rapid communication

Immunodepletion experiments suggest that acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) protein plays a major catalytic role in adult human liver, adrenal gland, macrophages, and kidney, but not in intestines

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Abstract The first acyl-coenzyme A:cholesterol acyltransferase (ACAT) cDNA cloned and expressed in 1993 is designated as *ACAT-1*. In various human tissue homogenates, ACAT-1 protein is effectively solubilized with retention of enzymatic activity by the detergent CHAPS along with high salt. After using anti-ACAT-1 antibodies to quantitatively remove ACAT-1 protein from the solubilized enzyme, measuring the residual ACAT activity remaining in the immunodepleted supernatants allows us to assess the functional significance of ACAT-1 protein in various human tissues. The results showed that ACAT activity was immunodepleted 90% in liver (83% in hepatocytes), 98% in adrenal gland, 91% in macrophages, 80% in kidney, and 19% in intestines, suggesting that ACAT-1 protein plays a major catalytic role in all of the human tissue/cell homogenates examined except intestines. Intestinal ACAT activity is largely resistant to immunodepletion and is much more sensitive to inhibition by the ACAT inhibitor Dup 128 than liver ACAT activity.—Lee, O., C. C. Y. Chang, W. Lee, and T-Y. Chang. **Immunodepletion experiments suggest that acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT-1) protein plays a major catalytic role in adult human liver, adrenal gland, macrophages, and kidney, but not in intestines. *J. Lipid Res.* 1998.** 39: **1722–1727.**

Supplementary key words cholesteryl esters • hepatocytes • membrane protein solubilization • detergents

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is an intracellular enzyme that catalyzes formation of cholesteryl esters from cholesterol and fatty acyl coenzyme A in various tissues (reviewed in ref. 1). It has been shown in animals that blocking ACAT activity by using various ACAT inhibitors causes a reduction in very low density lipoprotein (VLDL) synthesis and secretion in the liver and/or cholesterol absorption in the intestines (2–4). In addition, ACAT may play an important role in the differentiating monocytes and in forming the macrophage foam cells during the development of atherosclerosis (5, 6).

The ACAT cDNA was first cloned and functionally expressed in 1993 (7), and its homologues in various species have also been cloned (reviewed in ref. 1). This gene is now designated as ACAT-1. Recently, ACAT-1 gene knockout mice have been generated (8). The homozygous knock-out mice showed markedly reduced amounts of cholesteryl esters in adrenal glands and peritoneal macrophages. In contrast, liver ACAT activity was not reduced in these mice, suggesting that the structure of liver ACAT may be different from that in the adrenal glands and peritoneal macrophages. These and other results (9) have led to the suggestion that in mice, ACAT-1 gene product may not play a significant role in the liver and intestines. Liver and intestines are major organs involved in cholesterol synthesis and lipoprotein assembly/secretions in the body.

We had earlier developed a biochemical approach to assess the functional significance of ACAT-1 protein in various human cell lines (10). Using the specific polyclonal anti-ACAT-1 antibodies, immunoblot analysis showed that the antibodies cross-reacted with a single 50 kDa protein band in homogenates from various human cells. Anti-ACAT-1 antibodies quantitatively immunoprecipitated the ACAT-1 protein and effectively depleted more than 90% of deoxycholate (DOC)-solubilized ACAT activities from

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DOC, deoxycholate; PC, phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; CHO, Chinese hamster ovary.

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six different human cell lines, demonstrating that the 50 kDa protein is the major ACAT catalytic component in these cells (10). When we attempted to use this procedure in various human tissue homogenates, however, we found that DOC used at concentrations from 0.5% to 2% severely inactivated the ACAT activity. In this communication, we show that the zwitterionic detergent CHAPS with high salt (1 m KCl) effectively solubilizes the ACAT-1 protein from various human tissues with retention of enzymatic activity. This finding enabled us to assess the functional significance of ACAT-1 protein in various human tissues by immunodepletion.

MATERIALS AND METHODS

Materials

Chemicals, solvents, and tetradecane were purchased from Sigma (St. Louis, MO). Frozen human tissue samples were obtained from Anatomical Gift Foundation (Laurel, MD). Primary hepatocytes were purchased from Clonetics (Walkersville, MD).

