Lack of requirement for sterol carrier protein-2 in the intracellular trafficking of lysosomal cholesterol

William J. Johnson[1,*] **and Michael P. Reinhart**[2,†]

Department of Biochemistry,* The Medical College of Pennsylvania, 2900 Queen Lane, Philadelphia, PA 19129 and Eastern Regional Research Center,† United States Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19118

Abstract Previous work has established that the absence of peroxisomes, as occurs in Zellweger syndrome, is accompanied by the absence of cellular sterol carrier protein-2 (SCP_2). In the present study, Zellweger-syndrome fibroblasts and peroxisome-deficient CHO-ZR78 cells were used to study the role of SCP_2 in the intracellular transport of low density lipoprotein (LDL)-derived lysosomal cholesterol. By immunoblotting, peroxisome-deficient cells were confirmed to contain either no detectable SCP_2 or far less SCP_2 than corresponding normal cells. To monitor the transport of lysosomal cholesterol to the plasma membrane, we measured efflux of lysosomal cholesterol to HDL_3 or phospholipid vesicles. SCP_2-deficient cells, in comparison to normal cells, demonstrated little or no impairment in this efflux, suggesting that SCP_2 is not required for the efficient delivery of lysosomal cholesterol to the plasma membrane. To examine the role of SCP_2 in the delivery of lysosomal cholesterol to acyl-CoA:cholesterol acyltransferase (ACAT) in the rough endoplasmic reticulum (RER), the lysosomal and whole-cell cholesterol pools were differentially labeled, and then the ACAT-mediated esterification of each pool was measured in response to an 8-h incubation with native LDL. For both cholesterol pools, esterification was stimulated by LDL, and the responses in normal and Zellweger cells were similar, demonstrating that SCP_2 is required for neither the stimulation of ACAT that follows LDL uptake nor for the transport of lysosomal cholesterol to the RER. These findings suggest that some major aspects of lysosomal cholesterol trafficking in cells can occur by mechanisms not involving SCP_2.—**Johnson, W. J., and M. P. Reinhart.** Lack of requirement for sterol carrier protein-2 in the intracellular trafficking of lysosomal cholesterol. *J. Lipid Res.* 1994. **35:** 563-573.

Supplementary key words nonspecific lipid transfer protein • fibroblasts • Chinese hamster ovary cells • low density lipoprotein • high density lipoprotein • peroxisomes • low density lipoprotein receptor • Zellweger syndrome • sterol • intracellular transport • acyl-CoA:cholesterol acyltransferase • acid cholesteryl ester hydrolase

Cellular cholesterol homeostasis is maintained by a complex set of processes that include: *1)* sterol synthesis in the endoplasmic reticulum (ER); *2)* endocytosis and lysosomal degradation of cholesterol-rich low density lipoprotein (LDL); *3)* cholesterol efflux from the plasma membrane to high density lipoprotein (HDL); *4)* intracellular formation of cholesteryl ester catalyzed by acyl-CoA:cholesterol acyltransferase (ACAT); and *5)* sterol-responsive regulation of cholesterol synthesis, LDL receptor activity, and ACAT that ensures proper levels of free (unesterified) cholesterol (FC) in cellular membranes. The trafficking of cholesterol between various subcellular compartments is crucial to the proper functioning of this system. In most cells, the major pathways of sterol trafficking appear to be from lysosomes and the ER to the plasma membrane, and from lysosomes to the rough ER (RER), the site of ACAT. The movement of newly synthesized cholesterol from the ER to the plasma membrane appears to be mediated by vesicles (1). Little is known about the mechanisms of other aspects of intracellular sterol trafficking (for a recent review, see ref. 2).

