Esterase 22 and beta-glucuronidase hydrolyze retinoids in mouse liver

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Abstract  Excess dietary vitamin A is esterified with fatty acids and stored in the form of retinyl ester (RE) predominantly in the liver. According to the requirements of the body, liver RE stores are hydrolyzed and retinol is delivered to peripheral tissues. The controlled mobilization of retinol ensures a constant supply of the body with the vitamin. Currently, the enzymes catalyzing liver RE hydrolysis are unknown. In this study, we identified mouse esterase 22 (Es22) as potent RE hydrolyse highly expressed in the liver, particularly in hepatocytes. The enzyme is located exclusively at the endoplasmic reticulum (ER), implying that it is not involved in the mobilization of RE present in cytosolic lipid droplets. Nevertheless, cell culture experiments revealed that overexpression of Es22 attenuated the formation of cellular RE stores, presumably by counteracting retinol esterification at the ER. Es22 was previously shown to form a complex with beta-glucuronidase (Gus). Our studies revealed that Gus colocalizes with Es22 at the ER but does not affect its RE hydrolysis activity. Interestingly, however, Gus was capable of hydrolyzing the naturally occurring vitamin A metabolite retinoyl-beta-glucuronide. In conclusion, our observations implicate that both Es22 and Gus play a role in liver retinoid metabolism. — Schreiber, R., U. Taschler, H. Wolinski, A. Seper, S. N. Tamegger, M. Graf, S. D. Kohlwein, G. Haemmerle, R. Zimmermann, R. Zechner, and A. Lass. Esterase 22 and beta-glucuronidase hydrolyze retinoids in mouse liver. J. Lipid Res. 2009. 50: 2514–2523.

Supplementary key words  vitamin A • retinol • retinyl ester hydrolyase • endoplasmic reticulum

Retinoids (vitamin A and metabolites) are essential micronutrients in mammals (1). Dietary retinoids are readily absorbed by the intestine. In the intestinal lumen, retinyl esters (REs) are hydrolyzed to retinol by the action of pancreatic triglyceride lipase (2, 3). Within the enterocytes, retinol is reesterified (3–5) for the incorporation into chylomicrons and secreted (6). In the circulation, chylomicrons are depleted from triglycerides by lipoprotein lipase and are thereby transformed to chylomicron remnants (7). These remnants acquire apolipoprotein E and are then cleared mostly by parenchymal cells of the liver (i.e., hepatocytes) (8–10). In hepatocytes, REs are hydrolyzed, and unesterified retinol is associated with retinol-binding protein 4 (RBP4) for secretion (11, 12) or transferred to hepatic stellate cells for storage (10, 13, 14). These stellate cells store most of the total body vitamin A reserves (~80%) in the form of retinyl palmitate in cytosolic lipid droplets (14).

According to the body’s demand, stored retinoids are released from the liver to facilitate a constant supply. In the circulation, the biologically inactive retinol is transported bound to RBP4 and delivered to target tissues. There, retinol is converted into its biologically active metabolites 11-cis retinaldehyde and retinoic acids, which act as hormone acceptor in the visual cycle and as ligand of nuclear receptors, respectively.

The dynamic balance between synthesis and hydrolysis of RE determines the concentration of retinol in the circulation and also the availability of retinol for conversion in active metabolites in various cell types. Retinol is esterified by the action of acyl-CoA:retinol acyltransferase (ARAT) or lecithin:retinol acyltransferase (LRAT) for storage in lipid droplets. Much work has focused on the understanding of how REs are released from lipid droplets and which

Abbreviations: ARAT, acyl-CoA:retinol acyltransferase; BHT, butylated hydroxytoluene; CE, cholesteryl ester; CEL, carboxyl ester lipase; ER, endoplasmic reticulum; Es22, esterase 22; GFP, green fluorescent protein; Gus, beta-glucuronidase; KID, kidney; LacZ, beta-galactosidase; LRAT, lecithin:retinol acyltransferase; MOMA, macrophage marker antibody; RAG, retinol beta-glucuronide; RBP4, retinol binding protein 4; RE, retinyl ester; REH, retinyl ester hydrolyase; RFP, red fluorescent protein; a-SMA, alpha smooth muscle actin; TBST, Tris/NaCl/Tween-20; TG, triglyceride. 1To whom correspondence should be addressed.
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retinyl ester hydrolases (REHs) are involved in this process. A number of potential candidates for the hydrolysis of REs in the liver has been studied so far in more detail. For instance, bile salt-activated carboxyl esterase (CEL) (15, 16) has been demonstrated to hydrolyze RE. However, CEL-deficient mice generated by targeted disruption of the CEL gene failed to show any effect on RE metabolism in the liver, on serum levels of retinol and RBP4, or on levels of retinoids in various tissues (17). Two distinct bile salt-independent REHs, one active at neutral (18), the other at acid pH (19), have been characterized in rat liver; in addition, three rat liver carboxyl esterases [Es2 (20), Es4, and Es10 (21, 22)] exhibit REH activity in vitro. Besides their expression in liver, no convincing evidence has been reported that any of these enzymes may play a key role in the mobilization of REs.

