MICROSOMAL ACID RETINYL ESTER HYDROLASE: ISOLATION, CHARACTERIZATION, SUBSTRATE AND TISSUE SPECIFICITY

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Running title: ES-10 as a retinyl ester hydrolase

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Previous work demonstrated the presence of both acid and neutral, bile salt-independent retinyl ester hydrolase activities in rat liver homogenates. Here we present the purification, identification and characterization of an acid retinyl ester hydrolase activity from solubilized rat liver microsomes. Purification to apparent homogeneity was achieved by sequential chromatography using SP-Sepharose cation exchange, Phenyl-Sepharose hydrophobic interaction, Concanavalin A-Sepharose affinity and Superose 12 gel filtration chromatography. The isolated protein had a monomer molecular weight of approximately 62 kDa, as measured by mass spectrometry. Analytical gel filtration chromatography of the purified protein revealed a native molecular weight of approximately 176 kDa, indicating that the protein exists as a homotrimeric complex in solution. The purified protein was identified as carboxylesterase ES-10 (EC 3.1.1.1) by N-terminal Edman sequencing and extensive LC-MS/MS sequence analysis and cross reaction with an anti ES-10 antibody. Glycosylation analysis revealed that only one of two potential N-linked glycosylation sites is occupied by a high mannose-type carbohydrate structure. Using retinyl palmitate in a micellar assay system the enzyme was active over a broad pH range and displayed Michaelis-Menten kinetics with a $K_m$ of 86 µM. Substrate specificity studies showed that ES-10 is highly active toward retinyl palmitate, and is also able to catalyze hydrolysis of triolein to a minor degree. Cholesteryl oleate was not a substrate for ES-10 under these assay conditions. Real time RT-PCR and Western blot analysis revealed that ES-10 is highly expressed in liver and lung. Lower levels of ES-10 mRNA were also found in kidney, testis and heart. A comparison of mRNA expression levels in liver demonstrated that ES-10, ES-4 and ES-3 were expressed at significantly higher levels than ES-2, an enzyme previously thought to play a major role in retinyl ester metabolism in liver. Taken together these data indicate that carboxylesterase ES-10 plays a major role in the hydrolysis of newly-endocytosed, chylomicron retinyl esters in both neutral and acidic membrane compartments of liver cells, and may play a role in retinyl ester hydrolysis in other tissues as well.

INTRODUCTION

Higher eukaryotic organisms require dietary vitamin A (retinol), which gives rise to a variety of active metabolites. Retinoids are needed for important physiological processes such as vision, reproduction, growth, development and immune function. Vitamin A must be obtained from the diet either as preformed vitamin A in foods of animal origin or as provitamin A carotenoids in fruits and vegetables. Preformed vitamin A is found in the diet mainly in the form of long-chain fatty acid esters (retinyl esters), with minor amounts being found in the form of retinol and retinoic acid (1).
Following ingestion, dietary retinyl esters are hydrolyzed to retinol in the lumen of the small intestine. This activity was previously thought to be catalyzed by pancreatic carboxyl ester lipase (2); however, experiments with a carboxyl ester lipase knock-out mouse model indicated that the luminal hydrolysis of retinyl esters is catalyzed by pancreatic triglyceride lipase (3). Rigtrup et al. also identified an enzyme with alkaline retinyl ester hydrolase activity in intestinal brush border membranes that is thought to be phospholipase B (4, 5). Liberated retinol is subsequently absorbed by enterocytes, reesterified with long-chain fatty acids primarily by lecithin:retinol acyltransferase, incorporated into chylomicrons and secreted into the lymphatic system.

After transport to and uptake by the liver, retinyl esters are hydrolyzed to retinol by neutral and acid retinyl ester hydrolases (REH). Retinol is then transferred to the endoplasmic reticulum, where it binds to retinol binding protein (RBP) before secretion into circulation as a retinol-RBP complex. Excess retinol is transported by the liver by a yet unknown mechanism to hepatic stellate cells for storage as retinyl ester. Mobilization of these reserves during times of inadequate dietary intake again requires hydrolysis by neutral and/or acidic REHs (1).

The enzymatic hydrolysis of retinyl esters in liver plays an important role in maintaining constant plasma retinol levels and is a key step in the formation and utilization of hepatic vitamin A reserves. Several enzymes, such as pancreatic carboxyl ester lipase, pancreatic triglyceride lipase and members of the non-specific carboxylesterase gene family, have been identified in the past that can hydrolyze retinyl esters in vitro (6). Research has also focused on the enzyme(s) that catalyze this important reaction in liver cells. Harrison and Gad as well as Napoli and coworkers showed that rat liver homogenates contain both neutral and acid bile salt-independent REH activities (7-11). The purification of different proteins from liver has also been reported by a number of groups. Sun et al. isolated two bile-salt independent neutral REHs from rat liver microsomes and identified the purified proteins as rat liver carboxylesterase ES-2 (serum esterase) and ES-10 (pl 6.0/6.1 esterase) (12). Schindler et al. described the purification of a neutral REH from pig liver tissue and its close relationship to carboxylesterase ES-4, as judged by its structural, immunological and catalytic features (13). Sanjani et al. purified six proteins with neutral retinyl palmitate hydrolase activities from rat liver microsomal extracts, the major three of which had been previously characterized as rat liver carboxylesterases ES-10, ES-4 and ES-3 (14). There have been no reports of the purification of acid retinyl ester hydrolases from liver or other tissues.

