A second complementation class of cholesterol transport mutants with a variant Niemann-Pick type C phenotype

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Abstract We previously isolated Chinese hamster ovary cell mutants that were defective in the intracellular transport of low density lipoprotein (LDL)-derived cholesterol (Dahl, N. K., K. L. Reed, M. A. Daunais, J. R. Faust, and L. Liscum. 1992. *J. Biol. Chem.* **267:** 4889–4896). Several of the mutants exhibited the same biochemical phenotype as classical Niemann-Pick type C (NPC) fibroblasts. Complementation analysis between these mutants and other cholesterol transport mutants with a variant biochemical phenotype has defined two complementation classes. One class is characterized by expression of the classical NPC phenotype and may represent a true cholesterol transport mutant, while the second is characterized by expression of a variant NPC phenotype and may represent a signaling defect in LDL-sensitive homeostatic responses.—**Dahl, N. K., M. A. Daunais, and L. Liscum.** A second complementation class of cholesterol transport mutants with a variant Niemann-Pick type C phenotype. *J. Lipid Res.* 1994. **35:** 1839–1849.

Supplementary key words cholesterol • Niemann-Pick type C • lipoproteins • somatic cell mutants • complementation analysis

Niemann-Pick type C (NPC) is an autosomal recessive lysosomal storage disease characterized by the accumulation of unesterified cholesterol, sphingomyelin, glucosylceramide, and bis(monoacylglycero)phosphate (1). Variations in expression of the disease are characterized clinically by age of onset and development of symptoms, and biochemically by responsiveness to low density lipoprotein (LDL) (2, 3).

Biochemically, three subtypes of NPC are found. The classical phenotype shows levels of LDL-stimulation of esterification less than 10% of normal controls and increased lysosomal accumulation of unesterified cholesterol in response to LDL. A variant phenotype shows a slight defect in LDL-stimulation of cholesterol esterification and is indistinguishable from NPC heterozygotes (2). A third group appears intermediate between the classical and variant phenotypes (2). NPC patients with the most severe clinical presentation generally exhibit the classical biochemical phenotype while adult onset patients fall into the variant group (2). For the majority of patients, biochemical phenotype expression does not correlate well with severity or progression of NPC disease. However, expression of a phenotype seems restricted within families (2), which indicates that a genetic basis exists for the biochemical differences.

We have isolated Chinese hamster ovary (CHO) cell lines that are defective in the intracellular transport of LDL-derived cholesterol. Biochemical analysis shows that mutants 2-2 and 4-4 express a classical NPC phenotype (4). In addition to defective LDL-stimulation of cholesterol esterification, they show impaired LDL-suppression of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity, and defective movement of LDL-derived cholesterol to the plasma membrane. These defects in LDL-mediated regulatory responses and LDL-derived cholesterol movement are commensurate with a lysosomal accumulation of LDL-derived, unesterified cholesterol.

In this study, we performed complementation analysis between our mutants showing classical and variant phenotypes, as well as CT, a cholesterol transport mutant isolated by Cadigan, Spillane, and Chang (5). Our results show that there are at least two complementation classes of CHO cholesterol transport mutants. One mutant class is phenotypically similar to classic NPC, while the other is more representative of the variant NPC phenotype.

EXPERIMENTAL PROCEDURES

Materials

[9,10-^{3}H]oleic acid (10 Ci/mmol), cholesteryl [1-^{14}C]oleate (57 mCi/mmol), DL-3-hydroxy-3-[^{14}C]methylglutaryl-

Abbreviations: NPC, Niemann-Pick disease type C; LDL, low density lipoprotein; CHO, Chinese hamster ovary; HMG, 3-hydroxy-3-methylglutaryl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HAT^s oua^r, CHO cells that are sensitive to HAT medium and resistant to ouabain; [^{3}H]CL-LDL, LDL that is labeled with [^{3}H]cholesteryl linoleate; SUVs, small unilamellar vesicles.

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CoA (57.7 mCi/mmol), RS-[2-^{3}H]mevalonolactone (48.6 mCi/mmol), and [4-^{14}C]cholesterol (53.2 mCi/mmol) were purchased from DuPont-New England Nuclear. [1,2-^{3}H]cholesteryl linoleate (50 Ci/mmol) was obtained from Amersham. Mevinolin was a generous gift from A. Alberts (Merck Research Laboratory). 25-Hydroxycholesterol and cholesterol were added to media from ethanolic solutions. Thioguanine was dissolved in serum-free medium, and ouabain was dissolved in DMSO. Newborn calf serum and all other reagents were from Sigma or obtained as described previously (6).

Preparation of LDL, lipoprotein-deficient serum, [^{3}H]CL-LDL, media, and buffers

LDL was prepared by ultracentrifugation (7). Lipoprotein-deficient serum was prepared from newborn calf serum omitting the thrombin incubation (7). [^{3}H]CL-LDL was prepared essentially as described (8). Briefly, [^{3}H]cholesteryl linoleate (100 μCi) was evaporated with nitrogen, dissolved in 100 μl DMSO, and incubated with 2.5 mg of LDL at 40°C for 2 h in a final volume of 300 μl LDL buffer (150 mM NaCl, 0.3 mM EDTA, and 0.1 mM EGTA, pH 7.4). The reaction was dialyzed against LDL buffer and centrifuged (5 min, 4°C, 12,000 *g*). The supernatant averaged 18,000 cpm/nmol of total cholesteryl linoleate.

The following media were prepared: medium A [Ham's F-12 medium containing 5% (v/v) newborn calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES, pH 7.1]; medium B [medium A in which 5% (v/v) calf serum was replaced with 5% (v/v) lipoprotein-deficient serum]; medium C (medium B containing 20 μM mevinolin and 0.5 mM mevalonate); and medium D (medium A made with dialyzed newborn calf serum and containing 5 μg/ml thioguanine). Buffer A consisted of 150 mM NaCl and 50 mM Tris-chloride, pH 7.4.

Cultured cells

All cells were grown as monolayers in a humidified incubator (5% CO_2) at 37°C. A CHO cell line resistant to 5 μg/ml thioguanine and 1 mM ouabain was isolated as previously described (9), and is designated CHO (HAT^s oua^r). Resistance to thioguanine confers sensitivity to medium containing HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). Similarly, two 4-4 (HAT^s oua^r) cell lines were independently isolated and were resistant to 5 μg/ml thioguanine and 400 μM ouabain. A CT line resistant to 5 μg/ml thioguanine and 900 μg/ml Geneticin (Life Technologies, Inc.) was kindly provided by T. Y. Chang, Dartmouth University. CHO (HAT^s oua^r) cells were cultured in medium D containing 1 mM ouabain. 4-4 (HAT^s oua^r) cells were cultured in medium D containing 400 μM ouabain. CT cells were cultured in medium D containing 900 μg/ml Geneticin. Met-18b-2 cells were cultured in medium B containing 20 μM mevinolin and 200 μM mevalonate. All other cell lines were cultured in medium A. On day 0 of each experiment, monolayer stock flasks were trypsinized and seeded as indicated in the individual experiments.

