Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates

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Abstract **Two closely related enzymes with more than 50% sequence identity have been identified that catalyze the esterification of cholesterol using acyl-CoA substrates, namely acyl-CoA:cholesterol acyltransferase 1 (ACAT1) and ACAT2. Both are membrane-spanning proteins believed to reside in the endoplasmic reticulum of cells. ACAT2 has been hypothesized to be associated with lipoprotein particle secretion whereas ACAT1 is ubiquitous and may serve a more general role in cellular cholesterol homeostasis. We have prepared and affinity purified rabbit polyclonal antibodies unique to either ACAT enzyme to identify their cellular localization in liver and intestine, the two main lipoprotein-secreting tissues of the body, and for comparison, kidney and adrenal. In the liver, ACAT2 was identified in the rough endoplasmic reticulum of essentially all hepatocytes whereas ACAT1 was confined to cells lining the intercellular spaces among hepatocytes in a pattern typical of Kupffer cells. In the intestine, ACAT2 signal was strongly present in the apical third of the mucosal cells, whereas ACAT1 staining was diffuse throughout the mucosal cell, but with strong signal in goblet cells, Paneth cells, and villus macrophages. In the kidney, ACAT1 immunostaining was specific for the distal tubules and podocytes within the glomerulus. In the adrenal, ACAT1 signal was strongly present in the cells of the cortex, and absent from other adrenal cell types. No ACAT2 signal was identified in the kidney or adrenal. We conclude that only the cells of the liver and intestine that secrete apolipoprotein B-containing lipoproteins contain ACAT2, whereas ACAT1 is present in numerous other cell types. The data clearly suggest separate functions for these two closely related enzymes, with ACAT2 being most closely associated with plasma cholesterol levels.**—Lee, R. G., M. C. Willingham, M. A. Davis, K. A. Skinner, and L. L. Rudel. **Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates.** ***J. Lipid Res.*** **2000.** 41: **1991–2001.**

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Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is an integral membrane protein located in the rough endoplasmic reticulum (ER) that catalyzes a reaction in which a fatty acyl-CoA is esterified to a cholesterol molecule. This changes the physical properties of the cholesterol molecule, making it less soluble in the plasma membrane and facilitating cholesterol storage in cytoplasmic lipid droplets as cholesteryl esters.

There is strong evidence that ACAT plays an important role in the progression of atherosclerosis. Nonhuman primates fed diets rich in fat and cholesterol showed a high correlation between hepatic ACAT activity and coronary artery intimal area, a measure of the extent of atherosclerosis (1). In isolated liver perfusion studies of nonhuman primates, cholesteryl ester secretion rate was highly correlated to coronary artery atherosclerosis extent in the liver donor (2) and ACAT-specific inhibitors were effective in decreasing the rate of secretion of cholesteryl ester and apolipoprotein B (apoB) (3). Administration of an ACAT inhibitor, CI-976, which inhibits both intestinal and hepatic ACAT, to rabbits fed a hypercholesterolemic diet has been shown to decrease aortic foam cell area (4).

There are two known isoforms of ACAT, designated ACAT1 and ACAT2. The ACAT1 cDNA sequence was originally determined in the T-Y. Chang laboratory (5). Three different laboratories determined the ACAT2 sequence of three different animal species (mouse, monkey, and human) at about the same time (6–8). In vervet monkeys, ACAT1 consists of 551 amino acids, and ACAT2 consists of 526 amino acids (7). The first 100 amino acids at the N terminus of the two isoforms show no sequence similarity, whereas the remaining amino acids show 57% sequence identity. Northern blot analysis has shown that ACAT1 mRNA is found in highest levels in the adrenal tissue of

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; apoB, apolipoprotein B; FITC, fluorescein isothiocyanate; NGS, normal goat serum; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

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African green monkeys, but is also expressed in all of the other 18 tissues examined. However, ACAT2 mRNA was detected only in the liver and small intestine, and a similar outcome was obtained in mice (6).

Previous studies indicate that a serine residue essential for ACAT1 enzyme activity is on the cytosolic side of the ER membrane (9, 10), whereas computer topology models postulate that the same serine in ACAT2 is on the lumenal side of the ER (10). This, along with the tissue distributions of mRNA for ACAT1 and ACAT2, has led to speculation that ACAT1 functions in intracellular cholesterol homeostasis in many tissues, whereas in liver and intestine, ACAT2 is involved in the synthesis of cholesteryl esters destined for secretion in the core of apoB-containing lipoprotein particles. In the intestine, cholesterol absorbed from the lumen as free cholesterol is incorporated primarily as cholesteryl ester into chylomicron particles for secretion (11). In the liver, VLDL is secreted with varying amounts of cholesteryl esters in the core, depending on the cholesterol content of the liver (2).

The intracellular localization of ACAT1 has been studied by immunohistochemistry (12, 13). However, the localization of ACAT2 in the various cell types within human and nonhuman primate tissues has not yet been demonstrated. By developing polyclonal antibodies specific to ACAT1 and ACAT2 that allow us to determine the location of both ACAT1 and ACAT2 in nonhuman primate tissues, we can begin to establish the unique roles of the ACAT isoforms in cholesterol metabolism.

MATERIALS AND METHODS

Cell transfection

AC29, a Chinese hamster ovary cell line modified to have no ACAT activity (14), was a generous gift from T-Y. Chang (Dartmouth College of Medicine, Hanover, NH) and were maintained in Ham's F-12 medium supplemented with 2 mM L-glutamine, 1% Eagle's vitamins, penicillin (100 IU/ml), streptomycin (100 μg), and 10% (v/v) fetal bovine serum. Cells were grown to 50–70% confluence on 35-mm plates and transfections were carried out with FuGENE™ (Boehringer Mannheim Biochemicals, Indianapolis, IN) and African green monkey ACAT1 or ACAT2 cDNA in a pIRESneo vector purchased from Clontech (Palo Alto, CA) at a 6 μl:1 μg FuGENE™-to-DNA ratio. After selection with the drug geneticin (G418), cell colonies were isolated and characterized and colonies that stably expressed the desired ACAT gene were selected.

Primate tissue collection

A feral adult male African green monkey, who had been fed commercial chow in our colony for about 6 months, was killed, the carcass was flushed with saline, and tissue was collected and immediately flash frozen in liquid nitrogen. In addition, tissues from two adult male cynomolgus monkeys fed atherogenic diets were also collected in a similar manner and examined with no apparent difference due to species or dietary status. The tissue was subsequently stored at −80°C until use. A small 1 × 1 cm block of tissue was prepared for sectioning by immersion in imbedding oil at −24°C and was then sliced into 6-μm sections in a −24°C cryostat. Slices were mounted on a slide, fixed in 3.7% formaldehyde for 10 min at room temperature, and then washed and stored at 4°C in phosphate-buffered saline (PBS).