Retinyl ester hydrolysis in hepatocytes takes place after receptor mediated uptake of chylomicron remnants. Hagen et al. demonstrated in a cell culture model that most of the chylomicron remnant-derived retinyl ester is hydrolyzed in increasingly acidic endosomal compartments and that lysosomes do not contribute significantly to retinyl ester hydrolysis (15). Similar conclusions were drawn from experiments in intact rats by Harrison et al. (16). Even less is known about retinyl esters hydrolysis in hepatic stellate cells. Azais-Braesco and coworkers isolated retinyl ester-containing lipid droplets from liver stellate cells (17) and showed that an unidentified acid REH activity could use these lipid droplets as substrate for retinyl ester hydrolysis (18). The aim of the present investigation was to purify and characterize the bile-salt independent, acid REH from rat liver microsomes.

**EXPERIMENTAL PROCEDURES**

**Materials** — Frozen livers (average wet weight approximately 14 g) from male SD rats were obtained from Hilltop Lab Animals; retinyl-[1-14C]-palmitate (2.22 GBq/mmol), cholesteryl-[1-14C]-oleate (2.22 GBq/mmol) and [9,10-3H]-triolein (0.74 TBq/mmol) were purchased from American Radiolabeled Chemicals; Triton X-100, Triton X-100RS and octyl-β-D-glucopyranoside (OG) were from Sigma; Scintiverse-BD liquid scintillation liquid was from Fisher Scientific; SP Sepharose FF, Phenyl Sepharose HP, Concanavalin A Sepharose and Superose 12 were from Amersham/Pharmacia; Precision Protein Standards and Biosafe Coomassie Blue G-250 were from Biorad; Peptide: N-Glycosidase F and Endoglycosidase H were from New England BioLabs; BCA and BSA standards were from Pierce; Biomax 30 filters were from Millipore; H4 protein chips were from Ciphergen; tissue-specific
rat total RNA was from Ambion. All other reagents were of the highest purity available.

Isolation of hepatic microsomes — Rat liver microsomes were prepared as previously described by Gad and Harrison (10). Briefly, frozen rat livers were thawed and washed twice with an ice-cold sucrose/Tris buffer (250 mM sucrose, 20 mM Tris/HCl, pH 7.2). Individual livers were minced with a scalpel and processed through a tissue press. The minced liver tissue was resuspended in sucrose/Tris buffer and homogenized with three strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 10,000 x g. The supernatant was collected and the pellet was resuspended in sucrose/Tris buffer, homogenized and centrifuged again as described above. The combined supernatants were centrifuged for 60 min at 105,000 x g. The pelleted microsomes were resuspended with a Dounce homogenizer in sucrose buffer (250 mM) at a protein concentration of 2 mg/ml and stored in aliquots at –80°C.

Enzyme and protein assays — REH activity was measured with minor modifications by a radiometric method described previously by Harisson and Gad (7). In short, 10 µl of sodium acetate buffer (250 mM, pH 5.0) and 15 µl of enzyme source (freshly thawed subcellular or eluted fractions) were added together on ice. The enzyme reaction was initiated by the addition of retinyl-[1-14C]-palmitate (~12.5 nCi)) with tracer retinyl palmitate (500 pmol, with tracer carbon-14) to the test mixture at the following concentrations: 0.01, 0.1 and 1µg/ml. Protein concentrations were quantified by the bicinchoninic acid method using BSA as standard. All enzyme activity assays were performed three times in triplicates using ES 10 purified according to the previously described protocol. Enzyme kinetics were analyzed with the Origin 5.0 Professional software package.

Solubilization of acid REH activity from hepatic microsomal membranes — Freshly thawed microsomes were diluted with 250 mM sucrose to a protein concentration of 2 mg/ml. Triton X-100 was added to a final concentration of 0.1% (w/v) and the mixture was incubated for 60 min at 4°C. Following incubation the solution was centrifuged for 60 min at 4°C at 105,000 x g using a Ti 50.2 rotor (Beckman). The supernatant was collected and used as starting material for the purification of acid REH.