Protein determination

Cell protein was determined by the method of Lowry et al. (10) using bovine serum albumin as a standard.

Selection of somatic cell heterokaryons

Somatic cell fusions were performed as given (11, 12) with the following modifications. On day 0, 3×10^6 of each cell type were plated together on 100-mm dishes in medium A. On day 1, the cells were washed with Earle's buffered saline solution (EBSS), the medium was aspirated completely, and the cells were treated for 1 min at room temperature with 50% (w/v) polyethylene glycol 1000 (Baker) in 75 mM HEPES, pH 7.4. Cells were then washed 5–7 times with 7 ml EBSS prior to refeeding with medium A. The next day, cells were trypsinized and replated in 80-cm^2 tissue culture flasks in medium A containing HAT and the appropriate selective agent. After 2 days, cells were trypsinized and 20% of the cells were replated onto each of three 100-mm dishes. Heterokaryon selection continued for a total of 2 weeks with further replating and feeding as required. Heterokaryons of CHO (HAT^s oua^r) cells were selected in medium A containing HAT and 1 mM ouabain; heterokaryons of 4-4 (HAT^s oua^r) cells were selected in medium A containing HAT and 400 μM ouabain. Heterokaryons of CT cells were selected in medium A containing HAT and 900 μg/ml Geneticin. All heterokaryon stocks were kept in selective medium prior to assay for complementation. Each fusion was performed twice, and heterokaryons from each fusion were assayed twice for complementation. Complementation with one 4-4 (HAT^s oua^r #1) line was confirmed by analysis with a second independently derived 4-4 (HAT^s oua^r) line, 4-4 (HAT^s oua^r #3).

Assay for complementation

Complementation was assayed by examining the ability of LDL to stimulate cholesterol esterification in heterokaryons. Cholesterol esterification was measured by determining the incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate and [^{3}H]triglyceride (described below). Six mutants, each from an independently mutagenized pool, were selected for analysis. In addition, analysis was performed with CT, a cholesterol transport mutant developed in T. Y. Chang's laboratory (5) and met-18b-2 cells, a CHO-derived cell line that exhibits defective LDL receptor activity (13). Assays of the heterokaryons formed by fusion of cholesterol transport mutants with met-18b-2 cells served as a positive control for complementation. All heterokaryons of met-18b-2 fusions were induced and as-

sayed in medium B containing 20 μM mevinolin and 5 μM mevalonate. This lower mevalonate concentration was essential, as met-18b-2 cells express increased mevalonate uptake ability and show stimulation of cholesterol esterification in response to 500 μM mevalonate (13), whereas parental CHO cells and the cholesterol transport mutants do not.

Incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate and [^{3}H]triglyceride

On day 0, cells were seeded into 6-well plates (40,000/35-mm well) in 1.5 ml medium A. On day 1, all monolayers were washed with 2 ml EBSS and refed 1 ml medium B. On the evening of day 2, cells were fed medium C. Experiments were initiated on day 3. After incubations as indicated in the figure legends, monolayers were pulsed with 100 μM [^{3}H]oleate (14,500 cpm/nmol) bound to albumin (7). After 3 h, cells were washed once for 7 min with buffer A, before cholesteryl [^{3}H]oleate and [^{3}H]triglyceride were isolated (14). After the lipids were extracted, the monolayers were dissolved in 0.1 N NaOH and aliquots were removed for protein determination. Cholesterol esterification is expressed as the (incorporation of [^{3}H]oleate into cholesteryl ester/incorporation of [^{3}H]oleate into triglycerides) × 1000.

3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase activity

On day 0, cells were seeded in 60-mm dishes (52,000–65,000 cells/dish) in 3 ml medium A. On day 1, monolayers were washed once with 4 ml EBSS, and refed 3 ml medium B. Experimental additions were made on day 3 as described in the figure legends. At time of harvest, cells were washed with buffer A at 4°C once quickly, once for 7 min, and once quickly. Cells were scraped in 5 ml buffer A per dish. Aliquots of the cell suspension were removed for assay, subjected to centrifugation (10,000 *g*, 5 min, 4°C), and cell pellets were stored at −20°C. Cell pellets were thawed and solubilized as described (13). The conversion of DL-3-hydroxy-3-[^{14}C]methylglutaryl-CoA (185 μM, 5,237 cpm/nmol) to [^{14}C]mevalonate and protein content of detergent-solubilized extracts were measured as described (9). One unit of HMG-CoA reductase activity represents the formation of 1 nmol [^{14}C]mevalonate/min.

Movement of LDL-derived cholesterol to the plasma membrane

On day 0, cells were seeded in 6-well plates at 40,000 cells/well in 1.5 ml medium A. On day 1, cells were washed once with 2 ml EBSS and refed 2 ml medium B. On day 3, the cells were pulsed for 2 h with 15 μg/ml [^{3}H]CL-LDL in medium B, washed twice with EBSS, and refed 1 ml medium B containing 25 μl of small unilamellar vesicles (SUVs). SUVs were prepared with a cholesterol/phosphatidylcholine molar ratio of 0.7 (15); 25 μl delivers 1 μmol cholesterol and 1.45 μmol L-α-phosphatidylcholine. Media and cells were harvested at staggered times; cellular and medium [^{3}H]cholesterol were quantified as described (15).

RESULTS

Complementation analysis between mutants 2-2 and 4-4

Somatic cell mutants exhibiting defects in LDL-cholesterol movement from lysosomes to plasma membrane were selected as described (4). Our initial biochemical analysis focused on mutants 2-2 and 4-4, which were derived from two independently mutagenized pools (the first digit of the clone designation indicates the pool of the mutagenized cells from which the clone was derived; the second number indicates the clone number). Mutants 2-2 and 4-4 express a remarkably similar biochemical phenotype, resembling classical NPC. One minor difference between the lines is seen in the ability of LDL to suppress HMG-CoA reductase activity; 4-4 shows no suppression while 2-2 shows partial suppression of HMG-CoA reductase activity in response to LDL (4, 16). This may indicate that mutant 2-2 is a "leaky" version of 4-4 (representing allelic differences in expression of the defect) or that 2-2 and 4-4 are in different complementation classes. The experiment shown in **Fig. 1** was designed to select between these competing hypotheses. The activation of acyl CoA:cholesterol acyltransferase (ACAT), which catalyzes the esterification of cholesterol, was chosen as the complementation test because it is the homeostatic response that is most profoundly defective in the cholesterol transport mutants.