Maltose-binding protein/ACAT fusion protein preparation and purification

A 5′ ACAT1/MBP fusion protein and a 5′ ACAT2/MBP fusion protein were developed, using the protocol provided by New England BioLabs (Beverly, MA). The 5′ end of ACAT1 consisted of amino acids 1–113 encoded by the first 339 bases of the African green monkey ACAT1 cDNA sequence, and the 5′ end of the ACAT2 protein consisted of amino acids 8–92 encoded by bp 101–354 of the African green monkey ACAT2 cDNA sequence. Both cDNA sequences were cloned and expressed in pMal vectors that were then grown in *Escherichia coli*. Isopropyl-β-D-isothiogalactoside was used to induce protein production, and the fusion protein was purified using an amylose resin with elution in 10 mM maltose.

Initially, male New Zealand White rabbits were bled to collect a preimmune sample. Each animal was then given a subcutaneous injection of ~1.0 mg of either 5′ ACAT1/MBP or 5′ ACAT2/MBP in a 1:1 (v/v) mixture with Freund's complete adjuvant. The rabbits were given booster injections every 2 weeks for 6 to 8 weeks with the fusion protein (~0.5–1 mg) in a 1:1 (v/v) mix with Freund's incomplete adjuvant. Two weeks after the final boost the rabbits were bled from the ear vein (30 to 50 ml), with additional samples being collected at 2-week intervals for at least 8 weeks.

The IgG fraction from the antiserum was isolated by using an IgG purification kit purchased from Bio-Rad (Hercules, CA). The sample was then placed on the appropriate ACAT/MBP fusion protein column overnight at 4°C. The ACAT1 fusion protein column was composed of 60 mg of the 5′ ACAT1/MBP fusion protein bound to 5 ml of Affi-Gel 10™ (Bio-Rad) and the ACAT2 column was composed of 90 mg of 5′ ACAT2/MBP fusion protein bound to 5 ml of Affi-Gel 10™. After an overnight incubation to bind antibodies to the column, the column was washed with PBS and antibody was then eluted with 0.1 M glycine, pH 2.8, in 10% (v/v) ethylene glycol. For ACAT1 antibodies, an additional 3 M NaSCN elution was performed prior to the glycine elution to selectively remove a large portion of the antibodies specific for maltose-binding protein. The glycine eluate was dialyzed with multiple changes of PBS for 2 days. Immunopurified antibodies were stored in 0.1-ml aliquots at a final concentration of 0.5–1 mg/ml at −80°C.

Cell and tissue immunostaining

The technique for immunostaining was the same for both tissue and cells. Samples were taken out of PBS and blocked with 10% normal goat serum (NGS) plus 0.1% saponin in PBS for 5 min. A primary antibody solution, consisting of either ACAT1 antibody (10 μg/ml) or ACAT2 antibody (25 μg/ml) in 10% NGS plus 0.1% saponin in PBS, was added to the sample for 30 min, after which it was removed and the sample was washed three times with PBS. Affinity-purified rhodamine-labeled goat anti-rabbit IgG secondary antibody (25 μg/ml), purchased from Jackson ImmunoResearch (West Grove, PA) and suspended in 10% NGS plus 0.1% saponin in PBS, was then added to the sample for 30 min. The sample was then washed three times with PBS, and fixed with 3.7% formaldehyde for 10 min to cross-link the attached antibodies to the tissue, and the section was finally washed three times with PBS. A 10% PBS, 90% glycerol solution was used as a mounting medium. For double-staining experiments the procedure was repeated after the final formaldehyde fixation, using either anti-protein disulfide isomerase (PDI) or anti-

CD68 primary antibody and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody purchased from Jackson ImmunoResearch.

Mouse monoclonal antibodies to CD68 were purchased from Lab Vision (Fremont, CA), and were used at a 1:40 dilution in 1% bovine serum albumin (BSA) and 0.1% saponin in PBS. Mouse monoclonal antibodies to human PDI (15) were a gift from S-Y. Cheng (National Cancer Institute, NIH, Bethesda, MD) and were used at a concentration of 10 μg/ml.

Western blotting and antibody staining

Cells were trypsinized and pelleted, and mammalian protease inhibitor cocktail (Sigma, St. Louis, MO) was added to the pellet. The cells were then lysed at 37°C for 20 min in a solution containing 20 mM Tris, 1 mM ethylenediaminetetraacetic acid, 25 mM dithiothreitol, and 5% sodium dodecyl sulfate (SDS). The suspension was then briefly sonicated to break up DNA and aliquots were electrophoretically separated on a 10% polyacrylamide gel containing SDS. Proteins were transferred to nitrocellulose for 3 h at 100 V, using a Western blot apparatus. After transfer, the nitrocellulose was stained with Ponceau S and then blocked in 5% nonfat dry milk in TBST buffer [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.1% (v/v) Tween 20] overnight at 4°C. The nitrocellulose was washed with TBST buffer and then soaked in primary antibody at a concentration of about 3 μg/ml in TBST for 3 h at room temperature. The primary antibody was then removed, and the nitrocellulose was washed and soaked in secondary antibody at a 1:6,000 dilution for 1 h. After the removal of the secondary antibody, the blot was washed and developed with the Western blot chemiluminescence reagent from New England Nuclear (Boston, MA).

Isolation of tissue microsomes

African green monkey tissue was taken from the −80°C freezer and minced with a sterile razor blade. The tissue was then homogenized in isolation buffer with a Dounce homogenizer. The homogenate was then centrifuged at 13,000 rpm in an SW55 rotor in a Beckman (Fullerton, CA) ultracentrifuge for 15 min at 5°C. The supernatant was removed and subsequently centrifuged at 38,000 rpm for 60 min at 5°C in the SW55 rotor. The supernatant was removed and discarded, and the pellet was homogeneously resuspended in 1 ml of ACAT assay buffer (0.1 M K_2HPO_4, pH 7.4). The concentration of microsomal protein was measured by the method of Lowry et al. (16) and 0.1-ml aliquots at a concentration of about 5 mg/ml were stored at −80°C until use.

ACAT assay

Microsomes were thawed, an aliquot containing 200 μg of protein was mixed with 1 mg of BSA and 20 μl of a cholesterol-saturated solution of β-cyclodextrin, and the final volume was brought to 300 μl. The sample was equilibrated in a 37°C water bath for 30 min, and then [^{14}C]oleyl-CoA was added to the tube and incubated for 15 min. To stop the reaction, 6 ml of $CHCl_3$–methanol 2:1 was then added. KCl (1.2 ml) was then added and the sample was allowed to sit overnight at room temperature. The lower layer containing product was removed and evaporated to dryness under nitrogen. The residue was resuspended in 100 μl of chloroform containing carrier lipid and then applied to a thin-layer chromatography (TLC) plate with subsequent separation in hexane–ethyl ether–acetic acid 70:30:1. The portion of the TLC plate containing the cholesteryl esters was scraped and suspended in scintillation fluid, and radioactivity was determined. The data were expressed as specific activity (nanomoles per minute per milligram of microsomal protein).