Purification of REH by sequential chromatography — The detergent solubilized microsomes were adjusted with NaAc to pH 5.0 and a final concentration of 25 mM NaAc. The solution was clarified by centrifugation for 60 min at 105,000 x g and used as starting material for cation exchange chromatography. The clarified supernatant was directly applied to a SP-Sepharose FF column equilibrated with a NaAc buffer (25 mM NaAc, pH 5.0, 0.1% Triton X-100RS). After extensive washing, bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in sodium acetate buffer. Eluted fractions were immediately neutralized with a Tris/HCl buffer to pH 7.2 and brought to a final concentration of 50 mM Tris/HCl. The fractions with the highest specific activities were pooled, adjusted to 0.5 M ammonium sulfate (AS) and loaded onto a Phenyl Sepharose column, equilibrated with an AS/Tris buffer (0.5 M AS, 50 mM Tris/HCl, pH 7.2). After extensive washing with the same buffer, bound protein was eluted with a N-octyl-β-D-glucopyranoside (OG)/Tris buffer (25 mM OG, 50
mM Tris/HCl, pH 7.2). Fractions with the highest specific activity were pooled, adjusted to 0.5 M NaCl and applied to a Concanavalin A Sepharose column equilibrated with Con A buffer (30 mM Tris/HCl, pH 7.2, 500 mM NaCl, 25 mM OG, 1 mM Mn/CaCl₂). After extensive washing, specifically bound proteins were eluted with 0.5 M methyl-α-D-glucopyranoside in Con A buffer. Fractions with the highest specific activity were pooled, adjusted to 0.5 M NaCl and applied to a Superose 12 high resolution column equilibrated with a Tris/NaCl gel filtration buffer (20 mM Tris/HCl, pH 7.2, 150 mM NaCl, 25 mM OG). Fractions with the highest specific activity were concentrated and stored in frozen aliquots at -80°C.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and N-terminal sequence analysis** — SDS-PAGE analysis was carried out with the Tris/Tricine buffer system according to Schagger and Von Jagow (19). Proteins were separated on 10% SDS-PAGE mini gels (10 x 7 cm) and visualized by colloidal Coomassie Blue G-250 or silver staining according to standard protocols (20, 21). SDS-PAGE separated proteins were transferred electrophoretically onto a PVDF membrane using the Towbin transfer buffer system (25 mM Tris, 192 mM Glycine, pH 8.3, 20% methanol). Tranferred proteins were stained with Amido Black for 1 min, destained with diluted acetic acid for 5 minutes and washed extensively with MilliQ water. N-terminal Edman sequence analysis was carried out by the Protein Microchemistry/Mass Spectrometry Facility at the Wistar Institute (Philadelphia, PA) on an Applied Biosystems Model 494 Precise Protein Sequencer. Additional sequence information was obtained by in-gel tryptic digestion of purified REH and LC-MS/MS analysis at the Protein Microchemistry/Mass Spectrometry Facility at the Wistar Institute on an Finnigan LCQ Classic Ion Trap Mass Spectrometer with Nano-capillary HPLC.

**On-chip glycosylation analysis by SELDI-TOF mass spectrometry** — Purified REH was adjusted to 50% (v/v) acetonitrile and 0.1% (v/v) TFA and spotted onto the surface of a H4 protein chip pretreated with 100% acetonitrile. The sample was air dried and spotting was repeated two more times. After a brief wash with MilliQ water, the protein chip was placed in a 8-well bioprocessor. The samples were incubated either without or with 1 U PNGaseF (in 50 mM sodium phosphate, pH 7.5) or 1 U EndoH (in 50 mM sodium citrate, pH 5.5) in final volume of 50 µl. Samples were incubated for 2 h at 37°C. After the incubation, the protein chip was briefly washed with MilliQ water and air dried. One µl of a saturated solution of sinapinic acid in 50% (v/v) acetonitrile and 0.1% (v/v) TFA in MilliQ water was deposited on the individual spots of the protein chips. Glycosylated and deglycosylated REH was analyzed by time-of-flight mass spectrometry.

**cDNA synthesis and real time RT-PCR analysis** — cDNA was synthesized, in a total volume of 20 µL, from 5 µg rat total RNA (Ambion) in the presence of random primers and ImProm-II™ reverse transcriptase (Promega) according to the manufacturer’s protocol. mRNA expression was determined by real time RT-PCR on an ABI PRISM™ 7700 sequence detector (PE Applied Biosystems, Foster City, Ca). Probes and Primers (Table 1) were designed with the Primer Express 1.5 software program (Applied Biosystems, Foster City, Ca) and synthesized by Keystone DNA Biosource International (Biosource International, Camarillo, CA, USA). PCR amplification was performed in capped 96-well optical plates. PCR reactions were carried out with the cDNA equivalent of 100 ng RNA/well in a total volume of 50µl using the Stratagene Brilliant kit (La Jolla, CA). After an initial step of 2 min at 50°C and 10 min at 95°C, 40 thermal cycles were carried out for 15 sec at 95°C and 1 min at 60°C. Relative quantification of gene expression was performed by the Comparative Threshold Cycle (C_T) method (ABI PRISM 7700 sequence detection system, user bulletin #2) using the rat ribosomal protein L32 as standard for comparisons.

