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 hydrolase 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 countering 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. Taschner, 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.hv receptor 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 oleate; 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, monocytic-macrophage antibody; RAG, retinol beta-glucuronide; RBP4, retinol binding protein 4; RE, retinyl ester; REH, retinol ester hydrolyase; RFP, red fluorescent protein; aSMA, alpha smooth muscle actin; TBST, Tris/NaCl/Tween-20; TG, triglyceride.

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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 lipase (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 retinol 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. The resulting fusion constructs encoded GFP and RFP coding sequences were amplified using liver cDNA as template and respective primers: Es22_forward, 5’-ATCTGGAGCCGACATGTGCCCTCTGGTGCTAGCC-3’; Es22_reverse, 5’-TGGATTCGCAAGTCTAGTTGCTGCTGCGG-3’; Gus_forward, 5’-TCTCGAGGCCACATGCGGCGCTGTTGCTGTTG-3’; Gus_reverse, 5’-CGAATTCAGAGTGCGTAGCGGAGCTGCTGTT-3’. The amplified Es22 and Gus coding sequences were digested with restriction endonucleases XbaI and EcoRI and ligated into respective sites of the pEGFP-N1 vector (Takara Bio Inc., Otsu, Japan). The amplified RFP coding sequence was digested with EcoRI/ BamHI and ligated into respective sites of the pEFP-N1 vector (Takara Bio Inc., Otsu, Japan). The amplified RFP coding sequence was digested with EcoRI/ BamHI 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

Monkeys embryonic kidney cells (COS-7, ATCC CRL-1651) were maintained in DMEM (Gibco® from InvitrogenTM) 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 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, Düren, 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/well 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 and antibiotics. Cells were analyzed two days after transfection.

Preparation of COS-7 cell lysates

Cells were collected by trypsinization 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, 1 mM PMSF, and 1 mM β-mercaptoethanol) in a Dounce homogenizer. Homogenates were centrifuged at 10,000 g for 10 min at 4°C and supernatants were used for further analysis.
2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) by sonication (Vibrosonic 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 30 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 corrections. Substrate for REH assay contained 10 nmol retinol palmitate/assay and retinyl [9,10(n)-H]palmitate, 50,000 cpm/nmol as tracer) and was either emulsified in 100 mM potassium phosphate buffer pH 7.4, containing 90 µM phosphatidyl-choline/-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 33 nmol triglyceride/assay (TG; glycerol tri[9,10(n)-H]oleate, 40,000 cpm/nmol) or 20 nmol/assay of cholesteryl oleate (CE; cholesteryl [9,10(n)-H]oleate, 50,000 cpm/nmol). All lipid substrates were prepared by sonication on ice (Vibrosonic 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 transfected 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 trypsination, 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. (25).

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.

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TABLE 1. List of cloned genes
HPLC analysis of retinoids
Retinoids were separated on a reverse-phase Lichrospher® 125-4 5 µm C18 column (125 x 4 mm) preceded by a Super-spher® 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 at 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 ($\epsilon_{325\text{nm}} = 52,275 \text{ M}^{-1}\text{cm}^{-1}$) (26); RAG ($\epsilon_{1\text{cm}, 1\%} = 1065$ 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 $\text{Ca}^{2+}$ and $\text{SO}_4^{2-}$) opened by a vertical incision. Then, the liver was perfused via the portal vein with 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 ($\epsilon_{325\text{nm}} = 52,275 \text{ M}^{-1}\text{cm}^{-1}$) (26); RAG ($\epsilon_{1\text{cm}, 1\%} = 1065$ 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 $\text{Ca}^{2+}$ and $\text{SO}_4^{2-}$) 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), 2% 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 $\text{g}$ 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, Rodeløkka, 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 $\text{g}$ 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, BioRad Laboratories, Hercules, CA). The membrane was probed with [α-$\text{32P}$]dCTP-labeled cDNA specific for mouse Es22, Gus, or α-smooth muscle actin (α-SMA). The following probes were used: for Es22, the first 416 bp and for Gus, the first 606 bp of the coding region were obtained by extension digestion using BamHI and HindIII, respectively; for α-SMA, the fragment between 66 and 638 bp of the coding sequence was amplified using following primers: forward, 5′-CGTCCGTGTGATGATGCTC-3′; reverse, 5′-GTAACACGAGATCTGACCCTG-3′, ligated into pcCR2.1 vector (Invitrogen™) and isolated from the multiple cloning site of the vector. After hybridization and washing, signals were visualized by exposure to a PhosphorImager Screen (Apliotech, Freiburg, Germany) and analyzed using ImageQuant Software (Molecular Dynamics). As a loading control, RNA separated by the formaldehyde/agarose gel was visualized with ethidium bromide staining.