In this study, we compared the potency of several members of the mouse carboxyl esterase superfamily to hydrolyze REs. Among other enzymes, we identified esterase 22 (Es22) as a potent REH. Notably, expression of Es22 attenuated cellular RE accumulation, indicating that this enzyme affects retinol metabolism in living cells. The enzyme is highly expressed in liver cells and localizes to the endoplasmic reticulum (ER), suggesting that it counteracts the formation of RE by LRAT and ARAT. Es22 has been shown to interact with β-glucuronidase (Gus) at the ER (23). We found that Gus colocalizes with Es22 at the ER and is capable of hydrolyzing retinoyl β-glucuronide, a naturally occurring retinoid. In conclusion, our data indicate that both Es22 and Gus play a role in liver retinoid metabolism.

**MATERIALS AND METHODS**

**Materials**

All-trans-retinol, all-trans-retinyl-palmitate, and palmitic acid were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). All-trans-retinoyl β-glucuronide (RAG) was kindly provided by Prof. Arun B. Barua, Iowa State University, Ames, IA. pCRU, a self-made yeast expression vector containing a monomeric RFP sequence, was a kind gift from Julia Petschnigg, Kohlwein Laboratory, University of Graz, Graz, Austria. Retinyl [9,10-(n)-3H]palmitate was prepared according to Boechzelt et al. (24) using palmitic acid (2 mCi, 0.033 μmol, GE Healthcare, Piscataway, NJ) as radiolabel. All reagents were of per analysis grade.

**Animals**

Adult male C57BL/6 mice between 12 and 16 weeks of age were used in this study. Mice were maintained on a regular light-dark cycle (14 h light, 10 h dark) and fed a standard laboratory chow diet (4.5% wt/wt fat).

cDNA cloning of recombinant His, GFP-, or RFP-tagged proteins

Total RNA was isolated from mouse tissue using the Trizol® Reagent procedure according to the manufacturer’s instruction (Invitrogen™, Carlsbad, CA). Poly A’ RNA was isolated from liver total RNA using the Oligotex® mRNA Mini Kit from Qiagen GmbH (Hilden, Germany). Liver mRNA was transcribed into first-strand cDNA using SuperScript™ Reverse Transcriptase protocol from Invitrogen™. Second-strand cDNA was obtained from first-strand cDNA by addition of Escherichia coli DNA ligase buffer, E. coli DNA polymerase, E. coli DNA ligase (all chemicals from New England Biolabs, Inc., Beverly, MA), and deoxyribonucleotide triphosphates (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) to the mixture and subsequent incubation at 16°C for 3 h. Thereafter, T4 DNA polymerase (New England Biolabs) was added and further incubated for 20 min to give blunt end cDNA. The coding sequences of various genes (see Table 1) were amplified by PCR from liver cDNA using Advantage® cDNA Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA). Respective primers were designed to create 5’ and 3’ restriction endonuclease cleavage sites for in-frame ligation into expression vector pcDNA4/HisMax (Invitrogen™). A control pcDNA4/HisMax vector expressing β-galactosidase (LacZ) was provided by the manufacturer (Invitrogen™).

For generation of Es22-green fluorescent protein (GFP) and Gus-red fluorescent protein (RFP) fusion constructs full-length Es22 and Gus coding sequences were amplified using liver cDNA as template and respective primers: Es22_forward, 5’ ATCTCGAGCCACCATGTGCTCTCGTGCTAGTGCC-3’; Es22_reverse, 5’ CTCCATGCGAGGAGCTCGGAGTGCGTCG-3’; Gus_forward, 5’ TCTCCAGGCCACCATGTGCCCTCTGCTCTGCTGCC-3’; Gus_reverse, 5’ CGAGTTGCAGGCTGAGTGACCATAGCTCC-3’. RFP coding sequence was amplified from the pCRU vector with the following primers: RFP_forward, GGAATTCGCCTCTCGAGGGATGTCTAAT; RFP_reverse, GGAATTCGTTAGGCGCGGCGGTGAGTG. The PCR mixture contained 1 μl cDNA or pTR-CU vector (10 ng/μl), 10 pmol primers, 10 nmol dNTPs, 1 U Phusion™ High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland), and 6 μl Phusion™ high fidelity buffer in a total volume of 30 μl. The amplified Es22 and Gus coding sequences were digested with XhoI and ligated into respective sites of the pEGFP-N1 vector (Takara Bio Inc., Otsu, Japan). The amplified RFP coding sequence was digested with EcoRI and ligated into respective sites of the pEGFP-N1 vector already containing the Gus coding sequence. The resulting fusion constructs encoded GFP and RFP at the C terminus of Es22 (Es22-GFP) and Gus (Gus-RFP), respectively.