**Results**

**Purification of Acid Retinyl Ester Hydrolase** — Gad and Harrison have reported previously that rat liver microsomes contain both bile salt-independent, neutral and bile salt-independent, acid REH activities (10). Rat liver microsomes were therefore used as enzyme source for developing an AREH purification protocol. Initially, a number of different non-ionic
detergents were screened for their ability to effectively solubilize AREH activity from microsomal membranes. Triton X-100, Nonidet P40, Tween 20, OG and OTG were found to be equally effective solubilizers of AREH activity when used above their respective critical micellar concentrations (data not shown). AREH activity was subsequently purified from Triton X-100-solubilized microsomal membranes by sequential chromatography using cation ion exchange, hydrophobic interaction and lectin affinity chromatography. Trial experiments showed that binding of AREH activity to Concanavalin A Sepharose was inhibited by the presence of Triton X-100 in the binding buffer; however, increased affinity of AREH activity to Con A-Sepharose was observed when Triton X-100 was replaced by OG in the binding buffer. As a consequence, a detergent exchange was incorporated into the elution protocol of the hydrophobic interaction chromatography step. Purification to near homogeneity was achieved by a final gel filtration step with a calibrated Superose 12 HR10/30 column, which not only removed remaining impurities but also provided additional insight into the degree of complexation of AREH activity in solution. Analytical gel filtration estimated the native molecular weight of AREH to be approximately 176 kDa (Fig.1). The presence of octyl glucoside in the gel filtration buffer was necessary to prevent a significant loss of total retinyl ester hydrolase activity during this purification step. The purification of AREH activity was monitored by the increase in specific activity (Table 2) and by SDS-PAGE (Fig. 2). Table 2 demonstrates that Triton X-100 not only releases AREH from microsomal membranes but that it also stimulates total AREH activity approximately three-fold. Enrichment and yield of AREH activity were therefore calculated in respect to the solubilized supernatant of the microsomal membrane preparation to take this stimulatory effect into account. In contrast, AREH activity became increasingly unstable once it was solubilized from microsomal membranes which resulted in a loss of over 90% of total AREH activity during the subsequent four chromatographic purification steps (Table 2). Since the sensivity of AREH to serine and cysteine protease inhibitors was not known, these inhibitors were not included in the solubilization buffer. Furthermore, some proteasomes are associated with microsomal membranes (22) and might therefore be responsible for the loss of enzymatic activity observed after the solubilization and subsequent purification steps.

Identification of AREH as carboxylesterase ES-10 — In order to identify the AREH activity, the purified protein was subjected to in-gel tryptic digestion, followed by MALDI-TOF analysis of the resulting peptides. The resulting peptide mass fingerprint was then compared to the peptide mass fingerprints of all known rat proteins in the non-redundant NCBI database with the Mascot protein search software. The initial search successfully matched the tryptic peptide map to several different proteins of the highly homologous carboxylesterase family but failed to unambiguously identify a single protein. As a consequence, the in-gel tryptic digest of the purified protein was subjected to LC-MS/MS sequence analysis which identified the purified AREH activity as rat liver carboxylesterase ES-10. The N-terminus of mature ES-10 was identified by automated Edman degradation of the intact protein and was found to have the sequence: YPSSPPVVNTVKGKV (Table 3). A polyclonal antibody raised against the 13 C-terminal amino acids of ES-10 also reacted with the purified protein, further confirming the identification of AREH as ES-10 (data not shown).

Enzymatic deglycosylation of ES-10 — The analysis of the amino acid sequence of ES-10 revealed two potential N-linked glycosylation sites at positions 79 and 489. In order to analyze the glycosylation of ES-10 in more detail, the purified
protein was spotted onto the surface of a NP20 protein chip, incubated with either PNGase F or Endo H and analyzed by SELDI-TOF mass spectrometry. Deglycosylation of purified ES-10 with PNGase F reduced the MW from 62.0 kDa (Fig. 3A) to 60.3 kDa (Fig. 3B) whereas the deglycosylation of ES-10 with Endo H reduced the MW to approximately 60.4 kDa (Fig. 3C). In addition, the carbohydrate structure of ES-10 was probed with a number of different digoxigenin-labeled lectins that selectively recognize specific terminal sugars and thus distinguish between high mannose, complex and hybrid N-glycans and O-glycosidically linked carbohydrate structures. Of all lectins tested, ES-10 cross-reacted only with GNA which recognizes terminal mannose residues of high mannose N-glycans and has a similar binding specificity as Con A. None of the other lectins (SNA, MAA, PNA and DSA) tested were bound by the carbohydrate structure of ES-10 (data not shown).


**Enzymatic characterization of ES-10 as a retinyl ester hydrolase** — The enzymatic activity of purified rat liver carboxylesterase ES-10 was measured as a function of pH in the range of 2.5 to 10. Fig. 4a shows that ES-10 exhibited optimal activity at pH 8.0; however, it is noteworthy that ES-10 retained 80 to 90% of its maximal activity in the physiologically relevant pH range of 7.4 to 5.0 and that ES-10 had significant enzymatic activity down to pH 4. K_M and V_max were determined by increasing the substrate concentration in the assay while keeping the amount of detergent used to solubilize the substrate constant. Fig. 4b shows that ES-10 displayed Michaelis-Menten kinetics under the chosen assay conditions and that the rate of hydrolysis increased in a linear fashion up to a substrate concentration of 50 µM. Maximal rates of hydrolysis were observed at substrate concentrations in excess of 100 µM. The observed specific activities were analyzed by non-linear curve fitting to a one-site binding model. Under the chosen assay conditions, ES-10 had a K_M of 86 µM and a V_max of 742 nmol/h/mg (Table 3). Several different lipid substrates were tested in the micellar assay system, but only retinyl palmitate was found to be an efficient substrate for ES-10, while no activity was detected with cholesteryl oleate. Triolein was a substrate for ES-10, although at a much slower rate (Fig. 4c). Additional experiments were carried out to determine whether retinol binding proteins had any effect on the hydrolytic activity of ES-10. None of the proteins tested, including plasma RBP, recombinant apo-cRBP-I, β-lactoglobulin and albumin increased the rate of retinyl ester hydrolysis in the micellar assay system (data not shown). The addition of physiological levels of bile salts to the assay mixture also had no significant effect on the rate of retinyl palmitate hydrolysis by purified ES-10 (data not shown).

