Isolation and Characterization of Chinese Hamster Ovary Cell Mutants Defective in Intracellular Low Density Lipoprotein–Cholesterol Trafficking

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Abstract. This paper reports the isolation and characterization of Chinese hamster ovary cell mutants defective in low density lipoprotein (LDL)–cholesterol trafficking. The parental cell line was 25-RA, which possesses LDL receptors and various cholesterogenic enzyme activities that are partially resistant to down regulation by exogenous sterols (Chang, T. Y., and J. S. Limanek. 1980. *J. Biol. Chem.* 255:7787–7795). Because these cells accumulate a large amount of intracellular cholesteryl ester when grown in medium containing 10% fetal calf serum, mutagenized populations of 25-RA cells were grown in the presence of a specific inhibitor of acyl-coenzyme A: cholesterol acyltransferase (ACAT), which depleted their cholesteryl ester stores. Without this cholesterol ester storage, 99% of 25-RA cells die after 5-d growth in cholesterol starvation medium, while the mutant cells, which accumulate free cholesterol intracellularly, survived. In two mutant clones chosen for characterization, activation of cholesteryl ester synthesis by LDL was markedly reduced in the mutant cells compared with 25-RA cells. This lack of activation of cholesterol ester synthesis in the mutant cells could not be explained by defective uptake and/or processing of LDL or by a decreased amount of ACAT, as determined by in vitro enzyme activity. Mutant cells grown in the presence of LDL contain numerous cytosolic particles that stain intensely with the fluorescent compound acridine orange, suggesting that they are acidic. The particles are also stained with filipin, a cholesterol-specific fluorescent dye. Indirect immunofluorescence with a monoclonal antibody specific for a lysosomal/endosomal fraction revealed a staining pattern that colocalized with the filipin signal. The mutant phenotype was recessive. The available evidence indicates that the mutant cells can take up and process LDL normally, but the hydrolyzed cholesterol accumulates in an acidic compartment, probably the lysosomes, where it can not be transported to its normal intracellular destinations.

In mammalian cells, the enzyme responsible for intracellular cholesteryl ester synthesis, acylcoenzyme A: cholesterol acyltransferase (ACAT)[1] uses cholesterol and fatty acyl coenzyme A as substrates (18). In rat liver, ACAT has been localized to the rough endoplasmic reticulum (4, 30). In Chinese hamster ovary (CHO) cells and primary cultures of human fibroblasts, ACAT activity is highly regulated by exogenous sources of cholesterol, such as low density lipoprotein (LDL) (16, 23, 26, 35), and by endogenous cholesterol synthesis (15). All available evidence suggests that the enzyme is regulated by cholesterol at the posttranslational level (10, 16, 23, 24, 26). The major mechanism of regulation is probably the supply of the substrate cholesterol. In human fibroblasts, it has been shown that in order for LDL to exert its regulatory effects (i.e., down regulation of LDL receptors and cholesterol biosynthesis and activation of ACAT activity), the cholesteryl ester moiety of the liproprotein, which forms the majority of the LDL cholesterol, must be hydrolyzed by a specific acid lipase found in the lysosomes (26). Presumably, the free cholesterol then leaves the lysosome, and in the case of ACAT, activates the enzyme by providing an increased amount of cholesterol as substrate.

The mechanism of transfer of the liberated cholesterol from lysosomes to the ACAT enzyme is unknown. There are cellular proteins capable of transferring hydrophobic molecules between membranes in vitro. One of these proteins, sterol carrier protein$_2$, has been demonstrated to activate ACAT activity in vitro by stimulating the transfer of exogenously added cholesterol to the ACAT enzyme (25, 48). However, the physiological role of this protein in cholesteryl ester synthesis has not been established.

Obtaining mutants in which cholesterol movement is defective would provide a valuable tool for defining intracellular cholesterol transport pathways and serve as a first step towards identifying the factors involved. It has been shown that fibroblasts isolated from patients with Niemann-Pick type C (NP-C) disease accumulate free cholesterol when incubated with LDL (36, 44). These fibroblasts bind and internalize LDL at normal rates, and the LDL cholesteryl ester is hydrolyzed to free cholesterol. However, the normal

1. *Abbreviations used in this paper*: ACAT, acyl-coenzyme A: cholesterol acyltransferase; CT, cholesterol trafficking; DIC, differential interference contrast; LDL, low density lipoprotein; NP-C, Niemann-Pick, type C.

regulatory responses elicted by LDL were much slower compared with unaffected fibroblasts (37, 45). This has been shown to be caused by a decrease in the rate of movement of LDL-derived cholesterol out of the NP-C lysosomes (38). Double fluorescence studies with the cholesterol-specific stain filipin and a monoclonal antibody directed against a lysosomal membrane antigen have demonstrated colocalization of the accumulated intracellular cholesterol with lysosomes (7, 54). In addition, a strain of BALB/c mice with a neuropathological condition similar to NP-C disease has been described in which free cholesterol accumulates in the lysosomes (5, 40, 42, 52).

In this report, a highly efficient selection protocol for isolating CHO cell mutants resistant to cholesterol starvation is described. Biochemical analysis showed that the mutants possess a phenotype similar to the one described for NP-C fibroblasts (i.e., lack of activation of ACAT by LDL despite an intact LDL receptor pathway, and an accumulation of free cholesterol upon growth in medium containing LDL). Cell hybridization analysis demonstrated that the phenotype of the two mutant clones examined was recessive and that they belonged to the same complementation group. Whether the genetic locus affecting these CHO cell mutants is the same as the one(s) corresponding to the NP-C disease awaits further genetic analysis. These mutants should serve as unique tools for cloning and identifying the gene affected via DNA mediated gene transfer (1).

Materials and Methods

Reagents

[10-^{3}H]Oleic acid and [1,2,6,7-^{3}H]cholesteryl linoleate were purchased from Du Pont Company-Biotechnology Systems (Wilmington, DE). Oleyl coenzyme A was synthesized as described by Stadtman using oleyl anhydride (55) and [^{3}H]oleyl coenzyme A was synthesized by another method (6). Both preparations were quantitated as previously described (11, 12). Na^{125}I (carrier free in 0.1 N NaOH) and Na [1-^{14}C] acetate were from Amersham Corp. (Arlington Heights, IL). Compound 58-035 (3-[decyldimethylsily]-*N*-[2-(4-methylphenyl)-1-phenylethyl] propanamide) was provided by Dr. John Heider (Sandoz Inc., East Hanover, NJ). Melvinolin was a gift from Alfred Alberts (Merck, Rahway, NJ). 25-hydroxycholesterol was purchased from Steraloids Inc. (Wilton, NH), dextran sulfate from Pharmacia Inc. (Piscataway, NJ), and sodium taurocholate from Calbiochem Corp. (La Jolla, CA). Polyethylene glycol 4000 was obtained from EM Science (Cherry Hill, NJ). Chloroform, methanol, and isopropanol used for the cholesterol analysis were from Mallinckrodt Inc. (Paris, KY) or Fisher Scientific Co. (Pittsburgh, PA) and were nanograde and spectranalyzed grade, respectively. FITC-conjugated goat anti-mouse antibody was obtained from Cappell Laboratories (Malvern, PA) (No. 1211-0081). The antibody lgp58 was generously provided by Dr. Ira Mellman (Yale University School of Medicine, Department of Cell Biology, New Haven, CT). MOPC-21 antibody was a gift of Dr. Stanley C. Froehner (Department of Biochemistry, Dartmouth Medical School, Hanover, NH). Cholesterol oxidase was generously provided by Dr. Albert Chen (Beckman Instruments, Inc., Fullerton, CA). Horseradish peroxidase (P-6140), cholesterol esterase (C-1892), and phosphatidylcholine Type XI (P-2772, lot 67F-8410) and all other enzymes and biochemical reagents were from Sigma Chemical Co. (St. Louis, MO). Other organic solvents and chemicals were from Fisher Scientific Co. and were of reagent grade quality.

Cell Culture

All CHO cell lines described in this paper were grown as monolayers in tissue culture flasks or dishes purchased from Costar (Data Packaging Corp., Cambridge, MA) or Falcon (Becton, Dickinson & Co., Lincoln Park, NJ). During the mutant selections all cells were maintained in F-12 medium minus linoleic acid supplemented with antibiotics as previously described (11) plus 10% FCS (Sigma Chemical Co.). When delipidated FCS was used, it was prepared according to a published procedure (14), as modified by Chin and Chang (22). After the mutant selections, all of the CHO cell lines used in this report were maintained on a 1:1 mixture of the above mentioned F-12 medium and MEM (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM glutamine and antibiotics, plus 10% bovine calf serum (Hyclone Laboratories, Sterile Systems, Inc., Logan UT). Delipidated bovine calf serum was made using Cab-o-sil (Eastman Kodak Co., Rochester, NY) as previously described (37).

In this report (with the exceptions of Figs. 2 and 6), all experiments were performed with CHO cells that had been adapted to continuous growth in 10% delipidated bovine calf serum in the F-12/MEM mixture. The adaptation (which took only one passage) and all subsequent platings were only successful when using tissue culture flasks or dishes from Falcon Labware. Upon plating of cells into the delipidated serum medium for the first time, most of the cells attached but looked unhealthy and grew very slowly. After a few days, however, some of the cells (~10%) began to grow more rapidly. When these cultures reached confluency, they could be plated into fresh flasks where they attached, appeared healthy, and grew rapidly.

Originally cultures were maintained in 10% delipidated serum. However, it was observed that occasionally the monolayers tended to clump together in patches. We subsequently found the clumping to occur less frequently when the concentration of delipidated serum was changed to 5%. None of the data shown in this report were obtained from cultures with evident clumping. The human LDL (d = 1.019–1.063 g/ml) used in this report was prepared from plasma by sequential flotation in the presence of protease inhibitors as previously described (11).

Mutagenesis and Isolation of Mutants

The CHO cell clone 25-RA and the ACAT mutant AC29 were mutagenized with 125 μg/ml *N*-nitroso-*N*-ethylurea as previously described (11). 5 d after mutagenesis, AC29 cells were plated at a low density (1.5×10^5 cells per 20×100 mm dishes) in F-12 medium plus 10% FCS. Because large amounts of intracellular cholesterol ester interfered with the selection (see Results), mutagenized 25-RA cells were grown for 3 d in 10% FCS medium that contained the specific ACAT inhibitor, 58-035 (50) and then plated at low density in 10% FCS medium plus 58-035. The concentration of 58-035 used (200 ng/ml) inhibits cholesteryl ester synthesis >98% (11). 36–48 h after plating, the monolayers were washed twice with PBS and cultured in cholesterol starvation medium (F-12 medium plus 10% delipidated FCS, 35 μM oleic acid, 50 μM mevinolin, and 230 μM mevalonate). Mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (2), blocks synthesis of mevalonate and thus inhibits synthesis of steroidal and nonsteroidal isoprenoids. The small amount of mevalonate permits the synthesis of nonsteroidal isoprenoids (51). Medium changes were given on the 2nd, 3rd, and 4th d of growth in the cholesterol starvation medium. After 5–6 d almost all of the mutagenized 25-RA cells were dead or dying. At this time, the starved cultures were washed once with PBS and grown in 10% FCS medium plus 230 μM mevalonate. After an additional 5 d of growth, the largest colonies of surviving cells were located and potential mutants were identified by their distinct morphological appearance (see Results). Colonies were removed using cloning rings and then recloned by limiting dilution in 10% FCS medium. Sometimes, depending on the amount of nonmutant cell contamination in the populations removed with the cloning ring, it was necessary to subject the cells to another round of starvation selection before recloning.