Human tissue preparations for immunoblotting

Donors ranged from 15 to 62 years old. All the tissues were transplantation-quality and were rapidly frozen in liquid nitrogen. About 1 g of frozen tissues was homogenized at room temperature with 5 ml lysis solution (10% SDS, 50 mm DTT) using a Potter-Elvehjem grinder at medium speed for 1–2 min. Proteins were separated by 10% SDS-PAGE, electrotransferred onto a PVDF membrane, and immunoblotted with 3.2 μg/ml of affinity-purified anti-ACAT-1 antibodies as described (10).

Limited proteolysis of cloned ACAT-1 versus liver ACAT

Standard procedures (11) were used with minor modifications as described in Fig. 1B legend.

Human tissue preparations for immunodepletion

About 2–5 g of frozen tissues was homogenized on ice with 5–10 ml ice-cold buffer A (0.1 mm phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 50 mm Tris, 5 mm EDTA, pH 7.8) using a Potter-Elvehjem grinder at medium speed for 1–2 min. To obtain the microsomal fraction, sequential centrifugations at 4°C were performed at 1,000 *g* for 30 min to obtain post-nuclear supernatant, 10,000 *g* for 30 min for post-mitochondrial supernatant, and 100,000 *g* for 30 min for microsomal pellet.

Immunodepletions were performed at 4°C, following the procedures described by Chang et al. (10) with minor modifications. Briefly, whole cell homogenate or microsome protein concentration was adjusted to 5 mg/ml. Protein concentrations were determined as described (12). Solid KCl and 10% CHAPS (dissolved in H_2O) were added sequentially to obtain a final concentration of 1 m KCl and 2% CHAPS. After vortexing briefly several times, solubilized supernatant was prepared by ultracentrifuging at 100,000 *g* for 30 min at 4°C to discard any unsolubilized material. Four hundred fifty μl of the 100,000 *g* supernatants was incubated for 2 h with 80 μl of 0.1 m Tris-glycine buffer (pH 7) containing 10 μg of affinity-purified polyclonal anti-ACAT-1 antibodies or non-specific rabbit IgGs (as a negative control). Subsequently, 100 μl of pre-washed protein A-Sepharose was added to the mix and incubated for 1 h at 4°C. Immunodepleted supernatants were separated from the pellets by centrifugation at 12,000 *g* for 30 sec. Control experiments showed that the solubilized liver ACAT enzyme activities were not completely stable at 4°C. In three experiments, we found that the ACAT activities present in the 100,000 *g* supernatants gradually lost 10–30% of their original activities when incubated on ice for 3–4 h. For this reason, the immunodepletion experiments were always conducted within 3 h.

ACAT enzyme assay using reconstituted vesicles

Previously reported procedures (13) were used with minor modifications. Thirty μl of the solubilized enzyme was added to 188 μl of preformed vesicles (cholesterol–PC–tetradecane, molar ratio 0.4:1:1 ; Doolittle and Chang (14) have previously shown that tetradecane in vesicles enhances pig liver ACAT activity). Vesicles were prepared by the cholestyramine resin method (15). The solubilized enzyme extracts were incubated with the vesicles on ice for 10 min. For ACAT inhibitor studies, the ACAT enzyme in the vesicles was incubated on ice for 10 min with serial dilutions of the ACAT inhibitor Dup 128 (16) dissolved in ethanol. Final concentration of ethanol was less than 1%, and an equal amount of ethanol without any ACAT inhibitor was added to the controls. ACAT assays were performed in triplicate at 37°C for 30 min. For comparison, microsomal ACAT assays were performed in triplicate using 5 mg/ml microsomal extract with or without adding vesicles, and assayed for 5 min at 37°C.

Immunoblot analysis after immunodepletion

Standard procedures (10) were used with minor modifications. After immunodepletion, the proteins present in the supernatant fraction were precipitated by the choloroform–methanol extraction method (17) to remove the detergent and KCl and then resuspended in lysis solution (10% SDS, 20 mm DTT). Immunopellets were directly solubilized in the same lysis solution.

Sources of primary hepatocytes and macrophages

Human primary hepatocytes were purchased as monolayers on collagen matrix from Clonetics (Walkersville, MD) isolated by a modification of the published procedure (18) and cultured in Hepatocyte Maintenance Medium provided by the company. Cells were harvested within 4 days of the initial culture by the hypotonic shock method (19). Two percent CHAPS plus 1 m KCl was used to solubilize the whole cell extracts. Immunodepletions and reconstituted vesicle assays were performed as described. Human monocyte-derived macrophages, harvested on day 5 in culture as described previously (6), were used for immunodepletion and reconstituted vesicle assays as described.