**Expression of recombinant proteins in cultured cells**

Monkey embryonic kidney cells (COS-7, ATCC CRL-1651) were maintained in DMEM (Gibco® from Invitrogen™) containing 10% fetal calf serum (FCS) (Sigma-Aldrich Chemie GmbH) and antibiotics at 37°C in humidified air (89–91% saturation) and 5% CO2. The day before transfection, COS-7 cells were cultured in logarhythmic phase, seeded in 6-wells dishes at a density of 150,000 cells/well, and cultured overnight. Transient transfection of COS-7 cells with pcDNA4/HisMax encoding respective His-tagged recombinant proteins or as a control LacZ was performed with Metafectene™ (Biontex GmbH, Munich, Germany). One μg purified DNA (NucleoBond® AX, Macherey-Nagel GmbH and Co. KG, Duren, Germany) was mixed with 5 μl Metafectene™ in a total volume of 100 μl serum- and antibiotics-free DMEM and incubated for 20 min at room temperature to allow formation of the DNA/Metafectene™ complex. Then, 100 μl of the DNA/Metafectene™ mix was added to COS-7 cells and incubated for 4 h in serum and antibiotics-free DMEM. Thereafter, the medium was removed and cells were cultured in DMEM containing 10% FCS in serum and antibiotics-free DMEM. Thereafter, the medium was removed and cells were cultured in DMEM containing 10% FCS in serum and antibiotics-free DMEM. Cells were analyzed two days after transfection.

**Preparation of COS-7 cell lysates**

Cells were collected by trypsination and washed three times with PBS. Then, cells were disrupted on ice in buffer A (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptine, 10 μg/ml aprotinin, 1 mM PMSF). The resulting lysate was centrifuged at 13,000 × g for 1 h to remove cellular debris. The supernatant was collected and further processed as described.
2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) by sonication (Vinsonic 475, Virtis, Gardiner, NJ). Nuclei and unbroken cells were removed by centrifugation at 1,000 g at 4°C for 5 min.

**Determination of lipid hydrolase activities**

For determination of REH activity of recombinant proteins, 100 µl of COS-7 cell lysates (≥100 µg cell protein) containing recombinant proteins and 100 µl substrate was incubated in a water bath at 37°C for 60 min. The reaction was terminated by addition of 3.25 ml of methanol-chloroform-heptane (10:9:7, v/v/v) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. After vigorous vortexing and centrifugation (800 g, 10 min), 1 ml of the upper aqueous phase was aspirated and radioactivity was determined by liquid scintillation counting (Tri-Carb 2100TR, Packard Instrument Co., Downers Grove, IL). Blank incubation was performed with 100 µl buffer A under identical conditions as for cell lysates. Counts obtained for blank incubation were used for background correction. Substrate for REH assay contained 10 nmol retinyl palmitate/assay and retinyl [9,10-(n)-H]palmitate, 50,000 cpn/nmol as tracer) and was either emulsified in 100 mM potassium phosphate buffer pH 7.4, containing 90 µM phosphatidylcholine/-inositol (PC:PI; 3:1) or 100 mM Tris/Maleate buffer pH 8.0, containing 40 mM cholate. Other lipid substrates were prepared in 100 mM Tris/Maleate buffer pH 8.0, containing 40 mM cholate and either 35 nmol triglyceride/assay (TG; glycerol tri[9,10-(n)-H]oleate, 40,000 cpn/nmol) or 20 nmol/assay of cholesteryl oleate (CE; cholesteryl [9,10-(n)-H]oleate, 50,000 cpn/nmol). All lipid substrates were prepared by sonication on ice (Vinsonic 475, Virtis). In some cases, substrate was prepared using buffer systems and detergents as indicated in respective figure.

**Determination of RAG hydrolase activity**

Cell lysates containing recombinant Gus, Es22, or LacZ were incubated in 50 mM Tris/Maleate buffer pH 8.0, containing 25 µM RAG for 30 min at 37°C. Then, reaction was stopped by addition of methanol and RAGs were extracted using three different organic solvents (n-hexane, ethyl acetate, and chloroform). Extracts were combined and RAG content was determined by HPLC as described below.

**Intracellular retinyl palmitate accumulation**

COS-7 cells were plated in 6-well plates and transduced with Es22 or LacZ, as described above. After 48 h of incubation, the media was replaced with DMEM containing 10% FCS, antibiotics, 500 µM palmitic acid (10 mM, solubilized in sterile PBS, containing 50 mg defatted BSA/ml), and 100 µM all-trans-retinol (350 mM in ethanol). At various time points, cells were harvested by trypsinization, washed three times with PBS, and subjected to determination of retinyl palmitate content by HPLC.