ACAT Assays

Monolayers were pulsed with a [^{3}H]oleate/BSA solution and analyzed for incorporation of radiolabel into cholesteryl oleate as previously described (11, 12, 16). For the in vitro ACAT assays, cell homogenates were prepared by the hypotonic shock and scraping method (20) and used immediately. The microsomal assay has been described in detail previously (11, 16, 23). The reconstituted ACAT assay was performed as described in Cadigan and Chang (10).

LDL Pathway Analysis

LDL was iodinated using iodine monochloride as described by Goldstein et al. (29). After incubation of the monolayers with the indicated amounts of ^{125}I-LDL, the cells were assayed for dextran sulfate releasable and cell-associated ^{125}I-lipoprotein, and the medium was analyzed for ^{125}I-LDL degradation as previously described (11). Specific binding and cell uptake were calculated by subtracting the nonspecific value, determined by assay-

ing in the presence of 50-fold excess of unlabeled LDL (up to 500 μg/ml), from the total value (assayed with ^{125}I-LDL alone). The nonspecific values ranged between 12 and 25% of the total value.

For measurement of the hydrolysis and reesterification of LDL-derived cholesteryl linoleate, LDL was labeled with [1,2,6,7-^{3}H] cholesteryl linoleate by the method of Roberts et al. (49), using the lipoprotein free fraction (d >1.215) of fresh human serum as the source of cholesterol ester exchange protein. After incubation with medium containing the ^{3}H-cholesteryl linoleate-LDL, the cells were harvested in 0.2 M NaOH, neutralized with HCl, and the lipids extracted exactly as previously described for the [^{3}H]oleate pulse (11, 16). Unlabeled cholesterol, cholesteryl oleate and cholesteryl linoleate were added as carriers. The extracted lipids were separated from one another essentially as described by Goldstein et al. (28). Briefly, the extracted lipids were spotted in 80 μl ethyl acetate onto Silica G TLC plates (Analtech Inc., Newark, DE) impregnated with $AgNO_3$. The TLC plates were impregnated by dipping into a 6.7% solution (methanol/H_2O; 2:1) of $AgNO_3$ for 2 min followed by air drying for 30 min. The plates were then baked at 80°C for 1 h, cooled, and spotted. Plates were run in a benzene/hexane (1:1) solvent system, allowed to air dry overnight, sprayed with dichlorofluorescine (0.2% solution in ethanol), air dried, and visualized with short wave ultraviolet light to localize the relevant lipid species. The R_f values were: cholesterol ~0.1; cholesteryl linoleate ~0.3; and cholesteryl oleate ~0.6, respectively. Bands were scraped and counted in the presence of Betafluor scintillation fluid. An unidentified radioactive band (R_f ~0.75) was also found; its location on the plate made it likely that it was a cholesteryl ester with a saturated fatty acid moiety (28) and it was ~15% of the cpm found in the cholesterol oleate band. Since the ratio of the unknown band's cpm to the cholesterol oleate cpm remained roughly constant throughout the different conditions and different cell types, it was not included in the calculations of the data. Control experiments revealed an average recovery of 60% for cholesterol, cholesteryl oleate, and cholesteryl linoleate after extraction and TLC analysis.

Microscopy

Cells were grown on glass coverslips at a density of 5 × 10^4 cells per well in six well plates containing 1.5 ml of medium. It was found that the cells did not plate well on coverslips in medium containing delipidated bovine calf serum alone and it was necessary to plate in medium containing 0.5% bovine calf serum/4.5% delipidated bovine calf serum. This concentration of bovine calf serum was not enough to induce the lipoprotein-dependent morphological changes described in this paper. 1 d after plating, the cultures were washed two times with PBS and changed to medium containing 5% delipidated serum supplemented with 100 μg/ml LDL (sometimes with 200 ng/ml 58-035) or washed medium containing only delipidated serum. A medium change was given 2 d after plating. On day 3, coverslips were stained as indicated, mounted, and kept moist with Hank's balanced salt solution containing no phenol red. Immediately thereafter, the cells were viewed using a Zeiss universal microscope with either a 67× achromat oil immersion (Carl Zeiss, Inc., Thornwood, NY) phase-contrast objective with differential interference contrast (DIC) optics or a 40X achromat water immersion (Carl Zeiss, Inc.) phase-contrast objective. All micrographs were taken with TMAX-400 film (Eastman Kodak Co.) and developed according to instructions provided by the manufacturer.

Acridine orange staining was carried out according to Poole (47). The monolayers were incubated for 30 min in the appropriate media containing 10 μg/ml acridine orange. Cells were then incubated for 15 min in medium lacking acridine orange before mounting the coverslips as described above. During the staining process, cells were kept in a 37°C, 5% CO_2 incubator. A 1 mg/ml stock solution of acridine orange in DMSO was made fresh each time before use and diluted 100-fold into media. Acridine orange fluorescence was viewed with rhodamine fluorescence optics (No. 48 77 15; Carl Zeiss, Inc.).

For the filipin staining, cells were washed four times with PBS and fixed with 4 ml of 10% PBS formalin at room temperature for 1 h, rinsed four times with PBS, and stained with 1.5 ml of filipin solution for 1 h at room temperature (36) before washing and mounting for microscopy. The filipin solution was made fresh each time by dissolving 2.5 mg filipin in 1 ml dimethylformamide and then diluting into 50 ml of PBS. The fixed cells were examined with a UV filter package (No. 48 77 02; Carl Zeiss, Inc.).

When staining with the lysosomal/endosomal antibody, lgp58, cells were first stained with filipin as described above. lgp58 at 50–150 μg/ml was diluted 50-fold into goat serum, spun in a microfuge for 30 s, and diluted another 50-fold into a 0.1% solution of saponin in PBS. This solution was used to label cells for 30 min at room temperature. Cells were washed extensively before labeling with an FITC-conjugated goat anti-mouse second antibody solution for 30 min at room temperature. The FITC-conjugated antibody was diluted 500-fold into goat serum, spun in a microfuge for 30 s, and then diluted another 500-fold into 0.1% saponin in PBS before adding to the cells. Cells were rinsed with PBS and mounted for microscopy. Immunofluorescence was examined with FITC-fluorescence optics (No. 48 77 17; Carl Zeiss, Inc.). In control experiments, cells were labeled with IgG secreted by the myeloma cell line MOPC-21 at a similar concentration as with the antibody lgp58.

Other Analytical Procedures

Cellular cholesterol was determined using the fluorometric assay of Heider and Boyett (31) as described previously (12). Cholesteryl ester was calculated by subtracting the free cholesterol value from the total cholesterol value (determined after incubation of cellular lipids with cholesterol esterase). Cell hybridizations (11) and the [^{14}C]acetate pulse (11, 19) were performed as described previously. All protein determinations in this paper were made using the Peterson modification (46) of the method of Lowry et al. (39); TCA precipitation was performed only when determining the protein content of the cell extracts used for the in vitro ACAT assays.

Results

Isolation of Cholesterol Starvation Resistant Mutants

The rationale for the ability of certain CHO cell mutants to survive cholesterol starvation is depicted in Fig. 1. The cholesterol starvation medium consisted of 10% delipidated FCS plus 50 μM mevinolin (to inhibit endogenous cholesterol synthesis) and 230 μM mevalonate (to permit the synthesis of nonsteroidal isoprenoids essential for cell growth; see Materials and Methods). When grown in 10% FCS medium, 25-RA cells have previously been demonstrated to contain large amounts of cytosolic cholesterol ester droplets (11, 17). During cholesterol starvation, the hydrolysis of this cholesteryl ester apparently served as a reservoir of cellular cholesterol for the 25-RA cells, enabling them to survive (Fig.1 *a*). As shown in Fig. 2 *b*, 25-RA cells were able to grow for at least 5 d in the cholesterol starvation medium. In contrast, 25-RA cells treated with the specific ACAT inhibitor 58-035 (or ACAT deficient mutants derived from 25-RA cells) contain very little cholesteryl ester (11). Consequently, almost all of these cells die during cholesterol starvation (Fig. 1 *b* and Fig. 2 *b*). The same result using the ACAT deficient mutant AC29 was found (data not shown).

This cholesterol starvation procedure was originally designed for the isolation of revertants of the ACAT deficient mutants. However, these attempts were not successful because of the high frequency of a novel class of mutants that were resistant to cholesterol starvation. Grown in 10% FCS, these mutants (designated as cholesterol trafficking, or CT mutants) possess intracellular particles that are distinct from the cholesteryl ester droplets seen in 25-RA cells. As will be demonstrated in the following sections, the CT mutants accumulate a large amount of unesterified cholesterol when grown in medium containing 10% FCS or LDL. The storage of free cholesterol in a distinct cellular component enabled the CT mutants to survive the cholesterol starvation selection (Fig. 1 *c*). Three independent CT clones were isolated from three dishes (4.5 × 10^5 total cells) of mutagenized populations of AC29 and 11 independent clones possessing the CT phenotype were isolated from 11 dishes (16.5 × 10^5 total cells) of mutagenized populations of 25-RA cells (pretreated with 58-035 to remove intracellular cholesteryl esters). No CT mutants were found in four dishes (6 × 10^5 total cells)

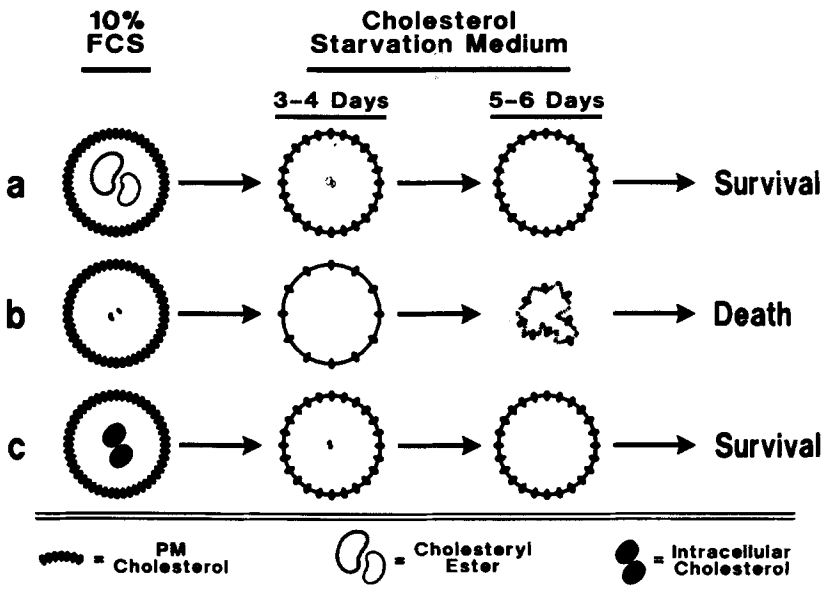

Figure 1. Depiction of 25-RA cells (*a*), ACAT-deficient mutants from 25-RA, or 25-RA cells pretreated with 58-035, a specific ACAT inhibitor (*b*), and CT mutants treated with 58-035 (*c*) during cholesterol starvation. After growth in 10% FCS medium, 25-RA cells contain a large amount of intracellular cholesteryl esters, while ACAT-deficient mutants or 25-RA cells treated with 58-035 contain very little. During cholesterol starvation, the cholesteryl esters in 25-RA hydrolyzed to free cholesterol can maintain a sufficient level of cholesterol for survival. Because they lack stored cholesterol, the ACAT-deficient mutants and 58-035-treated 25-RA cells die in starvation medium. When grown in 10% FCS medium the CT mutants contain a high level of intracellular free cholesterol. This stored cholesterol pool apparently provides the mutants with enough cholesterol to survive the starvation. The ACAT inhibitor has no effect on the accumulation of this free cholesterol in the CT mutants.

of unmutagenized 25-RA cells. The biochemical characterization of two cholesterol trafficking clones derived from 25-RA cells, CT52 and CT60, is described in this report.