Sterol carrier protein-2 (SCP_2, also called nonspecific lipid transfer protein) is a 13-kDa peptide that is found intracellularly in most mammalian tissues. Its subcellular distribution is a matter of controversy, although significant amounts appear to be located in the cytosol and on the cytosolic surfaces of intracellular organelles (3, 4). When isolated and studied in cell-free systems, it is able to accelerate the transfer of sterols and other lipids between bilayer membranes and to stimulate various biochemical processes involving sterols (for recent reviews

Abbreviations: ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; LDL, low density lipoprotein; r[^{3}H-CO]LDL, LDL reconstituted with [^{3}H]cholesteryl oleate; r[^{3}H-CB]LDL, LDL reconstituted with [^{3}H]cholesteryl butyrate; HDL, high density lipoprotein; FBS, fetal bovine serum; ACAT, acyl-CoA:cholesterol acyltransferase; FC, free (unesterified) cholesterol; SCP_2, sterol carrier protein-2 (also called nonspecific lipid transfer protein); CHO, Chinese hamster ovary (cell); SUV, small unilamellar vesicles; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CO, cholesteryl oleate; CB, cholesteryl butyrate.

[1]To whom correspondence should be addressed.

[2]Present address: Department of Physical Science, Philadelphia College of Textiles and Science, School House Lane and Henry Avenue, Philadelphia, PA 19129.

see refs. 5 and 6). Its involvement in the trafficking of sterols in intact cells has not been established. For reasons that are not yet clear, SCP_2 is not found in tissues from individuals with Zellweger syndrome, an inherited peroxisome-deficiency disorder (7). Recently, peroxisome-deficient CHO cell variants were obtained and these also were found to be severely deficient in SCP_2 (8). As suggested by subcellular fractionation of liver from Zellweger patients (9) and as shown by data in the present paper, peroxisome-deficient cells retain functional lysosomes. This combination of traits in peroxisome deficiency has provided intact-cell systems for testing the involvement of SCP_2 in the trafficking of lysosomally generated sterol. The objective of the present study was to test for the involvement of SCP_2 in the transport of LDL-derived lysosomal cholesterol to the plasma membrane and to the RER, and in the signaling events by which LDL uptake and degradation lead to the stimulation of ACAT activity. The strategy was to compare peroxisome-deficient cells (fibroblasts and CHO) to their normal counterparts, monitoring delivery to the plasma membrane by the availability of cholesterol for efflux to extracellular acceptors (HDL or phospholipid vesicles), and monitoring transport to the RER and the stimulation of ACAT by the formation of cholesteryl ester from both whole-cell and lysosomal cholesterol pools. The results indicate that SCP_2 is not required for the transport of lysosomal cholesterol to the plasma membrane and RER, or for the stimulation of ACAT that follows LDL uptake.

MATERIALS AND METHODS

Materials

Normal human fibroblasts (GM3468A, GM0970A, and GM0041B), Zellweger-syndrome fibroblasts (GM04340, GM00228, GM06256), infantile Refsum's-syndrome fibroblasts (GM08770), Leber congenital amaurosis fibroblasts (GM03852), and type C Niemann-Pick fibroblasts (GM03123) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts were between passages 6 and 36 when used in experiments. Wild-type CHO-K1 cells and the peroxisome-deficient CHO-ZR78 variant were obtained from Dr. Christian Raetz (Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ). Monolayer cultures were maintained and passaged by standard methods, as described previously (10, 11). Media were based on minimum essential medium (MEM) for fibroblasts or Hams-F12 medium for CHO cells. All growth and experimental media were supplemented with 50 μg/ml of gentamicin. Routine growth media also were supplemented with 10% fetal bovine serum (FBS).

