Adrenal and liver in normal and *cld*/*cld* mice synthesize and secrete hepatic lipase, but the lipase is inactive in *cld*/*cld* mice

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Abstract **Combined lipase deficiency (*cld*) is a recessive mutation in mice that causes a severe lack of lipoprotein lipase (LPL) and hepatic lipase (HL) activities, hyperlipemia, and death within 3 days after birth. Earlier studies showed that inactive LPL and HL were synthesized by *cld*/*cld* tissues and that LPL synthesized by *cld*/*cld* brown adipocytes was retained in their ER. We report here a study of HL in liver, adrenal, and plasma of normal newborn and *cld*/*cld* mice. Immunofluorescence studies showed HL was present in extracellular space, but not in cells, in liver and adrenal of both normal and *cld*/*cld* mice. When protein secretion was blocked with monensin, HL was retained intracellularly in liver cell cultures and in incubated adrenal tissues of both groups of mice. These findings demonstrated that HL was synthesized and secreted by liver and adrenal cells in normal newborn and *cld*/*cld* mice. HL activities in liver, adrenal, and plasma in *cld*/*cld* mice were very low, <8% of that in normal newborn mice, indicating that HL synthesized and secreted by *cld*/*cld* cells was inactive. Livers of both normal newborn and *cld*/*cld* mice synthesized LPL, but the level of LPL activity in *cld*/*cld* liver was very low, <9% of that in normal liver. Immunofluorescence studies showed that LPL was present intracellularly in liver of *cld*/*cld* mice, indicating that LPL was synthesized but not secreted by *cld*/*cld* liver cells. Immunofluorescent LPL was not found in normal newborn liver cells unless the cells were treated with monensin, thus demonstrating that normal liver cells synthesized and secreted LPL. Livers of both groups of mice contained an unidentified alkaline lipase activity which accounted for 34–54% of alkaline lipase activity in normal and 65% of that in *cld*/*cld* livers. Our findings indicate that liver and adrenal cells synthesized and secreted HL in both normal newborn and *cld*/*cld* mice, but the lipase was inactive in *cld*/*cld* mice. That *cld*/*cld* liver cells secreted inactive HL while retaining inactive LPL indicates that these closely related lipases were processed differently.**—Schultz, C. J., E. J. Blanchette-Mackie, and R. O. Scow. **Adrenal and liver in normal and *cld*/*cld* mice synthesize and secrete hepatic lipase, but the lipase is inactive in *cld*/*cld* mice.** ***J. Lipid Res.*** **2000.** 41: **214–225.**

Supplementary key words lipoprotein lipase • combined lipase deficiency • immunofluorescence microscopy • newborn mice

Lipoprotein lipase (LPL) and hepatic lipase (HL) are enzymes that have key roles in the metabolism of circulating lipoproteins. LPL is necessary for the uptake of triglycerides (TG) by extrahepatic tissues from chylomicrons and very low density lipoproteins (VLDL) (1–3) and uptake of chylomicron remnants by liver (4). HL is involved in the conversion of VLDL remnants to intermediate density (IDL) and low density (LDL) lipoproteins (5), conversion of high density lipoproteins$_2$ (HDL_2) to HDL_3 (6, 7), uptake of chylomicrons and VLDL remnants by liver (8, 9), and uptake of cholesteryl esters (CE) from HDL_2 by adrenal, liver and other cells (6, 10, 11).

LPL and HL are glycoproteins that belong to the same gene family (12, 13). LPL is synthesized and secreted by parenchymal cells of extrahepatic (adipose, muscle, and heart) tissue and is found at the luminal surface of adjoining capillaries (14–16). LPL is also synthesized by liver, but only in newborn rodents (17, 18). HL is synthesized and secreted by liver (19, 20) and is found on the surface of liver cells (21, 22), in the space of Disse (21) and at the luminal surface of liver sinusoids (21, 23, 24). HL is also found in adrenals and ovaries (25, 26), but it is generally thought that HL found in these tissues is synthesized by liver cells (6, 25–27).

The active forms of bovine LPL (3), rat LPL (28), mouse LPL (29), and human HL (30) are homodimers, whereas the active form of rat HL is a monomer in liver and a dimer in adrenal and ovary (31). The oligomeric form of active mouse HL is not known. Human HL has four N-linked oligosaccharide chains per subunit (32),

Abbreviations: AEBSF, 4-(2 aminoethyl)-benzenesulfonylfluoride; BSA, bovine serum albumin; *cld*, combined lipase deficiency; EDTA, ethylenediamine tetraacetate; ER, endoplasmic reticulum; HL, hepatic lipase; HDL, high density lipoproteins; LPL, lipoprotein lipase; PMSF, phenylmethyl-sulfonyl fluoride; RT-PCR, reverse transcriptase polymerase chain reaction; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; TG, triacylglycerol; VLDL, very low density lipoproteins.

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while rat HL (33) and mouse HL (34) have two. Trimming in ER of glucose residues from the oligoglycans is required for activation and secretion of LPL in rat (35) and mouse adipocytes (36) and HL in rat liver (37). Although active LPL (38, 39) and active HL (37) secreted by incubated rodent cells have endo H-resistant oligosaccharide chains, processing of their oligosaccharides to endo H-resistance is not required for synthesis or secretion of active forms of these lipases (37, 39). Thus, the oligosaccharide chains of secreted LPL and HL may be variable.

LPL and HL are bound to endothelial cells by heparan sulfate proteoglycans and both lipases can be released into the blood stream by injection of heparin (40). LPL has a higher affinity than HL for heparin (40). While the affinity of LPL for heparin is similar in several species, that of HL is lower in mouse than in rat or human (41). This low affinity of mouse HL may explain why HL activity in preheparin plasma is 25-fold higher in adult mouse than in adult rat, and the increase in plasma HL activity after heparin injection is much lower in mouse than in rat (1- to 3-fold vs. 100-fold) (41, 42). However, heparin injection had no effect in normal newborn mice on HL activity in plasma, while it increased LPL activity 76-fold (43).

Combined lipase deficiency (*cld*) is an autosomal recessive mutation on mouse chromosome 17 (44) that blocks synthesis of active LPL and HL (16, 17, 45). *Cld*/*cld* mice develop extreme hypertriacylglycerolemia and die within 3 days after birth if allowed to suckle (16, 44). The *cld* mutation does not affect the structural genes for LPL and HL (46, 47), which are located in mice on chromosomes 8 and 9, respectively (13). Brown adipocytes cultured from *cld*/*cld* mice synthesized LPL which had high mannose-type oligosaccharides, but it was inactive and retained in the ER (16). Brefeldin A (BFA), via translocation of Golgi components to ER, enabled in *cld*/*cld* brown adipocytes synthesis of LPL which was active and had partially endo H-resistant oligosaccharides (29). However, LPL synthesized in BFA-treated *cld*/*cld* cells was not secreted when BFA (which also blocks transport of protein from ER) was withdrawn (29). It was concluded that production of inactive LPL in *cld*/*cld* cells results from the inability of such cells to transport LPL from ER to Golgi, where LPL is thought to become active in normal cells (29, 38). Little is known of the effect of the *cld* mutation on HL, other than [^{35}S]methionine-labeled HL synthesized by *cld*/*cld* liver slices had high mannose-type oligosaccharide chains (45).