As shown in Fig. 2 *a*, CT52 and CT60 pretreated with 58-035 grow at comparable rates to 58-035 treated 25-RA in 10% delipidated serum, but unlike 25-RA they also grow in delipidated serum plus mevinolin and mevalonate (i.e., starvation medium) (Fig. 2 *b*). These cell lines also retain resistance to the cytotoxic effects of 25-hydroxycholesterol (data not shown), a hallmark of 25-RA (19) and the 25-RA derived ACAT mutants (11).

As mentioned above, CT52 and CT60 have a distinct morphological appearance when grown in 10% FCS medium. The appearance of the various cell lines viewed with DIC optics is illustrated in Fig. 3. In delipidated serum (Fig. 3, *a*, *d*, and *g*) all three cell types had a similar appearance. When grown in delipidated serum plus 100 μg/ml LDL for 48 h, 25-RA (Fig. 3 *b*) accumulated perinuclear droplets. These particles were similar to the ones previously observed in 25-RA grown in FCS medium and have been identified as cholesteryl esters (11, 12). CT52 (Fig. 3 *e*) and CT60 (Fig. 3 *h*) cells grown in LDL also have intracellular particles, which, unlike the droplets of 25-RA, extended beyond the perinuclear area. Using the DIC microscopy, the particles in both cell types appeared similar. However, if the cells were grown in LDL plus 58-035, the particles in 25-RA (Fig. 3 *c*) disappeared completely, while only a minor reduction occurred in the amount of particles in CT52 (Fig. 3 *f*) and CT60 (Fig. 3 *i*). This indicates that the majority of particles in the CT mutants are not intracellular cholesteryl ester droplets derived as a product of the ACAT reaction.

Cholesteryl Ester Synthesis and Cell Hybridization Analysis

In pilot experiments (data not shown), it was found that the rate of cholesteryl ester synthesis in 25-RA cells grown in delipidated serum did not reach the low levels previously observed in this laboratory for wild-type CHO cells (16, 23). This was probably due to the high rate of endogenous cholesterol biosynthesis in 25-RA cells compared with wild-type cells (11, 19). The addition of 10 μM mevinolin to the medium for 4 h before the pulse experiment using [^{3}H]oleate reduced [^{3}H]cholesteryl ester synthesis from a rate of 12.7 ± 1.6 pmol/min/mg to 2.9 ± 1.1 pmol/min/mg (data not shown). Therefore, all the experiments described in this paper used media containing 10 μM mevinolin (as well as 230 μM mevalonate) for the indicated times.

As shown in Fig. 4 *a*, CT52 and CT60 cells have a greatly reduced activation of cholesteryl ester synthesis compared to 25-RA cells as measured by [^{3}H]oleate pulse after 6 h of in-

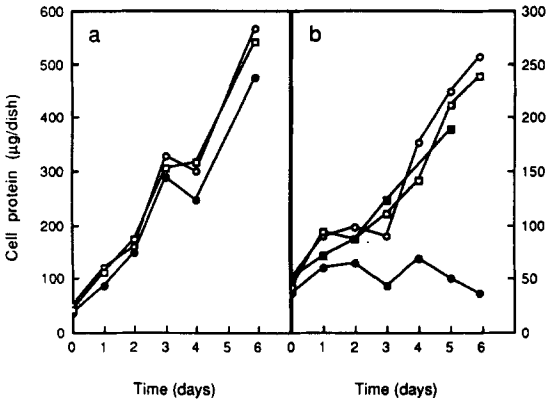

Figure 2. Growth of 25-RA (*solid circles*), CT52 (*open circles*), and CT60 (*open squares*) cells in delipidated serum medium with or without an inhibitor of cholesterol biosynthesis. 2.5 × 10^4 cells were plated in 9-cm^2 plates containing F-12 medium + 10% FCS and 200 ng/ml 58-035. After 48 h, the cultures were washed twice with sterile PBS and grown in F-12 medium supplemented with 10% delipidated FCS (*a*) or 10% delipidated FCS + 50 μM mevinolin and 230 μM mevalonate (*b*). Growth of 25-RA cells without the 58-035 treatment are also indicated in *b* (*solid squares*). The FCS was delipidated using Cab-o-sil (see Materials and Methods). The cultures were given fresh medium every day and were harvested in 0.2 M NaOH at the indicated times and aliquots were taken for protein analysis as described in Materials and Methods. Values are the average of duplicate dishes and ranged within 5% of the mean.

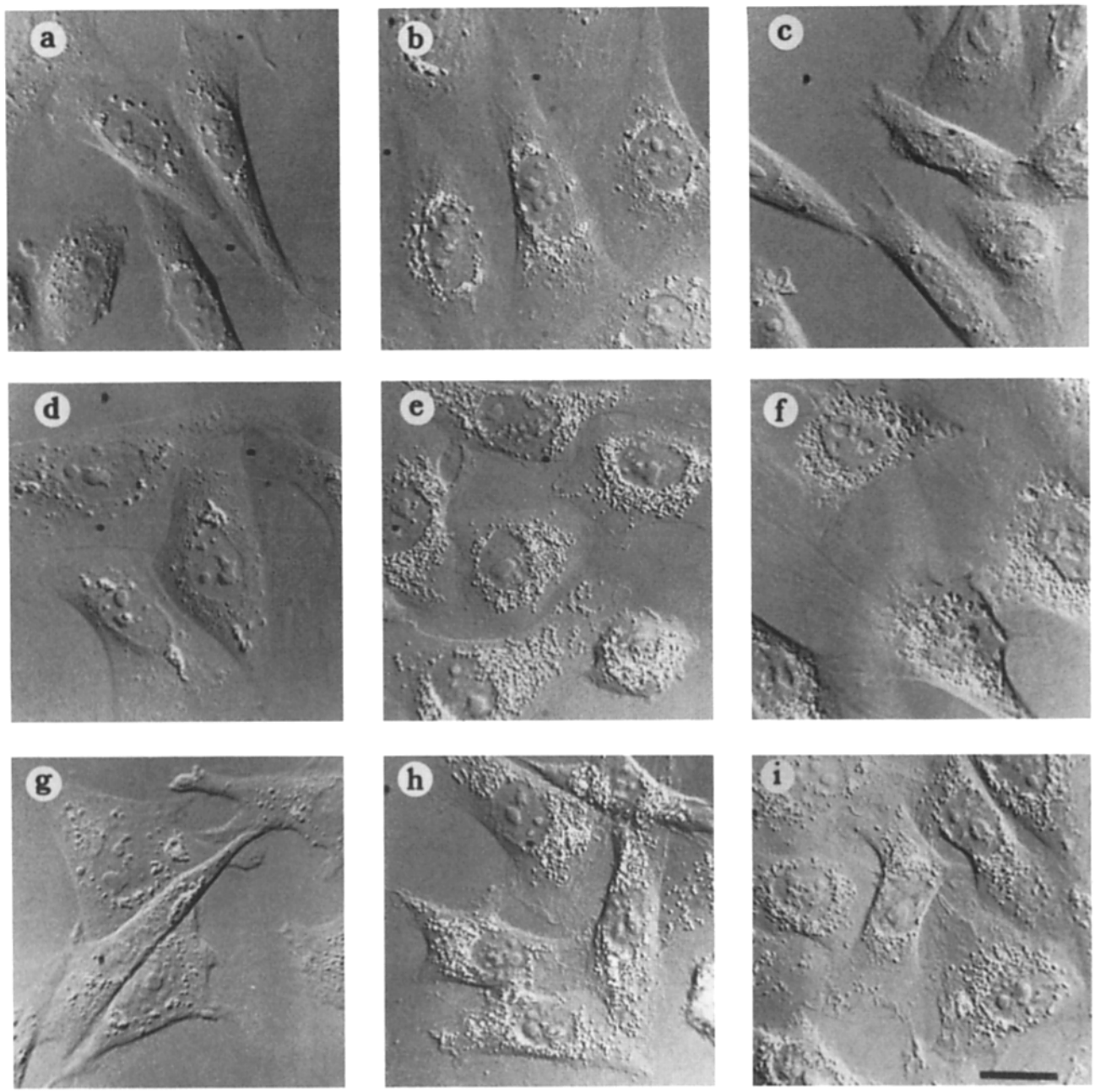

Figure 3. Appearance of 25-RA (*a–c*), CT52 (*d–f*), and CT60 (*g–i*) cells viewed with DIC microscopy. 5×10^4 cells were plated on glass coverslips in 9 cm^2 plates as described in Materials and Methods. Some cultures were plated in the presence of 200 ng/ml 58-035 (*c*, *f*, and *i*). After 24 h, the cultures were washed twice with PBS and switched to medium containing 5% delipidated bovine calf serum alone (*a*, *d*, and *g*), supplemented with 100 μg/ml LDL (*b*, *e*, and *h*), or 100 μg/ml LDL + 200 ng/ml 58-035 (*c*, *f*, and *i*). Cultures were fed fresh medium at 48 h and coverslips were mounted and viewed with DIC microscopy at 72 h as described in Materials and Methods. Bar, 20 μm.

cubation with various concentrations of LDL. In a parallel experiment without mevinolin, LDL activated the rate of cholesteryl ester synthesis (expressed as pmol/min/mg protein) in 25-RA from 11.1 to 51.6; in CT52 from 15.5 to 24.8; and in CT60 from 17.5 to 23.8. Fig. 4 *b* shows the activation of cholesteryl ester synthesis upon the addition of increasing amounts of 25-hydroxycholesterol to the medium. This oxysterol activates ACAT independently of the LDL receptor pathway (26, 35). 25-hydroxycholesterol activated cholesteryl ester synthesis to a lesser degree than LDL, but the activation was not defective in the CT mutants. The higher values observed in CT52 and CT60 compared to 25-RA cells may be due to their higher basal values in the absence of oxysterol.