RESULTS

A major goal for this experiment was to raise antibodies specific to the ACAT1 and ACAT2 proteins and that could be used to identify either enzyme in nonhuman primate tissue. A fusion protein strategy was developed, polyclonal antibodies were prepared, and AC29 cells transfected with ACAT1 or ACAT2 were used as the source of antigen to check the antibody preparations. Initially, cell homogenate from ACAT1 and ACAT2 stable cell lines and control AC29 cells was subjected to Western blotting (**Fig. 1**). ACAT1 cell homogenate characterized with ACAT1 antibody showed three specific bands that were not present in control lanes, that is, a dark, broad band at about 53 kDa, an additional band at about 30 kDa, and a lighter band at about 120 kDa. Previous observations suggest that the 30-kDa band is a proteolytic fragment of ACAT1 that is sometimes seen in cells overexpressing the enzyme, the 53-kDa band is apparently the full-size ACAT1 protein that has been identified in other cell lines, and the 120-kDa band may be a result of protein self-association (12). Homogenate from nontransfected

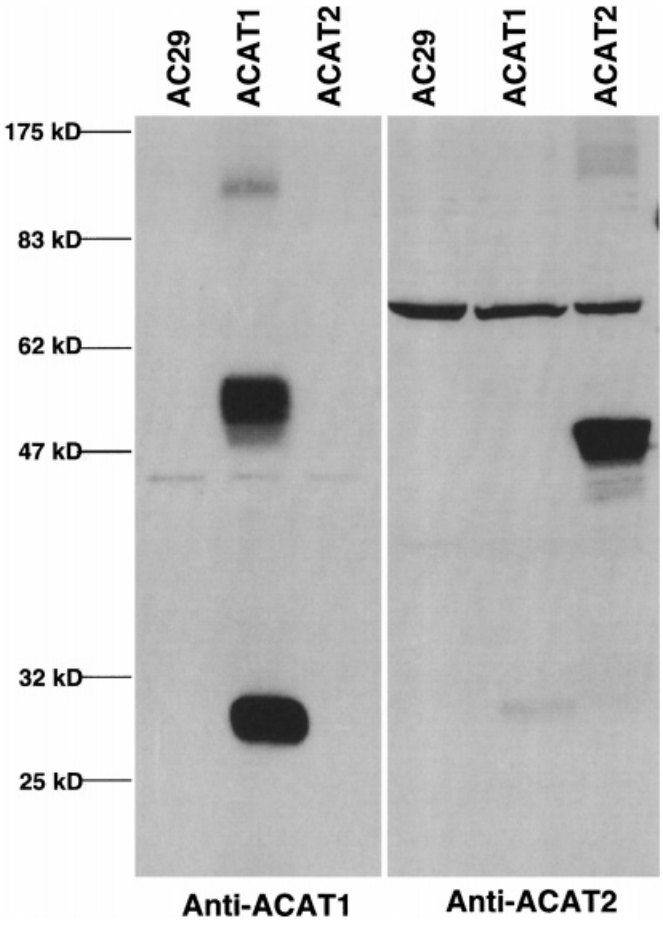

Fig. 1. Immunoblotting of ACAT1 and ACAT2 stable cell homogenates. The first three lanes are, respectively, immunoblots of AC29 and of ACAT1 and ACAT2 stable cell homogenates with ACAT1 antibodies. The remaining three lanes are immunoblots of the same cell homogenates with ACAT2 antibodies. The positions of molecular weight standards are shown on the left.

AC29 cells and from the ACAT2 stable cell line showed no signal with the ACAT1 antibodies.

Homogenate from the ACAT2 stable cell line characterized with the ACAT2 antibody showed a doublet running near 47 kDa. Some diffuse staining in the 175-kDa region was also seen. Homogenate from control AC29 cells and from ACAT1 cells stained with the ACAT2 antibody showed only the background band running at about 67 kDa. The size of the ACAT2 protein on this gel system was about 47 kDa, which was smaller than the band identified for ACAT1. Neither the ACAT1 nor the ACAT2 protein from these transfected cells ran during electrophoresis in SDS as a protein as large as the apparent molecular weight based on the deduced amino acid sequence from the cDNA. These data clearly demonstrate the specificity of the antibodies to both ACAT1 and ACAT2.