We report here studies of HL in liver, primary cultures of hepatocytes, adrenals, and plasma, and of LPL in liver and plasma of normal newborn and *cld*/*cld* mice. Our immunolocalization studies show that HL was synthesized and secreted by liver and adrenal cells in both normal and *cld*/*cld* mice. HL synthesized by *cld*/*cld* cells, however, was inactive. *Cld*/*cld* liver cells also synthesized inactive LPL, but did not secrete it. These results indicate that HL and LPL, although closely related, are processed differently in liver cells.

METHODS AND MATERIALS

Chemicals

Heparin was from Hynson, Wescott, and Dunning, Baltimore, MD. Pepstatin was from Boehringer-Mannheim. AEBSF and BSA were from ICN. Aprotinin, PMSF, trioleoylglycerol, bovine insulin, and l-ornithine were from Sigma. Tri[9, 10-^{3}H]oleoylglycerol was from Amersham. Gum arabic was from US Biochemicals. Dulbecco's modified Eagle's medium without arginine was from Specialty Media, Inc, Lavallette, NJ. Fetal calf serum was from Hyclone, Logan, UT. Penicillin, streptomycin, and amphotericin were from Biofluids, Rockville, MD. Rabbit anti-mouse albumin IgG was from Cappel, West Chester, PA. Monoclonal rat anti-mouse Golgi membrane was a kind gift from Dr. Thomas August, Johns Hopkins University, Baltimore. All affinity purified secondary antibodies were from Jackson Immunoresearch, West Grove, PA. All other reagents were highest grade commercially available.

Animals

The mice used in this study were derived from a colony bearing the autosomal recessive combined lipase deficiency (*cld*) mutation (44). The very high plasma triacylglycerol concentration in *cld*/*cld* mice suckled more than 12 h, >10,000 mg/dl, gives the blood a creamy pink color, in contrast to the non-creamy red color in unaffected mice (16). Because of the close association of the *cld* mutation with mutations affecting tail length, about 95% of the mice born with combined lipase deficiency had no tail (16, 44). Thus, suckled mice that were tailless and had creamy blood were classified as defective (*cld*/*cld*), whereas those that had a tail and non-creamy blood were classified as normal. The mice were raised in the animal facilities of NIDDK, NIH, Bethesda, MD, from stock kindly supplied by Dr. Karen Artzt of the Patterson Laboratory, University of Texas, Houston, TX. The mice were fed NIH Open Formula Rat and Mouse Ration (NIH-7).

Lipase antibodies

Rabbit antiserum against rat HL was kindly provided by Dr. Michael Schotz, VA Wadsworth Medical Center, Los Angeles. This antibody crossreacts with mouse HL (45). Chicken IgG against bovine LPL was kindly provided by Dr. Thomas Olivecrona, University of Umeå, Sweden. This antibody crossreacts with mouse LPL (17).

Lipase assays

Lipase activity was measured in homogenates of liver and adrenals and in (preheparin) plasma. Livers were excised from decapitated 1-d-old mice and immediately homogenized with ice-cold buffer A (25 mm ammonium chloride, 5 mm EDTA, 0.02% Triton X-100, 0.04% SDS, 40 μg/ml heparin, 1 μg/ml pepstatin A, 3.5 μg/ml aprotinin, and 0.1 mm PMSF, pH 8.1). Homogenates were centrifuged at 12,000 *g* for 10 min at 4°C. Adrenals (~0.5 mg/animal) were excised from decapitated 1-d-old mice and immediately frozen in liquid N_2. Adrenals pooled from several mice were later processed as above for liver homogenates. Chylomicron-free plasma was prepared by multiple centrifugation from blood pooled from several mice (40–50 μl /animal) and frozen in liquid N_2 until assay.

HL and LPL were assayed with different media, HL with a medium containing 1 m NaCl at pH 9.0, and LPL with a medium containing 0.1 m NaCl and 5% serum (as source of apolipoprotein C-II) at pH 8.5. The substrate emulsion was prepared fresh on the day of assay by a modification of the method of Hernell, Egelrud, and Olivecrona (48). A mixture of 125 μCi of tri[9,10-^{3}H]oleoylglycerol (1 Curie/mmol), 25 mg unlabeled trioleoylglycerol, 1.25 ml of 10 mm Tris-HCl (pH 8.5), 1.0 ml 10% (w/v) gum arabic, and 2.0 ml 0.83 m NaCl was emulsified by sonication

for a total of 8 min (30 sec on/15 sec off) with a 9.5 mm probe at maximum energy output (BiosonikIII, Bronvill Scientific, Rochester, NY) in a 4°C bath. The HL assay (1.0 M NaCl) medium was a mixture of 4.25 ml of the substrate emulsion, 2.5 ml of 667 mM Tris-HCl (pH 9.0), 2.6 M NaCl and 10% (w/v) BSA, and 1.58 ml of 5.35 M NaCl. The LPL assay (0.1 M NaCl + serum) medium was a mixture of 4.25 ml of the substrate emulsion, 2.5 ml of 667 mM Tris-HCl (pH 8.5) and 10% BSA, 0.74 ml of H_2O and 0.84 ml of heat-inactivated (56°C for 30 min) fasted rat serum. For assay, 100 μl of the appropriate assay medium (containing 3 μCi tri[9,10-^{3}H]oleoylglycerol, 340 nmol trioleoylglycerol, and 45 nmol BSA) was added to 100 μl of sample and incubated in a shaking water bath at 37°C. Fatty acids produced by lipolysis were extracted and measured as previously described (49). One milliunit of lipase activity represents production of 1 nmol of fatty acid/min.

Lipase activities were measured for 30 min in homogenate equivalents of 0.5 mg of liver or 2 mg of adrenal, or in 4 μl chylomicron-free plasma. The rate of production of fatty acids was linear with amount of sample analyzed. Lipase activities in samples of liver, adrenals, and plasma were immunoinhibited by incubating samples with antibodies against HL and/or LPL for 2 h on ice before assay. Maximal inhibition of HL and LPL was obtained with 5 μl of anti-HL serum and 40 μg of anti-LPL IgG, respectively, in liver and plasma samples.

Reproducibility between preparations of lipase substrates was monitored by assaying an aliquot of mouse postheparin plasma stored at −80°C. Interassay variations, expressed as the coefficient of variation, were ± 15% for HL assay conditions (mean 466 ± 70 (SD) mU/ml plasma, n = 8 assays) and ± 16% for LPL assay conditions (733 ± 122 mU/ml plasma, n = 7 assays). Normal and *cld/cld* samples were analyzed at the same time using the same preparation of substrate emulsion.

Culture of hepatocytes

Primary cultures of newborn mouse hepatocytes were prepared by a modification (42) of the method of Leffert and Paul (50) for culturing fetal rat liver cells. The culture medium consisted of high glucose (25 mM) arginine-free Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin, 1.7 μM insulin, and 0.4 mM l-ornithine. At 2 days in culture, more than 80% of the cells from both normal and *cld/cld* livers had the following characteristics of hepatocytes: *a*) relatively large size, *b*) polygonal shape, *c*) large, centrally located nuclei and nucleoli, *d*) dense appearance with granular cytoplasm, *e*) distinct borders, and *f*) contained albumin detected by immunofluorescence (data not shown). The other cells in the cultures were spindle-shaped and did not contain immunofluorescent albumin. Cultured normal and *cld/cld* hepatocytes were equally viable, in that they incorporated [^{35}S]methionine into intracellular and secreted proteins and [^{3}H]oleic acid into cellular lipids at similar rates (42).