**Extraction of retinoids**

All operations were carried out with precooled solvents; whenever possible, samples were placed on ice and protected from light. For the extraction of retinyl palmitate, 400 µl of COS-7 cells (2 mg cell protein/ml) suspended in buffer A were treated with 400 µl 100% methanol containing 0.1% butylated hydroxytoluene (BHT), 1 mM EDTA, and 800 µl water-washed n-hexane. Thereafter, the mixture was vortexed (15 s) and centrifuged at 2,000 g for 3 min. An aliquot of the supernatant (600 µl) was removed, dried down using a speedvac (Heto-Holten, Allered, Denmark), and resuspended in 100 µl of methanol. An aliquot of 20 µl was analyzed by HPLC. Extraction of RAG was performed according to the procedure as outlined by Barua et al. (21). Briefly, 200 µl of lysates were treated with 200 µl ethanol containing 0.1% BHT and 400 µl ethyl acetate. After vortexing (15 s) and centrifugation (2,000 g for 2 min), 200 µl of water was added and extraction was performed by vortexing and centrifugation as described. Then, the upper organic phase was saved and the aqueous phase was acidified with 5 µl of 10% glacial acetic acid in water. The aqueous phase was subsequently extracted with 200 µl ethyl acetate and then extracted with 200 µl n-hexane by vortexing and centrifugation as described above. All organic solvents were pooled and brought to dryness using a speedvac (Heto-Holten). The residue was dissolved in 100 µl methanol and 40 µl were analyzed by HPLC.

**TABLE 1. List of cloned genes**

<table>
<thead>
<tr>
<th>GI Number</th>
<th>Accession Number</th>
<th>Identifier</th>
<th>Description</th>
<th>Primer Forward/Reverse</th>
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<tbody>
<tr>
<td>117553603</td>
<td>NM_053290</td>
<td>Ces3</td>
<td>Carboxylesterase 3</td>
<td>5′-GGAATTC GCGCTCTCAACTCCCTCCTATGATGCC-3′</td>
</tr>
<tr>
<td>141802881</td>
<td>NM_172759</td>
<td>Ces5</td>
<td>Carboxylesterase 5</td>
<td>5′-GGAATTC TGGCCTCTTCTGTTCCAAGTCG-3′</td>
</tr>
<tr>
<td>10946841</td>
<td>NM_021456</td>
<td>Ces1</td>
<td>Carboxylesterase 1</td>
<td>5′-GGAATTC TGGCCTCTTCTGTTCCAAGTCG-3′</td>
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<tr>
<td>21362300</td>
<td>NM_144511</td>
<td>EG13</td>
<td>predicted gene, EG1309</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
</tr>
<tr>
<td>28279460</td>
<td>BC046327</td>
<td>ACE</td>
<td>Acetylcholinesterase</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
</tr>
<tr>
<td>22122766</td>
<td>NM_146213</td>
<td>BC026</td>
<td>cDNA seq, BC026374</td>
<td>5′-GGAATTC GCGCTCTCCTGCTCCATGCCCTG-3′</td>
</tr>
<tr>
<td>20886282</td>
<td>NM_146488</td>
<td>sim Ces2</td>
<td>similar to Ces 2</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
</tr>
<tr>
<td>124487349</td>
<td>NM_009738</td>
<td>Bchc</td>
<td>Butyrylcholinesterase</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
</tr>
<tr>
<td>145301632</td>
<td>NM_133660</td>
<td>Es22</td>
<td>sim rat Es3</td>
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<tr>
<td>21450338</td>
<td>NM_144930</td>
<td>TGH2</td>
<td>TGH2 (sim rat Es4)</td>
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</tr>
<tr>
<td>142348402</td>
<td>NM_007954</td>
<td>Es1</td>
<td>sim rat Es2</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
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<tr>
<td>146134463</td>
<td>NM_016903</td>
<td>Es10</td>
<td>Esterase 10</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
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<tr>
<td>6754097</td>
<td>NM_010368</td>
<td>Gus</td>
<td>β-Glucuronidase</td>
<td>5′-GGAATTC AGACTGGAACAAATTCATGCTCG-3′</td>
</tr>
</tbody>
</table>
HPLC analysis of retinoids

Retinoids were separated on a reverse-phase Lichrospher® 125-4 5 µm C18 column (125 × 4 mm) preceded by a Sphero® RP-18 guard column (Merck, Darmstadt, Germany). For the separation of retinyl palmitate 100% methanol and for RAG methanol-water (7.5:2.5, v/v) containing 10 mM ammonium acetate was used isocratically as the eluant at a flow rate of 1.5 and 1 ml/min, respectively. The HPLC system used was System Gold® from Beckmann Coulter Inc. (Fullerton, CA), consisting of a 125 solvent module, a 508 autosampler, and a 168 diode-array detector. Detections of retinyl palmitate and RAG were performed at 325 nm and 350 nm, respectively. The areas under peaks were standardized against known amounts of reference compounds. For the determination of molar concentrations of reference compounds, the following molar extinction coefficients were used: all-trans-retinyl palmitate (E$_{325\text{nm}}$ = 52,275 M$^{-1}$cm$^{-1}$) (26); RAG (E$_{1cm}$, 1% = 1065 at 360 nm) (27).

