Hydrolysis of Retinyl Esters by Pancreatic Triglyceride Lipase†

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Received November 29, 1999; Revised Manuscript Received February 1, 2000

ABSTRACT: Previously [van Bennekum, A. M., et al. (1999) *Biochemistry 38*, 4150−4156] we showed that carboxyl ester lipase (CEL)-deficient (CELKO) mice have normal levels of pancreatic, bile salt-dependent retinyl ester hydrolase (REH) activity. In the present study, we further investigated this non-CEL REH activity in pancreas homogenates of CELKO and wild-type (WT) mice, and rats. REH activity was detected in both the presence and absence of tri- and dihydroxy bile salts in rats, WT mice, and CELKO mice. In contrast, pancreatic cholesteryl ester hydrolase (CEH) activity was only detected in the presence of trihydroxy bile salts and only in rats and WT mice, consistent with CEL-mediated cholesteryl ester hydrolysis. Enzyme assays of pancreatic triglyceride lipase (PTL) showed that there was a colipase-stimulated REH activity in rat and mouse (WT and CELKO) pancreas, consistent with hydrolysis of retinyl ester (RE) by PTL. Pancreatic enzyme activities related to either CEL or PTL were separated using DEAE-chromatography. In both rats and mice (WT and CELKO), REH activity could be attributed mainly to PTL, and to a much smaller extent to CEL. Finally, purified human PTL exhibited similar enzymatic characteristics for triglyceride hydrolysis as well as for retinyl ester hydrolysis, indicating that RE is a substrate for PTL in vivo. Altogether, these studies clearly show that PTL is the major pancreatic REH activity in mice, as well as in rats.

In two recent studies, we showed that in carboxyl ester lipase (CEL)[1]-deficient mice both the intestinal (*1*) and hepatic (*2*) uptake of retinyl ester (RE, vitamin A) were normal. As expected, in CEL knockout (CELKO) mice, pancreatic bile salt-dependent cholesteryl ester hydrolase (CEH) activity was absent, and it was half the level of wild-type (WT) mice in heterozygous mice (*1, 2*). Similar results were observed for the hepatic activities (*2*). These results are consistent with the notion that CEL mediates cholesteryl ester (CE) hydrolysis (*3, 4*). However, when this assay (using millimolar concentrations of trihydroxy bile salt, typically detecting CEL) was employed using RE as a substrate, the bile salt-dependent retinyl ester hydrolase (REH) activities of liver and pancreas of WT, heterozygous, and homozygous CELKO mice were identical. This indicates that in mouse liver and pancreas there is an REH enzyme activity, which is active in the presence of bile salt and distinct from CEL.

The aim of the present study was to identify this non-CEL, pancreatic bile salt-dependent REH activity in WT, heterozygous, and CELKO mice. Studies were also conducted in rats, as in this species many aspects of retinoid (vitamin A) metabolism (*5*) and the role of CEL (*6*) have been well described. Three decades ago Erlanson and Borgström reported the partial separation of two different pancreatic REH activities in rat using a Sephadex G100 column (*7*). These two activities hydrolyzed different physical forms of the retinyl palmitate substrate; the early peak mainly hydrolyzed retinyl palmitate, which was dispersed in millimolar concentrations of taurodeoxycholate [a condition known to inhibit pancreatic triglyceride lipase (PTL) (*8*)], whereas the subsequent peak was more effective in hydrolyzing dispersed retinyl palmitate in the absence of bile salt. These two different REH elution patterns were consistent with CEL and PTL, respectively (*7*).

In the studies reported here, pancreatic REH of rat and mouse (WT, heterozygous, and CELKO) was explored by assessing REH activity under either CEL-optimal conditions (employing millimolar concentrations of trihydroxy bile salt) or PTL-optimal conditions (employing millimolar concentrations of dihydroxy bile salt in the presence of saturating concentrations of colipase). Also, we attempted to further separate those two enzyme activities in mouse and rat pancreas homogenates using DEAE-anion exchange chromatography. Using a buffer at neutral pH, CEL is expected to bind to DEAE and to be eluted using a KCl gradient (*9*), whereas PTL is expected to not bind (*10*). Last, the ability of PTL to hydrolyze RE was directly addressed by studying the enzymatic characteristics of purified human PTL (hPTL) using either triglyceride or RE as a substrate. The results of

† This work was supported in part by grants from the NIH (DK44498 and DK52444) and the W. W. Smith Charitable Trust.

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[1] Abbreviations: BBM, brush border membrane; CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; CEL, carboxyl ester lipase; CELKO, CEL-deficient; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; hPTL, human pancreatic triglyceride lipase; PLRP1, pancreatic lipase related protein 1; PLRP2, pancreatic lipase related protein 2; PTL, pancreatic triglyceride lipase; RE, retinyl ester; REH, retinyl ester hydrolase; TG, triglyceride; TGH, triglyceride hydrolase; WT, wild type.

10.1021/bi9927235 CCC: $19.00
Published on Web 03/30/2000

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MATERIALS AND METHODS

Preparation of Pancreas Homogenates and Protein Determination. Pancreati obtained from male and female WT, heterozygous, and CELKO mice [(*1, 2*), weighing approximately 25 g], and of male Sprague Dawley rats (Harlan, Madison, WI; weighing 350−375 g) were homogenized in 4 mL of 0.25 M sucrose per gram of tissue using a polytron homogenizer. Tissue protein levels were determined using a modified Lowry procedure (*11*). Protein levels averaged about 130 mg/g of tissue, and were not different for WT, heterozygous, and CELKO mice, or rats.