Immunolocalization of HL, LPL, Golgi, and albumin in tissues and cells

HL was immunolocalized in liver, adrenals, and cultured hepatocytes, LPL in liver, and albumin and Golgi membrane protein in cultured hepatocytes. All immunolocalizations were made with indirect double antibody techniques using unlabeled primary antibodies and fluorescein- or rhodamine-labeled secondary antibodies. The antibody combinations used were: rabbit antiserum to rat HL (diluted 1:400) and goat anti-rabbit IgG (40 μg/ml); chicken anti-bovine LPL IgG (5 μg/ml) and rabbit anti-chicken IgG (40 μg/ml); rabbit anti-mouse albumin IgG (5 μg/ml) and goat anti-rabbit IgG (40 μg/ml); and monoclonal rat anti-mouse Golgi membrane (5 μg/ml) and goat anti-rat IgG (40 μg/ml).

Liver used for immunolocalization was quickly removed from decapitated 1-d-old mice, minced into 1 mm^3 pieces and immediately fixed in either 3% paraformaldehyde and 0.2% glutaraldehyde, or 4% paraformaldehyde, at pH 7.4. After fixation at room temperature for 1 h, pieces of liver were infiltrated with 2.3 M sucrose in PBS at pH 7.4 for 2 h, mounted in a drop of 2.3 M sucrose on a cryopin, and rapidly plunged into liquid nitrogen. Semithin sections (1–2 μm) were cut at −80°C on a Reichert Ultracut E microtome with an FC4 attachment and transferred on drops of sucrose-gelatin solution to two-chambered microscope slides. The sections were washed in PBS, incubated for 45 min in quenching/blocking solution (PBS containing 0.2 M glycine and IgG (1 mg/ml) of the same species as the secondary fluorescent antibody), incubated for 60 min with antibody, preimmune serum, or non-immune IgG, washed three times (10 min each) in blocking solution, incubated for 60 min with labeled secondary antibody, and washed three times in blocking solution. The sections (or cultured cells as described below) were placed under a coverslip and viewed either with a Leitz epifluorescence microscope (Figs. 2, 4, and 5) or with a Bio-Rad MRC-6000 confocal imaging system (Figs. 1 and 3) with appropriate filters for detection of fluorescein or rhodamine.

Adrenal glands were quickly removed from decapitated 1-d-old mice and incubated in culture medium with 0 or 50 μM monensin for 1 h at 37°C. The glands were then fixed, cryosectioned, and processed the same as liver.

Liver cells cultured on plastic chamber slides (Nunc Lab Tek, Naperville, IL) were fixed in 3% paraformaldehyde at pH 7.4 for 1 h, washed in PBS, and stored in fresh PBS (0–12 h) at 4°C until immunostained. The cells were permeabilized with 1% saponin, which was present in all incubations after fixation. Fixed cells were washed and incubated first with quenching/blocking solution and then with antibody solutions as described above for cryosections of liver.

RESULTS

HL activity in liver, adrenals, and plasma

HL activity in liver, adrenals, and plasma was measured in the presence of 1 M NaCl at pH 9.0. Total lipase activity of liver under these conditions was 651 mU/g in newborn normal mice and 149 mU/g in *cld/cld* mice (**Table 1**). Addition of preimmune rabbit serum, which would be expected to contain LPL cofactor apoC-II, increased lipase activity 75 mU/g in normal liver and 52 mU/g in *cld/cld* liver, suggesting that LPL activity in mouse liver could be measured in the presence of 1 M NaCl. The lowering of lipase activity by addition of rabbit antiserum to HL indicated that HL accounted for 259 mU/g of lipase activity in normal liver and 16 mU/g of activity in *cld/cld* liver, 6% of that in normal liver. Lipolytic activity remaining after immunoinhibition with anti-HL serum, designated unknown lipase activity, was 392 mU/g in normal liver and 133 mU/g in *cld/cld* liver (Table 1).

Total lipase activity of adrenals measured in the presence of 1 M NaCl was 34.7 mU/g in newborn normal mice and 21.5 mU/g in *cld/cld* mice (**Table 2**). Addition of preimmune rabbit serum decreased lipase activity 5.3 mU/g in normal adrenals and 4.3 mU/g in *cld/cld* adrenals. The

TABLE 1. Lipase activities in liver of newborn and *cld/cld* mice

Group[b]	Addition[c]	Lipase Activity[a]					
		1.0 m NaCl			0.1 m NaCl + Serum		
		Total Lipase	HL	Unknown Lipase[d]	Total Lipase	LPL	Unknown Lipase[e]
		mU/g wet weight					
Normal	Pre-immune serum	726 ± 51	259	—	—	—	
	Anti-HL serum	467 ± 28	—	—	—	—	—
	Non-immune IgG	—	—	—	1019 ± 87	448	—
	Anti-LPL IgG	—	—	—	571 ± 29	—	—
	None	651 ± 35	—	392	1046 ± 139	—	339
cld/cld	Pre-immune serum	201 ± 7	16	—	—	—	—
	Anti-HL serum	185 ± 8	—	—	—	—	—
	Non-immune IgG	—	—	—	146 ± 12	36	—
	Anti-LPL Igg	—	—	—	110 ± 3	—	—
	None	149 ± 5	—	133	145 ± 11	—	93

[a] Lipase activity was assayed for 30 min at 37°C in the presence of either 1.0 m NaCl at pH 9.0 or 0.1 m NaCl and 5% heat-inactivated fasted rat serum at pH 8.5.
[b] Livers from three 1-d-old mice were pooled for each assay. Values are means ± SE of three assays.
[c] Samples were incubated with indicated serum (from rabbit) or IgG (from chicken) for 2 h on ice before being assayed. See Methods and Materials for details.
[d] Unknown lipase activity in the presence of 1.0 m NaCl was calculated as the difference between total lipase activity and HL activity.
[e] Unknown lipase activity in the presence of 0.1 m NaCl + serum was calculated as the difference between total lipase activity and the sum of HL and LPL activities.

lowering of lipase activity by addition of antiserum to HL indicated that HL accounted for 10 mU/g of lipase activity in normal adrenals and none of the activity in *cld/cld* adrenals. The lipase activity remaining after addition of anti-HL serum was 19.4 mU/g in both normal and *cld/cld* adrenals. This activity could be due to hormone-sensitive lipase, the adrenal cholesterol esterase which has hydrolytic activity against triacylglycerol (51). The findings suggest that adrenal cholesterol esterase was unaffected by the *cld* mutation.

Total lipase activity of chylomicron-free (pre-heparin) plasma measured in the presence of 1 m NaCl was 19.6 mU/ml in normal mice and 4.7 mU/ml in *cld/cld* mice (Table 2). Total lipase activity was increased in normal plasma, to 30.6 mU/ml, but not in *cld/cld* plasma by addition of pre-immune rabbit serum. The lowering of lipase activity when anti-HL serum was added, instead of pre-immune serum, indicated that HL accounted for 27.3 mU/ml of lipase activity in normal plasma and 1.7 mU/ml in *cld/cld* plasma (Table 2). These values for HL activity are in agreement with those reported earlier from our laboratory (43). Lipolytic activity remaining in plasma after addition of anti-HL serum was very small, about 3 mU/ml, in both groups of mice.