Western blotting analysis
Cell lysates were solubilized in SDS-PAGE sample buffer and proteins separated on a 10% SDS-PAGE gel using the Laemmli discontinuous buffer system (29). Then, proteins were transferred onto a polyvinylidene fluoride transfer membrane (Pall Life Sciences, Pensacola, FL). The membrane was blocked with 10% blocking grade milk powder (Carl Roth GmbH and Co. KG) in Tris/NaCl/Tween-20 (TBST) and incubated with mouse anti-keratin-18 monoclonal antibody (Progen, Heidelberg, Germany) at a dilution of 1:1,000 or rat anti-MOMA-2 antibody (Acris antibodies, Hidenhausen, Germany) at a dilution of 1:5,000. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-mouse (GE Healthcare) antibody at a dilution of 1:10,000 and with horseradish peroxidase-conjugated rabbit anti-rat (Dako, Glostrup, Denmark) antibody at a dilution of 1:1,000. After washing with TBST, the membrane was developed with enhanced chemiluminescence (ECL plus, GE Healthcare) and exposed to X-ray film (Hyperfilm™ ECL, GE Healthcare).

Live cell imaging
COS-7 cells were cultivated on 24 x 24 mm coverslips and transiently transfected with GFP-tagged Es22, RFP-tagged Gus, or 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.

Hydrolysis of retinoids in mouse liver
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 (H11601 1.3-fold), Es1 (H11601 1.7-fold), TGH2 (H11601 2.4-fold), and Ces5 (H11601 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 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 63x 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 555 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 (H11601 1.3-fold), Es1 (H11601 1.7-fold), TGH2 (H11601 2.4-fold), and Ces5 (H11601 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 an ~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.
Hydrolysis of retinoids in mouse liver

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.

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 RE as substrate (Fig. 3). Furthermore, we found a moderate but significant increase by 50% 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, (30 times less efficient) 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

**Fig. 4.** Es22 inhibits the accumulation of retinyl esters in COS-7 cells. COS-7 cells were transfected with Es22 or LacZ. After 48 h of transfection (time point = 0 h), cells were incubated with 500 µM palmitic acid and 100 µM all-trans-retinol for up to 20 h. At various times, cells were harvested and cellular retinyl palmitate content was determined by RP-HPLC. Data are mean ± SD of three determinations and are representative for three independent experiments. *P < 0.05; **P < 0.01.

**Fig. 5.** β-Glucuronidase hydrolyzes retinoyl β-glucuronide (RAG). COS-7 cells were transfected with plasmids encoding Gus, Es22, or LacZ. Lysates were subjected to activity assays using (A) retinoyl β-glucuronide or (B) retinyl (3H) palmitate as substrate. To investigate whether Gus affects the activity of Es22 and vice versa, mixtures of lysates containing Gus and Es22 or Gus and LacZ were incubated with the substrates. A: Activity assays were performed in 50 mM Tris/Maleate buffer, pH 8.0. After 30 min of incubation, the content of RAG was determined by RP-HPLC. Loss in RAG content was expressed as nmol/h/mg protein. Data are mean ± SD of three determinations and are representative for two independent experiments. *P < 0.05; **P < 0.01. B: β-Glucuronidase does not affect Es22 REH activity. Activity assays were performed with radiolabeled retinyl palmitate substrate in 50 mM Tris-maleate, pH 8.0 and 20 mM cholate. Data are mean ± SD of triplicate determinations and are representative for two independent experiments. **P < 0.01.
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 hydrolyase 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 hydrolyase 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.

**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 under-
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 represents an enormous threat for these populations. Yet, apart from its function in vision and as a ligand for nuclear receptors, little is known about mechanisms facilitating constant 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 pH 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 acetanilide (35, 36), a compound that exhibits analgesic and antipyretic properties (37). Our studies suggest that murine Es22 is a potent REH compared with the other members of the carboxyl esterase family investigated in this study. These observations are in contrast to the results obtained with rat homologs. Explanations for this discrepancy could be either 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 conditions 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 primary storage site of RE. Interestingly, the highest expression 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 experiments, 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 mechanisms. 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 demonstrate 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 exclusively at the ER, indicating that the enzyme affects RE synthesis by counteracting retinol esterifying enzymes, resulting in reduced RE formation. The liberated retinol most likely is subsequently secreted, converted into bioactive vitamin A metabolites, or reesterified. Thus, Es22 presumably 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 because, 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 mucopolysaccharidosis 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 possible 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 function of Gus.

**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.
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 retinoyl 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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REFERENCES

tate by enzymes of rat pancreas and liver. Differentiation of bile salt-dependent and bile salt-independent, neutral retinyl ester hy-
drolases in rat liver. J. Biol. Chem. 264: 17142–17147.

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