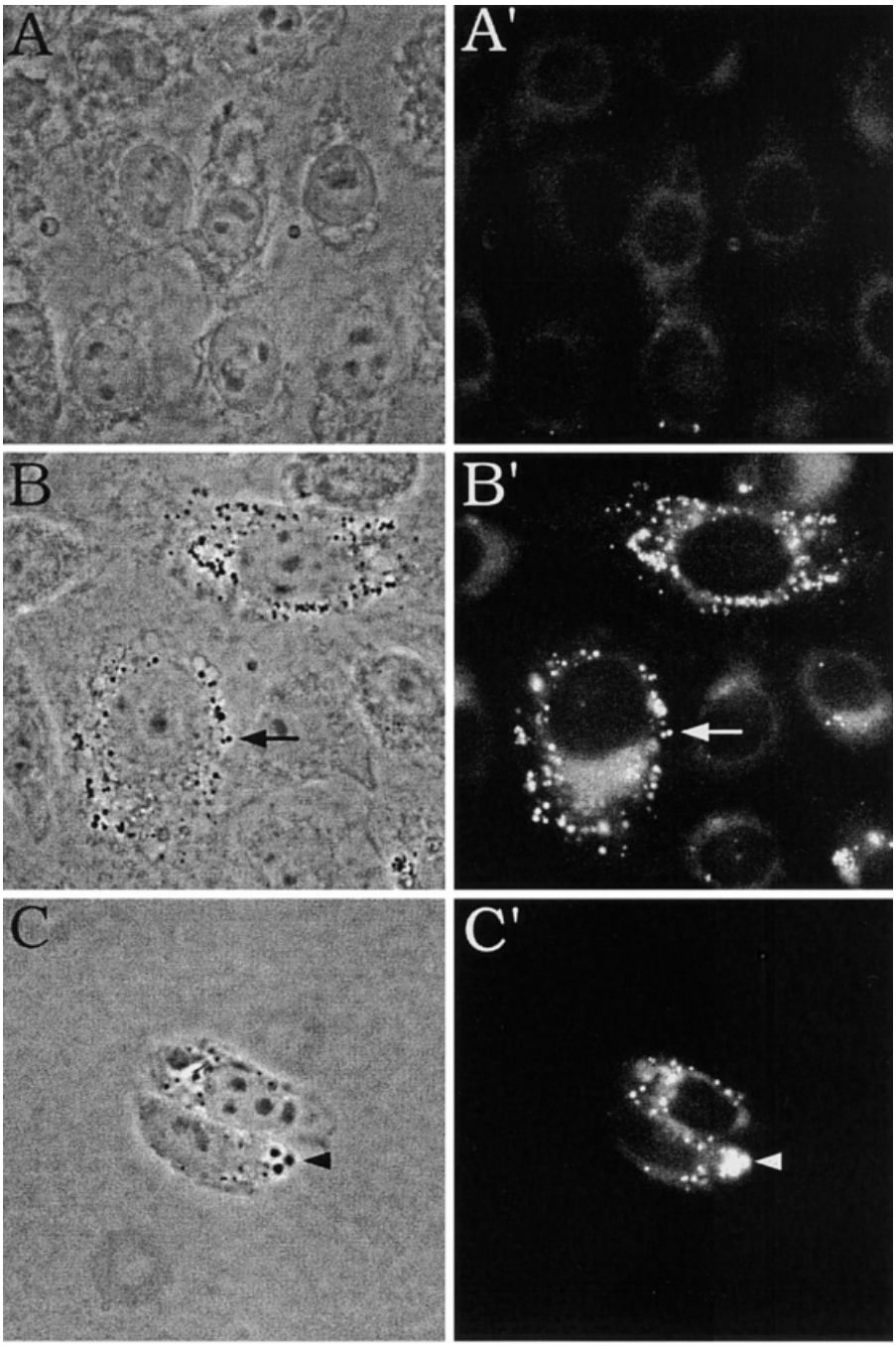

Fig. 2. Location of neutral lipid in transiently ACAT1- and ACAT2-transfected AC29 cells. (A and A′) Phase-contrast and rhodamine channel images, respectively, of AC29 cells stained with Nile red. (B and B′) Phase-contrast and rhodamine channel images of AC29 cells transfected with ACAT1 cDNA stained with Nile red. (C and C′) Phase-contrast and rhodamine channel images of AC29 cells transfected with ACAT2 cDNA and stained with Nile red. Arrows in (B) and (B′) and arrowheads in (C) and (C′) denote the position of neutral lipid bodies. Original magnification: ×690.

The data in **Fig. 2** show that, in contrast to the parent AC29 cells, both the ACAT1 and ACAT2 cells were filled with numerous lipid inclusions that show as black dots in the phase-contrast images of the stable cells. Nile red, which binds to neutral lipid, stained the numerous dark bodies in both ACAT1-transfected cells (Fig. 2B) and ACAT2-transfected cells (Fig. 2C) (compare Fig. 2B with B′, and Fig. 2C with C′), demonstrating that these structures represent lipid inclusions. These structures were not present in untransfected AC-29 cells (Fig. 2A) and no fluorescence on Nile Red staining was seen (Fig. 2A).

The data in **Fig. 3A** show that all the cells of the ACAT1 stable cell line seen in the phase-contrast image of Fig. 3A were brightly immunostained with ACAT1 antibodies. Likewise, all the cells of the ACAT2 stable cell line seen in the phase-contrast image (Fig. 3B) were strongly reactive with ACAT2 antibodies (Fig. 3B′). These data demonstrate the specificity and excellent reactivity of the polyclonal antibodies during immunostaining. Staining of the parent AC29 cells and both of the stable cell lines with PDI monoclonal antibodies (**Fig. 4A′**, **B′**, and **C′**) documented an ER distribution, and the same distribution within each of the cells as seen with the ACAT1 and ACAT2 antibodies, respectively (Fig. 4B and C). These data show that both ACAT enzymes had an ER-specific pattern of distribution in these cells.

To demonstrate any cross-reactivity of the antibodies between the two ACAT enzymes expressed in cells, negative control experiments were done. ACAT1 stable cell clones were immunostained with ACAT2 antibodies (**Fig. 5A**), and ACAT2 stable cell clones were immunostained with ACAT1 antibodies (Fig. 5B). The lack of a positive signal in both of the fluorescence channel images (Fig. 5A′ and B′) supports the Western blot data showing that the antibodies are specific and not cross-reactive with the other ACAT protein.

After determining the specificity of the antibodies for their respective ACAT isoform, as well as the intracellular location of the protein in stable cell clones, the next step was to immunostain tissues to determine the location and distribution of the two enzymes. The four tissues that were examined include the liver, the adrenal, the jejunum, and the kidney from monkeys. Staining of the liver showed two distinct patterns for each of the two ACAT isoforms (**Fig. 6**). ACAT1 was found in stellate cells located in the spaces between the chords of hepatocytes (Fig. 6B). These are apparently the Kupffer cells, which are macrophages that function as scavenger cells in the liver. ACAT2 was found in the hepatocytes (Fig. 6C), which make up 80–90% of the total cell mass of the liver. All hepatocytes in the tissue sections examined stained strongly positive and the pattern of staining was typical for an enzyme distributed in the rough ER. The control section of liver, stained only with the rhodamine-

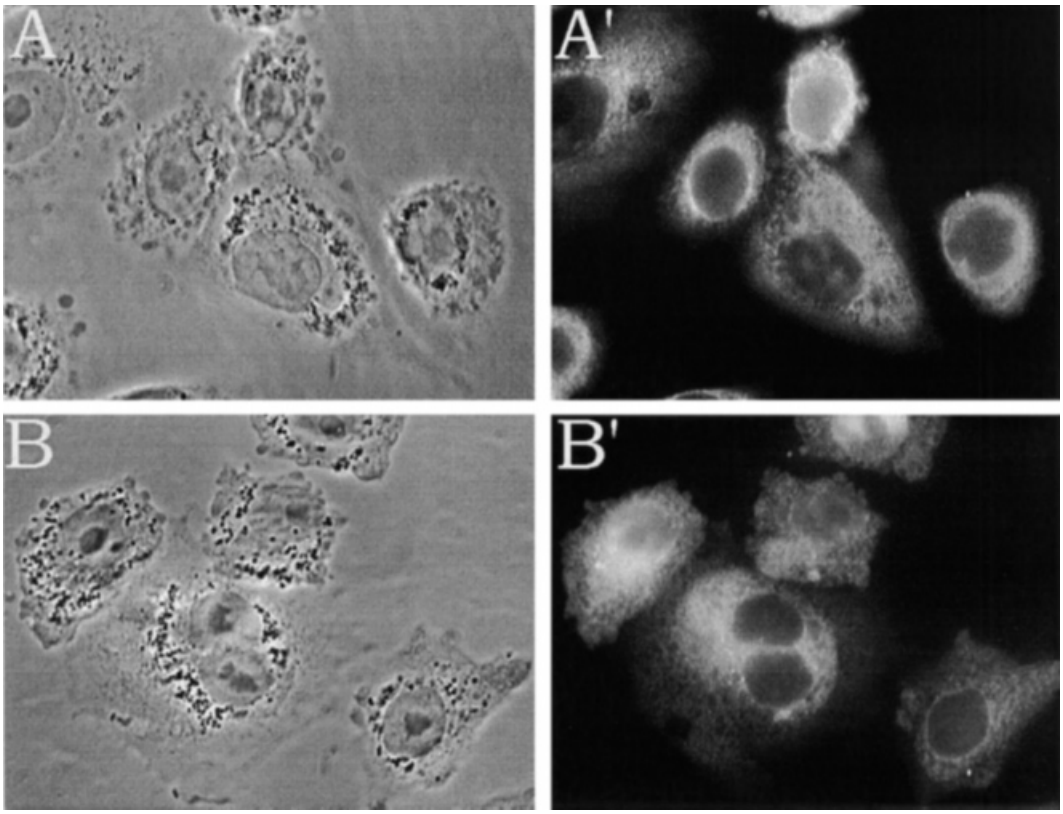

Fig. 3. Immunolocalization of ACAT1 and ACAT2 in stable cell lines. (A and A′) Phase-contrast and rhodamine channel images, respectively, of ACAT1 cells stained with anti-ACAT1 IgG (10 μg/ml) followed by rhodamine-labeled goat anti-rabbit IgG (25μg/ml). (B and B′) Phase-contrast and rhodamine channel images, respectively, of ACAT2 cells stained with anti-ACAT2 IgG (25 μg/ml) followed by rhodamine-labeled goat anti-rabbit IgG (25 μg/ml). Original magnification: ×800.