Nomenclature and Enzyme Assays. Four enzyme assays were used in this work and are referred to consistently as follows. PTL-TGH refers to assaying PTL with its standard TG substrate and reflects the activity of pancreatic triglyceride lipase. Likewise, CEL-CEH refers to assaying CEL with its standard substrate, namely, CE. Finally, PTL-REH and CEL-REH refer to assays of retinyl ester hydrolase activity (using retinyl palmitate as substrate) under conditions optimized for the assay of PTL and CEL, respectively. Thus, REH, CEH, and TGH refer to neutral lipid ester hydrolase activities that may be associated with one or more specific enzyme proteins, whereas PTL and CEL refer to specific enzymes.

Assay of Pancreatic CEH and REH Activities under CEL-Optimal Conditions. These assays were performed according to Harrison (*3*). Briefly, for assaying CEH activity, 20 μL of an appropriately diluted enzyme source was incubated at 37 °C for 60 min in an incubation mixture containing 50 mM Tris−maleate, pH 8.0, 20 mM sodium cholate, and 0.05 μCi of cholesteryl[1-^{14}C]oleate (54 mCi/mmol, Amersham Life Science Inc., Cleveland, OH). For REH, the incubation mixture consisted of identical components, except that 0.05 μCi of retinyl[1-^{14}C]palmitate [53 mCi/mmol, synthesized according to Azais-Braesco et al. (*12*)] was used as substrate. The final concentration for both types of substrate was 10 μM. When other bile salts were tested, 20 mM final concentrations of bile salt were used.

The reaction products [[1-^{14}C]oleic acid (for CEH) and [1-^{14}C]palmitic acid (for REH)] were extracted into an alkaline, aqueous upper phase according to Harrison (*3*), and an aliquot of this phase was counted in a liquid scintillation counter. The amount of oleic acid or palmitic acid released was determined from the partition coefficients of these compounds and the specific activity of the substrate (*3*).

Assay of Pancreatic Triglyceride Hydrolase (TGH) and REH Activities under PTL-Optimal Conditions. These assays were performed essentially according to Lowe (*13*) using a slight modification. Briefly, for assaying TGH activity, 20 μL of an appropriately diluted enzyme source was incubated at 23 °C for 60 min in an incubation mixture containing 27 mM Tris-HCl, pH 7.0, 19.8 mM sodium taurodeoxycholate, 0.1 mM $CaCl_2$, 3 μg/mL colipase (Sigma, from porcine pancreas), and 0.15 μCi of [9,10-^{3}H]triolein (20 Ci/mmol, Dupont NEN, Boston, MA). For REH, the incubation mixture consisted of identical components, except that 0.15 μCi of retinyl[1-^{14}C]palmitate was used as substrate. The final concentration for both types of substrate was 100 μM. Reaction products ([9,10-^{3}H]oleic acid and [1-^{14}C]palmitic acid) were extracted as described above.

DEAE-Chromatography of Solubilized Pancreas Homogenates. Pancreas homogenates obtained from mice (WT and CELKO) and rats were solubilized in a buffer containing 1% CHAPS, 50 mM Tris-HCl, pH 7.4, 5 mM glutathione, 3 mg/L leupeptin, 0.33 g/L EDTA, and 0.1 g/L sodium azide. Briefly, a part of the homogenate was mixed with an appropriate volume of the above-described buffer (without CHAPS), and subsequently, an appropriate volume of a concentrated CHAPS solution was added slowly (up to a final concentration of 1%) to the homogenate at 4 °C, while the homogenate was being vortexed gently. The resulting preparation was kept at 4 °C for 1 h with occasional stirring. Solubilized homogenates were centrifuged at 45 000 rpm for 30 min at 5 °C to obtain the soluble and pellet fractions. Enzyme activities in the pellet were determined after suspending the pellet in the original solubilization buffer. Recoveries of all activities studied in total pancreas homogenates after the solubilization procedure were at least 75%, and the recovery of soluble activity (as % of total) was greater than 65%.

Soluble fractions were applied to a DEAE-Sepharose CL-6B column (Pharmacia, Piscataway, NJ) previously equilibrated with 50 mM Tris-HCl, pH 7.4, 5 mM glutathione, 1 mg/L leupeptin, 0.33 g/L EDTA, and 0.1 g/L sodium azide (hereafter referred to as column buffer) at 4 °C. Unbound protein was washed off with 2 column volumes of column buffer, and bound protein was subsequently eluted with 6 column volumes of a gradient of 0−0.4 M KCl in column buffer. Under these chromatographic conditions, it is expected that PTL does not bind to the column (*10*), whereas CEL elutes during the salt gradient (*9*). Absolute recoveries of enzyme activities after DEAE-chromatography were variable, but the activity profiles among the animals tested were very similar.