Immunolocalization of HL in liver, cultured hepatocytes, and adrenals

HL was immunolocalized in tissues and cells with an indirect double antibody technique using primary antiserum to HL and secondary fluorescence-labeled antibodies.

Normal mice. Immunofluorescent HL was found extra-

TABLE 2. HL activity in adrenals and plasma of newborn normal and *cld/cld* mice

Group	Addition[d]	Lipase Activity in 1.0 m NaCl[a]			
		Adrenals[b]		Plasma[c]	
		Total Lipase	HL	Total Lipase	HL
		mU/g wet weight		*mU/ml*	
Normal	Pre-immune serum	29.4 ± 3.1	10	30.6 ± 3.0	27.3
	Anti-HL serum	19.4 ± 2.3	—	3.3 ± 0.4	—
	None	34.7 ± 2.6	—	19.6 ± 2.5	—
cld/cld	Pre-immune serum	17.2 ± 2.1	0	4.6 ± 0.4	1.7
	Anti-HL serum	19.4 ± 1.4	—	2.9 ± 0.1	—
	None	21.5 ± 2.8	—	4.7 ± 0.2	—

[a] Lipase activity was assayed for 30 min at 37 °C in the presence of 1.0 m NaCl at pH 9.0.
[b] Adrenals from 40 1-d-old mice were pooled for each assay. Values are means ± SE of three assays.
[c] Chylomicron-free plasma from 5 mice was pooled for each assay. Values are means ± SE of three assays.
[d] Samples were incubated with indicated serum (from rabbit) for 2 h on ice before being assayed. See Methods and Materials for details.

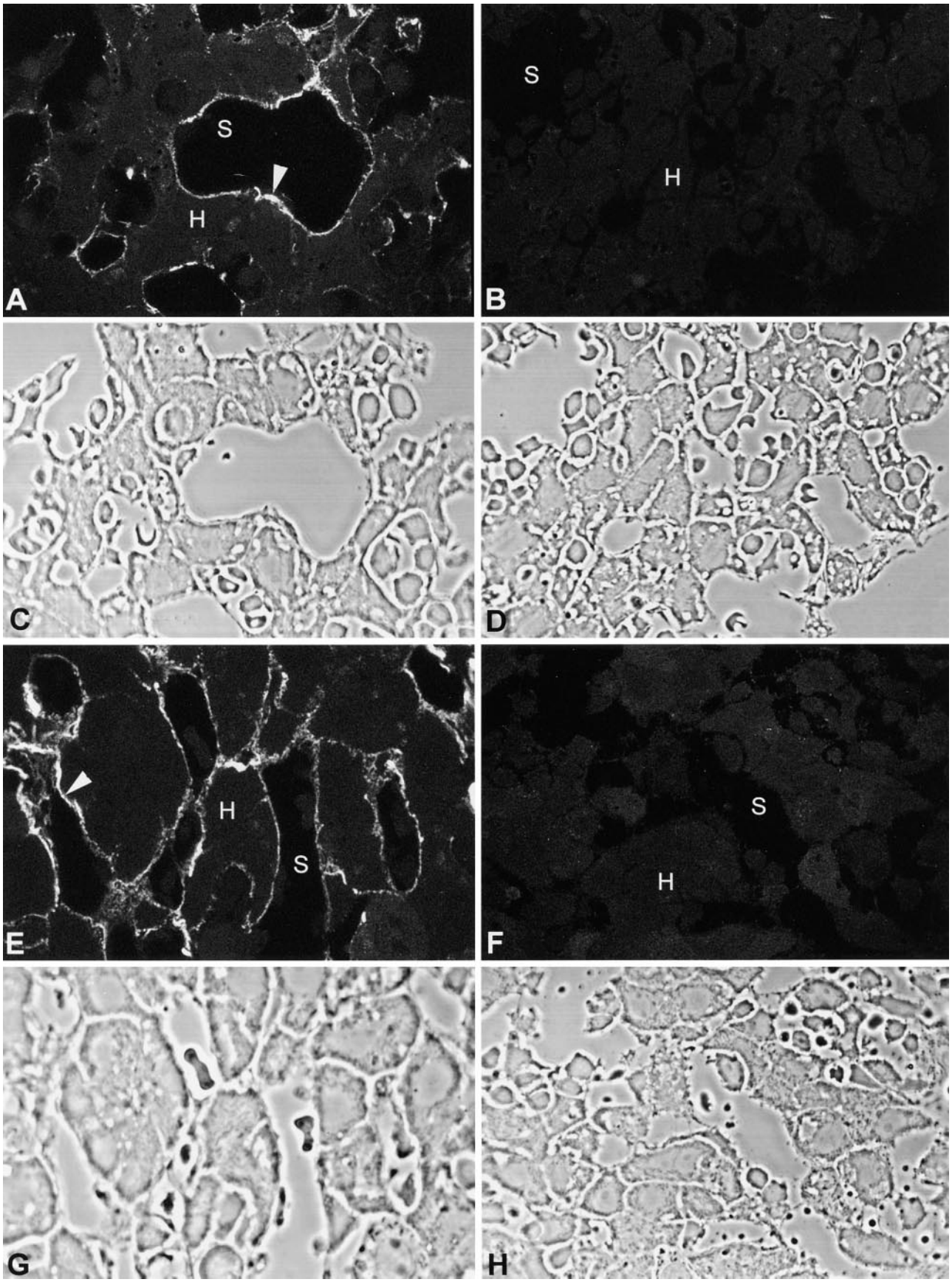

Fig. 1. Immunolocalization of HL in sections of liver of newborn normal and *cld/cld* mice. Livers were removed from 1-d-old mice and immediately fixed for morphological study. Immunofluorescent HL (arrowheads) was present extracellularly, in the space of Disse, in normal liver (A). HL was also present extracellularly, not inside cells, in *cld/cld* liver (E). Immunofluorescence was not observed in sections of normal (B) and *cld/cld* liver (F) processed with pre-immune serum. Phase micrographs of liver sections shown in panels A, B, E, and F are presented in panels C, D, G, and H, respectively. H, hepatocyte; S, sinousoid. Magnification × 450.

cellularly, not inside cells, in liver of normal newborn mice (**Fig. 1A**). HL was localized primarily in the space of Disse, not in sinusoids. No immunofluorescence was observed in normal liver specimens processed with preimmune serum instead of anti-HL serum (Fig. 1B).

Immunofluorescent HL was not seen in cultured normal hepatocytes (data not shown) unless they were treated with monensin, an inhibitor of protein transport in Golgi (52) (**Fig. 2A**). Distribution of HL in monensin-treated cells coincided closely with that of immunofluorescent Golgi membrane protein (Fig. 2B). These findings indicate that HL is synthesized and transported through Golgi in normal hepatocytes. The absence of immunofluorescent HL in untreated normal liver cells probably reflects rapid turnover of HL in these cells.