Isolation of various liver cell-types

Mice were anesthetized and the abdomen was surgically opened by a vertical incision. Then, the liver was perfused via the portal vein with Krebs-Henseleit buffer (without Ca$^{2+}$ and SO$_4^{2-}$) for 10 min followed by a perfusion with Krebs-Henseleit buffer containing 30 mg collagenase type II (0.2 mg/ml, Worthington Biochemical Corporation, Lakewood, NJ), 1% BSA, and 0.1 mM CaCl$_2$ for 10–15 min. Thereafter, the liver was excised, disrupted, and the cell suspension passed through gauze, followed by filtration through a 70 µm cell strainer. Parenchymal cells were separated from nonparenchymal cells by centrifugation at 50 g for 3 min at 4°C. The remaining supernatant was used for the isolation of various nonparenchymal cell-types using OptiPrep® self-forming density gradient solutions (Axis-Shield PoC AS, Rodelsøka, Norway) according to manufacturer’s instructions. Briefly, nonparenchymal cell suspension was adjusted to a density of 24% iodixanol, overlaid with 17%, 11.5%, 8.4%, and 0% iodixanol in Krebs-Henseleit buffer containing 1.25 mM CaCl$_2$ and 1.2 mM NaSO$_4$. After centrifugation at 1,400 g for 20 min at 4°C, Kupffer and stellate cells were isolated at 11.5/8.4 and 8.4/0% iodixanol interphases, respectively. RNA of hepatocytes and Kupffer cells was isolated immediately or after cultivation of the cells overnight. RNA of stellate cells was obtained from freshly prepared cells or after cultivation for 7 days [selective detachment according to Trøen et al. (28)]. Cell lysates for Western blotting analysis of different liver cells were washed twice with PBS after respective cultivation, scraped off, and disrupted as described for COS-7 cells.

Northern blotting analysis

Total RNA was extracted from various mouse tissues or liver cell types using Trizol® Reagent (Invitrogen™), separated by formaldehyde/agarose gel electrophoresis and blotted onto a Hybond-N+ membrane (GE Healthcare) by vacuum blotting (Bio-Rad 785, Rodejøl, Norway) according to manufacturer’s instructions. Briefly, nonparenchymal cell suspension was adjusted to a density of 24% iodixanol, overlaid with 17%, 11.5%, 8.4%, and 0% iodixanol in Krebs-Henseleit buffer containing 1.25 mM CaCl$_2$ and 1.2 mM NaSO$_4$. After centrifugation at 1,400 g for 20 min at 4°C, Kupffer and stellate cells were isolated at 11.5/8.4 and 8.4/0% iodixanol interphases, respectively. RNA of hepatocytes and Kupffer cells was isolated immediately or after cultivation of the cells overnight. RNA of stellate cells was obtained from freshly prepared cells or after cultivation for 7 days [selective detachment according to Trøen et al. (28)]. Cell lysates for Western blotting analysis of different liver cells were washed twice with PBS after respective cultivation, scraped off, and disrupted as described for COS-7 cells.

Fig. 1. Retinyl ester hydrolase activity of various carboxyl esterases using (A) phospholipids or (B) cholate as detergents for substrate preparation. Carboxyl esterases (see Table 1) were cloned into the mammalian expression vector pcDNA4/HisMax for expression in COS-7 cells. As a control, COS-7 cells were transfected with a plasmid encoding β-galactosidase (LacZ). Lysates of COS-7 cells (1,000 mg supernatant) were subjected to REH assays using radiolabeled retinyl (3H) palmitate as tracer. Data are mean ± SD of triplicate determination in relation to the activity detected in control transfected cells (LacZ) and are representative for three independent determinations. * P < 0.05; ** P < 0.01; *** P < 0.001.
with 2 µM Bodipy® 558/568 C12 for 30 min at 37°C. After staining, cells were washed twice with PBS. For microscopy, coverslips with attached cells were mounted on standard microscope slides. Microscopy was performed using a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) with spectral detection and a Leica 63× water immersion objective (HCX PL APO W Corr CS, 1.2 NA). GFP or RFP fluorescence was excited at 488 or 555 nm and detected in the range between 500 and 535 or 580 and 620, respectively. ER-Tracker Red™ fluorescence was excited at 543 nm and detected in the range from 600 to 650 nm. Bodipy® 558/568 C12 fluorescence was excited at 543 nm and detected in the range between 550 and 650 nm. Fluorescence emissions of GFP and RFP or ER Tracker Red™ or Bodipy® 558/568 C12 were detected simultaneously as indicated in the legend to Fig. 7.

**Determination of protein concentration**

Protein concentrations of cell lysates were determined with Bio-Rad protein assay according to manufacturer’s instructions (Bio-Rad Laboratories) using BSA as standard.

**Statistical analysis**

All data are expressed as means ± SD. Statistical significance was determined by the Student’s unpaired t-test (two-tailed). Group differences were considered significant for $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

**RESULTS**

**REH activities of mouse carboxyl esterases**

Some of the most promising enzymes thought to be involved in the hydrolysis of RE in the liver belong to the group of the carboxyl esterase super family (30). To compare REH activities of different carboxyl esterases, we cloned respective coding sequences of mouse carboxyl esterases (Table 1) into a mammalian expression vector and expressed the recombinant His-tagged proteins in COS-7 cells. A vector encoding LacZ was used for control transfections. REH activities were determined in cell lysates containing the respective recombinant proteins (1,000 g supernatant). Using retinyl palmitate emulsified with phospholipids as substrate, we found significantly increased REH activities in cells expressing Es22 (1.3-fold), Es1 (1.7-fold), TGH2 (2.4-fold), and Ces5 (1.2-fold), compared with LacZ expressing cells (Fig. 1A). To investigate whether the activity of carboxyl esterases is dependent on the presence of bile-salts, we also determined REH activities using retinyl palmitate emulsified in the presence of cholate. Under these conditions, expression of Es22 resulted in a 9-fold increase in REH activity compared with the LacZ control (Fig. 1B). A minor yet statistically significant increase was also observed for Es1 and TGH2.