Fig. 5 examines a time course of the activation of cholesteryl ester synthesis by LDL. Cholesteryl ester synthesis in the CT mutants was lower than 25-RA cells even after long periods of incubation with LDL. In the experiment

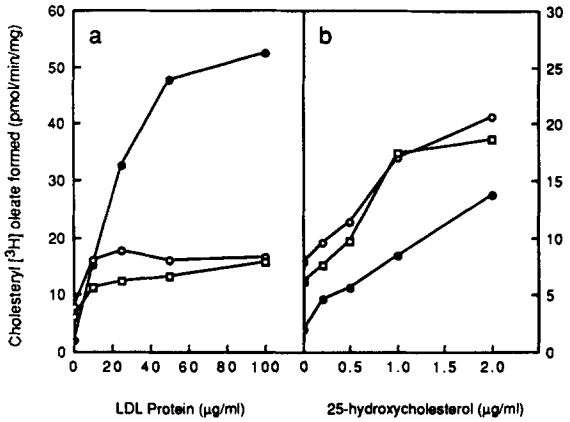

Figure 4. Effect of LDL and 25-hydroxycholesterol on the synthesis of cholesteryl esters in 25-RA (*solid circles*), CT52 (*open circles*), and CT60 (*open squares*) cells. Stock cultures were grown continuously in medium containing delipidated bovine calf serum for at least two passages as described in Materials and Methods. 4 × 10^5 cells were plated into 9 cm^2 plates in medium containing 10% delipidated bovine calf serum. Medium was changed 48 h after plating. 62 h after plating, the cultures were changed to 10% delipidated serum medium + 10 μM mevinolin and 230 μM mevalonate. At 66 h, the same medium supplemented with the indicated amounts of LDL (*a*) or 25-hydroxycholesterol (*b*) was added and 6 h later, the monolayers were pulsed with [^{3}H]oleate for 30 min. After the pulse, the cells were harvested and analyzed for incorporation of radiolabel into cholesteryl [^{3}H]oleate as described in Materials and Methods. Values are the average of duplicate dishes and ranged within 15% of the mean.

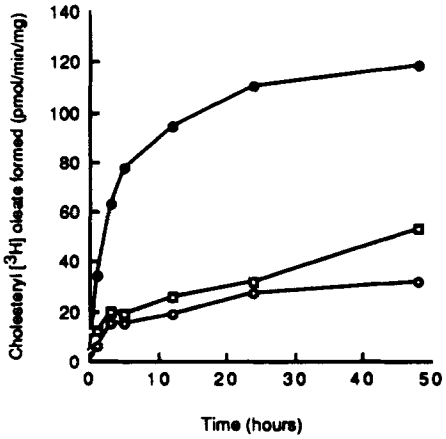

Figure 5. Time course of activation of cholesteryl ester synthesis by LDL in 25-RA (*solid circles*), CT52 (*open circles*), and CT60 (*open squares*) cells. Cells were plated as described in Fig. 4. 4 h before the addition of LDL, the monolayers were switched to 10% delipidated serum medium + 10 μM mevinolin and 230 μM mevalonate. Cultures were switched to delipidated serum medium plus mevinolin, mevalonate, and 100 μg/ml LDL at staggered times so that all the cultures could be pulsed with [^{3}H]oleate 72 h after plating. All cells were fed the appropriate fresh medium at 48 and 60 h after plating. After the pulse, the cells were harvested and analyzed for incorporation of radiolabel into cholesteryl [^{3}H]oleate as described in Materials and Methods. Values are the average of duplicate dishes and ranged within 15% of the mean.

shown in Fig. 5, the rate of incorporation of [^{3}H]oleate into triglycerides was found to be similar between all three cell types (data not shown).

The [^{3}H]oleate pulse provides an easy and quantitative method for distinguishing the 25-RA cell and the CT mutant phenotypes. All three cell lines were fused to a 25-RA cell clone bearing the azaguanine resistance (AG^r) and ouabain resistance (oua^r) markers. These genetic markers, one recessive (AG^r) and one dominant (oua^r) make these cells "universal fusion donors" that can be used to obtain heterokaryons with any cell type (3, 32). The hybrids were kept in selective medium until plating for the [^{3}H]oleate pulse and >95% of the cells in each population had the characteristic large size seen with CHO/CHO cell hybrids previously obtained in this laboratory (11). As seen in Fig. 6 *a*, the unfused controls grown in delipidated serum ± 100 μg/ml LDL for 6 h had rates of cholesteryl ester formation similar to those seen in Fig. 4. In contrast, the data shown in Fig. 6 *b* demonstrate that the cholesterol esterification in the hybrids were all activated to a similar degree as was observed with unfused 25-RA cells. These data show that the phenotype of mutants CT52 and CT60 is recessive.

In addition, complementation analysis was performed using a CT60 cell line bearing the azaguanine- and ouabain-resistant markers as the fusion donor. When grown in 10% delipidated serum and then switched to medium containing 100 μg/ml LDL, the CT60/25-RA hybrids increased their cholesteryl ester synthesis from 11.1 to 46.2 pmol/min/mg. The CT60/CT60 hybrid's rate of synthesis went from 20.5 to 24.6 pmol/min/mg, and the CT60/CT52 hybrid's from 13.2 to 18.4 pmol/min/mg. Thus CT60 and CT52 appear to belong to the same complementation group.

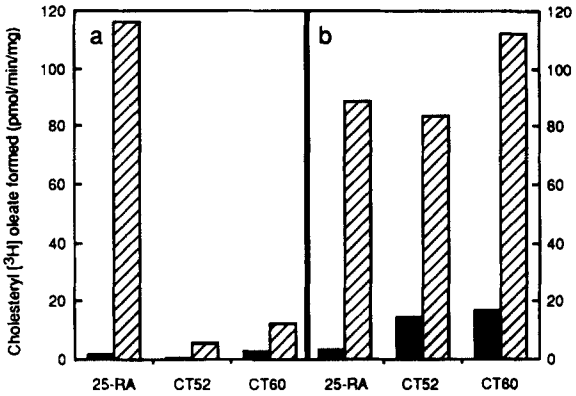

Figure 6. Cholesteryl ester synthesis in unfused CHO cells (*a*) and cell hybrids (*b*) grown in delipidated serum (*solid bars*) or delipidated serum plus LDL (*hatched bars*). Hybrids were prepared by fusing the indicated cell clones with the 25-RA cells that bear the azaguanine resistant and ouabain resistant markers (11). 5 × 10^4 unfused controls and 7.5 × 10^4 hybrids were plated in 9 cm^2 dishes containing medium plus 10% FCS. After 48 h, the monolayers were washed twice with PBS and switched to medium plus 10% delipidated FCS. Fresh medium was given 24 h later. At 86 h after plating, the cultures were changed to delipidated FCS medium plus 10 μM mevinolin and 230 μM mevalonate. At 90 h, the cells were given the above medium ± 100 μg/ml LDL and grown for an additional 6 h before being pulsed for 30 min with [^{3}H]oleate. Incorporation of radiolabel into cholesteryl [^{3}H]oleate was determined as described in Materials and Methods. Values are the average of duplicate dishes and ranged within 15% of the mean.

We next examined the in vitro ACAT activity in the relevant cell lines (Table I). The microsomal ACAT assay, like the [^{3}H]oleate pulse, utilizes cellular cholesterol as one of the ACAT substrates. The values obtained from cell homog-

Table I. Effect of Growth in LDL on the Microsomal and Reconstituted ACAT Activity of 25-RA, CT52, and CT60 Cell Extracts

Cell type	Time in LDL	ACAT specific activity	
		Microsomal	Reconstituted
	h	*pmol/min/mg*	
25-RA	0	18.9	82.3
	2	115.3	104.0
	48	131.5	98.7
CT52	0	25.1	136.6
	2	45.3	142.6
	48	98.0	154.2
CT60	0	19.8	78.6
	2	29.4	87.1
	48	174.4	94.0

3×10^6 cells were plated in 75 cm^2 flasks in 12 ml of medium and grown under identical conditions as described in Fig. 5. LDL was added to the cultures at the indicated times. 72 h after plating, the monolayers were harvested and the microsomal and reconstituted ACAT activities of the cell extracts determined as described in Materials and Methods. Values are the average of duplicate aliquots taken from each cell extract and ranged within 10% of the mean.

enates harvested after 2 h growth in LDL are reminiscent to those shown in Fig. 5, i.e., the CT mutants are defective in the activation of ACAT. However, the activities found from the extracts prepared from cultures grown in LDL containing medium for 48 h did not reflect the values obtained in the [^{3}H]oleate pulse. Under these conditions, and when cells are grown continuously in FCS medium (data not shown), the ACAT activities are as high or higher in the CT mutants compared with 25-RA.

Since the [^{3}H]oleate pulse and microsomal ACAT assay rely on cellular cholesterol, the results of these assays are influenced by the amount of cholesterol surrounding ACAT in addition to the absolute amount of enzyme. Therefore, reconstituted ACAT assays, where the enzyme activity is solubilized and then reconstituted into cholesterol-phosphatidylcholine vesicles of a defined concentration (10) were performed. There was no difference found between reconstituted ACAT activities in the cell extracts obtained from each cell line grown with or without LDL (Table I). This is consistent with previous work from this laboratory (10, 15, 16, 23) that suggested that changes in the ACAT protein content are not responsible for the regulation of enzyme activity by LDL. The reconstituted ACAT activities in CT60 and 25-RA cell extracts were found to be similar but the reconstituted ACAT activities in CT52 cell extracts were always ~50% greater than values obtained with 25-RA cell extracts. Since the mutants were isolated from mutagenized cells, it is possible that the CT52 mutant may contain a separate mutant locus, irrelevant to the CT locus addressed in this report, which may cause elevated ACAT activities in this particular cell line. Other possibilities can not be excluded. The data clearly show that in vitro ACAT activity is present in the CT mutants in normal or greater than normal amounts.