4-4 (HAT^s oua^r #1) cells were fused to both parental CHO and 2-2 cells. Heterokaryons were selected and assayed for the ability of LDL to stimulate cholesterol esterification (Fig. 1A). In medium C without LDL, the amount of cholesterol esterified was similar in all cell lines, ranging from 4 to 7. LDL at 40 μg/ml stimulated cholesterol esterification to 158 and 4 in CHO and mutant 4-4 cells, respectively. CHO/4-4 heterokaryons gave an intermediate value of 85, which suggests the defect in 4-4 is co-dominant. However, the maximal fold stimulation (esterification rate with 40 μg/ml LDL/esterification in medium C alone) was similar in CHO and CHO/4-4 cells (22.6 and 17.0, respectively), which suggests that the mutation in 4-4 is recessive.

In contrast to the stimulation of esterification seen in CHO/4-4, LDL did not stimulate cholesterol esterification in the 4-4/2-2 heterokaryons. The initial value of 4 in medium C remained unchanged with all additions of LDL, indicating that there is no complementation between mutants 2-2 and 4-4. In a separate experiment, CHO/2-2 cells gave an intermediate value exactly like

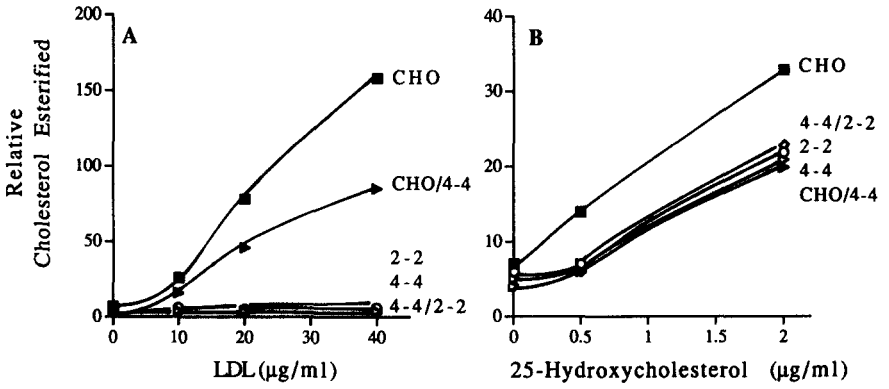

Fig. 1. Stimulation of cholesterol esterification by LDL (panel A) and 25-hydroxycholesterol (panel B). Parental CHO cells (■), mutant 2-2 (○), mutant 4-4 (▷), 4-4/CHO heterokaryons (▶), and 4-4/2-2 (◇) heterokaryons were grown as described under Experimental Procedures. On day 3, each monolayer received 1 ml medium C containing the indicated concentration of LDL or 25-hydroxycholesterol (with 10 μg/ml cholesterol). After 5 h incubation, cells were pulse-labeled with [^{3}H]oleate for 3 h. The cellular content of cholesteryl [^{3}H]oleate and [^{3}H]triglyceride was determined as described under Experimental Procedures. Relative cholesterol esterified represents the (incorporation of [^{3}H]oleate into cholesteryl oleate/incorporation of [^{3}H]oleate into [^{3}H]triglycerides) × 1000. Each data point represents the average of two wells.

CHO/4-4 (data not shown). Thus the lack of complementation between 2-2 and 4-4 is not due to the expression of a dominant defect in either mutant.

Heterokaryons from LDL receptor-defective met-18b-2 cells were used to validate the complementation assay. As met-18b-2 cells lack functional LDL receptors, LDL fails to elicit any regulatory responses in this mutant cell line. As both mutant 2-2 and 4-4 show normal uptake and hydrolysis of LDL (4), they are clearly defective at a different genetic locus than met-18b-2 and should show complementation with met-18b-2 cells. In three experiments, LDL at 40 μg/ml stimulated cholesterol esterification in CHO, 4-4, and met-18b-2 cells to 98.9 ± 15.2, 2.5 ± 0.4, and 2.7 ± 0.7, respectively. Cholesterol transport-defective 4-4 cells were fused to LDL receptor-defective met-18b-2 cells. LDL at 40 μg/ml stimulated cholesterol esterification in 4-4/CHO, 4-4/2-2, and 4-4/4-4 heterokaryons to 112, 6, and 6, respectively. LDL at 40 μg/ml stimulated cholesterol esterification in 4-4/met-18b-2 heterokaryons to 32, indicating that complementation can be detected by our assay.

25-Hydroxycholesterol stimulated cholesterol esterification in all cell lines and heterokaryons in this assay (Fig. 1B), indicating that the failure to demonstrate complementation was not due to a defect in ACAT activity in these cells.

4-4 (HAT^s oua^r #1) cells were fused to four additional mutants (1-2, 3-6, 5-1, and 10-3) from independently mutagenized pools (4). Mutants 1-2, 5-1, and 10-3 showed the same result as 2-2. None expressed dominant defects and none showed complementation with 4-4, as measured by the ability of LDL to stimulate cholesterol esterification (data not shown).

Complementation analysis between mutants 3-6 and 4-4

Mutant 3-6 was unusual among the mutants selected for analysis because it showed a slight increase in cholesterol esterification in response to LDL. If this slight amount of LDL-cholesterol transport or signaling was due to allelic differences in the gene defective in 4-4, no further increase in LDL-stimulation of esterification would be expected in 4-4/3-6 heterokaryons. The experiment shown in **Fig. 2** was designed to assess the complementation class of 3-6 relative to 4-4.

LDL-stimulation of cholesterol esterification is shown in Fig. 2A. All cell lines and heterokaryons show relatively similar cholesterol esterification in medium C without an addition of LDL, ranging from 4 to 12. Parental CHO cells show a stimulation of cholesterol esterification to 155 with 40 μg/ml LDL. Mutant 4-4 shows no LDL-stimulation of cholesterol esterification (to 4 with 40 μg/ml LDL) while 3-6 shows a slight response (to 30 with 40 μg/ml LDL).