RESULTS

CEL-CEH and CEL-REH Activities in Pancreas Homogenates from WT, Heterozygous, and CELKO Mice, and Rats, in the Absence or Presence of Different Types of Bile Salt. Figure 1 shows pancreatic CEH and REH activities assayed under CEL-optimal conditions (CEL-CEH and CEL-REH, respectively) in WT, heterozygous, and CELKO mice, as well as rats using various types of bile salt. CEL-CEH activity was detected in the presence of trihydroxy bile salts (20 mM cholate and taurocholate), but not in the presence of dihydroxy bile salts (20 mM deoxycholate, taurodeoxycholate) or CHAPS, or in the absence of bile salts in rats, WT, and heterozygous mice (Figure 1, top panel). No CEL-CEH activity was observed in pancreas homogenate of the CELKO mouse under any of the bile salt conditions employed (Figure 1, top panel). These observations are consistent with CEL-mediated cholesteryl ester hydrolysis (*3, 4*).

CEL-REH activity, however, was detected in the presence of both tri- and dihydroxy bile salts, and in the absence of bile salt in rats and in all three types of mice (Figure 1, bottom panel). Therefore, these data indicate that another bile salt-stimulated enzyme with REH activity must be

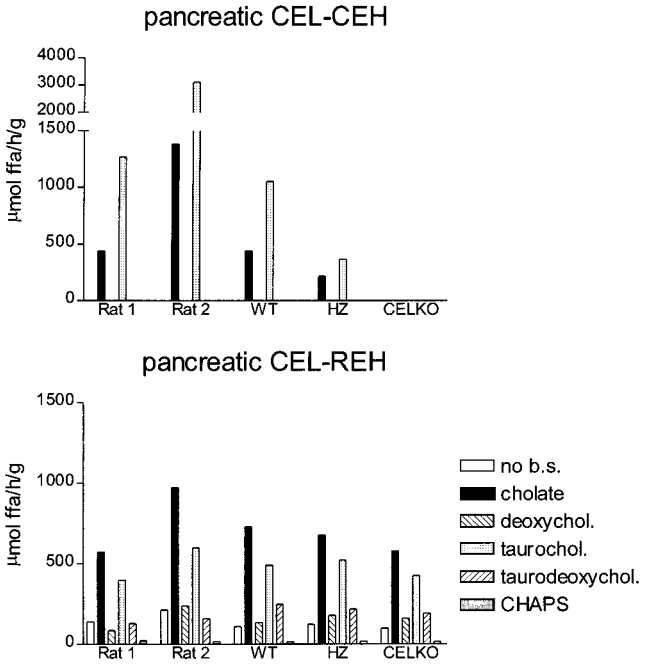

FIGURE 1: Effect of different bile salts on CEL-CEH (top panel) and CEL-REH (bottom panel) activities in pancreas homogenates of wild-type (WT), heterozygous (HZ), and CELKO mice and in rats. One mouse of each genotype and two rats were used. Assays were performed using either 20 mM sodium cholate (cholate), sodium deoxycholate (deoxychol.), sodium taurocholate (taurochol.), sodium taurodeoxycholate (taurodeoxychol.), CHAPS, or no bile salt (no b.s.). Values are expressed as μmol of ffa h^{-1} (g of tissue)$^{-1}$, and represent the average of duplicate measurements which, for most samples, varied by less than 10%.

present in the pancreas of CELKO mice (and possibly in the pancreas of WT mice and rats) which is not CEL. We hypothesized that this enzyme activity may be, at least partly, due to PTL, as PTL is enzymatically active against triglyceride in the presence of its cofactor colipase using millimolar amounts of dihydroxy bile salts (*8*).

PTL-TGH and CEL-REH Activities of Pancreas Homogenates from WT and CELKO Mice, and Rats, in the Absence or Presence of Colipase. Figure 2 shows the effect of substrate concentration on pancreatic TGH assayed under PTL-optimal conditions, and REH assayed under CEL-optimal conditions (PTL-TGH and CEL-REH, respectively) in a WT mouse, CELKO mouse, and rat. In all three groups of animals, a colipase-stimulated TGH activity was observed, consistent with PTL-mediated triolein hydrolysis (Figure 2, left panels). Under these assay conditions, a colipase-stimulated REH activity also was observed in these animals, whereas CEH activity was undetectable (data not shown). The TGH activity that remains in the absence of colipase (and in the presence of millimolar amounts of dihydroxy bile salt) in rat and mouse pancreas homogenates may point to the existence of yet another colipase-independent TGH activity (*14*).

Surprisingly, even when assayed under CEL-optimal conditions, a significant colipase-stimulation of REH activity was observed for rat, and WT and CELKO mice (Figure 2, right panels), whereas no such colipase-stimulation was observed for CEL-CEH activity in rat and WT mouse (data not shown). This colipase-stimulated REH activity cannot be solely due to CEL, as it was also present in CELKO mice. These data suggest that PTL is responsible for at least part of the CEL-REH activity observed in pancreas homogenates of rats and mice.

PTL-TGH, PTL-REH, CEL-CEH, and CEL-REH Activities after DEAE-Column Chromatography of Solubilized Pancreas Homogenates from WT Mouse, CELKO Mouse, and Rat. To further characterize the PTL-related REH activity in total pancreas homogenates, we subjected 1% CHAPS-solubilized pancreas homogenates obtained from rat, and WT and CELKO mice to DEAE chromatography. Figure 3, top left panel, shows the elution profile of PTL-TGH and PTL-REH activities of a WT mouse. A similar elution pattern for both activities was observed for rat and CELKO mouse (data not shown). The majority of PTL-TGH activity eluted in the unbound fraction, consistent with the properties of the PTL-protein. Also, the majority of PTL-REH activity eluted at this position (Figure 3, top left panel**)**, suggesting that PTL-REH activity is also due to PTL.