LDL Receptor Pathway Analysis

The binding, internalization, and degradation of ^{125}I-LDL was examined in 25-RA and the CT mutants (Fig. 7). The specific binding (Fig. 7 *a*) and internalization (Fig. 7 *b*) in the CT mutants were found to be ~1.6–2.0-fold higher than that of 25-RA cells. Total degradation of the radiolabeled

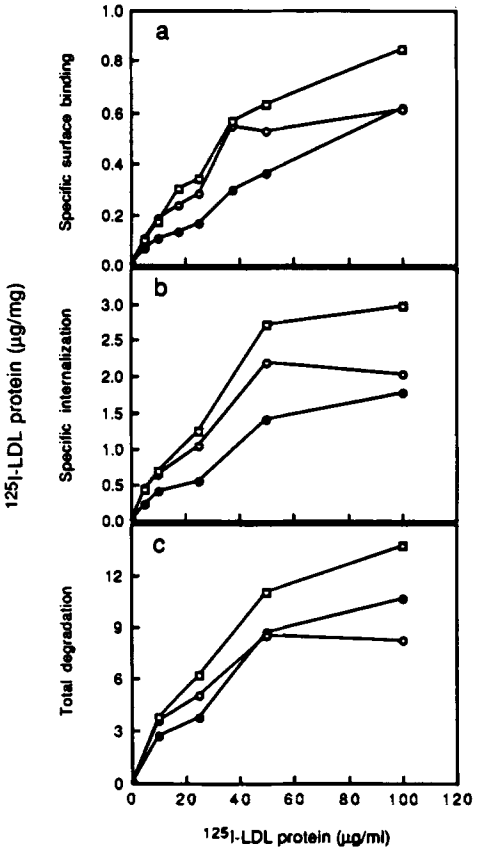

Figure 7. The specific binding (*a*), specific internalization (*b*), and total degradation (*c*) of ^{125}I-LDL in 25-RA (*solid circles*), CT52 (*open circles*), and CT60 (*open squares*) cells. Cells were plated and grown as described in Fig. 4, except that 5% delipidated serum was used. At 66 h after plating, media containing the indicated amounts of ^{125}I-LDL (343 cpm/ng) were added to the cells. After 6 h, the cells were analyzed for specific binding and internalization, and total degradation of the ^{125}I-LDL as described in Materials and Methods. Values are the average of duplicate dishes and ranged within 10% of the mean.

LDL was also measured (Fig. 7 *c*). Controls demonstrated that the addition of a large excess of unlabeled LDL (500 μg/ml) inhibited the degradation of the ^{125}I-lipoprotein by 82–93%, and the presence of 100 μM chloroquine, an inhibitor of lysosomal function (26, 28), did not block internalization but resulted in a 71 to 90% reduction in degradation (data not shown). Degradation of radiolabeled LDL in the CT mutants was similar or slightly higher than levels found in 25-RA cells.

Besides the degradation of the LDL protein moiety, the hydrolysis of LDL-cholesterol ester also occurs in the lysosomes, via a specific acid lipase (26, 28). Of the total cholesterol in LDL, 75–80% is esterified (53), and the hydrolysis of this sterol ester is necessary for the regulatory effects of LDL to be observed in human fibroblast (26, 28) and CHO cells (22). By labeling the LDL with [^{3}H]cholesteryl linoleate, it is possible to measure the hydrolysis of [^{3}H]cholesteryl linoleate to free [^{3}H]cholesterol.

The data in Fig. 8 illustrate the total LDL-[^{3}H]cholesteryl linoleate hydrolyzed (Fig. 8 *a*) and the LDL derived [^{3}H]cholesterol that is reesterified (Fig. 8 *b*) after addition of [^{3}H]cholesteryl linoleate-LDL to the cultures. CT52 cells hydrolyzed, on average, 2.1 times more LDL-[^{3}H]cholesteryl

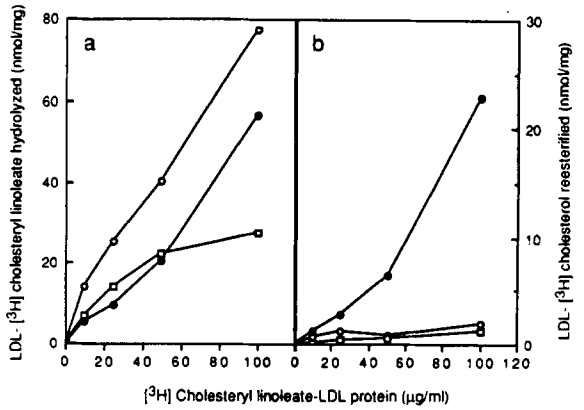

Figure 8. Hydrolysis (*a*) and reesterification (*b*) of LDL-[^{3}H]cholesteryl linoleate in 25-RA (*solid circles*), CT52 (*open circles*), and CT60 (*open squares*) cells. Cells were plated and grown as described in Fig. 7, except that after 62 h, all media were supplemented with 35 μM oleic acid. At 66 h after plating, medium containing the indicated amounts of [^{3}H]cholesteryl linoleate-LDL (3,180 cpm/nmol cholesteryl linoleate) were added to the cells. After 6 h, the cells were harvested and the lipid extracts were analyzed for [^{3}H]cholesterol, [^{3}H] cholesteryl linoleate, and [^{3}H]cholesteryl oleate as described in Materials and Methods. Hydrolysis of LDL-[^{3}H]cholesteryl linoleate was calculated by addition of the [^{3}H]cholesterol and [^{3}H]cholesteryl oleate values. The amount of reesterification of LDL-[^{3}H]cholesterol was calculated from the [^{3}H]cholesteryl oleate values. Values are the average of duplicate plates and ranged within 10% of the mean.

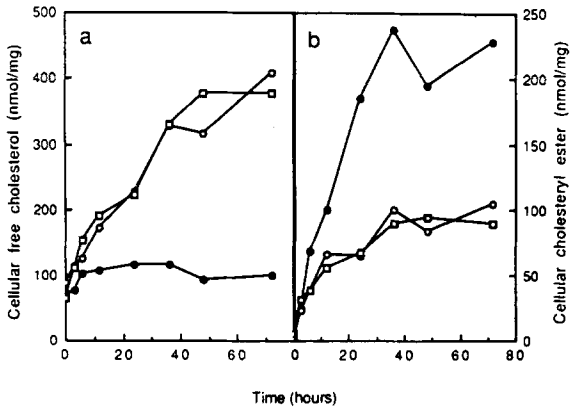

Figure 9. Time course of cholesterol (*a*) and cholesteryl ester (*b*) accumulation after addition of LDL. 25-RA (*solid circles*), CT52 (*open circles*), and CT60 (*open squares*) cells were plated and grown exactly as described in Fig. 5 except that 5% delipidated serum was used throughout the experiment. At 72 h after plating, the cultures were harvested and analyzed for cholesterol and cholesteryl ester as described in Materials and Methods. Values are the average of duplicate aliquots taken from cell extracts and ranged within 5% of the mean.

linoleate than 25-RA cells, while CT60 hydrolyzed between 50 and 140% of the values found for 25-RA. Despite this relatively normal or greater than normal hydrolysis of LDL-[^{3}H]cholesteryl linoleate in the CT mutants, they reesterified 11–19 times more LDL derived [^{3}H]cholesterol than CT52 and CT60 cells.

Control experiments similar to the ones performed during the ^{125}I-LDL experiment demonstrated that the amount of LDL-[^{3}H]cholesteryl linoleate hydrolyzed by the cells was inhibited 66–87% by excess unlabeled LDL. The addition of chloroquine to the medium caused a drastic increase in the amount of [^{3}H]cholesterol linoleate found in the cells and a large decrease (73–88%) in the percentage of radiolabel found in the [^{3}H]cholesterol and [^{3}H]cholesteryl oleate bands (data not shown).

Sterol Analysis

The data shown in Fig. 8 indicates that the cholesteryl ester moiety of LDL is hydrolyzed normally in the CT mutants but unlike the parental cell line 25-RA, the cholesterol is reesterified at a much diminished rate. To address the question of what happens to this liberated cholesterol, sterol analysis of 25-RA and the CT mutants was performed. Fig. 9 illustrates the changes in sterol content with increasing time (3–72 h) after the addition of 100 μg/ml LDL. The unesterified cholesterol level of the 25-RA cells remained relatively unchanged while the presence of LDL in the medium caused a large accumulation of cholesteryl ester in 25-RA cells and a smaller increase in the CT mutants, consistent with the activation of cholesteryl ester synthesis shown earlier (Figs. 4–6). In contrast, both CT mutants exhibited a dramatic time dependent increase in their cellular cholesterol content, which reached at maximum 4.5–5.0-fold above the levels observed in the absence of LDL. This accumulation was also dependent on the concentration of LDL in the medium and was seen when the cells were grown in the presence of the ACAT inhibitor 58-035 (data not shown).

In a parallel experiment to the one shown in Fig. 9, mevinolin was not included in the growth medium, and very similar results were obtained (data not shown), suggesting that endogenous synthesis of cholesterol did not contribute significantly to the accumulation of cellular cholesterol. Therefore, the large majority of accumulated cholesterol seen in the CT mutants must have come directly from LDL.

Localization of the Accumulated Intracellular Cholesterol

The accumulation of free cholesterol and the defective activation of cholesteryl ester synthesis after LDL addition in the CT mutants is very similar to the observations made in fibroblasts isolated from a strain of BALB/c mice with a neurological disorder (43), and human fibroblasts isolated from patients with NP-C disease (36, 44). Cells from these affected mice and humans have been shown to accumulate free cholesterol in their lysosomes in response to exogenous sterols such as LDL (5, 7, 38, 52, 54). Both CT52 and CT60 have been shown to accumulate a large amount of dark intracellular particles (Fig. 3) and free cholesterol (Fig. 9) when grown in the presence of LDL. It seemed that these two phenomena were linked, and the similarity between the CT mutants and the NP-C and mouse fibroblasts suggested that the cytoplasmic particles in the CT mutants may be lysosomes full of cholesterol.