CHO/3-6 heterokaryons were derived by fusing CHO (HAT^s oua^r) cells with 3-6 cells, while 4-4/CHO and 4-4/3-6 cells were derived by fusing 4-4 (HAT^s oua^r #1) cells with parental CHO and 3-6 cells. LDL at 40 μg/ml stimulates cholesterol esterification in CHO/3-6 and 4-4/CHO cells to 81 and 86, respectively, which is approximately 50% of the parental CHO response, suggesting that the mutations are co-dominant. However, when the basal rate of esterification was taken into account, the fold-stimulation of esterification was 12.9, 11.6, and 17.2 for CHO, CHO/3-6, and CHO/4-4 cells, respectively, suggesting that the 4-4 and 3-6 mutations are recessive.

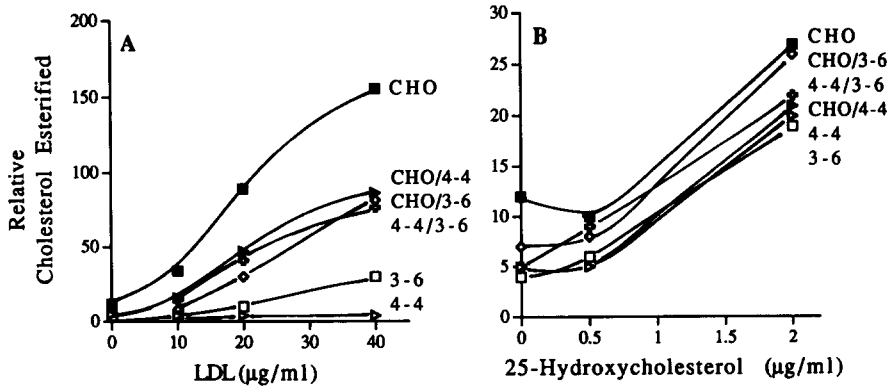

Fig. 2. Stimulation of cholesterol esterification by LDL (panel A) and 25-hydroxycholesterol (panel B). Parental CHO cells (■), mutant 3-6 (□), mutant 4-4 (▷), 4-4/CHO heterokaryons (▶), CHO/3-6 heterokaryons (◇), and 4-4/3-6 heterokaryons (open cross) were grown as described under Experimental Procedures. On day 3, each monolayer received 1 ml medium C containing the indicated concentration of LDL or 25-hydroxycholesterol (with 10 μg/ml cholesterol). After 5 h incubation, cells were pulse-labeled with [^{3}H]oleate for 3 h. The cellular content of cholesteryl [^{3}H]oleate and [^{3}H]triglyceride was determined as described under Experimental Procedures. Relative cholesterol esterified represents the (incorporation of [^{3}H]oleate into cholesteryl oleate/incorporation of [^{3}H]oleate into [^{3}H]triglycerides) × 1000. Each data point represents the average of two wells.

The 4-4/3-6 heterokaryons, by contrast, show greater LDL-stimulation of esterification than either 4-4 or 3-6 alone (76, 30, and 4 at 40 μg/ml LDL for the 4-4/3-6, 3-6, and 4-4, respectively), indicating complementation between the two lines. The LDL-stimulation of esterification is virtually identical in 4-4/3-6 compared to CHO/3-6 or 4-4/CHO, indicating maximal complementation of the defect in each line.

25-Hydroxycholesterol at 2 μg/ml stimulates cholesterol esterification in all cell lines and heterokaryons (Fig. 2B), indicating that ACAT is functional in all of the cell lines.

Confirmation of complementation analysis between 4-4 and 3-6

To confirm that 4-4 and 3-6 are in different complementation groups, identical fusions were performed with an independently mutagenized and selected 4-4 cell line resistant to thioguanine and ouabain (4-4 HAT^s oua^r #3). 4-4 (HAT^s oua^r #3) cells were fused to parental CHO cells and mutants 3-6, 1-2, and 5-1; heterokaryons were assayed for LDL-stimulation of cholesterol esterification (**Fig. 3A**). All heterokaryons showed similar cholesterol esterification in medium C without an addition of LDL, ranging from 3 to 4. LDL at 40 μg/ml stimulated esterification in 4-4/CHO, 4-4/1-2, and 4-4/5-1 cells to 62, 4, and 4, respectively. This indicates that mutants 1-2 and 5-1 are in the same complementation group as 2-2 and 4-4. However, 40 μg/ml LDL stimulated esterification to 36 in 4-4/3-6 cells, indicating that complementation occurs between 4-4 and 3-6.

25-Hydroxycholesterol at 2 μg/ml stimulates esterification virtually identically in all cell lines (Fig. 3B) indicating that ACAT is functional in all heterokaryons.

Complementation analysis between CT and 4-4

Complementation analyses were also performed between our mutants and CT, an independently derived cholesterol transport mutant obtained from T. Y. Chang (5). CT was fused to parental CHO, 2-2, 4-4, and met-18b-2 cells. The CT/met-18b-2 heterokaryons are a positive control, as met-18b-2 cells are defective in LDL-receptor activity (13) and express a defect that is distinct from CT, a cholesterol transport mutant that shows normal LDL uptake and hydrolysis (5). LDL-stimulation of cholesterol esterification in the heterokaryons is shown in **Fig. 4A.** All heterokaryons show a cholesterol esterification of 10 in medium C alone. CT/CHO heterokaryons show cholesterol esterification of 129 with 40 μg/ml LDL, while CT/2-2 and CT/4-4 heterokaryons express values of 18 and 20, respectively. In contrast, CT/met-18b-2 heterokaryons show LDL-stimulation of cholesterol esterification to 82.

25-Hydroxycholesterol at 2 μg/ml stimulates esterification virtually identically in all cell lines (Fig. 4B), indicating that ACAT is functional in all heterokaryons.

Complementation analysis between CT and 3-6

Next we assessed complementation in CT/3-6 heterokaryons to confirm that 4-4 and 3-6 are in distinct complementation groups, as earlier experiments indicated that 4-4 and CT were in the same group. Additionally, complementation in CT/3-6 heterokaryons would verify that our results were not an artifact of the selection procedure, as CT/3-6 heterokaryons required different selection conditions than 4-4/3-6 heterokaryons. The results of this assay are shown in **Fig. 5.**