Laboratory reagents, radioisotopic cholesterol, solvents, tissue culture media, bovine sera, and antibiotics were from sources noted previously (12). [9,10-^{3}H]oleic acid (sp act 10 Ci/mmole) was from New England Nuclear (Boston, MA). Butyryl and oleoyl anhydrides (used to synthesize esters of [^{3}H]cholesterol) were from Sigma (St. Louis, MO). ITLC-SA thin-layer chromatography plates were from Gelman Sciences (Ann Arbor, MI). Human lipoproteins and lipoprotein-deficient plasma were prepared by differental centrifugation (13) from fresh blood plasma that had been treated with 5 mM N-ethylmaleimide to inactivate lecithin:cholesterol acyltransferase (10). To prepare lipoprotein-deficient serum, lipoprotein-deficient plasma was treated with thrombin and then centrifuged to remove the protein clot (11). Reconstituted LDL with [^{3}H]cholesteryl oleate (r[^{3}H-CO]LDL) was prepared by the potato-starch method of Krieger et al. (14), with minor variations as described previously (12). The specific activity of [^{3}H]cholesteryl oleate ([^{3}H]CO) in this preparation was approximately 0.17 mCi/mg. LDL reconstituted with [^{3}H]cholesteryl butyrate (r[^{3}H-CB]LDL) was prepared identically, except that the radiolabeled tracer in this case was [^{3}H]cholesteryl butyrate. The major mass component of the core in r[^{3}H-CB]LDL was unlabeled cholesteryl oleate. Small unilamellar vesicles were prepared by sonication from egg phosphatidylcholine (PC) (15).

Methods

For the analysis of cellular SCP_2 content, cells were grown in T75 flasks, harvested with trypsin, rinsed by repeated pelleting and resuspension in phosphate-buffered saline, and then dissolved in SDS sample buffer. Aliquots containing 100 μg cellular protein were subjected to SDS-polyacrylamide gel electrophoresis (16) using gels of 15% acrylamide. Parallel gels were either stained with Coomassie blue or blotted onto nitrocellulose membranes. SCP_2 on the blots was visualized by treatment first with a rabbit polyclonal antiserum raised against rat liver SCP_2 (17), and then with goat anti-rabbit IgG conjugated to horseradish peroxidase, followed by incubation with 4-chloro-1-naphthol and H_2O_2 (Bio-Rad, procedures described in Bio-Rad Bulletin 170-6534). Anti-rat SCP_2 antiserum has been shown previously to react with human and hamster SCP_2 (8, 18). A yellow filter and Tri-X film (Kodak) were used for photography of gels and blots.

The labeling of cellular cholesterol and efflux conditions were essentially those described previously (12). Briefly, this involved: *1)* plating of cells in 22-mm tissue culture wells; *2)* a 2-day incubation with unesterified [4-^{14}C]cholesterol ([^{14}C]FC) dispersed with either 10 mg protein/ml of lipoprotein-deficient serum plus 5 μg/ml of PC (for fibroblasts) or 1% FBS (for CHO cells); *3)* the labeling of cells with r[^{3}H-CO]LDL by a 5-h incubation at 15°C with 5–10 μg protein/ml of the reconstituted lipoprotein; *4)* extensive rinsing at 4°C; and *5)* efflux at 37°C with 0.5 ml/well of media containing either human HDL_3

(1 mg protein/ml) or egg PC-SUV (1 mg/ml) as the sterol acceptor. During the efflux period (4–24 h), we monitored the hydrolysis of [^{3}H]CO in cells and the efflux of both [^{3}H]FC and [^{14}C]FC.

Chemical assays (for protein, cholesterol, and phospholipid) and liquid scintillation counting were as described previously (10). For chromatographic separations involving only free cholesterol and cholesteryl oleate, thin-layer chromatography was performed with ITLC-SA plates (Gelman Sciences) and a development solvent of toluene (R_f values of 0.3 and 0.95 for free cholesterol and cholesteryl oleate, respectively). For separations also involving cholesteryl butyrate (see Fig. 4), the development solvent was petroleum ether–toluene 65:35 (v/v). This solvent provided good separation of free cholesterol, cholesteryl butyrate, and cholesteryl oleate (R_f values of 0.02, 0.6, and 0.8, respectively). All incubations were performed at least in triplicate. Values are the means ± 1 SD of replicate (≥ 3) determinations.