The fluorescent stain acridine orange is a membrane permeant weak base that accumulates in acidic cellular compartments (47) where it emits red light (>620 nm) upon excitation. As seen in Fig. 10, *g* and *h*, CT52 cells grown in LDL before acridine orange staining contain a fluorescence signal pattern that colocalized with the dark cytoplasmic particles

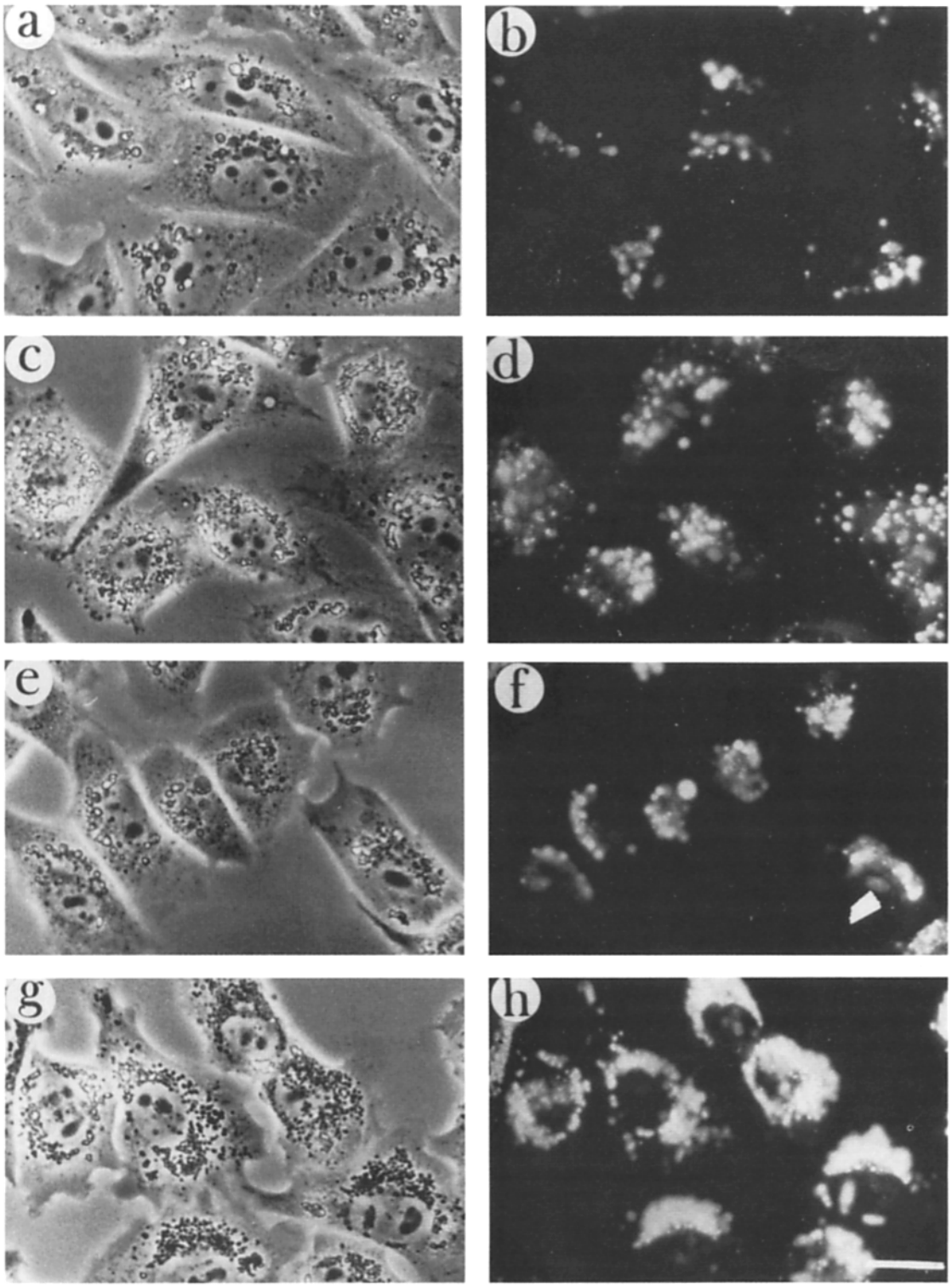

Figure 10. Localization of acidic compartments with acridine orange in 25-RA (*a–d*) and CT52 (*e–h*) cells. Cells were grown as described in Fig. 3 in medium containing 5% delipidated serum (*a*, *b*, *e*, and *f*) or medium containing 5% delipidated serum supplemented with 100 μg/ml of LDL (*c*, *d*, *g*, and *h*). Cells were stained with acridine orange as described in Materials and Methods before viewing with phase-contrast (*a*, *c*, *e*, and *g*) or fluorescence (*b*, *d*, *f*, and *h*) microscopy. Bar, 20 μm.

visualized with phase-contrast microscopy. In contrast, the lighter, perinuclear particles seen in the 25-RA cells treated with LDL did not colocalize with the acridine orange signal (Fig. 10, *c* and *d*). For CT52 cells, there was a large increase in the amount of acridine orange signal in cells grown in LDL-containing medium compared to cells grown in delipidated serum medium alone (Fig. 10, *f* and *h*), while no increase was observed in 25-RA cells (Fig. 10, *b* and *d*).

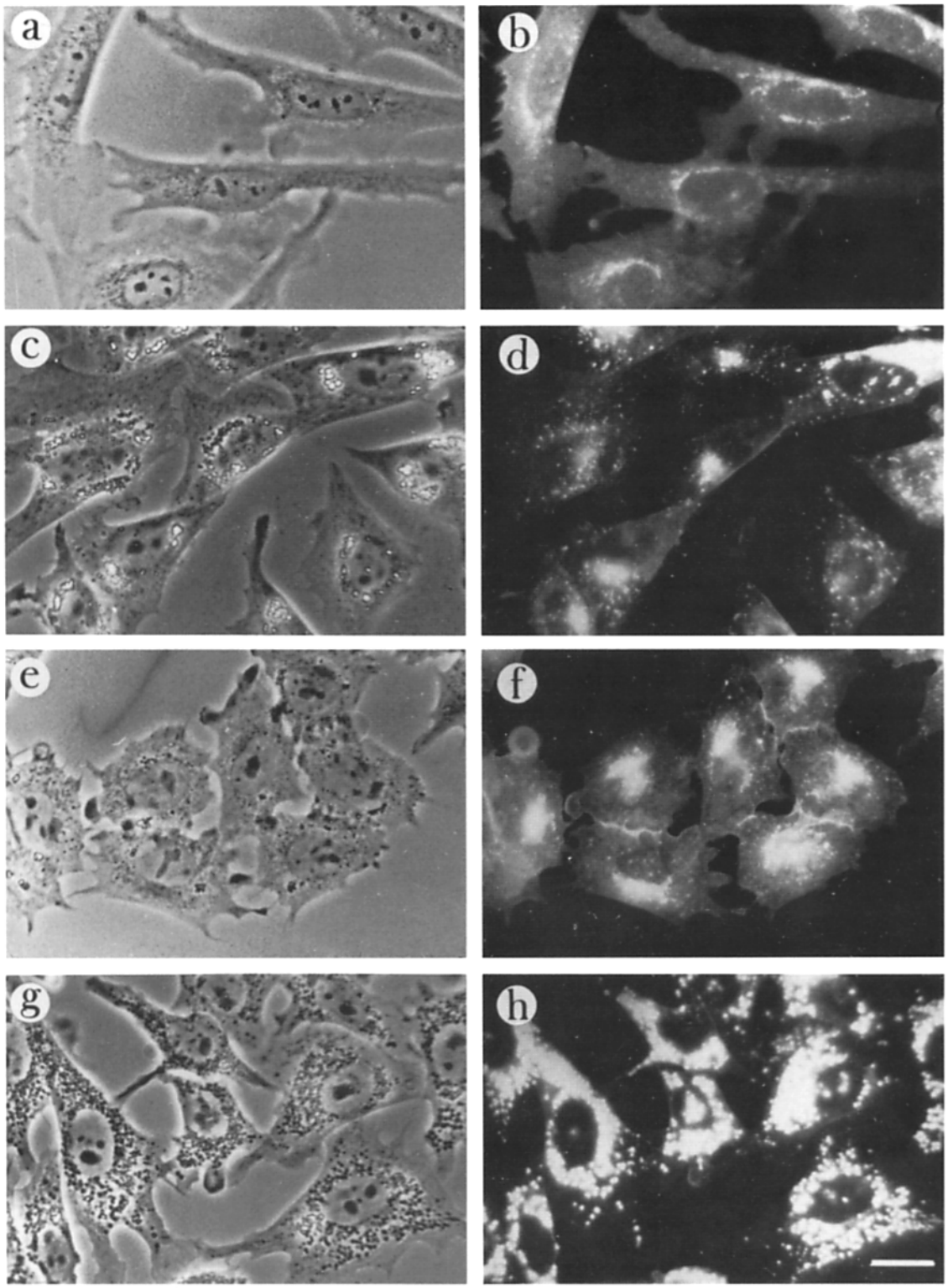

Figure 11. Localization of intracellular cholesterol with filipin in 25-RA (*a–d*) and in CT52 (*e–h*) cells. Cells were grown as described in Fig. 3 in medium containing 5% delipidated serum (*a*, *b*, *e*, and *f*) or medium containing 5% delipidated serum supplemented with 100 μg/ml of LDL (*c*, *d*, *g*, and *h*). Cells were fixed and stained with filipin as described in Materials and Methods before viewing with phase-contrast (*a*, *c*, *e*, and *g*) or fluorescence (*b*, *d*, *f*, and *h*) microscopy. Bar, 20 μm.

Results similar to those seen in CT52 cells were obtained with mutant CT60 (data not shown). To rule out the possibility that acridine orange is partitioning into a nonacidic site within the cell, the weak base ammonium chloride was used to neutralize the lysosomes/endosomes. When cells were incubated for 45 min with 50 mM ammonium chloride before staining with acridine orange, the staining pattern seen in the presence of LDL was abolished (data not shown). The cells

exhibited a uniform fluorescence that was faint and diffuse, thus the fluorescence pattern seen in Fig. 10 would appear to be from the accumulation of acridine orange in the cellular acidic compartments.

When CT52 cells were grown in LDL and stained with filipin, a fluorescent compound known to bind specifically to cholesterol (8), a strong filipin signal was seen that colocalized with dark perinuclear particles (Fig. 11, *g* and *h*). In contrast, the modest amount of filipin staining observed in 25-RA cells grown in LDL (Fig. 11, *c* and *d*) did not colocalize with its cytosolic, lipid droplet particles. As with the acridine orange staining experiment, after addition of LDL, there is a large increase in filipin fluorescence in CT52 mutants compared to CT52 grown in delipidated serum alone (Fig. 11, *f* and *h*). A much smaller increase was seen in 25-RA cells (Fig. 11, *b* and *d*). When these experiments were performed with CT60 cells, identical results were obtained (data not shown).

To further examine the intracellular location of the accumulated cholesterol, CT60 cells were costained with filipin and lgp58, a monoclonal antibody prepared against a CHO cell lysosomal/endosomal fraction (Mellman, I., unpublished data and personal communication). As seen in Fig. 12, *a* and *b*, the filipin (Fig. 12 *a*) signal colocalized with the signal obtained with the lysosmal/endosomal antibody via indirect immunofluorescence (Fig. 12 *b*). When the double staining experiment was performed with cells grown in delipidated serum medium alone, the filipin staining for 25-RA and CT60 resembled that shown in Fig. 11, while the antibody staining had a very similar and faint fluorescence for both cell types (data not shown). Results using CT52 cells were very similar to those shown using CT60 cells (data not shown). Control experiments with the nonspecific antibody MOPC-21 revealed faint uniform staining over the entire cell in all three cell types.