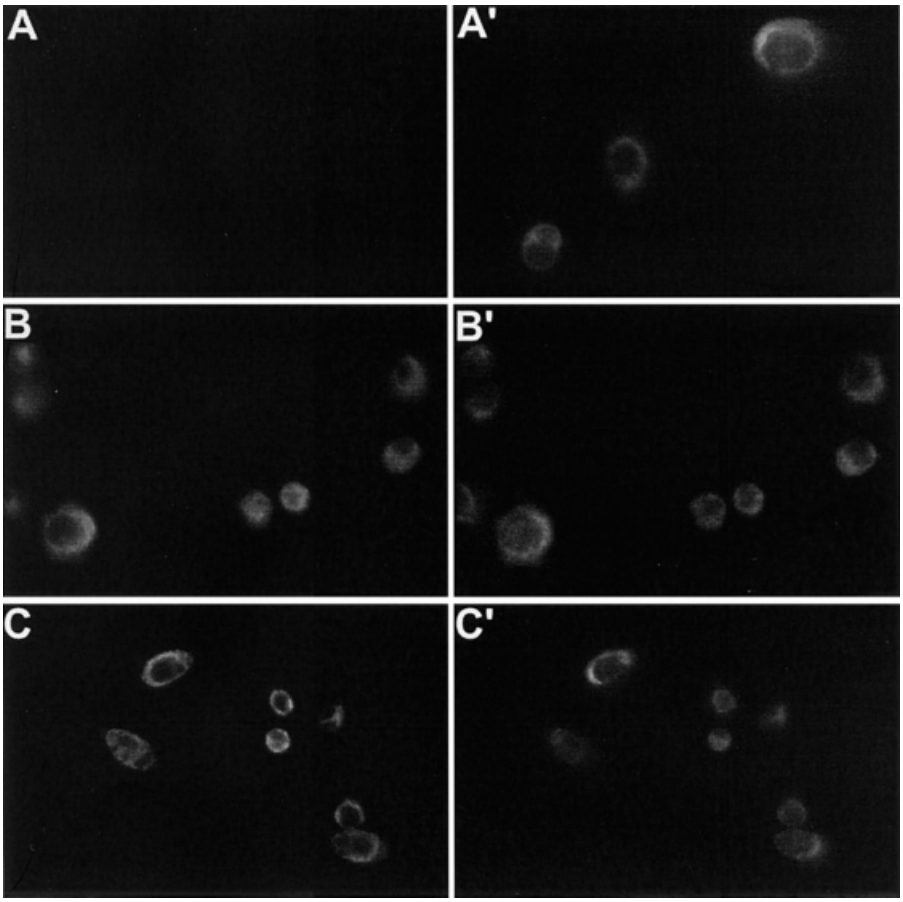

Fig. 4. Double staining of stable cell lines with ACAT antibody and PDI antibody. (A and A′) Rhodamine and fluorescein channel images, respectively, of AC29 cells stained with rhodamine-labeled goat anti-rabbit secondary antibody only, followed by anti-PDI mouse monoclonal antibody (10 μg/ml) and FITC-labeled goat anti-mouse IgG secondary antibody. (B and B′) Rhodamine and fluorescein channel images of ACAT-1 stable cells double stained with anti-ACAT1 rabbit primary antibody and rhodamine-labeled goat anti-rabbit IgG secondary antibody followed by anti-PDI mouse monoclonal antibody and FITC-labeled goat anti-mouse IgG secondary antibody. (C and C′) Rhodamine and fluorescein channel images of ACAT2 stable cells double stained with anti-ACAT2 rabbit primary antibody and rhodamine-labeled goat anti-rabbit IgG secondary antibody followed by anti-PDI mouse monoclonal antibody and FITC-labeled goat anti-mouse IgG secondary antibody. Original magnification: ×430.

conjugated secondary antibody, did not show significant fluorescence (Fig. 6A).

To make sure that the pattern of staining by the ACAT1 antibody represented Kupffer cell staining, double-staining experiments were done (**Fig. 7**) on liver sections with antibodies to ACAT1 and CD68, a macrophage cell marker that should identify Kupffer cells. The data show that the signals specific for both ACAT1 antibodies (Fig. 7C) and CD68 (Fig. 7D) colocalize to the same cells in the section, indicating that the cell types stained by the ACAT1 antibodies are indeed Kupffer cells. Figure 7A demonstrates that the fluorescein signal for CD68 secondary antibody (Fig. 7B) does not appear in the rhodamine channel in a head-to-head comparison.

In the adrenal, staining only with the secondary antibody did not show appreciable fluorescence (Fig. 6D). The reaction product with the antibody to ACAT1 filled essentially every cell within the adrenal cortex (Fig. 6E) and yet was absent from the capsule and from the medulla (data not shown). ACAT2 staining was not found in any compartment of the adrenal (Fig. 6F). Immunostaining of ACAT1 in the adrenal cortex resulted in the most intense immunofluorescence seen in any of the tissues examined.

In the jejunum, appreciable immunofluorescence in the presence of only the secondary antibody was not detected (**Fig. 8A**). ACAT1 staining was found in at least four cell types: goblet cells, Paneth cells, interstitial macrophages (data not shown), and mucosal cells (Fig. 8B and C). Goblet cells, which are named for their distinct morphology, are known to secrete mucus into the lumen of the jejunum, and had a characteristic staining pattern that extends deep into the outer cell border of the villus (Fig. 8B, arrow). Paneth cells are found in small groups on the basolateral side of the crypts (Fig. 8C, arrow), and are believed to secrete antimicrobial peptides, such as alpha defensins, into the lumen of the crypt. Although the staining of ACAT1 was strong in both of these cell types, a function for ACAT1 in these cells is unknown. ACAT2 was