**Es22 REH activity is highest in the neutral pH range and affected by the use of different detergents**

For further characterization of Es22 REH activity, we determined the pH optimum of the enzyme using RE emulsified with cholate as substrate. As shown in Fig. 2A, Es22 REH activity is highest in the neutral pH range and affected by the use of different detergents. **Fig. 2.** Dependence of Es22 REH activity on pH (A) and on various detergents (B). A: For the determination of pH dependence, lysates of COS-7 cells expressing Es22 were incubated with retinyl (3H) palmitate substrate emulsified in cholate using the following buffer systems: 100 mM Na-acetate buffer (pH 4.5 and 5), 100 mM Tris/Maleate buffer (pH 5 to 8), and 100 mM Glycin/NaOH buffer (pH 8 to 9.5). B: REH activity of Es22 in 100 mM Tris-maleate, pH 8.0 using the following detergents (final concentration): CHAPS (30 mM), cholate (20 mM), deoxycholate (20 mM), taurodeoxycholate (2 mM), and Triton X-100 (0.4%). In A and B, the activity detected in control transfected cells (LacZ) under identical conditions was set as blank to account for endogenous activity in COS-7 cells. Data are mean ± SD of triplicate determination (LacZ) and are representative for three independent determinations. *** $P < 0.001$.

**Fig. 3.** Es-22 specifically hydrolyzes retinyl palmitate. COS-7 cells were transfected with pcDNA4/HisMax encoding Es22 or LacZ. Then, lysates were prepared and incubated with the following substrates containing radiolabeled substrates as tracer: triolein (TG), cholesteryl oleate (CE), and retinyl palmitate. All substrates were prepared in 100 mM Tris-maleate, pH 8.0 and 40 mM cholate as detergent. Data are mean ± SD and representative for at least three independent experiments. * $P < 0.05$; *** $P < 0.001$. 

**Fig. 4.**
Hydrolysis of retinoids in mouse liver

PALMQUITIC ACID was necessary to promote retinyl palmitate synthesis. First, COS-7 cells were transfected with either Es22 or LacZ and subsequently incubated with retinol and palmitic acid. The formation of intracellular retinyl palmitate content was determined by HPLC. Incubation of LacZ transfected COS-7 cells with retinol and palmitic acid led to a linear increase in the cellular RE content over a period of 20 h (Fig. 4). In contrast, under identical conditions, Es22 transfected COS-7 cells exhibited decreased RE accumulation by 50% (Fig. 4). This effect was more pronounced during the initial 6 h of incubation.

Es22 specifically hydrolyzes RE

To determine the substrate specificity of Es22, lysates of COS-7 cells expressing Es22 or LacZ were prepared and incubated with CE, TG, or retinyl palmitate as substrate. All substrates were emulsified in 20 mM cholate and contained the respective radiolabeled compounds as tracer. Compared with LacZ, Es22 exhibited substantial activity using CE as substrate whereas no difference was observed with TG as substrate. These results indicate that Es22 specifically hydrolyzes retinyl palmitate and, to a much lower extent, cholesteryl oleate.

Es22 inhibits accumulation of RE in COS-7 cells

To demonstrate that Es22 affects retinol metabolism in living cells, we used COS-7 cells as a model system. HPLC analysis of endogenous RE content indicated that this cell line does not contain detectable amounts of this compound (detection limit ~0.5 µM). Therefore, artificial loading of COS-7 cells by incubation with retinol and palmitic acid was necessary to promote retinyl palmitate synthesis. First, COS-7 cells were transfected with either Es22 or LacZ and subsequently incubated with retinol and palmitic acid. The formation of intracellular retinyl palmitate content was determined by HPLC. Incubation of LacZ transfected COS-7 cells with retinol and palmitic acid led to a linear increase in the cellular RE content over a period of 20 h (Fig. 4). In contrast, under identical conditions, Es22 transfected COS-7 cells exhibited decreased RE accumulation by 50% (Fig. 4). This effect was more pronounced during the initial 6 h of incubation.