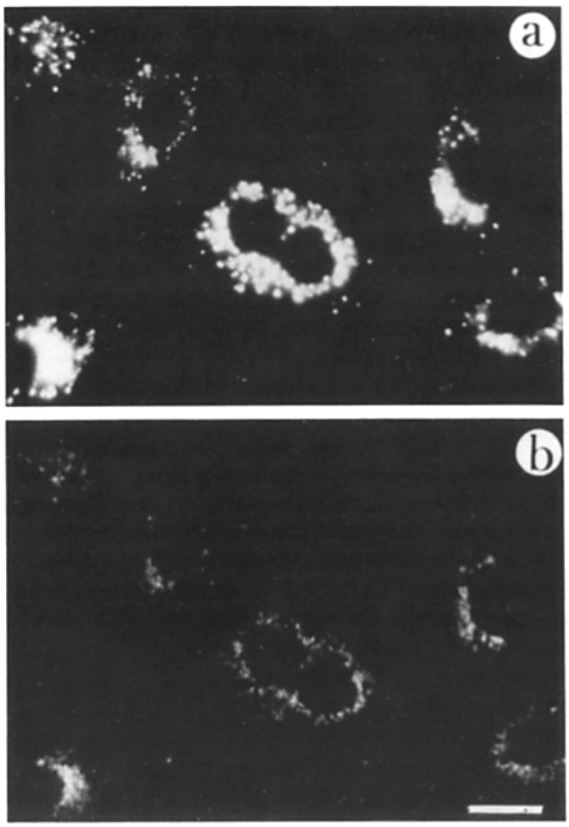

Figure 12. Indirect immunofluorescence of filipin-stained CT60 cells using a lysosomal/endosomal specific antibody. CT60 cells were grown as described in Fig. 3 in medium containing 5% delipidated serum plus 100 μg/ml of LDL. Cells were fixed, stained with filipin, and then stained by indirect immunofluorescence with lgp58 as detailed in Materials and Methods. *a* and *b* show filipin and lgp58 fluorescence, respectively. Bar, 20 μm.

Discussion

This report describes a selection procedure for isolating mutant cells that are resistant to cholesterol starvation. These mutants, which we have termed cholesterol trafficking, or CT mutants, were shown to accumulate a large amount of unesterified cholesterol when grown in medium containing LDL (Fig. 9) or 10% FCS. The data indicate that the excess cholesterol is located in intracellular particles shown by DIC (Fig. 3) and phase-contrast microscopy (Fig. 11). These particles are lysosomes and/or endosomes based on fluorescent microscopy studies with acridine orange (Fig. 11) and a lysosomal/endosomal monoclonal antibody (Fig. 12). The high cholesterol levels seen in the CT mutants when they were grown in LDL were found to decrease dramatically after 2 d of growth in delipidated serum medium (data not shown). This observation suggests that the cholesterol was able to leave the lysosomes of the CT mutants, perhaps via a nonspecific process (i.e., monomer diffusion, membrane recycling, etc.). The ability of the cholesterol to escape from the lysosomes probably provided the CT cells with the cholesterol needed for survival during the starvation selection.

In the selection protocol, the number of mutagenized cells present in each dish at the start of the cholesterol starvation was $\sim 1.5 \times 10^5$. Since every dish had at least one CT-like colony, the lowest possible frequency of this mutant phenotype was $\sim 6.6 \times 10^{-6}$. However, more than one CT-like colony was always seen and our protocol only identified the largest and most obvious surviving colonies. The actual frequency was probably between 2×10^{-5} (three independent CT mutants/dish) and 1×10^{-4} (15 CT mutants/dish). These frequencies are 2–12 times higher than the frequency of obtaining ACAT revertants using an alternative method (12), which might explain why no ACAT revertants were found in the starvation selections.

The frequencies of mutation in mutagenized CHO cells have commonly been found to range from 1×10^{-3} to 5×10^{-5} for single genes (13, 21, 34, 41, 56), and 1×10^{-6} to 2×10^{-7} for genes that are functionally diploid (11, 21, 34, 41). Thus, it appears that the generation of the CT mutant phenotype may only have required the mutation of a single gene. The data in Fig. 6 indicated that the mutations in CT52 and CT60 are recessive, suggesting that there is only one active CT gene in the CHO cell line, 25-RA.

One phenomena which occurred frequently throughout the biochemical analysis of the CT mutants was the higher than normal (i.e., 25-RA) activities of some of the commonly assayed processes of cholesterol metabolism, such as LDL receptor (Figs. 7 and 8) and ACAT activity (Table I), and the incorporation of labeled acetate into cholesterol (data not shown). The elevated reconstituted ACAT activity seen in

CT52 cell extracts (Table I) may be due to a secondary mutation since it is never observed in CT60 cells. Unlike ACAT, the LDL receptor has been demonstrated to be subject to stringent regulation at the level of transcription (27), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a rate limiting enzyme in cholesterol biosynthesis, has been shown to be highly regulated at both the transcriptional and posttranlational levels by cholesterol (27). It is possible that a mutation in a cholesterol transport process such as the one affected in the CT mutants may alter this complicated regulatory apparatus and cause an elevation of these activities. Once revertants of the CT mutants have been isolated and characterized, these possibilities can be more directly examined.

The microsomal ACAT specific activity of cell extracts normally reflects the values obtained in intact cells with the [^{3}H]oleate pulse (15, 16). In Table I this was true for the cell extracts harvested after growth in delipidated serum medium or after 2 h in medium containing LDL. However, CT mutants grown for 48 h in LDL had microsomal ACAT specific activities as high or higher than 25-RA cell extracts, in contradiction to the [^{3}H]oleate pulse data (Fig. 5). These high activities may be an artifact of the preparation of the cell extracts, which involved hypotonic shock (20) that may have disrupted the cholesterol loaded lysosomal/endosomal compartments in the CT cells. This cholesterol could then act as a substrate for ACAT. This type of phenomena has been reported in NP-C fibroblasts (54).

Previously, it has been shown that the suppression of the LDL receptor and cholesterol biosynthesis by LDL occurred with a significant lag in NP-C fibroblasts compared to unaffected fibroblasts (37, 45), consistent with the defective movement of cholesterol out of the NP-C lysosomes (38). These experiments were attempted with the CT mutants but the results were ambiguous because the parental cell line, 25-RA, is partially resistant to suppression of cholesterol biosynthesis and the LDL receptor pathway by exogenous sterols (19, and data not shown). Perhaps a significant lag in the kinetics of LDL-mediated suppression of these pathways would be evident if the CT mutants were in a wild-type CHO cell genetic background. It should be pointed out that the LDL receptor pathway is two to three times more active in 25-RA cells treated with 58-035 than wild-type cells (11). Therefore, a CT mutant derived from a wild-type cell might not accumulate as much intracellular unesterified cholesterol and thus would not survive as well (if at all) during the starvation selection.

The exact biochemical process defective in the CT mutants is not known. One obvious candidate would be an inactivated intracellular cholesterol transporter. Sterol carrier protein$_2$ is a 13-kD protein originally identified in rat and bovine liver cytosol. This protein has been shown to transfer cholesterol between membranes in vitro and to activate ACAT activity in vitro (25, 48). Sterol carrier protein$_2$ has been localized predominantly to peroxisomes in liver (33, 57) but it is present in smaller amounts in the endoplasmic reticulum and cytosol (33). The development of a reliable assay for this protein in CHO cells would allow direct testing of whether sterol carrier protein$_2$ activity is present in CT mutant cell extracts.

The defect in the CT mutants does not necessarily have to be in a cytosolic cholesterol transporter. There could be a defect in the mobilization of the newly hydrolyzed cholesterol in the lysosomal interior, or a defect in a receptor molecule that receives the cholesterol after transport from the lysosomes. More knowledge concerning the structure and polypeptide composition of the cholesterol loaded lysosomes, and the normal pathways of LDL-derived cholesterol transport out of the lysosomes will be beneficial towards understanding the exact molecular defect in the CT mutants.

In conclusion, this paper reports the isolation of CHO cells in which the intracellular movement of LDL-derived cholesterol is defective, leading to an accumulation of cholesterol in acidic compartments that are most likely lysosomes. The phenotype of the CT mutants is almost identical to that of the NP-C fibroblasts and a strain of BALB/c mice with a similar neurological disorder. The value of the CT mutants is that CHO cells are much more amenable to genetic manipulation than primary cultures of human fibroblasts or mice. The isolation of a large number of recessive CT mutants will allow complementation analysis to determine if the inactivation of more than one gene can generate a CT phenotype. The ability to fuse CHO cells with human fibroblasts and the subsequent segregation of the human chromosomes could provide a means for identifying the chromosome where the human homologue of the CT gene (or genes) is located. In addition, fusions between the CT mutants and NP-C firboblasts and biochemical analysis of the hybrids could determine if the same locus is affected (no complementation observed). The final advantage of obtaining the cholesterol trafficking phenotype in a CHO genetic background, and perhaps the most important, is that exogenous DNA can be introduced into CHO cells via DNA mediated gene transfer (1). The complementation of the CT mutation via transfection of exogenous DNA should be possible provided a suitable selection system can be developed. Once transformants are obtained, the cloning of the complementing gene should be possible. Such work is currently underway in our laboratory.

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References

1. Abraham, I. 1985. DNA-mediated gene transfer. *In* Molecular Cell Genetics. M. M. Gottesman, editor. John Wiley & Sons, Inc., New York. 181–210.
2. Alberts, A. W., J. Chen, G. Kuron, V. Hunt, J. Huff, C. Hoffman, J. Rothrock, M. Lopez, H. Joshua, E. Harris, A. Patchett, R. Moneghan, S. Currie, E. Stapley, G. Albers-Schonberg, O. Hensons. J. Hirshfield, K. Hoogsteen, J. Liesch, and J. Springer. 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc. Natl. Acad. Sci. USA.* 77:3957–3961.
3. Baker, R. M., D. M. Brunette, R. Mankovitz, L. H. Thompson, G. H. Whitmore, L. Siminovitch, and J. E. Till. 1974. Ouabain-resistant mutants of mouse and hamster cells in culture. *Cell.* 1:9–21.
4. Balasubramaniam, S., S. Venkatesan, K. A. Mitropoulus, and T. J. Peters. 1978. The submicrosomal localization of acyl-coenzyme A-cholesterol acyltransferase and its substrate, and of cholesteryl esters in rat liver. *Biochem. J.* 174:863–872.
5. Bhuvaneswaran, C., M. D. Morris, H. Shio, and S. Fowler. 1982. Lysosome lipid storage disorder in NCTR-BALB/c mice. III. Isolation and