Figure 5A shows the results of LDL-stimulation of cho-

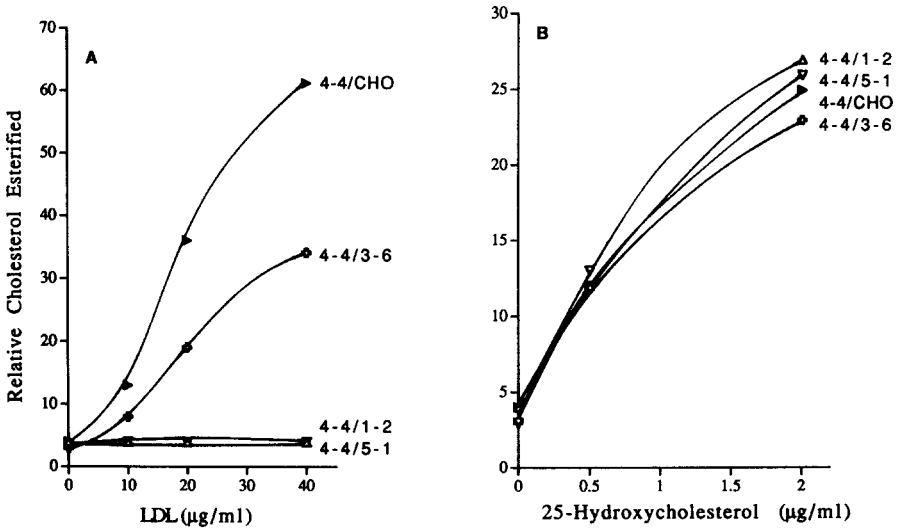

Fig. 3. Stimulation of cholesterol esterification by LDL (panel A) and 25-hydroxycholesterol (panel B). 4-4/CHO heterokaryons (▶), 4-4/3-6 heterokaryons (open cross), 4-4/1-2 heterokaryons (△), and 4-4/5-1 heterokaryons (▽) were grown as described under Experimental Procedures. On day 3, each monolayer received 1 ml medium C containing the indicated concentration of LDL or 25-hydroxycholesterol (with 10 μg/ml cholesterol). After 5 h incubation, cells were pulse-labeled with [^{3}H]oleate for 3 h. The cellular content of cholesteryl [^{3}H]oleate and [^{3}H]triglyceride was determined as described under Experimental Procedures. Relative cholesterol esterified represents the (incorporation of [^{3}H]oleate into cholesteryl oleate/incorporation of [^{3}H]oleate into [^{3}H]triglycerides) × 1000. Each data point represents the average of two wells.

lesterol esterification in CHO, CT, 3-6, and CT/3-6 heterokaryons. All cell lines and heterokaryons showed similar cholesterol esterification in medium C alone, ranging from 4 to 13. Cholesterol esterification was stimulated to 142 with 40 μg/ml LDL in the parental CHO line. However, the response to 40 μg/ml LDL was less in 3-6 and CT cells, at 31 and 62, respectively. The CT lines, while expressing a clear defect, always showed a greater LDL-mediated response than mutants 2-2, 4-4, or 3-6, consistent with the results of Cadigan et al. (5). LDL-

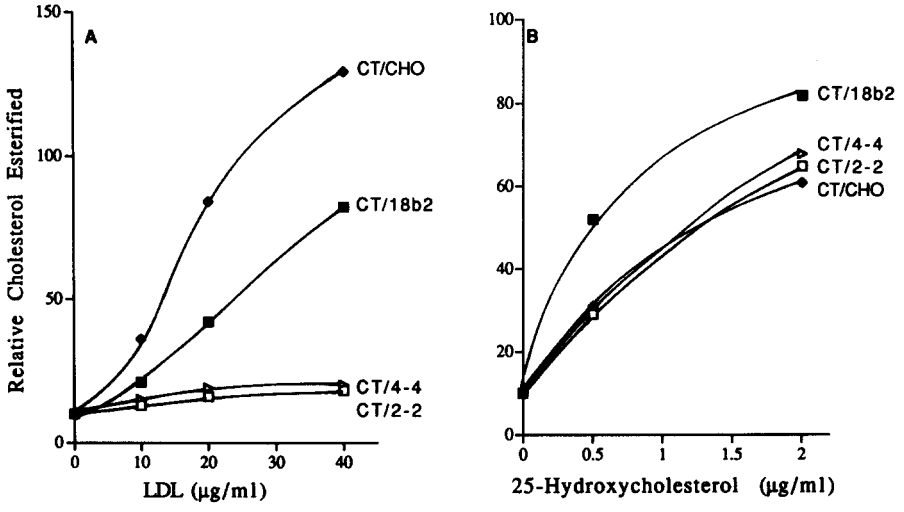

Fig. 4. Stimulation of cholesterol esterification by LDL (panel A) and 25-hydroxycholesterol (panel B). CT/CHO heterokaryons (♦), CT/met-18b-2 heterokaryons (■), CT/4-4 heterokaryons (▷), and CT/2-2 heterokaryons (□) were grown as described under Experimental Procedures. On day 3, each monolayer received 1 ml medium C containing the indicated concentration of LDL or 25-hydroxycholesterol (with 10 μg/ml cholesterol). After 5 h incubation, cells were pulse-labeled with [^{3}H]oleate for 3 h. The cellular content of cholesteryl [^{3}H]oleate and [^{3}H]triglyceride was determined as described under Experimental Procedures. Relative cholesterol esterified represents the (incorporation of [^{3}H]oleate into cholesteryl oleate/incorporation of [^{3}H]oleate into [^{3}H]triglycerides) × 1000. Each data point represents the average of two wells.

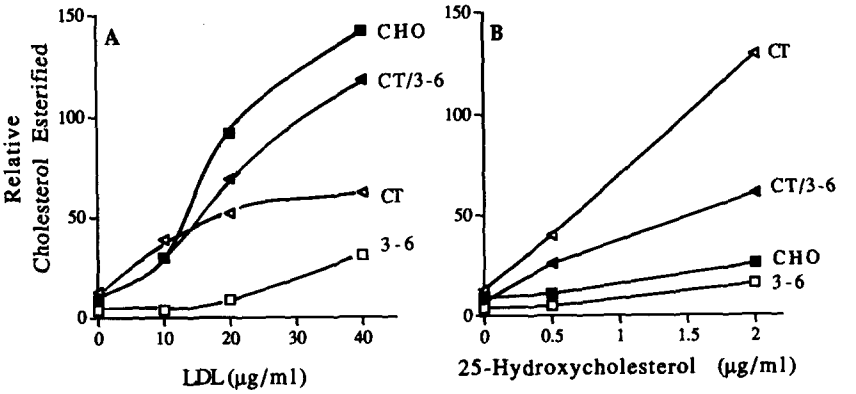

Fig. 5. Stimulation of cholesterol esterification by LDL (panel A) and 25-hydroxycholesterol (panel B). Parental CHO cells (■), mutant 3-6 (□), mutant CT (◁), and CT/3-6 heterokaryons (◀) were grown as described under Experimental Procedures. On day 3, each monolayer received 1 ml medium C containing the indicated concentration of LDL or 25-hydroxycholesterol (with 10 μg/ml cholesterol). After 5 h incubation, cells were pulse-labeled with [^{3}H]oleate for 3 h. The cellular content of cholesteryl [^{3}H]oleate and [^{3}H]triglyceride was determined as described under Experimental Procedures. Relative cholesterol esterified represents the (incorporation of [^{3}H]oleate into cholesteryl oleate/incorporation of [^{3}H]oleate into [^{3}H]triglycerides) × 1000. Each data point represents the average of two wells.

stimulation of cholesterol esterification to 118 in CT/3-6 heterokaryons indicated complementation between 3-6 and CT lines.