RESULTS

Immunoblotting and limited proteolysis

The SDS-PAGE immunoblot analysis showed that the anti-ACAT-1 antibodies recognize a single 50 kDa protein band in various human tissue homogenates (**Fig. 1A**), its size being identical to the human ACAT-1 protein cloned and expressed in CHO cells (10). To further identify its nature, we isolated the 50 kDa protein present in the human liver microsome, carried out limited proteolysis using V8 protease, and compared the partial digestion pattern with that of the cloned human ACAT-1 protein isolated from CHO cell line K1 (10). This method has been used by many investigators to probe the structural differences between closely related proteins (for a few examples, see refs. 20–22). The partial proteolytic patterns of these two different samples were indistinguishable (Fig. 1B). Under the premise that SDS-PAGE combined with limited proteolysis can resolve subtle structural differences between

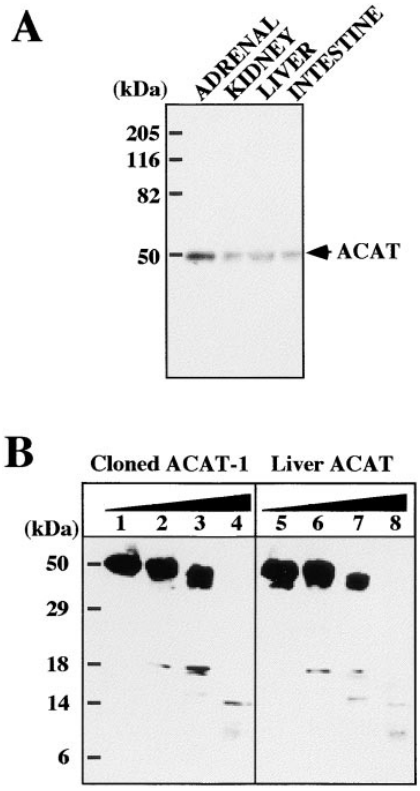

Fig. 1. A: Multiple human tissue immunoblot. Frozen human tissue samples were homogenized in lysis solution (10% SDS, 50 mm DTT). One hundred μg protein of various indicated tissue lysates was loaded per lane for 10% SDS-PAGE. Polyclonal ACAT-1 antibodies were used to detect the 50 kDa ACAT-1 protein. B: Limited proteolysis. Whole cell extracts of KI cells (a CHO cell line expressing cloned human ACAT-1 protein (10)) and human liver microsomes were solubilized by adding an equal volume of lysis solution (20% SDS, 100 mm DTT), with final protein concentration at approximately 5 mg/ml. Two hundred μg of lysates was loaded per lane for 10% SDS-PAGE. After the electrophoresis, the gel was cut near the 50 kDa region. Slices of gel cubes were loaded into the stacking wells of a 15% polyacrylamide gel. Twenty μl of 20% glycerol mix, followed by 10 μl of 10% glycerol mix (11) containing 0.005, 0.05, 0.5, or 5 μg of V8 protease was overlayered on top of the gel slices. The samples were in-gel digested for 2 h. The electrophoresis was completed, and anti-ACAT-1 antibodies were used to immunoblot the partial proteolytic patterns.

two related proteins, we interpreted the results obtained here as indicating that the 50 kDa protein recognized by the anti-ACAT-1 antibodies in the human liver is essentially identical to the cloned human ACAT-1 protein expressed in CHO cells.

Solubilization of ACAT in human tissues

We searched for a detergent that could provide solubilization of human liver microsomal ACAT-1 protein with retention in ACAT enzyme activity. The enzyme activities were monitored by the reconstituted vesicle assay (13). We tested deoxycholate, triton X-100, octyl glucoside, NP-40 at various concentrations and found that none was able to retain high ACAT activity after solubilization and reconstitution. In contrast, treating liver microsomes with the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (from 0.5% to 2%), along with high salt (1 m KCl), did retain high ACAT activity. **Figure 2A** shows the results of a typical experiment: 2% CHAPS in the presence of 1 m KCl resulted in the optimal ACAT activity (specific activity of 94 pmol/min per mg); this specific activity was much higher than microsomal ACAT activity without adding vesicles (18 pmol/min per mg). The procedures described here have been found to