**Tissue specificity of ES-10 gene and protein expression** — Tissue specific primers and probes (Table 1) were designed to measure the relative gene expression of ES-10 in various tissues by real time RT-PCR. Fig. 5 shows that ES-10 mRNA is highly expressed in liver and that comparable levels of ES-10 mRNA are also found in the lung as well. Kidney, testis and heart expressed only about 10-15% of the level of ES-10 mRNA in liver, whereas the expression of ES-10 in brain, ovary and intestine was less than 3% that of liver. A number of tissues were probed with an anti-ES 10 antibody to complement the gene expression experiments. Western blot analysis revealed high levels of ES-10 protein expression in liver, lung and testis, whereas the expression of ES-10 protein in kidney and heart was not detected with this antibody (Fig. 6) possibly due to the C-terminal specificity of this antibody. Additional probes and primers were designed to compare the gene expression of different carboxylesterases in rat liver. Fig. 7 shows that the expression of ES-10 in liver was significantly higher than that of ES-2, an enzyme previously thought to play a major role in liver retinyl ester metabolism (12).

**DISCUSSION**

The enzymatic hydrolysis of retinyl esters in liver plays an important role in maintaining retinol homeostasis and is a key step in the formation and mobilization of hepatic vitamin A reserves. Hepatic vitamin A metabolism begins with the uptake of retinyl ester containing chylomicron remnants by LDL-receptor mediated endocytosis (23). Blomhoff et al. demonstrated in a previous study by subcellular fractionation in density gradients that endocytosed chylomicron remnant [1H]retinyl esters and 125I-asialofetuin are initially located in low density endosomes of rat liver parenchymal cells. However, unlike 125I-
asiolofetuin, which was subsequently transported to and degraded in late endosomal/lysosomal compartments, radioactively labeled retinoid was probably transferred to the endoplasmic reticulum (ER), as indicated by the co-migration of [3H]retinoid and the ER marker enzymes glucose-6-phosphatase and rotenone-insensitive NADPH-cytochrome c reductase (24). These studies were later extended by Harrison et al. who showed that newly delivered, radioactively labeled retinyl ester co-localized with bile-salt independent, neutral and acid retinyl ester hydrolase activities in enriched plasma membrane/endosomal fractions (7). Despite the details provided by these studies, two intriguing questions remain largely unanswered: a) does retinyl ester hydrolysis occur in rat liver parenchymal cells before or after retinoid transport to the ER and b) how is retinol/retinyl ester selectively transferred to the ER while cholesteryl esters and triacylglycerols derived from endocytosed lipoproteins are transported to lysosomes for degradation (25). The goal of this study was therefore to purify the enzymes that are able to hydrolyze retinyl esters in the increasingly acidic environment of the endocytic pathway. The identity of these proteins might then provide important new clues to the possible localization of retinyl ester hydrolysis in hepatocytes.

Our approach to the purification of the bile salt-independent acid retinyl ester hydrolase was to monitor the enrichment in specific activity at pH 5.0 and to exclude bile salts from the in vitro assay mixture. The purified protein was subsequently identified as carboxylesterase-10 (also referred to as pI 6.0/6.1 esterase) by N-terminal Edman sequencing of the intact protein and a combination of MALDI-TOF and LC-MS/MS sequence analysis of tryptic peptides. These results were further supported by the observation that the purified acid retinyl ester hydrolase cross reacted with a polyclonal anti-ES-10 antibody directed at the C-terminus of ES-10. Analytical gel filtration (Fig. 1) showed that the active protein exists as a homotrimeric complex in solution, as had been described for ES-10 previously (26,27). ES-10 is a member of the non-specific carboxylesterase supergene family (28) and is able to hydrolyze both xenobiotic and lipid substrates (27). The ability of ES-10 to hydrolyze retinyl ester at neutral pH in the absence of bile salts or bile salt derivatives has been reported previously (12) and is supported by our own results (Fig. 4a). These data demonstrate that ES-10 has the catalytic properties that would enable it to function both in the neutral and acidic endosomal environments. The addition of recombinant apo-CRBP1 to the micellar assay system did not result in an increased rate of retinyl ester hydrolysis by purified ES-10. In contrast, Boerman and Napoli described the stimulation of cholate-independent retinyl ester hydrolysis by apo-CRBP1 of endogenous retinyl esters of rat liver microsomes (11). These two opposing observations are possibly due to the different assay methodologies used to measure retinyl ester hydrolase activity: a) micellar, detergent-solubilized retinyl palmitate versus phospholipid-bound, endogenous retinyl esters and b) highly purified ES-10 versus microsomes. These differences indicate that the stimulatory effect was highly dependent on the presence of phospholipid-bilayer membranes in the assay mixture (29). The strong stimulatory effect of apo-cRBP on retinyl ester hydrolysis was not observed at higher cholate concentrations (> 8 mM, cmc of cholate: ~7-8 mM at pH 8), probably due the solubilization of microsomes into cholate-phospholipid micelles. The same authors speculated that the stimulatory effect of apo-cRBP on retinyl ester hydrolysis is possibly due to a conformational change of retinyl ester hydrolase as a result of a direct protein-protein interaction between apo-CRBP and retinyl ester hydrolyase (11). While we cannot rule out that the detergents used in our test system possibly interfered with such a suggested interaction, we would point out that CRBP-I is a cytosolic protein, whereas ES-10 is most likely located in the lumen of the endoplasmic reticulum and/or endosomes. The different subcellular locations of these two proteins would therefore preclude such an interaction. In addition, two recent studies of retinol turnover in liver of wild-type and in cRBP1 knock-out mice also suggest that cRBP1 facilitates retinyl ester synthesis by LRAT rather than hydrolysis by REH and that cRBP1 might play an important role in retinol transport between hepatocytes and stellate cells (30, 31). The observed differences between our and the previous report illustrate the need for additional research to define the intracellular protein-protein interactions of both cRBP and retinyl ester hydrolase in greater
The recent development of novel protein-protein interaction screens in conjunction with the availability of recombinant cRBP should facilitate this research.