Next, the elution profile of PTL-REH was compared with that of CEL-REH. As we had observed colipase-stimulation of CEL-REH in total pancreas homogenates (Figure 2), we assumed that at least some of CEL-REH could be mediated by PTL. In WT mouse, two CEL-REH activity peaks ("peak II, peak III") eluted during the salt gradient in the expected CEL range (Figure 3, top right panel). To our surprise, however, the majority of CEL-REH eluted in the unbound fraction ("peak I"). Similar findings were observed for CELKO mouse and rat (Figure 3, bottom panels). Therefore, for both rat and mouse, the majority of CEL-REH activity does not appear to be due to enzyme activities eluting in the CEL range (i.e., during the KCl gradient), but appears to be consistent with PTL-mediated hydrolysis.

In rat, about one-third or less of CEL-REH activity was included in "peak III" eluting at about halfway along the KCl gradient. This peak coeluted exactly with the major CEL-CEH peak and, hence, is consistent with CEL-mediated retinyl ester hydrolysis (*15*). Also, a much smaller "CEL" activity peak eluted during the salt gradient ("peak II"), exhibiting the same ratio in specific activity against CE and RE as did "peak III" (Figure 3, bottom right panel). In WT mice, on the other hand, "peak II" eluting early during the KCl gradient was the predominant CEL-related peak exhibiting CEH and REH activities. CEL identity was confirmed by the absence of "peak II"- and "peak III"-CEL-CEH activity in CELKO mice (Figure 3, bottom left panel). Despite "peak II and III"-CEL-CEH being deficient in samples from the CELKO mouse, the CEL-REH activity of "peak II" (and some of that of "peak III") remained present in this mouse. This indicates that, at least in mouse pancreas, another CEL-REH activity exists which is neither CEL nor PTL.

As expected (*9*), the majority of CEL-CEH activity eluted during the KCl gradient in rat and WT mouse. However, a minor non-CEL CE hydrolytic activity was detected eluting in the unbound fraction in both rat, and WT and CELKO mice (Figure 3, top right panel and bottom panels). The reason this activity, amounting to about 20% of the total CEL-CEH activity recovered from the colum, was not detected in whole pancreas homogenates of CELKO mice (Figure 1) is not known. This activity cannot be due to PTL, as this enzyme does not appear to use CE as a substrate (*16*). Also, this activity cannot be CEL as this activity was present in CELKO mouse, and as the ratio of CEL-CEH to CEL-