Gus hydrolyzes RAG

It has been shown that Es22 can form a complex with Gus, thereby sequestering Gus at the ER. The interaction

REH activity exhibited a rather broad pH maximum, giving the highest rates in the neutral range. Next, we determined whether Es22 REH activity is dependent on the detergent cholate or whether the enzyme is also active in the presence of ionic or nonionic detergents. As shown in Fig. 2B, Es22 REH activity was highest with the detergent CHAPS (~150%) and decreased to 36, 53, and 52% when the detergents deoxycholate, taurodeoxycholate, or Triton X-100 were used, respectively. These data indicate that Es22 REH activity is affected by the use of different detergents. REH activity was also detected in the presence of the nonionic detergent Triton X-100, which indicates that enzyme activity is not critically dependent on the presence of bile-salts.
of Es22 and Gus raised the question whether Gus might be involved in retinoid metabolism by hydrolyzing RAGs, which are naturally occurring metabolites of vitamin A. For the determination of glucuronide hydrolase activity, we incubated COS-7 cell lysates containing Gus with RAG as substrate. Before and after incubation, the RAG concentration was determined by HPLC. As shown in Fig. 5A, Gus containing lysates exhibited increased RAG hydrolyzing activity as compared with cells expressing LacZ or Es22. Coincubation of Gus and Es22 did not affect glucuronide hydrolase activity of Gus (Fig. 5A). Conversely, coincubation of Gus and Es22 had no effect on REH activity of Es22 (Fig. 5B). However, the complex forming nature of these enzymes suggests that Es22/Gus act in concert in mobilizing retinol from RE and RAG.

**Es22 is predominantly expressed in hepatocytes**

Tissue expression patterns of Es22 and Gus were analyzed by Northern blotting using respective DNA probes. As shown in Fig. 6A, Es22 was found to be highly expressed in liver compared with other tissues, including white/brown adipose tissue, skeletal/cardiac muscle, and kidney. No signal was detected in the brain. In contrast, Gus expression levels were comparable in all tissues examined. Next, we investigated Es22 mRNA expression levels in specific liver cell types, such as hepatocytes, Kupffer, or stellate cells. These liver cell types were isolated from collagenase perfused mouse livers using iodixanol self-forming density gradient. Subsequently, RNA was isolated immediately (noncultivated cells) or after cultivation of cells and used for Northern blotting experiments. As depicted in Fig. 6B, in noncultivated liver cells, Es22 mRNA expression level was highest in hepatocytes and was barely detectable in Kupffer and stellate cells. Interestingly, after cultivation of liver cells, Es22 mRNA expression diminished in Kupffer and stellate cells. As a control, we also used a probe for α-SMA, which is known to be highly selective for stellate cells. α-SMA positively hybridized only with RNA obtained from stellate cells, confirming purity of the stellate cell preparations (Fig. 6B). Similarly, purity of hepatocyte and Kupffer cell preparations were analyzed using α-keratin 18 and monocytomonocyte-macrophage antibody 2 (MOMA-2) as marker proteins, respectively. As depicted in Fig. 6C, Western blotting analyses of cell preparations showed that keratin-18 and MOMA-2 were exclusively expressed in hepatocytes and Kupffer cells, respectively, demonstrating purity of these cell preparations.

**Es22 and Gus colocalize at the ER**

To determine the subcellular localization of Es22, a GFP fusion protein (Es22-GFP) was expressed in COS-7 cells. Laser scanning microscopy revealed that the Es22-GFP fusion protein distributed throughout the cell, thereby forming a network-like structure (Fig. 7 upper panel, left picture). To investigate if this structure represents the ER, we stained cells with an ER specific dye (ER-tracker). As shown in Fig. 7 (upper panel), the signals obtained for Es22-GFP and ER-tracker resulted in a perfect overlay, demonstrating that Es22 localizes to the ER. We also studied any localization of Es22-GFP at lipid droplets using the neutral-lipids staining dye Bodipy®. However, we could not detect any association of Es22 with lipid droplets, as evident from the merge (Fig. 7, middle panel). Next, we coexpressed Es22-GFP and Gus-RFP in COS-7 cells. As shown in Fig. 7 (lower panel), Es22 and Gus were found to mainly colocalize at the ER whereas Gus also localized to circular structures, presumably lysosomes (31).

**DISCUSSION**

Vitamin A has been known for almost a century as a fat soluble factor that is essential for normal growth (32, 33) and affects multiple physiological processes, including vision, cell differentiation, reproduction, and immunity. The retinoid 11-cis retinaldehyde functions as the active chromophore in rhodopsin and is crucial for vision. The metabolites all-trans and 9-cis retinoic acid interact with a number of nuclear receptors of the retinoic acid receptor and the retinoid X receptor family. These receptors function as ligand-activated factors and are involved in the regulation of gene expression. Up-to-date chronic unders-
supply of vitamin A affects millions of people, especially in
the “third world” countries. The deleterious effects of chronic vitamin A deficiency include, most importantly, increased susceptibility to infectious diseases, which repre-
sents an enormous threat for these populations. Yet, apart
from its function in vision and as a ligand for nuclear re-
ceptors, little is known about mechanisms facilitating con-
stant supply of the body, in particular the release of retinol
from the liver.