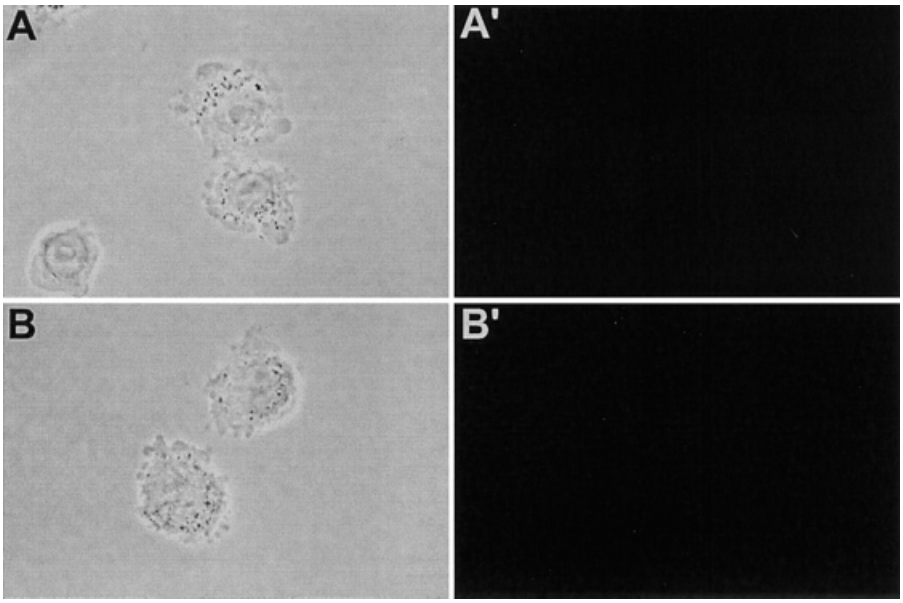

Fig. 5. Negative control experiment demonstrating specificity of ACAT1 and ACAT2 antibodies for protein of interest. (A and A′) Phase-contrast and rhodamine channel images, respectively, of ACAT1 stable cells stained with anti-ACAT2 rabbit primary antibody and rhodamine-labeled goat anti-rabbit secondary antibody. (B and B′) Phase-contrast and rhodamine channel images, respectively, of ACAT2 stable cells stained with anti-ACAT1 rabbit primary Ab and rhodamine-labeled goat anti-rabbit secondary antibody. Original magnification: ×680.

found most predominantly in the outer third of the mucosal cells of the jejunum (Fig. 8D); other cells appeared to contain little if any staining for ACAT2. The portion of the cell in which chylomicron particles are assembled is the apical region of the mucosal cell, the same site in which ACAT2 resides. In the location of goblet cells, no ACAT2 reaction product was seen (arrow in Fig. 8D), a finding in contrast to the location of the ACAT1 enzyme.

African green monkey kidney sections were also stained only with the secondary antibody and showed no appreciable fluorescence (Fig. 8E). When kidney sections were stained with the ACAT1 antibody, immunofluorescence was found in two distinct locations: *1*) near the nuclear envelope of cuboidal cells of the walls of the distal tubules (Fig. 8F) and *2*) in the ER of podocytes (Fig. 8G). The podocytes of the glomerulus extend foot processes around the glomerular capillaries and appear to act as a filter for the blood entering into Bowman's space; however, the functions of the cells displaying ACAT1 that would require cholesterol esterification are unknown. No appreciable fluorescence was seen when the ACAT2 antibody was used to stain the kidney, indicating an absence of appreciable ACAT2 isoform in this tissue.

To determine if ACAT activity in tissue roughly approximated the distribution of the enzyme, ACAT assays were run with tissue microsomes prepared from the same tissues analyzed immunochemically. The assays showed that specific activity (nanomoles of cholesteryl oleate formed per minute per milligram of microsomal protein), from highest to lowest, was adrenal (3.7), liver (1.35), small intestine (0.87), and kidney (0.15). These data do not distinguish between ACAT1 and ACAT2 activity, and include microsomes from all cell types within a tissue, but show general agreement with the intensity of the immunostaining patterns that were observed in the various tissues.

DISCUSSION

As anticipated from mRNA abundance measurements (7), each of the tissues examined in this study had ACAT1, and the liver and intestine also had ACAT2. The staining patterns shown here indicate that both ACAT isoforms reside in the ER of each of the cells in which they were identified, including the stable cell lines for both ACAT1 and ACAT2. Within a tissue, only some of the cells have ACAT1 or ACAT2 and few cells were found that have both enzymes. Interestingly, the topology for the two enzymes within the ER membrane has also been shown to be different (10), a finding that could be related to the fact that the two enzymes have separate functions and are not commonly colocalized within the same cell.

ACAT1 was found in numerous cell types including the Kupffer cells of the liver, goblet cells, Paneth cells, and macrophages of the intestine, cells of the adrenal cortex, and the distal tubular cells and podocytes of the kidney. The distribution of ACAT2 was limited to cells that are known to secrete lipoprotein particles, namely the hepatocytes of the liver and mucosal cells of the small intestine. Hepatocytes are known to secrete very low density lipoprotein (VLDL) particles containing cholesteryl esters in their core and ACAT inhibitors have been shown to limit VLDL apoB and cholesteryl ester secretion by isolated, perfused monkey livers (3). The pattern of immunostain-