RESULTS

Western blot analysis of SCP_2 in cells

To confirm the presence of SCP_2 in normal cells and its absence in peroxisome-deficient cells, detergent extracts of cell monolayers were analyzed by SDS-PAGE and Western blotting, followed by visualization of blots with a polyclonal antiserum to SCP_2. The cell types analyzed were normal human skin fibroblasts, several types of mutant human skin fibroblasts (Zellweger syndrome, infantile Refsum syndrome, Leber congenital amaurosis, and type C Niemann-Pick disease), wild-type CHO-K1 cells, and the peroxisome-deficient variant CHO-ZR78. Infantile Refsum's syndrome and Leber congenital amaurosis are inherited peroxisome deficiency disorders, with some similarities to Zellweger syndrome (19). Fibroblasts from these patients were of interest as additional possible SCP_2-deficiency models. Type C Niemann-Pick disease is a lysosomal lipid storage disease in which the primary defect appears to be reduced efficiency of lysosomal cholesterol transport (2). As indicated by staining of gels with Coomassie blue (**Fig. 1A**), all of the fibroblast lines had similar protein profiles, and the two CHO lines were similar to each other. As indicated by staining of blots with anti-SCP_2 antiserum (Fig. 1B), normal fibroblasts (GM3468A), congenital amaurosis fibroblasts (GM03852), and Niemann-Pick C fibroblasts (GM03123) contained similar and easily visualized amounts of the mature 13-kDa form of SCP_2, whereas Zellweger fibroblasts (GM04340) and infantile Refsum's-syndrome fibroblasts (GM08770) contained no immunodetectable protein at