In this study, we identified murine Es22 as a potent liver
REH. Earlier studies suggested that the rat homolog of
Es22, esterase 3 (Es3, 93% homology), might possess REH
activity. Rat Es3 was first identified as “esterase with pi 5.6”
and originally found to hydrolyze retinyl acetate and, to a
much lower extent, retinyl palmitate (21). In another
study, Es3 was shown to hydrolyze REs with low efficiency
compared with rat Es4 and Es10 (34). In addition to its
putative role in retinoid metabolism, two studies suggested
that Es3 is involved in the detoxification of xenobiotics.
Rat Es3 has been found to hydrolyze acetylamide (35, 36),
a compound that exhibits analgesic and antipyretic prop-
erties (37). Our studies suggest that murine Es22 is a po-
tent REH compared with the other members of the
carboxy esterase family investigated in this study. These
observations are in contrast to the results obtained with rat
homologs. Explanations for this discrepancy could be ei-
ther species differences in enzyme specificity or the use of
different assay conditions. For example, the influence of
assay conditions on Es22 activity is apparent from assays
using cholate or phospholipids as detergent. Es22 was
highly active using cholate or CHAPS but exhibited only
poor activity when retinyl palmitate was emulsified in the
presence of phospholipids. Thus, different assay condi-
tions substantially affect enzyme activity of Es22 and may
explain the discrepancy in REH activities of the rat and
mouse homologs.

Es22 is highly expressed in the liver, which is the pri-
mary storage site of RE. Interestingly, the highest expres-
sion was found in hepatocytes and, to a much lesser extent,
in Kupffer and hepatic stellate cells, which contain most of
the body’s vitamin A reserves. Yet, in cell culture experi-
ments, expression of Es22 attenuated the accumulation of
REs, suggesting that the enzyme affects the deposition of
cellular RE stores. In general, RE hydrolyzing enzymes can
attenuate RE accumulation by two independent mecha-
nisms. First, increased RE hydrolase activity at the ER can
counteract the esterification of retinol by ARAT or LRAT.
Second, enzymes capable of binding to lipid droplets can
increase the hydrolysis of stored RE. Our studies demon-
strate that Es22 does not attach to lipid droplets, implying
that the enzyme does not play a role in the mobilization of
RE stores contained in lipid droplets. Es22 is localized ex-
clusively at the ER, indicating that the enzyme affects RE
synthesis by counteracting retinol esterifying enzymes, re-
sulting in reduced RE formation. The liberated retinol
most likely is subsequently secreted, converted into bioac-
tive vitamin A metabolites, or reesterified. Thus, Es22 pre-
sumably inhibits RE accumulation in hepatocytes and
possibly other cell types. From this point of view, the low
expression of Es22 in stellate cells is not surprising be-
cause, specifically, these cells deposit large amounts of RE
in lipid droplets.

Es22 was first identified 30 years ago and named egasyn
(from the Greek root meaning “to hold together”) (38).
This name was chosen because it was found to sequester
up to 25% of total Gus at the ER. The rest is delivered to
lysosomes. Lysosomal Gus plays an important role in the
catabolism of glycosaminoglycans as evident from the mu-
copolysaccharidase type VII (MPS VII) mouse (39) and
from human patients with Gus deficiency (MPS VII or Sly
syndrome) (40). The physiological role of microsomal Gus
is less understood. Yet, some evidence suggests a pos-
sible role in the hydrolysis of endogenous and xenobiotic
glucuronides at the ER (41, 42). Our data demonstrate
that Gus colocalizes with Es22 at the ER. In addition, we
found that RAGs represent an additional physiological

![Fig. 7. Es22 and Gus colocalize to the endoplasmic reticulum. COS-7 cells were transfected with plasmids encoding either Es22-GFP or both Es22-GFP and Gus-RFP fusion proteins. The next day, cells expressing Es22-GFP alone were incubated with 400 µM olate for 18 h to promote lipid droplet formation. In some cases, Es22-GFP expressing cells were incubated with ER-Tracker™ Red dye (upper panels) or Bodipy® 558/568 C12 (middle panels) for staining of the ER or lipid droplets, respectively. Localization of Es22-GFP (green), Gus-RFP (red), and the ER/lipid droplets staining (red) as well as colocalizations (yellow) were visualized by confocal laser scanning microscopy. The right column displays a three-dimensional picture of respective cells as obtained by differential interference contrast microscopy. Bars = 10 µm.](image-url)
substrate for Gus. β-Glucuronides of all-trans retinol and of all-trans retinoic acid have been shown to be formed by liver microsomes in vitro (43) and in vivo (25, 42, 45) by the action of microsomal UDP-glucuronosyl transferase (45). Retinoid β-glucuronides have been detected in various mouse tissues (44) and in the circulation (46), suggesting that these compounds could represent a water-soluble transport form of vitamin A channeling retinol through the ER or through the circulation. In analogy to the formation and hydrolysis of RE, Gus could counteract the formation of retinol β-glucuronides by UDP-glucuronosyl transferase at the ER.

In summary, our studies demonstrate that murine Es22 and Gus are capable of hydrolyzing retinoids and colocalize at the ER. Our data indicate that these enzymes affect retinoid storage and mobilization by counteracting the formation of RE and retinoid glucuronides at the ER.

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mines the subcellular distribution of beta-glucuronidase, has es-