analysis of storage inclusions from liver. *Am. J. Pathol.* 102:160–170.
6. Bishop, J. E., and A. K. Hajra. 1980. A method for the chemical synthesis of ^{14}C-labeled fatty acyl coenzyme A's of high specific activity. *Anal. Biochem.* 106:344–350.
7. Blanchette-Mackie, E. J., N. K. Dwyer, L. M. Amende, H. S. Kruth, J. D. Butler, J. Sokol, M. E. Comly, M. T. Vanier, J. T. August, R. O. Brady, and P. G. Pentchev. 1988. Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. *Proc. Natl. Acad. Sci. USA.* 85:8022–8026.
8. Bornig, H., and G. Geyer. 1974. Staining of cholesterol with the fluorescent antibiotic "filipin." *Acta Histochem.* 50:110–115.
9. Butler, J. D., M. E. Comly, H. S. Kruth, M. Vanier, M. Filling-Katz, J. Fink, N. Barton, H. Weintroub, J. M. Quirk, T. Tokoro, D. C. Marshall, R. O. Brady, and P. G. Pentchev. 1987. Niemann-Pick variant disorders: comparisons of errors of cellular cholesterol homeostasis in group D and group C fibroblasts. *Proc. Natl. Acad. Sci. USA.* 84:556–560.
10. Cadigan, K. M., and T. Y. Chang. 1988. A simple method for reconstitution of CHO cell and human fibroblast ACAT activity into liposomes. *J. Lipid Res.* 29:1683–1692.
11. Cadigan, K. M., J. G. Heider, and T. Y. Chang. 1988. Isolation and characterization of Chinese hamster ovary cell mutants deficient in acyl-coenzyme A: cholesterol acyltransferase activity. *J. Biol. Chem.* 263: 274–282.
12. Cadigan, K. M., C. C. Y. Chang, and T. Y. Chang. 1989. Isolation of Chinese hamster ovary cell lines expressing human acyl-coenzymeA/cholesterol acyltransferase activity. *J. Cell Biol.* 108:2201–2210.
13. Campbell, C. E., and R. G. Worton. 1979. Evidence obtained by induced mutation frequency analysis for functional hemizygosity at the emt locus in CHO cells. *Somatic Cell Genet.* 5:51–65.
14. Cham, B. E., and B. R. Knowles. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* 17:176–181.
15. Chang, C. C. Y., and T. Y. Chang. 1986. Cycloheximide sensitivity in regulation of acyl coenzyme A: cholesterol acyltransferase activity in Chinese hamster ovary cells. II. Effect of sterol endogenously synthesized. *Biochemistry.* 25:1700–1706.
16. Chang, C. C. Y., G. M. Doolittle, and T. Y. Chang. 1986. Cycloheximide sensitivity in regulation of acyl coenzyme A: cholesterol acyltransferase activity in Chinese hamster ovary cells. I. Effect of exogenous sterols. *Biochemistry.* 25:1693–1699.
17. Chang, T. Y., and C. C. Y. Chang. 1982. Revertants of a Chinese hamster ovary cell mutant resistant to suppression by an analogue of cholesterol: isolation and partial biochemical characterization. *Biochemistry.* 21: 5316–5323.
18. Chang, T. Y., and G. M. Doolittle. 1983. Acyl coenzyme A: cholesterol O-acyltransferase. *In* The Enzymes. Vol. 16. P. D. Boyer, editor. Academic Press, Inc., New York. 523–539.
19. Chang, T. Y., and J. S. Limanek. 1980. Regulation of cytosolic acetoacetyl coenzyme A thiolase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and mevalonate kinase by low density lipoprotein and by 25-hydroxycholesterol in Chinese hamster ovary cells. *J. Biol. Chem.* 255:7787–7795.
20. Chang, T. Y., J. S. Limanek, and C. C. Y. Chang. 1981. A simple and efficient procedure for the rapid homogenization of cultured animal cells grown in monolayer. *Anal. Biochem.* 116:298–302.
21. Chasin, L. A. 1974. Mutations affecting adenine phosphoribosyl transferase activity in Chinese hamster cells. *Cell.* 2:37–41.
22. Chin, J., and T. Y. Chang. 1981. Evidence for coordinate expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase and low density lipoprotein binding activity. *J. Biol. Chem.* 256:6304–6310.
23. Doolittle, G. M., and T. Y. Chang. 1982. Acyl-CoA: cholesterol acyltransferase in Chinese hamster ovary cells. Enzyme activity determined after reconstitution in phospholipid/cholesterol liposomes. *Biochim. Biophys. Acta.* 713:529–537.
24. Drevon, C. A., D. B. Weinstein, and D. Steinberg. 1980. Regulation of cholesterol esterification and biosynthesis in monolayer cultures of normal adult rat hepatocytes. *J. Biol. Chem.* 255:9128–9137.
25. Gavey, K. L., B. J. Noland, and T. J. Scallen. 1981. The participation of sterol carrier protein$_2$ in the conversion of cholesterol to cholesterol ester by rat liver microsomes. *J. Biol. Chem.* 256:2993–2999.
26. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* 46:897–930.
27. Goldstein, J. L., and M. S. Brown. 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. *J. Lipid Res.* 25:1450–1461.
28. Goldstein, J. L., S. E. Dana, J. R. Faust, A. L. Beaudet, and M. S. Brown. 1975. Role of lysosomal acid lipase in the metabolism of plasma low density lipoprotein. *J. Biol. Chem.* 250:8487–8495.
29. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* 98:241–260.
30. Hashimoto, S., and A. M. Fogelman. 1980. Smooth microsomes. A trap for cholesteryl ester formed in hepatic microsomes. *J. Biol. Chem.* 255:8678–8684.
31. Heider, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* 19:514–518.
32. Jha, K. K., and H. L. Ozer. 1976. Expression of transformation in cell hybrids. I. Isolation and application of density-inhibited Balb/3T3 cells deficient in hypoxanthine phosphoribosyltransferase and resistant to ouabain. *Somatic Cell Genet.* 2:215–223.
33. Keller, G. A., T. J. Scallen, D. Clarke, P. A. Maher, S. K. Krisans, and S. J. Singer. 1989. Subcellular localization of sterol carrier protein-2 in rat hepatocytes: its primary localization to peroxisomes. *J. Cell Biol.* 108:1353–1361.
34. Kingsley, D. M., and M. Krieger. 1984. Receptor-mediated endocytosis of low density lipoprotein: somatic cell mutants define multiple genes required for expression of surface-receptor activity. *Proc. Natl. Acad. Sci. USA.* 81:5454–5458.
35. Krieger, M., M. S. Brown, and J. L. Goldstein. 1981. Isolation of Chinese hamster cell mutants defective in the receptor-mediated endocytosis of low density lipoprotein. *J. Mol. Biol.* 150:167–184.
36. Kruth, H. S., M. E. Comly, J. D. Butler, M. T. Vanier, J. K. Fink, D. A. Wenger, S. Patel, and P. G. Pentchev. 1986. Type C Neimann-Pick disease. Abnormal metabolism of low density lipoprotein in homozygous and heterozygous fibroblasts. *J. Biol. Chem.* 261:16769–16774.
37. Liscum, L., and J. R. Faust. 1987. Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Neimann-Pick type C fibroblasts. *J. Biol. Chem.* 262:17002–17008.
38. Liscum, L., R. M. Ruggietto, and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts. *J. Cell Biol.* 108:1625–1636.
39. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
40. Morris, M. D., C. Bhuvaneswaran, H. Shio, and S. Fowler. 1982. Lysosome lipid storage disorder in NCTR-BALB/c mice. I. Description of the disease and genetics. *Am. J. Pathol.* 108:140–149.
41. Nalbantoglu, J., O. Goncalves, and M. Meuth. 1983. Structure of mutant alleles at the *aprt* locus of Chinese hamster ovary cells. *J. Mol. Biol.* 167:575–594.
42. Pentchev, P. G., A. E. Gal, A. D. Booth, F. Omodeo-Sale, J. Fouks, B. A. Leumeyer, J. M. Quirk, G. Dawson, and R. O. Brady. 1980. A lysosomal storage disorder in mice characterized by a dual deficiency of sphingomyelinase and glucocerebrosidase. *Biochim. Biophys. Acta.* 619:669–679.
43. Pentchev, P. G., A. D. Boothe, H. S. Kruth, H. Weintroub, J. Stivers, and R. O. Brady. 1984. A genetic storage disorder in BALB/c mice with a metabolic block in esterification of exogenous cholesterol. *J. Biol. Chem.* 259:5784–5791.
44. Pentchev, P. G., M. E. Comly, H. S. Kruth, M. T. Vanier, D. A. Wenger, S. Patel, and R. O. Brady. 1985. A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. *Proc. Natl. Acad. Sci. USA.* 82:8247–8251.
45. Pentchev, P. G., M. E. Comly, H. S. Kruth, T. Tokoro, J. Butler, J. Sokol, M. Filling-Katz, J. M. Quirk, D. C. Marshall, S. Patel, M. T. Vanier, and R. O. Brady. 1987. Group C Niemann-Pick disease: faulty regulation of low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 1:40–45.
46. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* 83:346–356.
47. Poole, A. R. 1977. The detection of lysosomes by vital staining with acridine orange. *In* Lysosomes: A Laboratory Handbook. 2nd Ed. J. T. Dingle, editor. Elsevier/North-Holland, Amsterdam. 313–316.
48. Poorthuis, B. J. H. M., and K. W. A. Wirtz. 1982. Increased cholesterol esterification in rat liver microsomes by purified non-specific phospholipid transfer protein. *Biochim. Biophys. Acta.* 710:99–105.
49. Roberts, D. C. K., N. E. Miller, S. G. L. Price, D. Crook, C. Cortese, A. LaVille, L. Masana, and B. Lewis. 1985. An alternative procedure for incorporating radiolabeled cholesteryl ester into human plasma lipoproteins in vitro. *Biochem. J.* 226:319–322.
50. Ross, A. C., K. J. Go, J. G. Heider, and G. H. Rothblat. 1984. Selective inhibition of acyl coenzyme A: cholesterol acyltransferase by compound 58-035. *J. Biol. Chem.* 259:815–819.
51. Sege, R. D., K. Kozarsky, D. L. Nelson, and M. Kreiger. 1984. Expression and regulation of human low-density lipoprotein receptors in Chinese hamster ovary cells. *Nature (Lond.).* 307:742–745.
52. Shio, H., S. Fowler, C. Bhuvaneswaran, and M. D. Morris. 1982. Lysosome lipid storage disorder in NCTR-BALB/c mice. II. Morphologic and cytochemical studies. *Am. J. Pathol.* 108:150–159.
53. Skipski, V. P. 1972. Lipid composition of lipoproteins in normal and diseased states. *In* Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism. G. J. Nelson, editor. John Wiley & Sons, Inc., New York. 471–583.
54. Sokol, J., E. J. Blanchette-Mackie, H. S. Kruth, N. K. Dwyer, L. M. Amende, J. D. Butler, E. Robinson, S. Patel, R. O. Brady, M. E. Comly,

M. T. Vanier, and P. G. Pentchev. 1988. Type C Niemann-Pick disease. Lysosomal accumulation and defective intracellular mobilization of low density lipoprotein cholesterol. *J. Biol. Chem.* 263:3411–3417.
55. Stadtman, E. R. 1957. Preparation and assay of acylcoenzyme A and other thiol esters: use of hydroxylamine. *Methods Enzymol.* 3:931–941.
56. Urlaub, G., and L. A. Chasin. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. USA.* 77:4216–4220.
57. Van der Krift, T. P., J. Leunissen, T. Teerlink, G. P. H. Van Heusden, A. J. Verkleij, and K. W. A. Wirtz. 1985. Ultrastructural localization of a peroxisomal protein in rat liver using the specific antibody against the non-specific lipid transfer protein (sterol carrier protein 2). *Biochim. Biophys. Acta.* 812:387–392.