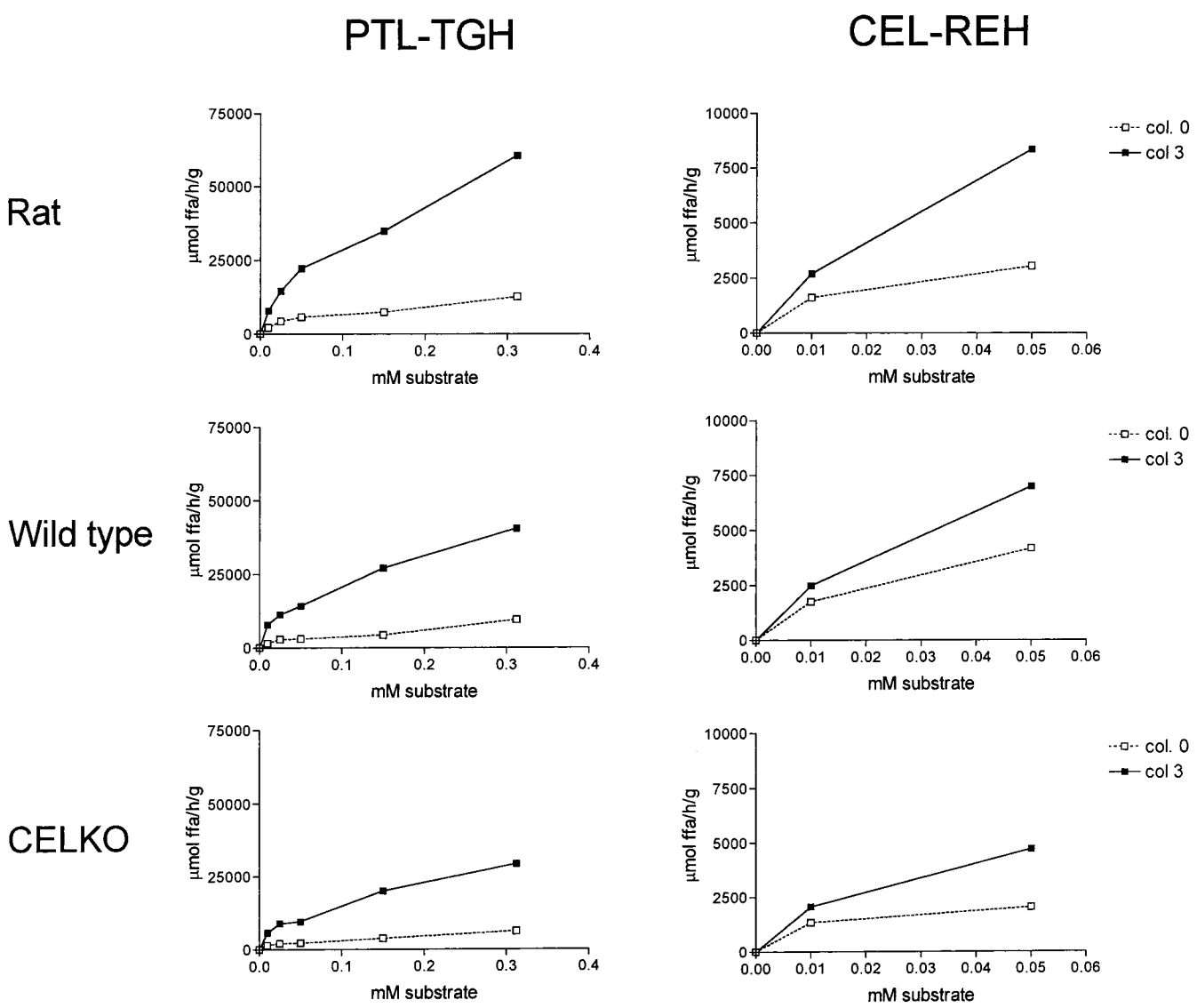

FIGURE 2: PTL-TGH and CEL-REH activities in pancreas homogenates of rats and wild-type and CELKO mice. Results from one animal of each type are presented. PTL activity assays were performed using 19.8 mM sodium taurodeoxycholate, in the absence (closed symbols) or presence (open symbols) of 3 μg/mL colipase. CEL activity assays were performed using 20 mM sodium cholate (also in the absence or presence of 3 μg/mL colipase). The results represent a 60 min incubation. Values are expressed as μmol of ffa h^{-1} (g of tissue)$^{-1}$, and represent the average of two measurements (each at one dilution in the linear range) which varied by less than 10% for most samples.

REH of "peak I" is different from that of "peak II and III" for both species, for example, a ratio of about 0.3 and 1.5, respectively, in WT mouse. Thus, these data provide further support that PTL is responsible for a considerable part of pancreatic CEL-REH activity in mouse, as well as rat.

Enzymatic Characteristics of Purified Human PTL toward Triglyceride (TG) and RE. The enzyme characteristics of purified human PTL (hPTL, Calbiochem, La Jolla, CA) either against its classical substrate TG or against RE were investigated. Figure 4 shows enzyme characteristics of hPTL using either triolein or retinyl palmitate as a substrate.

For PTL-TGH (Figure 4, left panels), triolein hydrolysis was not saturated up to 0.3 mM substrate (Figure 4, left panel, A), and triolein hydrolysis was completely dependent on colipase (in the presence of 20 mM bile salt). These data are in agreement with enzyme characteristics of purified rat and human PTL (*16*). They also agree with total pancreatic homogenate data (Figure 2), although in pancreas homogenates of rat and mouse a TGH activity also was observed in the absence of colipase (Figure 2). Colipase stimulation of hPTL is saturating at 1 μg/mL (Figure 4, left panel, B), which is similar to what we observed in rat and mouse pancreas homogenates (data not shown). Also, purified hPTL exhibits the typical bile salt inhibition, which is alleviated by the presence of colipase (Figure 4, left panel, C), as has been described for rat and porcine PTL (*8*).

To our surprise, almost identical characteristics were observed regarding colipase-dependence, colipase-saturation, and bile salt-inhibition when retinyl palmitate was used as a substrate (Figure 4, right panels). The substrate concentration curve (Figure 4, right panel, A) showed that PTL-REH activity was starting to saturate at 0.3 mM retinyl palmitate. The specific activity of PTL against triolein was about 10-fold higher than that against retinyl palmitate. These data show that retinyl palmitate is a substrate for purified hPTL.

To compare the results obtained with purified hPTL with those from pancreas homogenates or DEAE-fractions, the activity of hPTL against retinyl palmitate and triolein in the "PTL-assay" as well as hPTL's activity toward retinyl palmitate and cholesteryl oleate in the "CEL-assay" were