Figure 5B shows the results of 25-hydroxycholesterol stimulation of cholesterol esterification in these cell lines and heterokaryons. CHO and 3-6 cells showed esterification levels comparable to those presented in Figs. 1 and 2; however, CT showed much higher 25-hydroxycholesterol stimulation of cholesterol esterification than any other line, perhaps reflecting the higher ACAT activity in CT cells (5). CT/3-6 heterokaryons reflected an intermediate value of stimulation of cholesterol esterification, between the CT level and the 3-6 level, confirming that these cells are true heterokaryons of the two cell lines.

Movement of LDL-derived cholesterol to the plasma membrane

Mutant 3-6 is clearly in a different complementation class from mutant 4-4 or CT. We wanted to characterize other aspects of LDL-derived cholesterol transport in 3-6 to determine which aspects are similar to the classic NPC phenotype. Both classic NPC (15) and the 2-2 and 4-4 mutants (4) showed delayed movement of LDL-derived cholesterol from lysosomes to the plasma membrane. Thus, we wanted to assess this bulk-flow movement in the new class of cholesterol transport mutant. In addition, assaying movement of LDL-derived cholesterol to the plasma membrane allowed us to determine whether 3-6 is defective in LDL receptor activity or cholesteryl ester hydrolysis.

The relative rate of movement of LDL-derived [^{3}H]cholesterol to the plasma membrane was assessed in parental and mutant CHO cells (15). Cells were pulsed for 2 h with [^{3}H]CL-LDL followed by chase incubations for various times. Movement of [^{3}H]cholesterol from lysosomes to the plasma membrane was determined by quantitating the amount of [^{3}H]cholesterol desorbed into the medium and trapped by SUVs during the chase incubations.

We found that all three cell lines internalized and hydrolyzed similar amounts of [^{3}H]CL-LDL during the 2-h pulse (**Table 1**). Despite similar rates of LDL uptake and lysosomal hydrolysis, the amount of [^{3}H]cholesterol that desorbed from the cells to the medium varied in the three cell lines (**Fig. 6**). In CHO cells, there was a time-dependent increase in LDL-derived [^{3}H]cholesterol in the medium such that 8.6% of the [^{3}H]cholesterol had desorbed by 5 h. Consistent with our previous results (4), there was only a modest increase in desorption of [^{3}H]cholesterol from mutant 4-4 during the 5-h time course. However, cholesterol movement in mutant 3-6 was

TABLE 1. Cellular content of LDL-derived [^{3}H]cholesteryl ester and [^{3}H]cholesterol

Cell Line	Cholesteryl Ester	Cholesterol	Total (CE + C)	Hydrolysis
	nmol/mg			%
CHO	1.63	1.28	2.91	44
4-4	2.01	1.53	3.54	43
3-6	1.43	1.05	2.48	42

Parental CHO cells, mutant 4-4, and mutant 3-6 were grown as described under Experimental Procedures. On day 3, cells were pulsed with 15 μg/ml [^{3}H]CL-LDL in medium B for 2 h. Cellular [^{3}H]cholesteryl ester and [^{3}H]cholesterol were determined as described under Experimental Procedures, and are expressed as nmol/mg of cellular protein. The hydrolysis of LDL-derived [^{3}H]cholesteryl ester is calculated as [^{3}H]cholesterol/([^{3}H]cholesteryl ester + [^{3}H]cholesterol), and is expressed as a percentage.

greater than that in 4-4 cells, with 7.8% of the LDL-[^{3}H]cholesterol desorbed by 5 h. Thus mutant 3-6 is not defective in LDL uptake or cholesterol ester hydrolysis, and is only slightly defective in transporting LDL-derived cholesterol to the plasma membrane.

Suppression of HMG-CoA reductase activity by LDL and 25-hydroxycholesterol

LDL leads to the suppression of HMG-CoA reductase activity in parental CHO cells. This LDL-suppression is completely defective in 4-4 cells and is impaired in 2-2 cells (4, 16), both of which express allelic gene defects. As 3-6 represents another class of cholesterol transport mutant, we wanted to assay the ability of LDL to suppress HMG-CoA reductase activity in these cells.

Figure 7 demonstrates the ability of LDL to suppress HMG-CoA reductase activity in CHO, 3-6, and 4-4 cells. HMG-CoA reductase activity was very similar in CHO, 4-4, and 3-6 cells, at 2.48, 2.71, and 2.59 units/mg, respectively. LDL does not suppress HMG-CoA reductase activity in 4-4 cells at any concentration, in agreement with previous results (4). Both CHO and 3-6 cells showed a clear concentration-dependent suppression of HMG-CoA reductase activity, reaching maximal suppression with 8 μg/ml LDL. However, CHO cells plateaued at 30% maximal activity while 3-6 cells plateaued at 60% maximal activity.

LDL leads to the suppression of HMG-CoA reductase by two mechanisms, acceleration of degradation of existing protein and suppression of gene transcription. The partial response seen in the 3-6 cells is highly evocative of the response to sterols seen in SRD-3 (17) and TR-36 (18) cells, which suppress HMG-CoA reductase activity only at the level of acceleration of protein turnover. The partial suppression seen in 3-6 may be due to activation of only one pathway or to a partial defect in both pathways leading to suppression. Mutant 2-2, an allelic variant of 4-4, shows partial LDL-suppression of HMG-CoA reductase activity which is solely reflective of acceleration of protein turnover (16). Mutant 3-6 may be similar to 2-2 in this regard suggesting that two gene mutations that profoundly affect LDL-cholesterol signaling to ACAT have no effect on LDL-cholesterol-mediated turnover of HMG-CoA reductase.

In the same experiment, 0.1 μg/ml of 25-hydroxycholesterol suppressed HMG-CoA reductase activity to 19, 34, and 37% of control in CHO, 3-6, and 4-4 cells, respectively. Clearly then, the differences in LDL-suppression of HMG-CoA reductase activity in 4-4 and 3-6 are LDL-specific and are not due to inherent differences in the enzyme as both respond nearly identically to 25-hydroxycholesterol (37% vs. 34%). It is unclear why there is a modest difference between the response of the parental CHO cells and the two mutant lines. In previous experiments, CHO and 4-4 cells responded nearly identically to 25-hydroxycholesterol suppression of HMG-CoA reductase (4). The difference seen here may reflect subtle differences in seeding or growth of the cells.