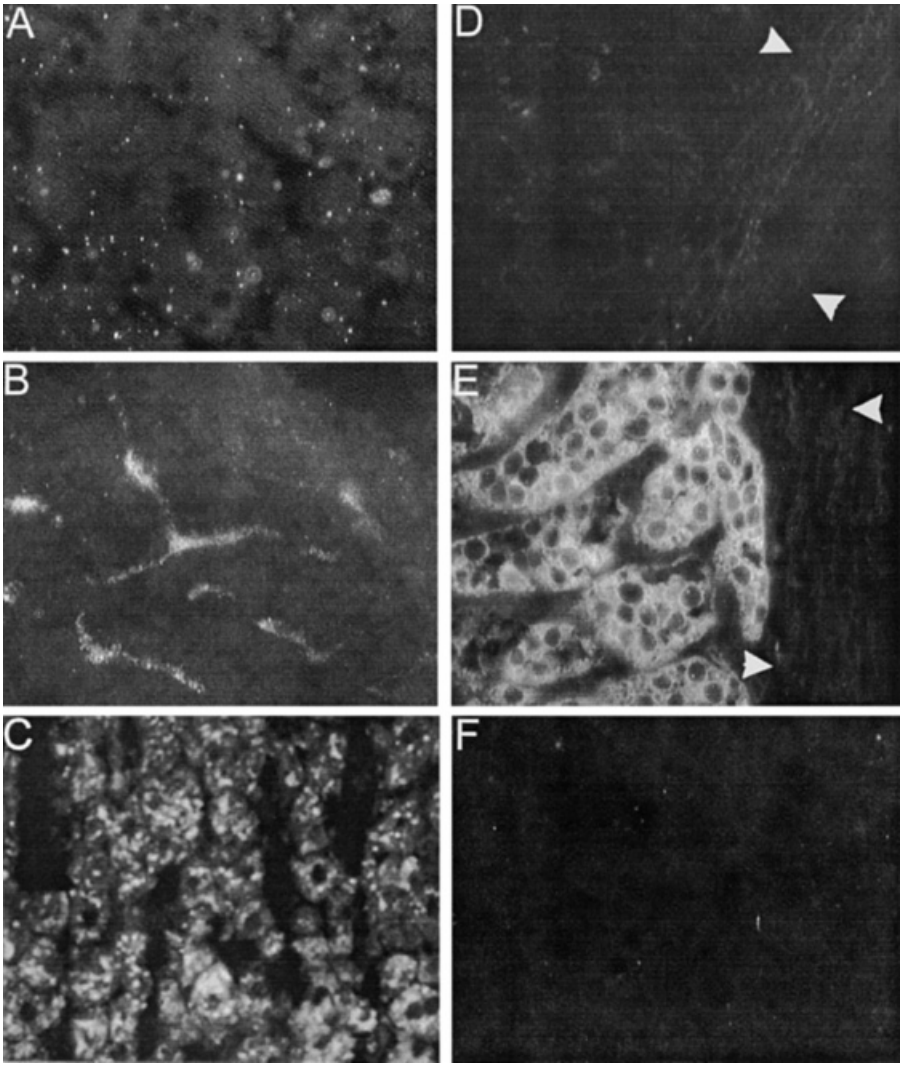

Fig. 6. ACAT1 and ACAT2 staining of African green monkey liver and adrenal gland. (A) Liver section stained with secondary Ab only. (B) Liver section stained with ACAT1 primary antibody. (C) Liver section stained with ACAT2 primary antibody. (D) Adrenal gland stained with secondary antibody only. Arrowheads denote capsule region. (E) Adrenal gland stained with ACAT1 primary antibody. Arrowheads denote capsule region. (F) Adrenal gland stained with ACAT2 primary antibody. Original magnification: ×430.

ing for ACAT2 in the hepatocytes was consistent with the possibility that most of the enzyme was present in the rough ER, where VLDL particle assembly is known to occur (17). Intestinal mucosal cells make and secrete chylomicrons, which then enter the body by way of the lymphatic system. The mucosal cells appeared to be the one cell type that may have both ACAT1 and ACAT2, although the ACAT1 signal was diffuse with a different localization within the cell. ACAT2 immunostaining of the small intestinal mucosal cell showed that the majority of this protein was found in the apical one-third of the cells, where triglyceride resynthesis and chylomicron particle assembly takes place (18). The fact that ACAT2 was seen only in lipoprotein-secreting cells of both liver and intestine emphasizes the possibility that this enzyme may somehow interact with the lipoprotein particle assembly machinery to facilitate lipoprotein particle formation.

Because the cell types within tissues that contain ACAT2 are only those important in lipoprotein particle secretion, the possibility that cholesteryl ester formation catalyzed by ACAT2 supports lipoprotein particle formation is consistent with these data. However, in the transfected AC29 cells, both ACAT1 and ACAT2 cells accumulated cytoplasmic lipid droplets. The fact that ACAT1 is of limited presence in hepatocytes also suggests that cholesteryl ester formation for inclusion in cytoplasmic lipid droplets, as is often seen in hepatocytes, could also occur via ACAT2. In such a case, access of the cholesteryl ester product to cell compartments on either side of the membrane is suggested. Perhaps the cholesteryl ester product initially locates into the inner hydrophobic portion of the lipid bilayer of the ER membrane, from which it can either be moved, perhaps by microsomal triglyceride transfer protein to the newly forming lipoprotein particle (19), or become part of a budding cytoplasmic lipid droplet.

ACAT1 was found in all tissues examined, and, for the

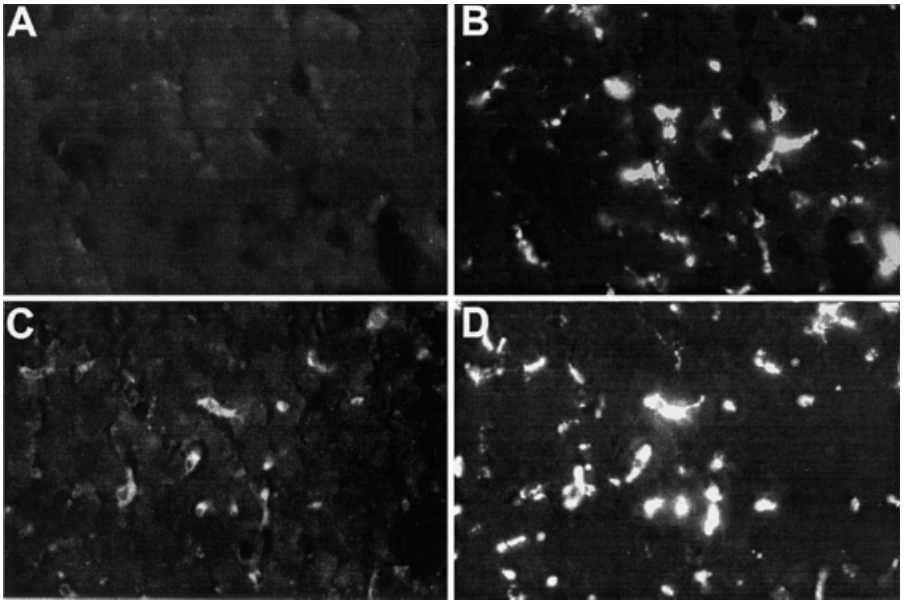

Fig. 7. Double staining of African Green monkey liver with anti-ACAT1 and anti-CD68. (A and B) Rhodamine and fluorescein channel images, respectively, of liver stained with rhodamine-labeled goat anti-rabbit IgG secondary antibody only, followed by anti-CD68 mouse monoclonal antibody and FITC-labeled goat anti-mouse secondary antibody. (C and D) Rhodamine and fluorescein channel images, respectively, of liver stained with anti-ACAT1 primary antibody and rhodamine-labeled goat anti-rabbit IgG secondary antibody followed by anti-CD68 primary antibody and FITC-labeled goat anti-mouse IgG secondary antibody. Original magnification: ×220.

most part, was limited to cells not involved in lipoprotein particle secretion. Perhaps because of its different membrane topology, ACAT1 cannot as efficiently provide cholesteryl esters to support lipoprotein particle formation. In any case, the different pattern of distribution supports the hypothesis that in contrast to ACAT2, ACAT1 is involved in intracellular cholesterol homeostasis, perhaps through maintenance of the appropriate level of unesterified cholesterol within cells for membrane stability. The latter possibility is supported by the fact that inhibition of ACAT1 in cholesterol-laden macrophages leads to cell toxicity (20). Some cell types within the tissues examined apparently did not have either ACAT enzyme. We can only speculate that these cell types either had low levels of the enzyme that escaped detection or have other means of maintaining cholesterol availability appropriate for that cell type.