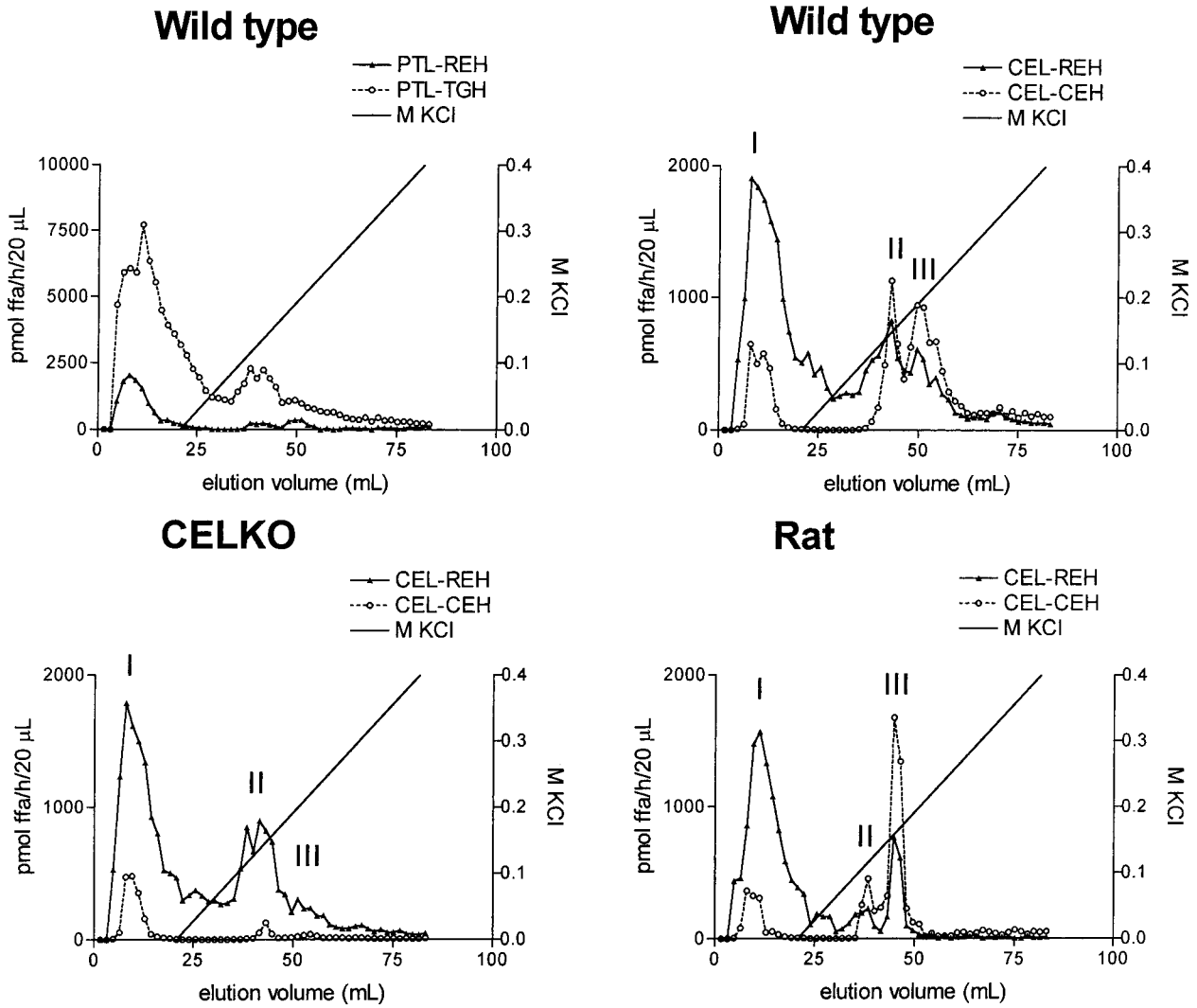

FIGURE 3: Elution profiles of PTL-TGH, PTL-REH, CEL-CEH, and CEL-REH activities of solubilized pancreas homogenates of WT mouse, CELKO mouse, and rat after DEAE-chromatography. Solubilized fractions of pancreas homogenates of WT mouse, CELKO mouse, and rat were applied to a 1 × 20 cm DEAE-Sepharose CL-6B column equilibrated in column buffer (see Materials and Methods). Bed volume was 11 mL, and 1 mL of sample (containing approximately 5.6 mg of protein) was loaded. Fractions of 0.32 mL were collected, and aliquots of 20 μL were directly used for enzyme assays. The column was washed with 2 column volumes (approximately 22 mL) of column buffer, followed by 6 column volumes (approximately 66 mL) of a gradient of 0−0.4 M KCl in column buffer. For CEL and PTL assays, 10 and 100 μM substrate were used, respectively, during a 60 min incubation. PTL assays were carried out in the presence of 3 μg/mL colipase. Values are expressed as pmol of ffa h^{-1} (20 μL)$^{-1}$, and represent single determinations.

assessed in a separate experiment. Using 100 μM triolein in the presence of 3 μg/mL colipase, 15 600 μmol of oleic acid was formed per gram of protein during a 60 min incubation. Using 100 μM retinyl palmitate, 1810 μmol of palmitic acid was formed by hPTL. This difference in specific activity agrees with the data shown before in Figure 4 (top panels). When pancreas homogenates were assayed using the "PTL-assay", the activity of the enzyme to release fatty acids from triolein was about 10-fold higher than that from retinyl palmitate at 100 μM substrate in both rat, and WT and CELKO mice (data not shown). Under CEL-assay conditions (using 10 μM substrate in the absence of colipase), we did not detect any CEH activity of hPTL [<190 μmol of oleic acid h^{-1} (g of protein)$^{-1}$], which is consistent with the notion that PTL does not use CE as a substrate (*16*). However, when hPTL was incubated with 10 μM retinyl palmitate, 1970 μmol of palmitic acid was formed per hour per gram of protein. This indicates that, even under CEL-assay conditions, purified PTL is active in hydrolyzing RE. As also shown above for hPTL, in total pancreas homogenates the hydrolytic activity against retinyl palmitate in the classical CEL-assay was of the same order of magnitude as its activity against retinyl palmitate under PTL-assay conditions in the presence of saturating colipase (Figure 2, and data not shown). When hPTL was treated with 1% CHAPS and chromatographed on the same DEAE-column as the rat and mouse solubilized pancreas homogenates, PTL-TGH and CEL-REH activity eluted in the same unbound fraction (data not shown). These observations provide further support for the hypothesis that a major part of CEL-REH activity in both rats and mice is due to the activity of PTL.