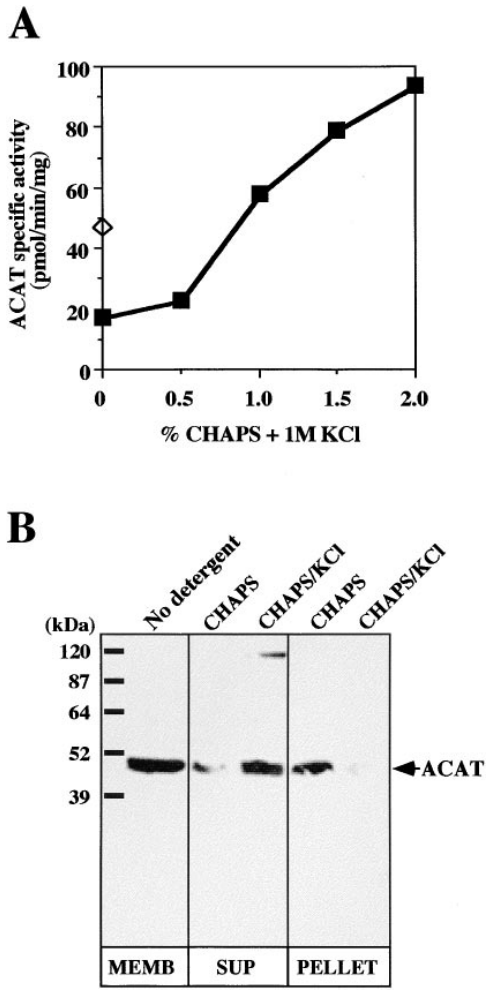

Fig. 2. Solubilization of the human liver microsome using CHAPS + 1 m KCl. A: Liver microsomal fraction was solubilized in various indicated concentrations of CHAPS + 1 m KCl. The samples were vortexed briefly before proceeding to reconstituted vesicle assay (see Materials and Methods). A parallel experiment showed that the specific activity of the microsomal (not detergent-treated) ACAT was 18 pmol/min per mg. When 188 μl of preformed vesicle (at cholesterol:PC:tetradecane molar ratio of 0.4:1:1) was added to 30 μl of unsolubilized microsomes, the specific activity observed was 48 pmol/min/mg (◇). B: Immunoblot of solubilized supernatant versus pellet. Liver microsomes were treated with 2% CHAPS in the presence or absence of 1 m KCl. The treated material was centrifuged at 100,000 *g* for 30 min, and 1/5 (by volume) of the supernatants (lanes SUP) and pellets (lanes PELLET) were loaded for SDS-PAGE; 1/5 (by volume) of the untreated microsomes were also loaded (lane MEMB). In the supernatant fraction, detergent and KCl were removed by using the chloroform–methanol extraction method (17) before SDS-PAGE. Immunoblotting with anti-ACAT-1 antibodies was performed as described (10).

effectively solubilize and maintain high ACAT activitites from microsomes prepared from human intestines, mouse liver, and rabbit liver (results not shown). In human liver microsome, CHAPS alone (at 2%) was not able to efficiently solubilize the ACAT-1 protein, while CHAPS combined with high salt (1 m KCl) efficiently solubilized the ACAT-1 protein (Fig. 2B).

Immunodepletions

We prepared CHAPS/KCl-solubilized 100,000 *g* supernatants from whole cell homogenates of various human tissues or cells and found that anti-ACAT-1 antibodies quantitatively immunoprecipitated ACAT-1 protein from the supernatants (**Fig. 3A**). In contrast, non-specific rabbit IgGs failed to immunoprecipitate ACAT-1 protein at the detectable level. For each tissue or cell source indicated, at least two different experiments were performed and the same results were obtained. Measuring residual ACAT activity remaining in the immunodepleted supernatants allowed us to assess the functional significance of ACAT-1 protein in various tissues or cells indicated. The results (Fig. 3B, 3C) indicate that ACAT activity was depleted 90% in liver (n = 5; n denotes the numbers of donors), 83% in hepatocytes (n = 2), 98% in adrenal gland (n = 2), 91% in macrophages (n = 1), 80% in kidney (n = 2), and 19% in intestines (n = 5). These results suggest that the ACAT-1 protein plays a major catalytic role in homogenates of all the human tissues and cells examined except intestines. The same results described here were obtained when microsomal fractions, instead of whole cell homogenates, prepared from various human tissues were used as the enzyme source for immunodepletion experiments (results not shown). The average specific activity of the intestinal ACAT was 51.1 ± 28 pmol/min per mg, which was higher than the average value for the liver ACAT (8.4 ± 1.2 pmol/min per mg).