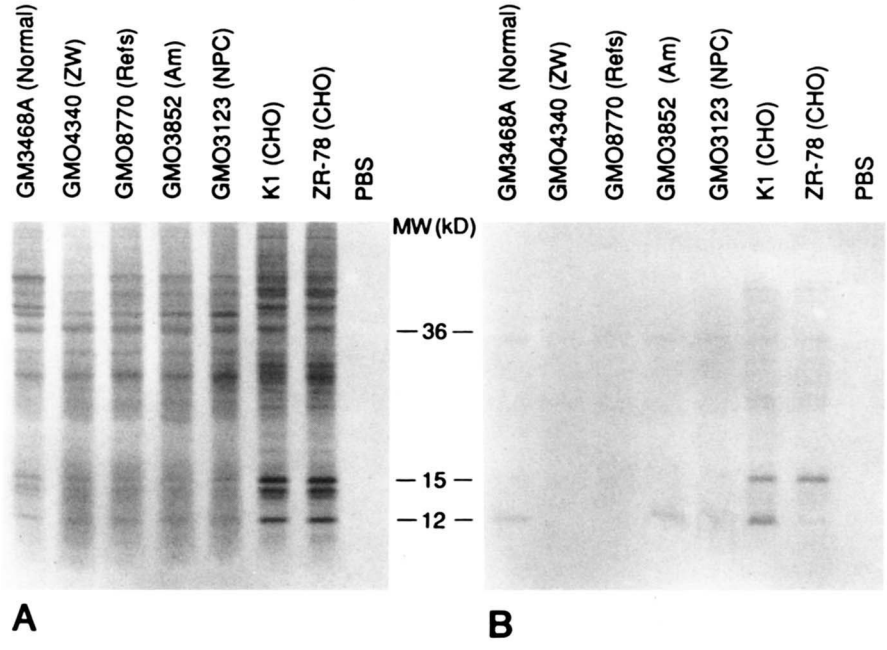

Fig. 1. SDS-PAGE and Western blot analyses of fibroblasts and CHO cells for SCP_2. Cell monolayers were harvested by trypsinization, rinsed with phosphate-buffered saline, and then dissolved in SDS sample buffer. Aliquots containing 100 μg protein were subjected to SDS-PAGE on 15% acrylamide gels. Identically prepared gels were either stained with Coomassie blue (A) or blotted onto nitrocellulose sheets and then probed with polyclonal anti-rat SCP_2 antiserum (B). Bound antibodies were visualized with goat anti-rabbit IgG conjugated to horseradish peroxidase (see Methods). Fibroblast lanes: GM3468A (normal human skin fibroblasts), GM04340 (Zellweger-syndrome fibroblasts), GM08770 (infantile Refsum syndrome fibroblasts), GM08352 (Leber congenital amaurosis fibroblasts), GM03123 (type C Niemann-Pick fibroblasts). CHO lanes: K1 (fully functional "wild-type" cells), ZR78 (peroxisome-deficient cells). The sample in lane marked PBS was a blank control prepared by combining SDS sample buffer with a volume of phosphate-buffered saline equivalent to that of a typical cell pellet.

this molecular mass. All of the fibroblast lines contained a 36-kDa protein that was visualized with the anti-SCP_2 antiserum. In other comparisons, two other Zellweger fibroblast lines (GM00228 and GM06256) contained no detectable SCP_2 (data not shown). The Western blot analysis of the CHO lines showed prominent staining of a 13-kDa protein in CHO-K1, with relatively faint staining at this molecular mass in CHO-ZR78 (Fig. 1B). Both CHO lines contained a prominent immunoreactive band at 15 kDa and several lightly reactive bands at higher molecular masses, including 36 kDa. The 15-kDa band probably was pro-SCP_2, which is thought to be the immediate precursor of mature SCP_2 (18). These results confirm that levels of the mature 13-kDa form of SCP_2 are considerably below normal in Zellweger syndrome fibroblasts and in CHO-ZR78 cells, although it appears that the latter cell contains significant amounts of the 15-kDa precursor of SCP_2. These findings validate comparisons between normal and Zellweger syndrome fibroblasts as a means of testing for the involvement of SCP_2 in the transport and efflux of lysosomal cholesterol. The validity of comparing the normal and peroxisome-deficient CHO cells is less certain, as the pro form of SCP_2 exhibits transfer activity for cholesterol (20), and thus its presence may compensate for the lack of mature SCP_2.

Cholesterol efflux in normal and SCP_2-deficient cells

For cholesterol efflux studies, cells usually were preincubated for a 2-day period with [^{14}C]cholesterol to label whole-cell (mostly plasma-membrane) cholesterol and then pulsed with r[^{3}H-CO]LDL to initiate the labeling of lysosomes. This was followed by the 37°C efflux incubation, during which we monitored the hydrolysis of [^{3}H]cholesteryl oleate in lysosomes and the efflux of ^{3}H-labeled and ^{14}C-labeled free cholesterol. Any observed differences in the efflux of lysosomally derived [^{3}H]FC might have been due to differences in either intracellular sterol transport or desorption of sterol from the plasma membrane. To control for possible differences in desorption from the plasma membrane, measurements of efflux of lysosomal cholesterol ultimately were normalized to the efflux of [^{14}C]FC.

Fig. 2 illustrates a time-course comparison between normal GM3468A fibroblasts and a Zellweger line, GM04340. As the data show, the two cells appeared to be similar with respect to the fractional efflux of whole-cell [^{14}C]FC (Fig. 2A), the kinetics of degradation of endocytosed LDL (Fig. 2B), and the fractional release of the lysosomally generated [^{3}H]FC (Fig. 2C). In this experiment, the degradation of endocytosed LDL was somewhat more efficient in normal cells than in the Zellweger cells. This difference was not a consistent finding. In most experiments, approximately 75% of endocytosed LDL was degraded after 8 h in both types of fibroblasts. At

