate specificity of ES-4

To substantiate further the identification of the cloned enzyme on the basis of its catalytic properties, we expressed the E-HNEL cDNA in parallel with the cDNAs encoding ES-10 [10] and ES-3 [11] in COS-cells. The activities of the extracts were then compared on the commonly used model substrates (Table 1). With *o*-nitrophenyl acetate as substrate ES-4 is by far the most active, but its K_m value is approx. 4-fold that of ES-10; a nearly first-order kinetics is obtained with ES-3 which shows the lowest activity at substrate concentrations below 1 mM. In contrast, with acetanilide as substrate, ES-3 is the most active and follows Michaelian kinetics. The activity of ES-4 is lower by one order of magnitude and is linearly related to substrate concentration. ES-4 is the only enzyme that cleaves palmitoyl-CoA; the activity increases with substrate concentration up to a maximum at 20–25 μM and then decreases sharply. The activity of the three enzymes on these substrates was nearly abolished (below 10 %) by preincubation at pH 7.4 for 30 min at 37 °C with 1 mM bis-*p*-nitrophenyl phosphate. These catalytic properties confirm that the sequence reported in this paper corresponds to ES-4 (microsomal palmitoyl-CoA hydrolase).

As ES-4 differs from the other isoenzymes by a fifth, unbridged Cys residue, we explored the possibility that Cys^{336} might be involved in the catalytic mechanism. The protein was carboxymethylated by treatment with iodoacetamide and passed over Sephadex G10. No significant loss of activity resulted from this treatment. Expression of a cDNA mutated to substitute Cys^{336} with Phe, the amino acid residue occurring at this position in all other cloned mammalian carboxylesterases [30], yielded a product which differed from ES-4 only by a 2-fold higher thioesterase activity. These results rule out the implication of a free thiol group in the hydrolysis of palmitoyl-CoA.

It is likely that the kidney enzyme (esterase B) of Yan et al. [12]

has similar kinetic properties and contributes to the microheterogeneity of ES-4. One may also wonder if this is not the case for the liver enzyme (esterase C) of the same laboratory [14]. This enzyme has been identified as esterase RL-2 [4] on the basis of its high activity on α-naphthyl acetate as compared to nitrophenyl acetate. This identification conflicts with the fact that RL-2 has a pI of 5.5, markedly different from the calculated pI value (6.17) of the encoded esterase. Furthermore, RL-2 appears to be identical with egasyn, which has only approx. 72 % identity with esterase C [11].

As a conclusion, it appears that the expression of various forms of ES-4 in liver, and most likely also in kidney, accounts for the microheterogeneity of this enzyme, which is not explained by a difference in the degree of glycosylation.

This work was financially supported by the following Belgian organizations: Fonds de la Recherche Fondamentale Collective (Grant 2.4591.93) and Services fédéraux des affaires scientifiques, techniques et culturelles (Pôles d'Attraction Interuniversitaires, Grant 44).

REFERENCES

1 Mentlein, R., Ronai, A., Robbi, M., Heymann, E. and von Deimling, O. (1987) Biochim. Biophys. Acta **913**, 27–38
2 Mentlein, R., Heiland, S. and Heymann, E. (1980) Arch. Biochem. Biophys. **200**, 547–559
3 Robbi, M. and Beaufay, H. (1983) Eur. J. Biochem. **137**, 293–301
4 Hosokawa, M., Maki, T. and Satoh, T. (1987) Mol. Pharmacol. **31**, 579–584
5 Morgan, E. W., Yan, B., Greenway, D., Petersen, D. R. and Parkinson, A. (1994) Arch. Biochem. Biophys. **315**, 495–512
6 Mentlein, R. and Heymann, E. (1984) Biochem. Pharmacol. **33**, 1243–1248
7 Mentlein, R., Suttorp, M. and Heymann, E. (1984) Arch. Biochem. Biophys. **228**, 230–246
8 Long, R. M., Satoh, H., Martin, B. M., Kimura, S., Gonzalez, F. J. and Pohl, L. R. (1988) Biochem. Biophys. Res. Commun. **156**, 866–873
9 Takagi, Y., Morohashi, K.-I., Kawabata, S.-I., Go, M. and Omura, T. (1988) J. Biochem. (Tokyo) **104**, 801–806
10 Robbi, M., Beaufay, H. and Octave, J.-N. (1990) Biochem. J. **269**, 451–458
11 Robbi, M. and Beaufay, H. (1994) Biochem. Biophys. Res. Commun. **203**, 1404–1411
12 Yan, B., Yang, D., Brady, M. and Parkinson, A. (1994) J. Biol. Chem. **269**, 29688–29696
13 Yan, B., Yang, D., Brady, M. and Parkinson, A. (1995) Arch. Biochem. Biophys. **316**, 899–908
14 Yan, B., Yang, D. and Parkinson, A. (1995) Arch. Biochem. Biophys. **317**, 222–234
15 Robbi, M. and Beaufay, H. (1992) Biochem. Biophys. Res. Commun. **183**, 836–841
16 Robbi, M. and Beaufay, H. (1994) in Esterases, Lipases and Phospholipases. From Structure to Clinical Significance (Mackness, M. I. and Clerc, M., eds.), pp. 47–56, NATO ASI Series vol. 266, Plenum Publishing Corporation, New York
17 Robbi, M. and Beaufay, H. (1991) J. Biol. Chem. **266**, 20498–20503
18 Medda, S. and Proia, R. L. (1992) Eur. J. Biochem. **206**, 801–806
19 Zhen, L., Baumann, H., Novak, E. K. and Swank, R. T. (1993) Arch. Biochem. Biophys. **304**, 402–414
20 Medda, S., Takeuchi, K., Devore-Carter, D., von Deimling, O., Heymann, E. and Swank, R. T. (1987) J. Biol. Chem. **262**, 7248–7253
21 Mentlein, R., Berge, R. K. and Heymann, E. (1985) Biochem. J. **232**, 479–483
22 Takebe, Y., Seiki, M., Fujisawa, J.-I., Hoy, P., Yokota, K., Arai, K.-I., Yoshida, M. and Arai, N. (1988) Mol. Cell. Biol. **8**, 466–472
23 Bradford, M. M. (1976) Anal. Chem. **72**, 248–254
24 Beaufay, H., Amar-Costesec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J. (1974) J. Cell Biol. **61**, 188–200
25 Berge, R. K. (1979) Biochim. Biophys. Acta **574**, 321–333
26 Akao, T. and Omura, T. (1972) J. Biochem. (Tokyo) **72**, 1245–1256
27 Kozak, M. (1986) Cell **44**, 283–292
28 von Heijne, G. (1983) Eur. J. Biochem. **133**, 17–21
29 Bause, E. (1983) Biochem. J. **209**, 331–336
30 Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K. and Doctor, B. P. (1993) Protein Sci. **2**, 366–382
31 Alexson, S. E. H., Mentlein, R., Wernstedt, C. and Hellman, U. (1993) Eur. J. Biochem. **214**, 719–727
32 Tsujita, T. and Okuda, H. (1993) J. Lipid Res. **34**, 1773–1781
33 Robbi, M. and Beaufay, H. (1988) Biochem. J. **254**, 51–57
34 Haugejorden, S. M., Srinivasan, M. and Green, M. (1991) J. Biol. Chem. **266**, 6015–6018

Received 24 April 1995/11 September 1995; accepted 2 October 1995