DISCUSSION

We recently showed that in CEL-deficient mice the intestinal absorption of CE was partly decreased, but that of RE was unaffected (*1*), indicating that CEL is not involved in RE absorption. Lipid ester (such as CE, TG, RE) hydrolysis is essential for lipid uptake from the intestinal

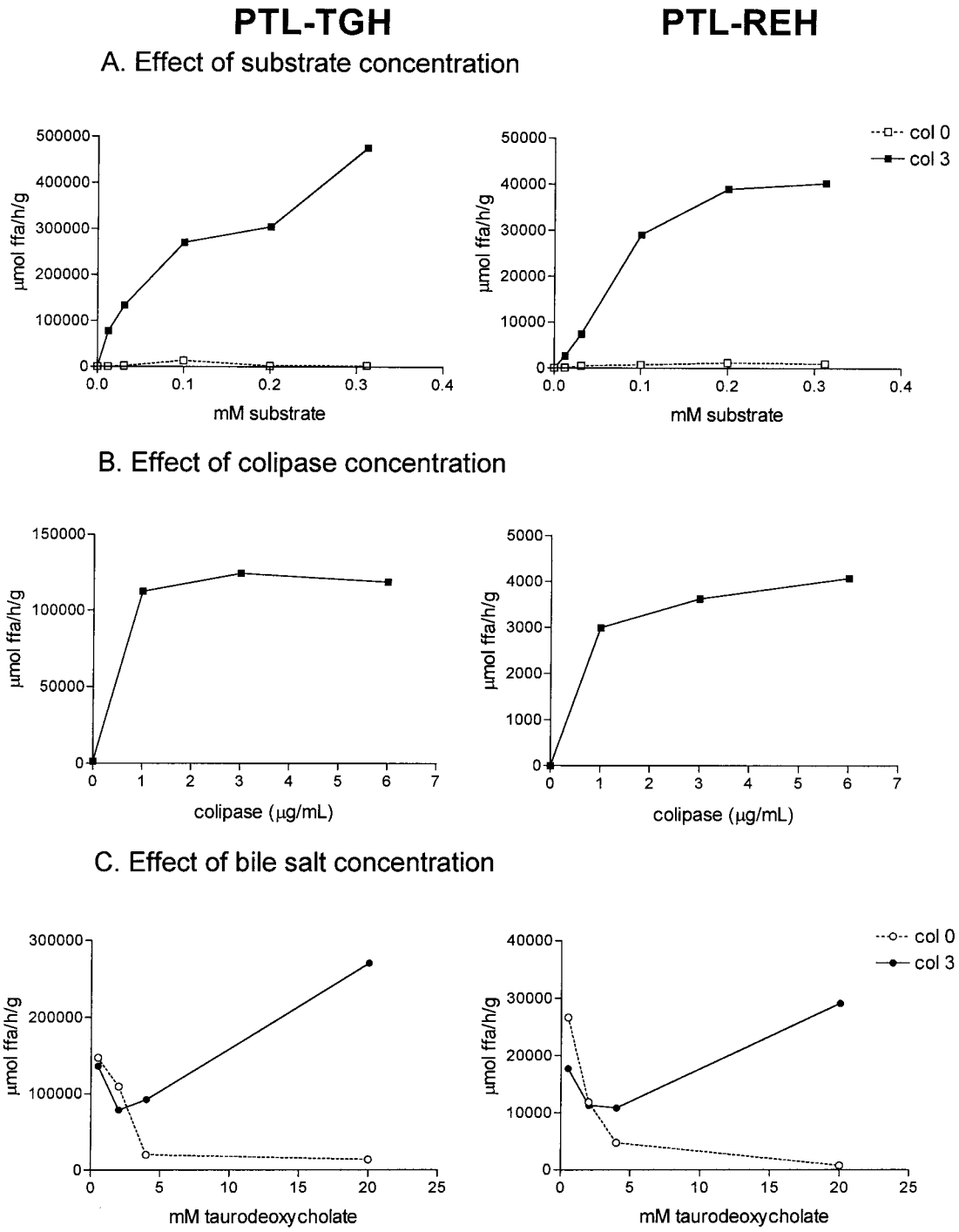

FIGURE 4: Characteristics of PTL-TGH and PTL-REH activities of purified hPTL. The effects of substrate concentration (A) and bile salt concentration (C) were assessed in the absence (open symbols) or presence (closed symbols) of 3 μg/mL colipase. For (B) (effect of colipase concentration) and (C) (effect of bile salt concentration), 100 μM substrate was employed. Assays were performed using a 60 min incubation under conditions where enzyme activity was directly proportional to the amount of enzyme added. Values are expressed as μmol of ffa h^{-1} (g of protein)$^{-1}$, and represent single measurements of one representative dilution measured in the linear range.

lumen by the intestinal brush border membrane (BBM)(*17*). Therefore, if intestinal RE absorption is unaffected in CELKO mice (*1*), one or more other RE hydrolytic enzymes must be present in the gut lumen or enterocyte.