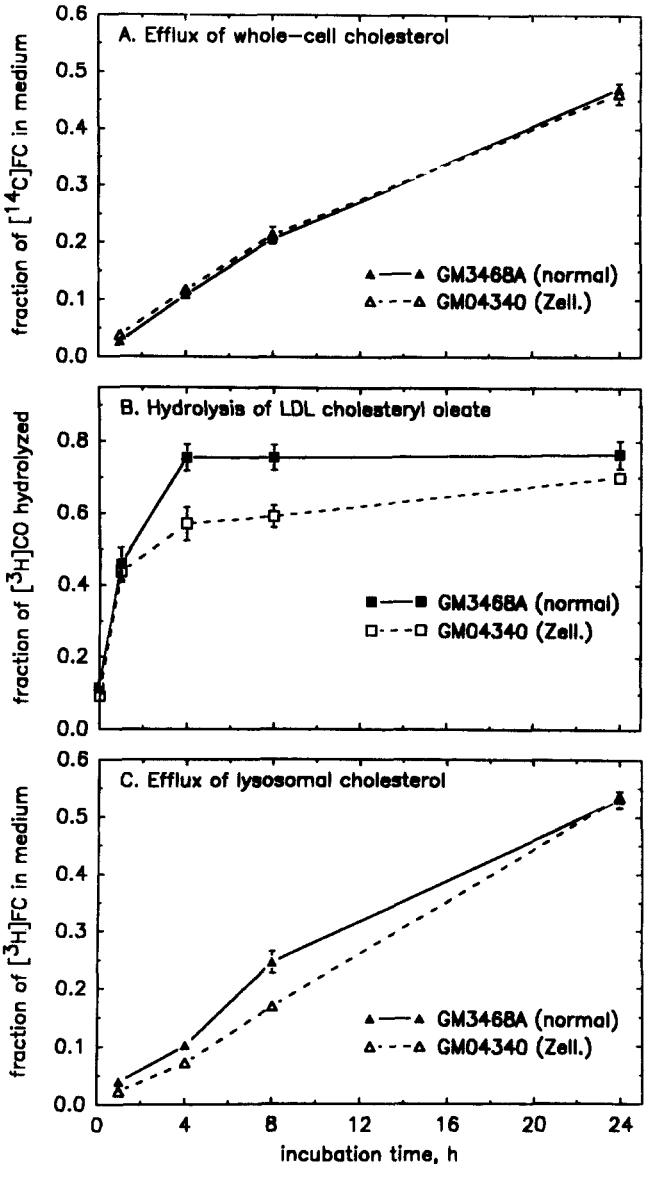

Fig. 2. Time courses of efflux of whole-cell and lysosomal cholesterol from normal and Zellweger fibroblasts to HDL_3. Cells were prepared in 22-mm tissue culture wells as described in Materials and Methods. Initial cell parameters for normal (GM3468A) fibroblasts were as follows: protein, 36.8 ± 0.4 μg/well; total [^{14}C]cholesterol, 7863 ± 167 cpm/well (FC/TC = 0.998 ± 0.001); total [^{3}H]cholesterol, 3137 ± 385 cpm/well (FC/TC = 0.115 ± 0.012). Initial cell parameters for Zellweger (GM04340) fibroblasts were as follows: protein, 28.1 ± 1.5 μg/well; total [^{14}C]cholesterol, 5901 ± 207 cpm/well (FC/TC = 0.997 ± 0.002); total [^{3}H]cholesterol = 6169 ± 919 cpm/well (FC/TC = 0.092 ± 0.005). Efflux medium (0.5 ml/well) contained 0.2% BSA and 1 mg protein/ml of human HDL_3. Panel A: Fractional efflux of whole-cell cholesterol was calculated by using the initial [^{14}C]FC in cells as the divisor. Panel B: Fractional hydrolysis of LDL cholesteryl oleate was calculated by summing the [^{3}H]FC in cells and medium at a given time and then dividing by the initial total [^{3}H]cholesterol in the cells. Panel C: Fractional efflux of [^{3}H]FC was calculated by dividing the [^{3}H]FC in medium at a given time by the total production of [^{3}H]FC measured at t = 24 h. Abbreviations: CO, cholesteryl oleate; Zell., Zellweger; FC, free (unesterified) cholesterol; TC, total cholesterol.