To date, four different proteins of the non-specific carboxylesterase supergene family have been identified that are able to hydrolyze retinyl esters in vitro: ES-2, ES-3, ES-4 and ES-10, (12-14,32). Sequence analysis of these proteins reveals that ES-3, ES-4 and ES-10 share the tetrapeptide sequence, HXEL, at their respective C-termini. The sequence HXEL is a variation of the eukaryotic ER retention signal, KDEL, and has been shown to be an efficient retention sequence that localizes carboxylesterases, including ES-10, to the lumen of the ER (33). In contrast, ES-2 (also called serum carboxylesterase) lacks this C-terminal consensus sequence and probably represents a secretory form of the carboxylesterases (34).

The glycosylation pattern, more specifically the extent and complexity of glycosylation, can be used to gain additional insight into the subcellular localization and intracellular transport of glycoproteins. Since we did not obtain direct sequence information of the ES-10 C-terminus by LC-MS/MS analysis we investigated the glycosylation pattern of ES-10 in more detail. ES-10 has two potential N-glycosylation sites at residues N79 and N479 and is sensitive to deglycosylation with PNGase F and Endo H (Fig. 3). Deglycosylation with PNGase F and Endo H reduced the molecular weight of the purified protein from 62 kDa to approximately 60.1 kDa and 60.2 kDa respectively, in good agreement with the theoretical average protein molecular weight of 60.05 kDa for mature ES-10. The substrate specificity of PNGase F and Endo H suggests that ES-10 contains high mannose-type carbohydrate structures (35). The molecular weight difference of less than 2 kDa between the glycosylated and deglycosylated form of mature ES-10 suggests that only one of two N-linked glycosylation sites in ES-10 is used and that the carbohydrate structure is most likely of the high mannose type. This is further supported by our observation that ES-10 can be purified with immobilized Con A Sepharose, which is known to bind Asn-linked oligosaccharides of biantennary complex-type and high mannose type, but not complex triantennary and tetraantennary carbohydrate structures (36).

Probing of purified ES-10 with several different lectins such as SNA, MAA, PNA and DSA that recognize sialic acid, galactose β(1-3) N-acetylgalactosamine and Gal β(1-4)GlcNAc carbohydrates structures respectively also failed to detect a more complex glycosylation pattern. The absence of a more complex glycosylation pattern indicates that ES-10 does not traverse the Golgi compartment and is indeed retained in the ER. In contrast, serum carboxylesterase (ES-2) is resistant to deglycosylation with Endo H and glycosylation contributes as much as 20% to the apparent molecular weight of this secreted protein (34).

The relative contribution of the different carboxylesterases to the overall hepatic retinyl ester turnover is difficult to assess due to the variety of assay conditions used to measure the specific enzymatic activities. Sanghani et al recently estimated that ES-10 is responsible for approximately 60% and ES-4 for approximately 34% of the total neutral retinyl ester hydrolysis activity in rat liver microsomes, as judged by the catalytic efficiency of these two enzymes in the presence of high concentrations of bile salts (14). However, it remains unclear whether ES-4 does indeed contribute to a large extent to hepatic retinyl ester turnover. Two recent papers demonstrated that Acyl-CoA thioesters are the preferred lipid substrates for ES-4 (37,38) and there are conflicting reports about the ability of ES-4 to hydrolyze retinyl esters in the absence of bile salts (13,28). In contrast, ES-10 showed a substrate preference for retinyl esters over triglycerides in a bile salt-free assay system, with no activity toward cholesteryl esters (Fig. 4c). These observations point toward ES-10 as the most prominent retinyl ester hydrolase in rat liver, especially if one also takes the relative levels of gene expression of ES-10 into account (Fig. 6). The special role of ES-10 in hepatic retinyl ester metabolism is further underscored by a recent proteomic analysis of rat hepatic stellate cells in which ES-10 was among the 150 stellate cell proteins identified and the only protein with known retinyl ester hydrolase activity (39).