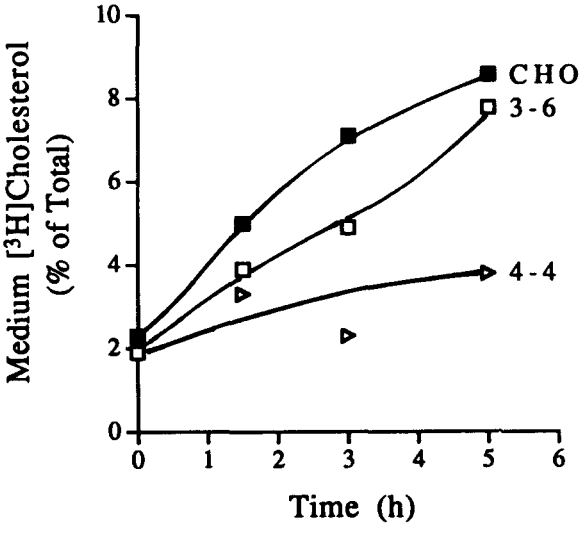

Fig. 6. Movement of LDL-derived cholesterol to the plasma membrane. Parental CHO cells (■), mutant 3-6 (□), and mutant 4-4 (▷) were grown as described under Experimental Procedures. On day 3, cells were pulsed with 15 μg/ml [^{3}H]CL-LDL in medium B for 2 h. Monolayers were then washed and refed 1 ml medium B containing 25 μl of SUVs. The media were removed and cells were harvested at staggered times. Cellular [^{3}H]cholesterol and medium [^{3}H]cholesterol were determined as described under Experimental Procedures. The LDL-derived, medium [^{3}H]cholesterol is expressed as a percentage of the total cellular and medium [^{3}H]cholesterol at each time point. Each data point represents the average of three wells.

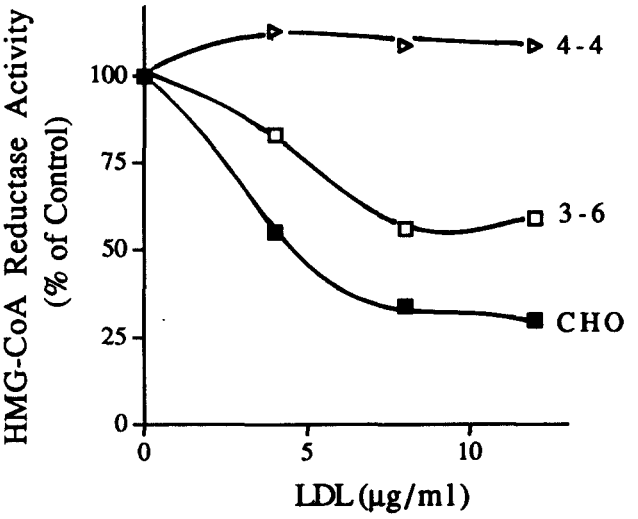

Fig. 7. LDL-mediated suppression of HMG-CoA reductase activity. *Parental CHO cells* (■), *mutant 3-6* (□), *and mutant 4-4* (▷) were grown as described under Experimental Procedures. On day 3, each monolayer received 3 ml medium B containing the indicated concentration of LDL. After 8 h, cells were harvested, and HMG-CoA reductase activity was measured as described under Experimental Procedures. Each data point represents the average of two dishes. The control values were 2.48, 2.71, and 2.59 units/mg for CHO, 4-4, and 3-6 cells, respectively.

DISCUSSION

Previously we described a selection protocol developed to isolate CHO cell mutants that are defective in transporting LDL-derived cholesterol from lysosomes to the plasma membrane (4). CHO cells were chosen for this study because they are functionally haploid for many genes (19). The hemizygosity may be due to gene inactivation, segregation, or deletion of loci. The ease of mutant selection, reversion, and complementation analysis makes them among the most widely used cell line for somatic cell genetics of cholesterol metabolism (20). The cell lines that we selected most likely express mutations in haploid genes.

The battery of mutants isolated included two mutants, 2-2 and 4-4, with a biochemical phenotype identical to the classical NPC phenotype. Classical NPC fibroblasts show LDL-stimulation of cholesterol esterification that is less than 10% of normal (2). In addition, they show other delayed or absent regulatory responses specific to LDL-derived cholesterol (6, 15, 21, 22) and delayed movement of LDL-derived cholesterol to the plasma membrane (15). The impaired regulatory responses and bulk movement are commensurate with an accumulation of LDL-derived, unesterified cholesterol within lysosomes (15, 22), suggesting that the classical NPC phenotype may be due to the inability of LDL-derived cholesterol to exit lysosomes. The lysosomal accumulation results in intense filipin staining of intracellular compartments (2, 23, 24). Genetic linkage analysis has localized the NPC mutation to human chromosome 18 (25).

Recently, an NPC variant has been described (2, 3) in which the biochemical phenotype resembles an intermediate state between normal and classical NPC fibroblasts. Fibroblasts from NPC patients expressing the variant biochemical phenotype show partial LDL-mediated stimulation of cholesterol esterification and partial down-regulation of cholesterol synthesis (3). In addition, LDL-derived cholesterol is transported normally to the plasma membrane in these cells as measured by cholesterol oxidase treatment of glutaraldehyde-fixed cells (3). Biochemically, fibroblasts from variant NPC patients resemble those of NPC heterozygotes, who are not clinically affected; however, the disease progression of variant NPC patients is identical to that of patients expressing the classical NPC phenotype (2).

Among our collection of cholesterol transport mutants are a minority that do not resemble the classical NPC phenotype. Some, such as 3-6, more closely resemble the variant NPC phenotype, which is relatively rare. Eighty-six % of a population of 125 patients expressed the classic phenotype while only about 7% expressed the variant phenotype (2). Thus, our mutant population may reflect the prevalence of these two defective genotypes within the NPC patient population.