The above results suggest that the observed ACAT activities in the intestines may be largely due to the presence of a different ACAT protein. To test this possibility, we tested the sensitivities of the human liver and intestinal ACAT enzyme against different ACAT inhibitors. We found that the intestinal ACAT is much more sensitive than the liver ACAT to inhibition by at least one of the specific ACAT inhibitors tested, Dup 128 (**Fig. 4**). The IC_{50} was approximately 8×10^{-9} m for the intestinal enzyme versus 7×10^{-8} m for the liver enzyme.

DISCUSSION

The results of this study suggest that the human ACAT-1 protein plays a major role in contributing to the overall ACAT enzyme activity found in homogenates of the fol-

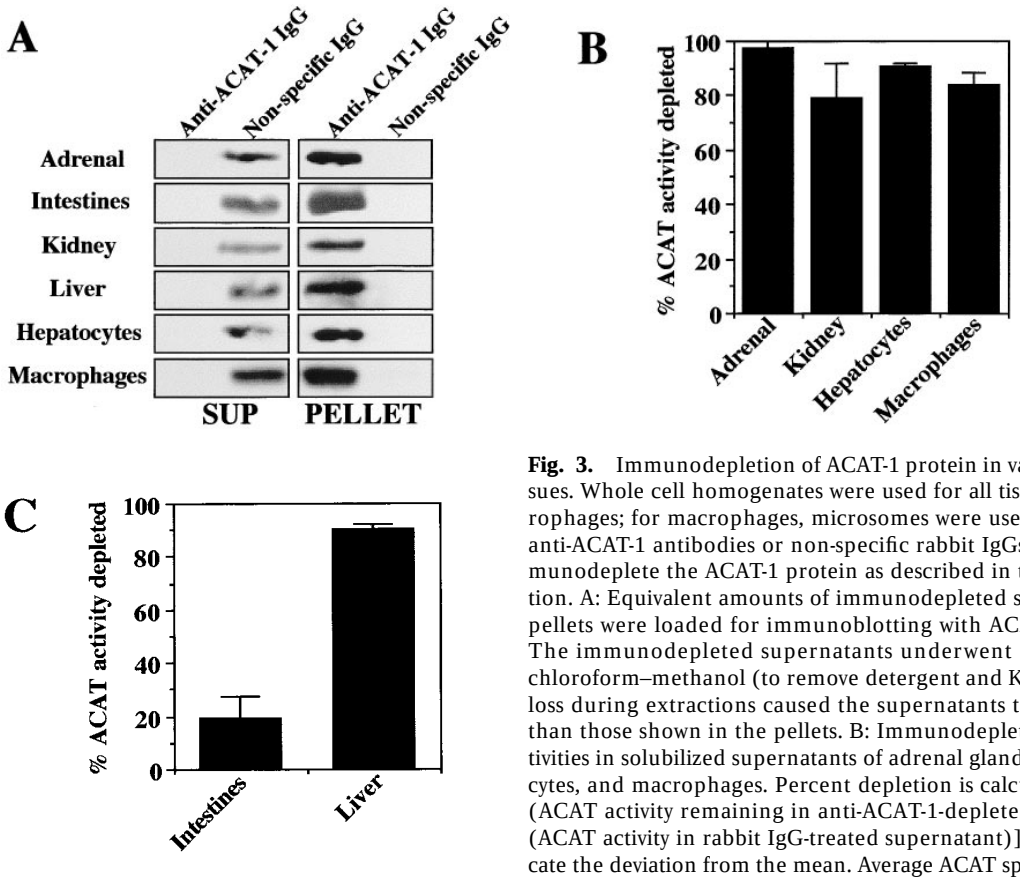

Fig. 3. Immunodepletion of ACAT-1 protein in various human tissues. Whole cell homogenates were used for all tissues except macrophages; for macrophages, microsomes were used. Ten μg of the anti-ACAT-1 antibodies or non-specific rabbit IgGs was used to immunodeplete the ACAT-1 protein as described in the Methods section. A: Equivalent amounts of immunodepleted supernatants and pellets were loaded for immunoblotting with ACAT-1 antibodies. The immunodepleted supernatants underwent extractions with chloroform–methanol (to remove detergent and KCl); the material loss during extractions caused the supernatants to be slightly less than those shown in the pellets. B: Immunodepletion of ACAT activities in solubilized supernatants of adrenal gland, kidney, hepatocytes, and macrophages. Percent depletion is calculated as [100 − (ACAT activity remaining in anti-ACAT-1-depleted supernatant)/(ACAT activity in rabbit IgG-treated supernatant)]; error bars indicate the deviation from the mean. Average ACAT specific activities ± deviation from the mean were as follows: adrenal, 79.7 ± 15.4 pmol/min per mg (n = 2; n denotes number of donors); kidney, 7.0 ± 1.0 pmol/min per mg (n = 2); primary hepatocytes, 3.2 ± 1.1 pmol/min per mg (n = 2); macrophages, 590 pmol/min per mg (n = 1). C: Immunodepletion of ACAT activities in the solubilized supernatants of liver and intestines. Error bars indicate sizes of standard error. Average specific ACAT activities ± standard error were as follows: liver, 8.4 ± 1.2 pmol/min per mg (n = 5); intestines, 51.1 ± 28.0 pmol/min per mg (n = 5).

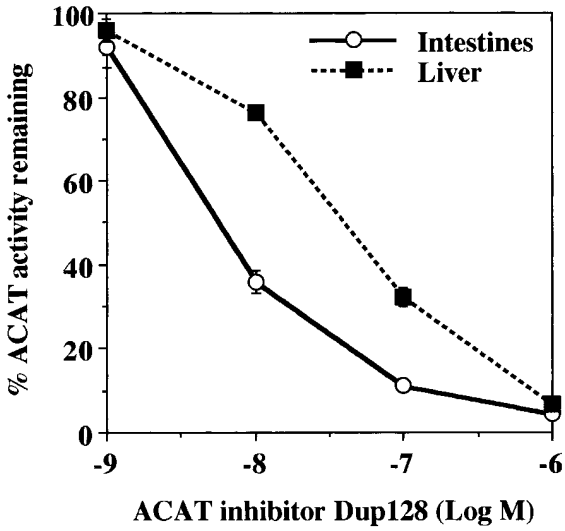