HL was also found extracellularly in sections of adrenal cortex of normal newborn mice (**Fig. 3A**). Monensin treatment caused retention of HL inside cortical cells in incu-

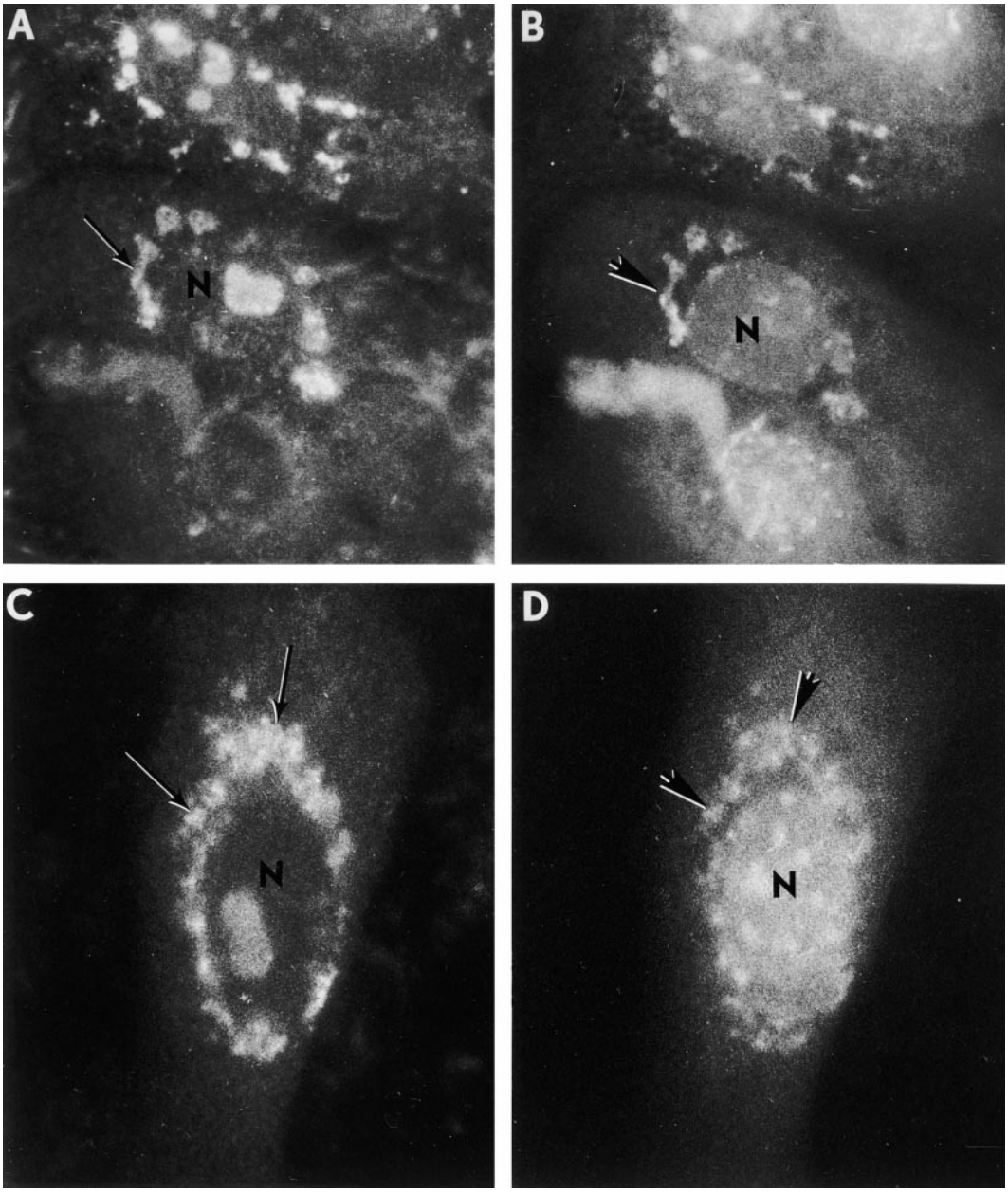

Fig. 2. Co-immunolocalization of HL with Golgi membrane in cultured normal and *cld*/*cld* hepatocytes treated with monensin. Hepatocytes were cultured for 2 d and then incubated 1 h with 50 μm monensin (to prevent transport of protein from Golgi) before being processed for immunofluorescence microscopy with both antibodies to HL (A, C) and antibodies to Golgi membrane protein (B, D). Micrographs in panels A and B are of the same normal cells and those in panels C and D are of the same *cld*/*cld* cell. HL was found inside (arrows) normal (A) and *cld*/*cld* (C) hepatocytes treated with monensin. The distribution of Golgi membrane (arrowheads) in normal (B) and *cld*/*cld* (D) hepatocytes was similar to that of HL. N, nucleus. Magnification: panels A and B, × 680; panels C and D, × 1040.

bated normal adrenal tissue (Fig. 3B). Immunofluorescence was not observed in monensin-treated adrenal tissue processed with non-immune serum instead of anti-HL serum (Fig. 3C). These findings indicate that adrenal cells of normal newborn mice synthesized and secreted HL.

Cld/cld mice. Immunofluorescent HL was found extracellularly, not inside cells, in liver of *cld/cld* mice (Fig. 1E), as in liver of normal mice (Fig. 1A). HL in *cld/cld* liver was attached to structural components in the extracellular space, the same as in normal liver, indicating that defec-