We performed complementation analysis with six of our cholesterol transport mutants, as well as with CT, a cholesterol transport mutant developed in T. Y. Chang's laboratory (5). Six of the seven mutants are in the same complementation class and express the classical NPC phenotype. The remaining mutant 3-6 represents a separate complementation class with the biochemical characteristics of fibroblasts from a NPC heterozygote. None of the mutants express dominant gene defects, as hybrids derived from fusing the mutants to parental CHO cells all show LDL-stimulation of esterification. However, it is unclear whether the defects are co-dominant or recessive. Comparison of the maximal level of esterification attained in response to LDL suggests that 3-6 and 4-4 mutations are co-dominant. This may indicate that the gene product is rate-limiting or that the presence of mutant gene product interferes with the function of the normal gene product (26). However, comparison of the maximal fold stimulation of esterification, which accentuates subtle differences in the basal level of cholesterol esterification, suggests that both mutations may be recessive. Further knowledge of the defective proteins in these cell lines will resolve this issue.

Could 3-6 be expressing a "leaky" version of the classical NPC phenotype seen in 4-4 due to allelic differences at the same genetic locus rather than expression of two unique genetic loci? If 3-6 and 4-4 (or 3-6 and CT) were expressing allelic variants of the same gene, their hybrids should only show the level of LDL-stimulation of cholesterol esterification of the "leakiest" parent line. In the case of 4-4/3-6 hybrids, this would mean stimulation of cholesterol esterification to the level seen in 3-6. In CT/3-6 hybrids, this would mean stimulation of esterification to the level seen in CT. However, both the 4-4/3-6 and CT/3-6 hybrids show LDL-stimulation of cholesterol esterification that is substantially higher than that seen in the leakiest parent, which indicates that true complementation is occurring.

Mutant 3-6 exhibits profoundly defective LDL-stimulation of cholesterol esterification, impaired LDL-suppression of HMG-CoA reductase, and near normal movement of LDL-derived cholesterol from lysosomes to plasma membranes. The most likely explanation for these intermediate responses is that 3-6 is a CHO model for the variant NPC phenotype. As variant NPC patients are clinically affected similar to classical NPC patients, serious alterations in cell function must result from the subtle transport defect combined with impaired regulatory functions.

The results presented in this paper are of a partial analysis of the entire collection of mutants. To date we have only found one mutant that is in the 3-6 complementation class. Thus, our conclusions about the phenotype of this complementation class are based on analysis of only one mutant. Further characterization of other mutants in this class will determine whether 3-6 is truly

representative of this new class, or whether the allelic variations in 3-6 mask the true phenotype of this class. Complementation analysis between CHO mutants and NPC fibroblasts expressing classical and variant biochemical phenotypes would conclusively determine whether mutant 4-4 shares the same gene defect as classical NPC, and whether mutant 3-6 shares the same gene defect as variant NPC. This analysis would also determine whether two mutations cause NPC. Complementation between immortalized CHO cells and primary human fibroblasts are technically difficult as human chromosomes are preferentially shed from these heterokaryons (27). Protocols that overcome this difficulty are currently being investigated.

Why would two proteins (encoded by the two different genetic loci) be required for the proper intracellular transport of LDL-derived cholesterol? Part of the answer may be supplied by examining the filipin staining pattern of cells expressing the variant NPC phenotype. These cells show a filipin fluorescence pattern that is intermediate between normal and classical NPC fibroblasts (2, 3). In normal fibroblasts, LDL enriches filipin staining in lysosomes and the *cis,* medial, and *trans* faces of the Golgi (28) perhaps reflecting the mixing of LDL-cholesterol with other cellular pools and the overall cholesterol enrichment of the cells. In classical NPC fibroblasts, in contrast, after incubation with LDL there is excess accumulation of cholesterol in lysosomes, and an earlier enrichment in the Golgi associated with the *trans* face (28). This may suggest that in classical NPC the primary defect is in the Golgi, and that a block in this stage of cholesterol transport leads to an accumulation in lysosomes, or that a bulk flow pathway for cholesterol from lysosomes is defective and a lower capacity pathway associated with the Golgi becomes saturated.

Mutant 4-4, which phenotypically resembles classical NPC, may be defective in a protein required for cholesterol efflux from lysosomes, possibly a cholesterol transporter or packager. The slight LDL-cholesterol transport seen in 2-2 (16) may reflect a more moderate defect or a greater compensation by a secondary cholesterol transport pathway. In classical NPC both bulk flow of LDL-cholesterol and signaling in response to LDL are defective. However, expression of the variant NPC phenotype clearly demonstrates that there is independent regulation of bulk flow and signaling, as 3-6 shows nearly normal bulk flow (as demonstrated by movement of LDL-derived cholesterol to the plasma membrane) coupled to an almost completely defective signaling response (LDL-activation of ACAT).

Variant NPC cells incubated with LDL show an intermediate filipin fluorescence pattern with a modest accumulation of lysosomal cholesterol (2, 3). This suggests that the primary defect in variant cells may not be due to the inability of unesterified, LDL-derived cholesterol to exit lysosomes. Rather, the milder lysosomal cholesterol accumulation within these compartments may be due to a block further along the transport pathway (outside of the lysosome), which results in a backup in the transport pathway and subsequent accumulation in lysosomes. The defect expressed in 3-6 could reflect the need for a protein to direct the effluxed cholesterol to proper intracellular targets or to relay intracellular regulatory signals.

One model proposed previously (16) is that two pathways exist for intracellular movement of LDL-derived cholesterol. One may be a protein-mediated, high capacity/bulk movement pathway responsible for cholesterol movement to the plasma membrane. The other may be a low capacity/signaling pathway responsible for eliciting regulatory responses. When LDL is internalized by normal cells, the high capacity pathway may transport LDL-cholesterol from lysosomes to the plasma membrane. An increase in cellular cholesterol pools (29) may activate the low capacity/signaling pathway. Defects in bulk movement leads to cholesterol accumulation in lysosomes and Golgi membranes, as well as impaired ACAT activation and transcriptional control. This is represented by mutants such as 4-4 which express the classical NPC phenotype.

Mutant 3-6, with a variant NPC phenotype, may also be defective in some aspect of bulk cholesterol movement. It may be that the 3-6 and 4-4 proteins act coordinately to regulate cholesterol transport and/or homeostasis. However, as signaling is more profoundly impaired than transport in 3-6, it is more likely that this mutant may be defective in the low capacity/signaling pathway. Thus LDL-cholesterol may reach the plasma membrane, but the signal that basal cholesterol levels are rising is not sent to ACAT and other homeostatic barometers. While the defective 3-6 protein may play a dual role in cholesterol homeostasis and transport, its impact on maintaining homeostasis would be greater than its contribution to intracellular cholesterol transport.

A final intriguing possibility must be considered. The defective protein in 3-6 may be the cholesterol sensor or receptor in cells. It may be the protein through which the signal to activate ACAT or suppress HMG-CoA reductase is mediated.

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