Fig. 4. Comparison of the sensitivity towards the ACAT inhibitor Dup128 between intestinal and liver ACAT. Solubilized supernatants of whole cell homogenates of intestines and liver were used as the enzyme source. Error bars indicate sizes of standard error. The specific activities were 15.8 pmol/min per mg for the intestinal ACAT, and 9.6 pmol/min per mg for the liver ACAT.

lowing human tissues/cells: liver, hepatocytes, adrenal gland, macrophages, and kidney. Intestines also contain the ACAT-1 protein. However, the observed ACAT activities in the intestines may be largely due to the presence of a different ACAT protein. In general, these results are consistent with the results obtained in the ACAT-1 gene knockout mice, suggesting that the ACAT-1 protein plays a major role in adrenal glands and macrophages, but not in intestines. An important difference is that while the ACAT-1 protein may not play a major role in mouse liver (8, 9), it does play a major role in human liver. In humans and other mammalian species, approximately 65% of liver is comprised of parenchymal hepatocytes, with the remaining 35% being non-parenchymal cells (endothelial cells, Kupffer cells, lipocytes, etc.) (23); parenchymal hepatocytes, but not non-parenchymal cells, are involved in cholesterol synthesis, lipoprotein assembly and secretion. To ascertain our results obtained in the whole liver, we performed immunodepletion experiments in primary human hepatocytes. The results again showed that the ACAT-1 protein plays a major role in primary human hepatocytes.

Our results also show that intestines contain the highest residual ACAT activity resistant to immunodepletion, suggesting the observed ACAT activities in the intestines may be largely due to the presence of a different ACAT protein. This interpretation is supported by the finding that the human intestinal ACAT is much more sensitive to inhibition by the ACAT inhibitor Dup 128 than the human liver ACAT. While the differences in the sensitivities towards Dup 128 in these two tissues are consistent with the hypothesis that the intestinal ACAT may be different from the liver ACAT, other interpretations cannot be ruled out at present. Definitive proof requires the molecular identification of the intestinal ACAT distinct from the cloned ACAT-1. Such efforts are in progress in our laboratory. A second ACAT gene has recently been cloned by Cases et al. (24) and by Sturley, Oelker, and Behari (25). When the antibodies against the second ACAT protein become available, it will be interesting to assess its functional significance in different species including humans.

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