The role that ES-10 plays in retinyl ester metabolism will most likely depend on the subcellular localization of this reaction. If retinyl esters are transported to the ER before hydrolysis, they will most likely be hydrolyzed by ES-10 and,
perhaps to a lesser degree, by ES-4. This takes into account that ES-10 and ES-4 are predominantly localized in the ER and that they contribute most of the total carboxylesterase activity detected in the ER. On the other hand, if retinyl esters remain in early endosomes they could also be hydrolyzed by ES-2, which has been localized in plasma membrane/endosomal fractions (12). It is conceivable that ES-2 undergoes a recycling process that localizes it to early endosomal compartments after endocytotic uptake alongside chylomicron remnants. A similar recycling process has been described recently for ApoE (40). The identification of the relevant retinyl ester hydrolysis pathway will require more detailed information about a) intracellular retinol trafficking in hepatocytes and b) the subcellular localization of ES-2, ES-3, ES-4 and ES-10. Progress in this area has been hampered due to the lack of specific antibodies that can differentiate between the highly related carboxylesterases. However, with the recent development of gene silencing by RNA interference to study protein function, we now have a tool that should help us to address these important questions.
REFERENCES


Footnotes

* This work was supported in part by the National Institute of Health Grant DK044498 and by the USDA-Agricultural Research Service, Project Number: 1235-51000-040-03.

The abbreviations used are: AREH, acid retinyl ester hydrolase; AS, ammonium sulfate; Con A, concanavalin A; DSA, Datura stramonium agglutinin; Endo H, β-endo-N-acetylglucosaminidase H; MAA, Maackia amurensis agglutinin; MALDI-ToF, matrix assisted laser desorption ionization-time of flight; MG, methyl-α-D-glucopyranoside; MS, mass spectrometry; NaAc, sodium acetate; OG, octyl-β-D-glucopyranoside; PNA, peanut agglutinin; PNGase F, peptide N-glycanase F; REH, retinyl ester hydrolase; SELDI-TOF, surface enhanced laser desorption ionization-time of flight; SNA, Sambucus nigra agglutinin; TFA, trifluoroacetic acid
FIGURE LEGENDS

Fig. 1. Gel filtration analysis of purified rat liver AREH. Pooled and concentrated fractions of the Con A column were applied to a Superose 12 HR10/30 gel filtration column and eluted isocratically as described under experimental procedures. Fig. 1 shows the calibration curve of the Superose12 HR10/30 column. MW standards used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 KDa).

Fig. 2. SDS-PAGE analysis of AREH during protein purification. Approximately 2.5-3 µg total protein were applied to each lane and separated under reducing conditions on a 10% Tris-Tricine SDS-PAGE gel. Proteins were visualized by colloidal Coomassie Blue staining or silver staining. Lane 1, microsomes; lane 2, solubilized microsomes; lane 3, SP-Sepharose pool, lane 4, Phenyl-Sepharose pool, lane 5, Con A-Sepharose pool; lane 6, Superose 12 pool; lane 7 silver stained Superose 12 pool.

Fig. 3. Deglycosylation of purified ES-10. Purified ES-10 was incubated on the surface of a H4 chip with either PNGase F or Endo H and then subsequently analyzed by SELDI-TOF MS as described under experimental procedures. Fig. 3a, untreated ES-10; Fig. 3b, purified ES-10 after treatment with PNGase F; Fig. 3c, purified ES-10 after treatment with Endo H.

Fig. 4. Enzymatic characterization of purified ES-10 as a retinyl ester hydrolase. Fig. 4a, effect of pH on purified AREH activity. Assays were conducted with 50 mM buffers at the indicated pHs. Fig. 4b, effect of substrate concentration on AREH activity. Assays were conducted in the presence of rising substrate concentrations while keeping the concentration of Triton X-100 constant. Fig. 4c, substrate specificity of ES-10 in a micellar assay system at a substrate concentration of 100 µM at the indicated pH value.

Fig. 5 Tissue specificity of ES-10 gene expression. ES-10 gene expression in different tissues was compared with the relative threshold cycle method using rat L32 as a housekeeping gene. For relative gene quantification the level of ES-10 gene expression in liver was set to 1.

Fig. 6 Tissue specificity of ES10 protein expression. Total tissue protein extracts were separated on a 10% Tris-Tricine SDS-PAGE gel and blotted onto PVDF membranes. ES-10 was detected with an anti ES-10 antibody and visualized with the SuperSignal West Dura Extented Duration Substrate kit (Pierce).

Fig. 7 Relative gene expression of ES-2, ES-3, ES-4 and ES-10 in liver. The gene expression of different carboxylesterases in liver was compared with the relative threshold cycle method using rat L32 as housekeeping gene. For relative gene quantification the level of ES-10 expression was set to 1.
### Table 1

Rat probe and primer sequences used for real time RT-PCR experiment. Optimized probe and primer concentrations are indicated in brackets.