In a publication by Sakashita and colleagues (13), the immunolocalization of ACAT1 in human tissues was similar in many respects to the data shown here, although some aspects were different. Both laboratories found detectable levels of ACAT1 protein in the cortical cells of the adrenal, the mucosal epithelial cells of the gut, and the Kupffer cells of the liver. However, Sakashita et al. also stated that there were detectable levels of ACAT1 in the hepatocytes of the liver, which we did not confirm. Conversely, we found detectable levels of ACAT1 in the goblet cells and Paneth cells of the jejunum, as well as in the podocytes of the glomerli, whereas Sakashita et al. apparently did not identify ACAT1 in these cell types. There are several possible explanations for these differences. First and most obvious is the species difference. It would be surprising if major differences occurred in basic cellular functions between nonhuman primates and humans, but it is possible that the relative abundance of ACAT1 may be different. The antibodies used by the two laboratories are different, but should include many similar epitopes in a similar region of the protein. There were also differences in the method of obtaining and preparing the tissue for staining. Finally, the method of detection of the antibodies was different. We used immunofluorescence whereas Sakashita et al. used a horseradish peroxidase stain. Whereas these differences in methodology may have led to some differences in detection, the clear-cut nature of the differential cellular association of ACAT1 and ACAT2 using controlled detection technology has not been attempted by others. The concept of different functions for the two enzymes is supported by the observations reported here, and although the data are not sufficient to define function in either case, an association of ACAT2 with lipoprotein particle secretion is strongly supported by its unique cellular location.

The initial evidence that led to the discovery of the ACAT2 isoform was that Acact-1 (the mouse homolog of ACAT1) knockout mice showed little to no decrease in cholesterol esterification activity in the liver, and had no effect on intestinal cholesterol absorption (21), suggesting that there was another cholesterol esterification enzyme. The demonstration that at least two ACAT-related enzymes are present in yeast (22) set the stage for sequence searches to identify additional ACAT enzymes, and at this point the ACAT gene family appears to contain three relatives,

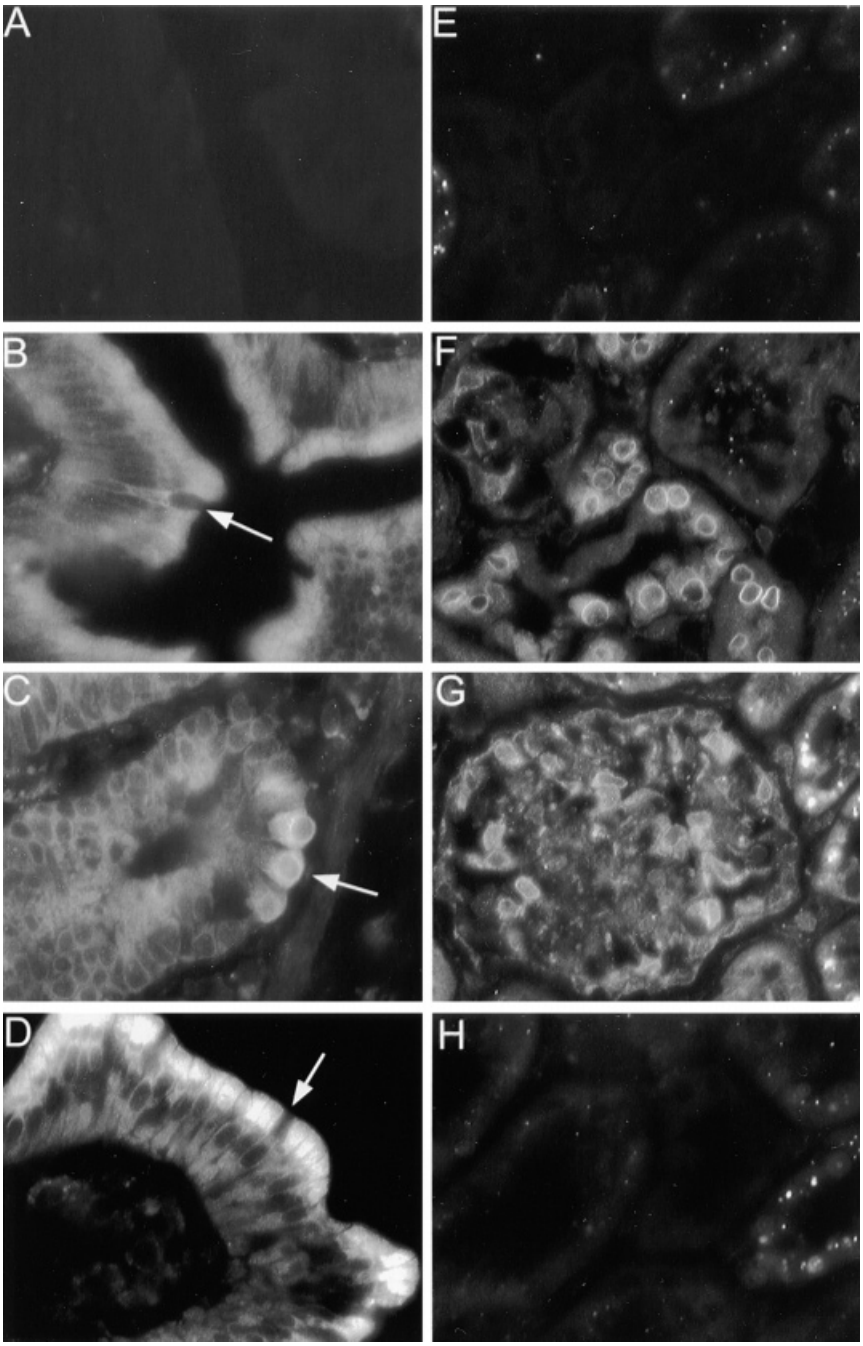

Fig. 8. ACAT1 and ACAT2 immunolocalization in African Green monkey jejunum and kidney. (A) Jejunum stained with secondary antibody only. (B and C) Jejunum stained with ACAT1 primary antibody. In (B), arrow denotes position of goblet cells; in (C), arrow denotes position of Paneth cells. (D) Jejunum stained with ACAT2 primary antibody. Arrow denotes position of a goblet cell. (E) Kidney stained with secondary antibody only. (F and G) Kidney stained with ACAT1 primary antibody. (H) Kidney stained with ACAT2 primary antibody. Original magnification: ×325.

ACAT1, ACAT2, and acyl CoA:diacylglycerol acyltransferase (6, 8, 23, 24). This article shows for the first time that the ACAT1 and ACAT2 isoforms are distributed uniquely among cells of the body, setting the stage for discovery of distinct functions for either enzyme. We have speculated about potential functions based on cell distribution and other circumstantial evidence, but much more work will be needed to identify the reasons that the two ACAT isoforms are distributed in such a unique and systematic fashion.

In work that appeared while this article was under review, Chang et al. (25) examined the distribution of ACAT1 and ACAT2 in human liver and intestine. Their data suggest that the primary enzyme in human liver is ACAT1 and in human intestine is ACAT2, but no distribution among cell types in the liver and intestine comparable to

that shown here was attempted by these authors. Therefore, it is difficult to tell whether the distribution we have described in the monkey applies to humans as well. Clearly, the pattern of distribution defined here would lead us to suspect that ACAT2 in human liver is the primary form of the enzyme in hepatocytes, but we have no data describing the level of ACAT2 enzyme in human liver compared with that in monkey. When comparing total ACAT activity in liver microsomes, we find that the activity in human liver is about 10% of that in monkey liver, and this suggests that the level of ACAT2 in human liver would be much lower. Futher work will be needed to define the cellular distribution of ACAT1 and ACAT2 in human liver.

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