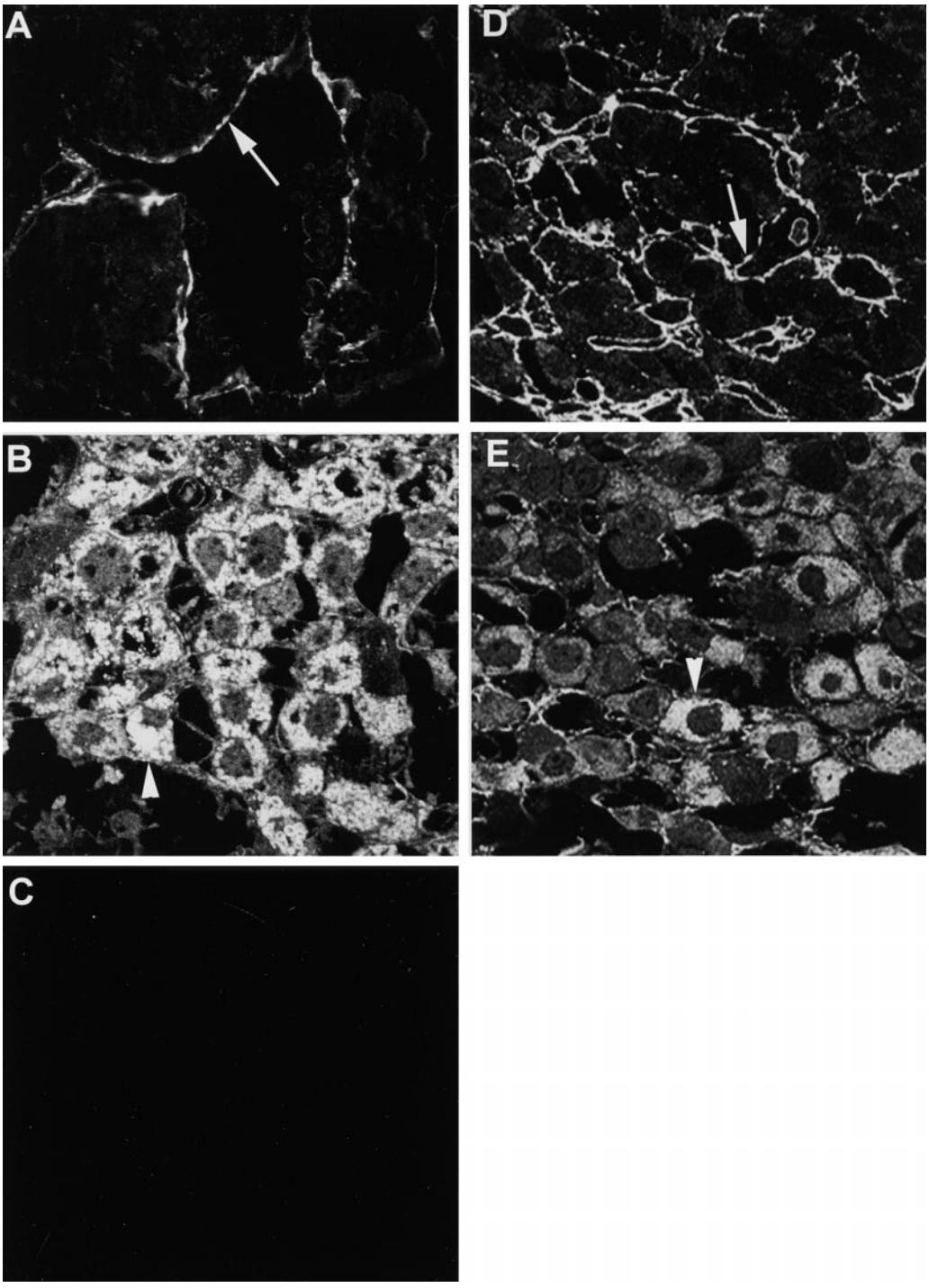

Fig. 3. Immunolocalization of HL in sections of adrenal cortex of newborn normal and *cld/cld* mice. Adrenals were removed from 1-d-old mice and incubated for 1 h at 37°C without monensin (A, D) or with 50 μm monensin (B, C, E) and then immediately fixed for morphological study. HL was present extracellularly (arrows), not inside cells, in untreated normal (A) and untreated *cld/cld* (D) adrenals. Monensin treatment caused retention of HL inside cells (arrowheads) in both normal (B) and *cld/cld* (E) adrenals. Immunofluorescence marking HL was not found in sections of monensin-treated normal adrenals processed with non-immune serum (C). Magnification: panels A and D, × 450; panels B, C, and E, × 470.

tive HL in *cld/cld* liver had the characteristics needed to be bound extracellularly in liver. Immunofluorescence was not observed in *cld/cld* liver processed with preimmune serum instead of anti-HL serum (Fig. 1F).

Immunofluorescent HL was not seen in cultured *cld/cld* hepatocytes (data not shown) unless they were treated with monensin (Fig. 2C). Distribution of HL in monensin-treated *cld/cld* cells coincided closely with that of immunofluorescent Golgi membrane protein (Fig. 2D). These findings indicate that HL is synthesized and transported through Golgi in *cld/cld* hepatocytes. The absence of immunofluorescent HL in untreated *cld/cld* liver cells probably reflects rapid turnover of HL in these cells.

Immunofluorescent HL was also found extracellularly in sections of adrenal cortex of *cld/cld* mice (Fig. 3D). As in normal adrenal tissue, monensin treatment caused retention of HL inside cortical cells in incubated *cld/cld* adrenal tissue (Fig. 3E). Immunofluorescence was not observed in monensin-treated adrenal tissue processed with non-immune serum instead of anti-HL serum (data not shown). The findings indicate that adrenal cells of *cld/cld* mice synthesized and secreted HL. In view of the absence of HL activity in *cld/cld* adrenals (Table 2), HL secreted by *cld/cld* adrenals was probably inactive.

LPL in liver

That isolated hepatocytes of normal neonatal rat (53) and liver of *cld/cld* mice (17, 45) can synthesize LPL suggested we could study in a single cell type, newborn mouse hepatocytes, the effect of the *cld* mutation on two closely related enzymes, HL and LPL.

LPL activity. LPL activity of liver and plasma was measured in the presence of 0.1 m NaCl and 5% serum at pH 8.5. Total lipase activity of newborn normal liver under these conditions was 1046 mU/g and was unaffected by addition of non-immune chicken IgG (Table 1). The lowering of lipase activity by addition of chicken anti-LPL IgG demonstrated that LPL accounted for 448 mU/g of lipase activity in normal liver. Assuming that HL activity was unaffected by the presence of 0.1 m NaCl + serum, the unknown lipase activity of normal liver under such conditions would be 339 mU/g, about 86% of that measured in the presence of 1 m NaCl.

Total lipase activity of *cld/cld* liver in the presence of 0.1 m NaCl + serum was 145 mU/g, <14% of that in normal liver, and was unaffected by addition of non-immune chicken IgG (Table 1). Reduction of total lipase activity by addition of anti-LPL IgG indicated that LPL accounted for 36 mU/g of lipase activity in *cld/cld* liver, 8% of that in normal liver. Unknown lipase activity in *cld/cld* liver was 93 mU/g in the presence of 0.1 m NaCl + serum, 28% of that in normal liver.

The values for LPL activity in normal newborn liver and for unknown lipase activity in normal newborn and *cld/cld* livers presented in Table 1 are several times larger than those reported earlier from our laboratory (43). Although the substrate components used in the two studies were similar, the mixtures were prepared differently. Also, the homogenizing techniques and solutions were different.

Immunolocalization of LPL. Immunofluorescent LPL was not found in sections of normal liver (**Fig. 4A**) or in 7-d cultured normal hepatocytes (**Fig. 5A**). LPL was found, however, inside 7-d cultured normal hepatocytes when the cells were treated with monensin (Fig. 5B), indicating that newborn mouse hepatocytes synthesize LPL.