The aim of our studies was to identify the non-CEL pancreatic REH activity that appeared to be present in CELKO mice (*2*), as well as to investigate this activity in WT mice and in rats. Several lines of evidence suggest that this activity is due to PTL. First, the dependence of pancreatic REH on different types of bile salt was investigated. When CEL-CEH is assayed utilizing different bile salt conditions, activity is detected only in the presence of trihydroxy bile salts (Figure 1), consistent with CEL-mediated CEH activity (*3*, *4*). CEL-REH activity, however, is detected not only in the presence of trihydroxy bile salts, but also in the presence of dihydroxy bile salts (as well as in the absence of bile salt or in the presence of CHAPS, a bile salt analogue). The finding that CEL-REH activity is supported by dihydroxy bile salts is consistent with PTL-mediated hydrolysis. Second, when total pancreas homogenates obtained from rat, and WT and CELKO mice are assayed for PTL-TGH, the colipase-stimulated TGH activity indicates the presence of PTL, as

would be expected (Figure 2). When CEL-REH activity was assayed, a considerable stimulation of the activity by colipase was observed (Figure 2). This indicates that PTL may be responsible for at least part of the pancreatic bile salt-dependent REH activity. Finally, when pancreas homogenates are subjected to DEAE-chromatography, the majority of CEL-REH activity appears to coelute with PTL-TGH activity in the unbound fraction in rats and in WT and CELKO mice, with a minor peak coeluting with CEL activity during the KCl gradient in both species (Figure 3). This further supports our notion of PTL-mediated RE hydrolysis, and suggests that PTL may be the main CEL-REH activity in the pancreas of both mice and rats.

Although our data strongly suggest that PTL may be a major REH in rat and mouse pancreas, they do not provide final proof for this concept. Some PTL-TGH was observed in the absence of colipase in pancreas homogenates from both rats and mice (Figure 2), which may point to the presence of other enzyme activities, such as pancreatic lipase related protein 2 [PLRP2 (*14*)]. This enzyme (which also hydrolyzes phospholipids) is 65% identical to PTL, and shows activity toward TG in the classical PTL assay (*14*). At present, we cannot definitely assess the percent contribution of PLRP2 to pancreatic bile salt-dependent REH. Also, yet another pancreatic lipase related protein (PLRP1) has been cloned which is 68% homologous to PTL, but for which the substrate is still unknown (*18*). PTL-TGH activity in the absence of colipase may also be due to endogenous colipase present in the pancreas homogenates.

Because of the possibility of multiple proteins being involved in pancreatic RE hydrolysis, we studied the enzymatic characteristics of purified hPTL, using either triolein or retinyl palmitate as a substrate (Figure 4). Both REH and TGH activities of hPTL were completely dependent on the presence of colipase. In addition, identical patterns of bile salt inhibition of hPTL hydrolytic activity and colipase alleviation were observed using either triolein or retinyl palmitate as a substrate (Figure 4). Moreover, this purified enzyme also exhibited CEL-REH activity.

In summary, it appears that several distinct enzymes participate in pancreatic bile salt-dependent REH activity in rats and mice in vivo. First, the majority of CEL-REH appears to be due to PTL. Second, CEL is most likely responsible for another part of CEL-REH. Furthermore, a third REH enzyme activity appears to exist which is neither PTL nor CEL (Figure 3). In addition to pancreatic bile salt-dependent REH activities, an REH activity intrinsically located in the brush border of the absorptive enterocytes was shown by Rigtrup et al. in rat (*19*) and human (*20*) intestines. These authors (*19*) showed that rat BBM, isolated from rats in which the common duct had been ligated for 2 days (thus, prohibiting contamination of BBM with any pancreatic secretions such as CEL or PTL), had a greatly decreased hydrolytic activity against short-chain RE (in the presence of trihydroxy bile salts), and a small (30%) decrease in the activity against long-chain RE (such as retinyl palmitate), as compared with sham-operated rats. Therefore, they suggested that short-chain REH was mainly from pancreatic origin (and could be due to CEL), whereas the majority (70%) of long-chain REH was intrinsic to the brush border. The remaining 30% of REH activity, however, could be due to PTL, as this REH activity was detected in the presence of both trihydroxy and dihydroxy bile salts (*19*). To determine which of the above-mentioned enzymes is most critical in intestinal RE digestion and absorption, it will become essential to perform RE absorption experiments in the appropriate knock-out mice strains.

As expected, the majority of CEL-CEH activity in rat and mouse eluted during the KCl gradient of DEAE-chromatography, consistent with CEL-mediated CE hydrolysis (*3, 4*). However, in both species a minor CEL-CEH activity eluted in the unbound fraction (Figure 3). At present, we have no further data about its identity. The activity cannot be related to CEL, as it was also detected in CELKO mice. A PTL identity is also unlikely, as purified hPTL does not appear to have any measurable CEH activity [see Results and (*16*)]. The presence of this non-CEL CEH activity in the pancreas of mouse could explain the incomplete ablation of CE absorption in CELKO mice (*1, 21*).

ACKNOWLEDGMENT

We thank Mrs. Kathleen Popoff for expert technical assistance, Dr. Marilyn S. Jorns and Dr. Francoise Strasser for their valuable discussion and suggestions, and Dr. Michael C. Phillips and Dr. Mahmood Hussain for critical reading of the manuscript.

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BI9927235