<table>
<thead>
<tr>
<th>Target (Gene Bank)</th>
<th>Probes (conc.)</th>
<th>Forward primer (conc.)</th>
<th>Reverse Primer (conc.)</th>
</tr>
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<tbody>
<tr>
<td>DNA</td>
<td>5’-FAM-3’BHQ</td>
<td>5’-3’</td>
<td>5’-3’</td>
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<tr>
<td>L-32 (NM_013226)</td>
<td>CAGCACAGCTGCGCATC</td>
<td>CCGAAAAGCCATCG</td>
<td>CTCGCGGTGGGGAT</td>
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<td></td>
<td>AGAGTCAC (200nM)</td>
<td>TAGAAAGA (300nM)</td>
<td>TGG (900nM)</td>
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<tr>
<td>ES-10 (NM_133295)</td>
<td>TGGGCTATCCACTCTCC</td>
<td>AGCAAGAGTTGGCT</td>
<td>AGAGGATTTGGCT</td>
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<tr>
<td></td>
<td>GAAGGCA (200nM)</td>
<td>GGATCAT (300nM)</td>
<td>GTTTTCTG (900nM)</td>
</tr>
<tr>
<td>ES-4 (X81825)</td>
<td>CTGGATGTGAACCCACCG</td>
<td>CAGCCGCTAAGCAA</td>
<td>ACGAGGCAGTGAAC</td>
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<td>ACATCTGCC (200nM)</td>
<td>ATTGCT (300nM)</td>
<td>AATGA (900nM)</td>
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<tr>
<td>ES-3 (NM_031565)</td>
<td>ATCCCCATGGACAGGG</td>
<td>CTGGGCAAACCTGTGC</td>
<td>TTTGGTCTACTCT</td>
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<td>CCTGCC (200nM)</td>
<td>TAGGAA (900nM)</td>
<td>GGCAATG (900nM)</td>
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<td>ES-2 (NM_133586)</td>
<td>CACGGAAACACCCACA</td>
<td>GCCCGACGAACTGA</td>
<td>GAATAAGCAGGAG</td>
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<td>TAGCTGGCTG (200nM)</td>
<td>GAACTG (900nM)</td>
<td>CCCAAAGAG (900nM)</td>
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### Table 2

Purification of a bile salt-independent AREH from solubilized rat liver microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein conc.</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Enrichment</th>
<th>Yield</th>
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<tbody>
<tr>
<td>Microsomes</td>
<td>50</td>
<td>12.8</td>
<td>640</td>
<td>704</td>
<td>1.1</td>
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<td></td>
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<tr>
<td>Solubilized microsomal supernatant</td>
<td>320</td>
<td>0.5</td>
<td>160</td>
<td>2080</td>
<td>13</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>SP Sepharose Pool</td>
<td>50</td>
<td>0.6</td>
<td>30</td>
<td>897</td>
<td>29.9</td>
<td>2.3</td>
<td>43</td>
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<tr>
<td>Phenyl Sepharose Pool</td>
<td>20</td>
<td>0.3</td>
<td>6</td>
<td>247</td>
<td>41.3</td>
<td>3.2</td>
<td>11.8</td>
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<td>ConA Sepharose Pool</td>
<td>18</td>
<td>0.18</td>
<td>3.2</td>
<td>203</td>
<td>63.5</td>
<td>4.9</td>
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<td>Superose 12 Gel Filtration</td>
<td>8</td>
<td>0.32</td>
<td>2.56</td>
<td>172</td>
<td>67.4</td>
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<td>8.3</td>
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Table 3

Enzymatic properties of carboxylesterase ES 10 as a bile salt-independent REH

<table>
<thead>
<tr>
<th>Molecular Weight (kDa)a</th>
<th>Glycosylated ES 10</th>
<th>62</th>
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<tr>
<td></td>
<td>Deglycosylated ES 10b</td>
<td>60.1</td>
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<tr>
<td>Carbohydrate structure of N-glycan</td>
<td>high mannose type</td>
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<tr>
<td>N-terminal amino acid</td>
<td>19Tyr</td>
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<tr>
<td>K_m (µM)c</td>
<td>86±12 (N=3)</td>
<td></td>
</tr>
<tr>
<td>V_max (nmol h⁻¹ mg⁻¹)c</td>
<td>724±48 (N=3)</td>
<td></td>
</tr>
<tr>
<td>Specific activity (nmol h⁻¹ mg⁻¹)c</td>
<td>437</td>
<td></td>
</tr>
</tbody>
</table>

a As determined by MALDI-ToF MS
b After PNGase F treatment
c At pH 5.0
d At a substrate concentration of 100 µM
Fig. 1

![Graph showing molecular weight vs. elution volume ratio for various proteins: β-Amylase, AREH, Alcohol Dehydrogenase, Albumin, Carbonic Anhydrase, Cytochrome C.](image)

Fig. 2

![Image of gel electrophoresis showing molecular weight markers and lanes labeled 1 to 6 and 7 with standards.](image)
Fig. 4b

![Graph showing retinyl ester hydrolase activity vs. substrate concentration. The x-axis represents substrate concentration (µM) ranging from 0 to 200, and the y-axis represents retinyl ester hydrolase activity (nmol/mg x h) ranging from 0 to 700. The activity increases with substrate concentration.]

Fig. 4c

![Bar chart showing ES substrate activity in % for retinyl palmitate, triolein, and cholesteryl oleate at pH 5 and pH 7. The bar for retinyl palmitate is significantly higher than for the other substrates at both pH levels.]

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Fig. 5

![Bar chart of tissue relative expression](image)

Liver, Spleen, Kidney, Heart, Lung, Brain, Ovary, Testis

Fig. 6

![Image of Western blot analysis](image)

Liver, Pancreas, Small Intest., Kidney, Spleen, Heart, Lung, Skin, Brain, Eye, Ovary, Testis, Placenta

ES10

Fig. 7

![Bar chart of relative expression](image)

ES 10, ES 4, ES 3, ES 2

Relative Expression