LPL was found intracellularly in sections of untreated

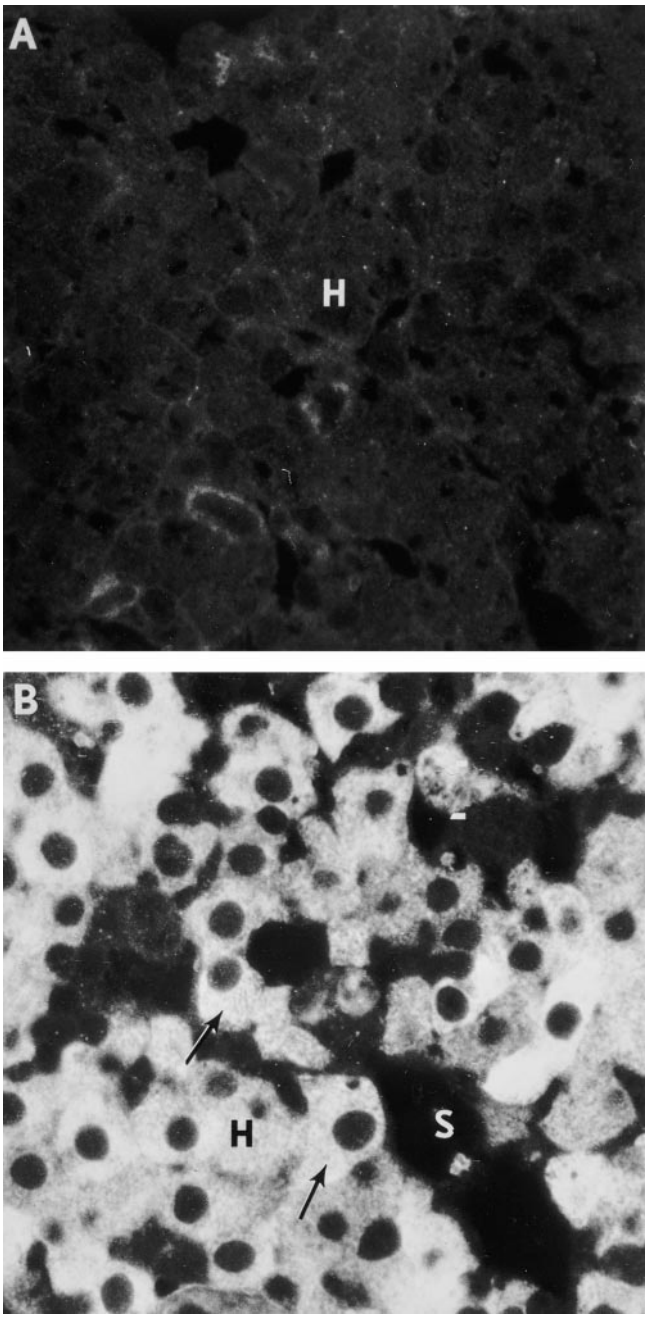

Fig. 4. Immunolocalization of LPL in sections of liver of newborn normal (A) and *cld/cld* (B) mice. Livers were removed from 1-d-old mice and immediately fixed for morphological study. Immunofluorescent LPL was not found in normal liver (A). The low level of fluorescence seen outside of normal cells was found also in tissue incubated with nonspecific IgG (data not shown). LPL was abundantly present inside hepatocytes (arrows) in *cld/cld* liver (B). H, hepatocytes: S, sinusoid. Magnification: × 470.

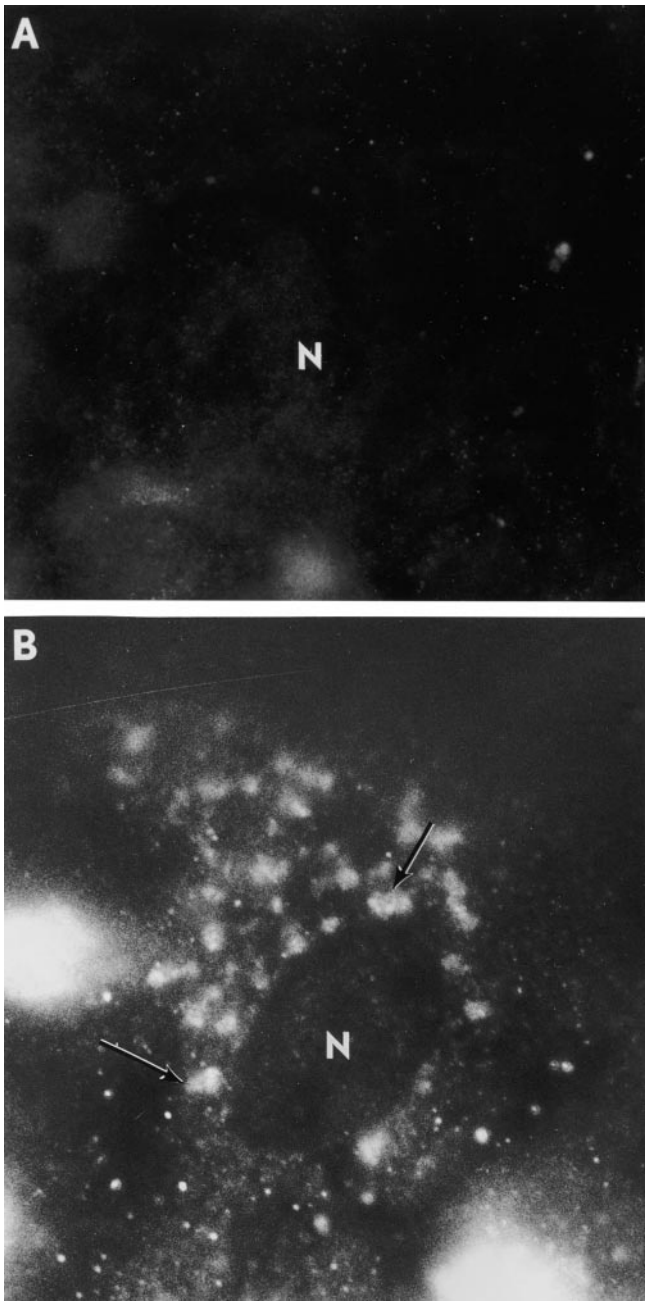

Fig. 5. Immunolocalization of LPL in cultured normal newborn hepatocytes treated with monensin. Isolated hepatocytes were cultured for 7 d and then incubated with 0 (A) or 50 (B) μm monensin before being processed for immunofluorescence microscopy. Immunofluorescent LPL (arrows) was found inside monensin-treated hepatocytes (B), but not inside untreated hepatocytes (A). N, nucleus. Magnification: × 1040.

cld/*cld* liver (Fig. 5B). LPL in *cld*/*cld* hepatocytes appeared to be distributed in a reticular pattern, suggesting it was located primarily in endoplasmic reticulum. The high level of immunofluorescence of LPL in *cld*/*cld* liver precluded precise localization of LPL in the cells. Earlier studies showed that *cld*/*cld* liver synthesized [^{35}S]methionine-labeled LPL (45). Our finding that LPL was present in untreated *cld*/*cld* hepatocytes shows that the *cld* mutation caused retention of LPL in these cells, as in adipocytes (16). That HL was secreted while LPL was retained by *cld*/*cld* liver cells indicates that these similar lipases are processed differently in liver cells.

DISCUSSION

HL activity is found in liver, adrenals, ovaries, and plasma (6) (Tables 1 and 2). Rat liver cells are known to synthesize and secrete active HL (19, 20). Our immunocytochemical findings in mouse liver cells treated with and without monensin demonstrate that mouse hepatocytes can also synthesize and secrete HL. Because neither HL mRNA nor HL synthesis could be demonstrated in rat adrenals and ovaries (19), it has been assumed that active HL found in these organs is transported by the blood stream from liver to these organs. Recently, a truncated form of HL mRNA was identified in rat adrenal and ovary using RT-PCR (27). This truncated mRNA translated in adrenals into a protein lacking the N-terminal part of liver HL, the signal sequence, and the 5-loop, which normally covers the catalytic pocket (27, 54). This form of HL would lack catalytic activity.

Our finding of immunofluorescent HL inside cells in the cortex of normal mouse adrenals treated with monensin and outside cells in untreated adrenals demonstrates for the first time that HL is synthesized and secreted by adrenal cortical cells. We did not determine whether HL synthesized by adrenal cells was active. That HL was retained in cells treated with monensin, an inhibitor of protein transport between *medial* and *trans* Golgi, indicates that HL is secreted through Golgi in normal adrenals. Studies using immunoblotting showed that HL in rat adrenals had the same M_r as mature sialyated HL purified from rat liver (19), indicating that HL present in rat adrenals had complex type oligosaccharides. That HL is secreted through Golgi in adrenal cells suggests that HL synthesized by normal mouse adrenals would be glycosylated and, perhaps, have complex type oligosaccharide chains.

Earlier studies showed that HL in liver is found on the surface of liver cells (21, 22), in the space of Disse (21), and at the luminal surface of sinusoidal endothelium (21, 23, 24). We report here immunolocalization of HL in the space of Disse of liver and in the extracellular space of adrenals in newborn mice. Thus, HL located in these spaces could act on lipoproteins and lipoprotein remnants transported through the fenestrated sieve plates of sinusoidal endothelium in liver (55), and act on HDL and LDL transported across capillary endothelium in adrenals.

LPL activity is present in liver of neonatal mice and rats (43, 53, 56) (Table 1). Synthesis of LPL protein was demonstrated in neonatal liver by incorporation of [^{35}S] methionine into LPL in newborn mouse liver in vivo (17) and in perfused newborn rat liver (56). Synthesis of active LPL has been reported in isolated neonatal rat liver cells (53). Our finding that immunofluorescent LPL was re-

tained intracellularly in 7-d cultured newborn normal liver cells when treated with monensin indicates that LPL is synthesized and secreted via Golgi in neonatal mouse hepatocytes. Absence of immunofluorescent LPL in untreated liver cells probably reflects rapid turnover of LPL, as observed in cultured adipocytes (16).

An unknown lipase activity, which was not inhibited by antibodies against either HL or LPL, accounted for one-third to two-thirds of the alkaline lipolytic activity in normal and *cld*/*cld* livers (Table 1). Only 10% of the lipolytic activity in normal plasma was unidentified (Table 2), suggesting that the unknown lipase activity was not secreted into the blood stream. This unknown lipase activity in liver was not lysosomal because lysosomal lipase has an acidic optimal pH range (57). Nonlysomal lipase activity has been demonstrated in microsomes of rat liver (58) and porcine liver (59). A lipase has been purified from porcine liver microsomes which hydrolyzed long-chain TG at pH 8.2 and did not bind to heparin (59). That this enzyme could not be released from microsomes by either salt wash or mild detergent treatment (59) suggests it could not account for the unknown lipase activity found here in homogenates of mouse liver (Table 1).

Our immunofluorescence studies showed that *cld*/*cld* liver and adrenals synthesized and secreted HL. The very low levels of HL activity found in liver, adrenals, and plasma of *cld*/*cld* mice (Tables 1 and 2), which confirmed earlier reports (43–45), indicate that HL secreted by *cld*/*cld* tissues was probably inactive.

Earlier studies demonstrated that *cld*/*cld* liver synthesized [^{35}S]methionine-labeled LPL (17, 45). Our immunofluorescence studies showed an abundance of LPL protein in untreated *cld*/*cld* liver cells (Fig. 5), indicating retention of LPL in these cells. Other studies showed that inactive LPL was retained in ER of cultured *cld*/*cld* brown adipocytes (16). The very low LPL activity in liver of *cld*/*cld* mice (Table 1) suggests that LPL synthesized in *cld*/*cld* liver was inactive. A very low level of LPL activity in post-heparin plasma of *cld*/*cld* mice (43) suggests that secretion of active LPL into blood was negligible in *cld*/*cld* mice. These findings indicate that liver in *cld*/*cld* mice synthesized LPL, but the lipase was inactive and not secreted. Other studies showed that about 40% of inactive LPL in *cld*/*cld* brown adipocytes was dimerized (29).

It is intriguing that the *cld* mutation inhibited activation of both LPL and HL, yet blocked secretion of only LPL. Although the two lipases belong to the same gene family, the enzymes differ in cofactor requirements, optimal reaction conditions, heparin affinity, and substrate specificity (60). Another difference is the oligomeric state of the active form of the enzymes. Active LPL in several species (human, bovine, rat, and mouse) is a dimer, whereas active HL varies with species. Based on radiation inactivation, active human HL is a dimer (30), whereas rat HL is a monomer in liver and a dimer in adrenal and (31). The oligomeric state of active mouse HL is not known.

The synthesis of active mouse LPL requires glycosylation of the protein, removal of glucose residues from the oligosaccharides and dimerization in ER, processing of oligosaccharides to complex type in Golgi, and transport of the lipase to the cell surface (38, 39). Processing of oligoglycans in Golgi, however, is not necessary for activation and secretion of mouse LPL (36). Dimerization of LPL is required for catalytic activity (3, 28, 29), but dimerization alone does not result in active mouse LPL (29). There are several reports suggesting that LPL becomes active in ER (61–63), while others (29, 38, 64) suggest that LPL becomes active in Golgi. Based on our findings in newborn mouse brown adipocytes, we proposed that activation of mouse LPL occurs in Golgi and requires some modification other than oligoglycan processing in Golgi (29), e.g., sulfation (65), phosphorylation, proteolysis or acylation (66, 67).

Activation of LPL in *cld*/*cld* brown fat cells by treatment with BFA, which blocks transport of protein from ER and translocates Golgi components to ER, did not enable transport of LPL from ER after withdrawal of BFA. Retention of LPL in ER of both treated and untreated *cld*/*cld* cells may result from a specific block of the LPL-transport system or an inability of LPL synthesized by *cld*/*cld* cells to interact with the LPL-transport system. We concluded that the *cld* mutation blocked transport of LPL from ER to Golgi and, thereby, prevented activation of LPL in *cld*/*cld* cells. Thus, the effect of the *cld* mutation on activation of LPL was secondary to its inhibitory effect on LPL transport.

Synthesis in liver of active rat HL also requires glycosylation of the protein, removal of glucose residues from the oligosaccharides in ER, processing of oligosaccharides to complex type in Golgi, and transport of the lipase to the cell surface (19, 20, 37). Like rat LPL, processing of oligoglycans in Golgi is not required for synthesis and secretion of active rat HL (37). The site of activation of HL is not known.

Less is known about synthesis of mouse HL. It has been shown that mouse HL synthesized in newborn mouse liver is glycosylated and its oligoglycan chains are processed to complex type (45). The role of dimerization in activation of mouse HL is not known. Recent findings indicate that active rat HL is a monomer in liver and a dimer in adrenal and ovary. Mouse HL, as discussed above, has a much lower affinity for heparin than rat HL (41). Heparin injected intraperitoneally had no effect on HL activity in plasma of normal newborn mice, yet it increased LPL activity 75-fold (43).

Studies by others (45) showed that *cld*/*cld* liver (slices) synthesized high mannose-type HL while normal newborn liver synthesized complex type HL (45), and our immunofluorescence studies showed that *cld*/*cld* liver cells secreted HL through Golgi. It is difficult to determine with the present findings how the *cld* mutation directly blocked activation of HL. It is possible that dimerization, in ER, is required for activation of mouse HL and that the *cld* mutation blocked this process.

Our findings indicate that HL and LPL, although closely related, are processed differently. The *cld* mutation in mice blocked transport of LPL from ER to Golgi and, thereby, prevented activation and secretion of LPL. Al-

though the *cld* mutation blocked activation of HL, it did not affect secretion of HL.

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