early time points in this experiment, the efflux of lysosomal cholesterol appeared to be moderately suppressed in the Zellweger cells. This finding was consistent from experiment to experiment, although in many cases the difference was not as large as in Fig. 2C (see below, Table 1). In most subsequent comparisons, we chose an incubation time of 4 h to examine these processes. This provided for near maximal degradation of LDL and was within the period of essentially constant rate of cholesterol release.

Table 1 summarizes several comparisons between the normal GM3468A fibroblasts and the GM04340 Zellweger line, in which efflux of whole-cell and lysosomal cholesterol to either HDL_3 (1 mg protein/ml) or egg PC small unilamellar vesicles (SUV, 1 mg/ml) was determined after incubations of 4 h. When the results under each condition were averaged, the trend that emerged was that the fractional efflux of whole-cell cholesterol was approximately 15% greater in Zellweger cells than in normal cells, whereas the fractional efflux of lysosomal cholesterol was depressed in the Zellweger cells by a similar percentage (Table 1, columns 4 and 5). As a consequence, the normalized efflux of lysosomal cholesterol (fractional efflux of lysosomal cholesterol divided by fractional efflux of whole-cell cholesterol) was significantly depressed by approximately 25% (Table 1, last column). In experiments with two other Zellweger lines (GM00228 and GM06256), the average values for normalized efflux of lysosomal cholesterol were 0.73 and 0.65, respectively. These values, although similar to the average obtained with GM04340 cells, were not significantly different from the corresponding GM3468A values, possibly due to small sample size (total n was only 3 for each of these comparisons). These results suggested the possibility of a moderate impairment in the delivery of lysosomal cholesterol to the plasma membrane in the Zellweger cells.

Fig. 3 illustrates a comparison between the SCP_2-containing CHO-K1 line and an SCP_2-deficient variant, CHO-ZR78. As described in the figure, the uptake and degradation of LDL were similar in the two cell types, whereas the fractional efflux of both whole-cell and lysosomal cholesterol were approximately 30% less in the ZR78 cell than in the normal K1 cells. As a consequence, the normalized efflux of lysosomal cholesterol was not significantly different between the two cells.

The above data suggested that cells deficient in SCP_2 exhibited either a moderate impairment or no impairment in the transport of cholesterol from lysosomes to the plasma membrane. This conclusion was based on an indirect assessment of sterol delivery to the plasma membrane, namely the availability of the internally generated sterol for efflux from the cell surface. Because of the indirect nature of this assessment, it was important to show that the methods used in these studies were adequate for demonstrating authentic defects in the transport of cholesterol from lysosomes to the plasma membrane. To address this issue, two types of control experiments were performed: *1)* comparisons between normal fibroblasts and the transport defective Niemann-Pick type C fibroblast (21), and *2)* experiments on the effect of the sterol transport inhibitor U18666A (22) on efflux of lysosomal

TABLE 1. Normalized efflux of lysosomal cholesterol from normal and Zellweger fibroblasts

Cell	Acceptor[a]	Number of Experiments	Cholesterol Efflux, Fraction/4 h: Whole-Cell[b] (^{14}C)	Lysosomal[b] (^{3}H)	Normalized[c] ($^{3}H/^{14}C$)
GM3468A[d]	HDL3	3	0.11	0.10	0.92 ± 0.10
GM3468A	PC-SUV	2	0.072	0.068	
GM04340[e]	HDL3	3	0.13	0.086	0.69 ± 0.06[f]
GM04340	PC-SUV	2	0.084	0.058	

[a]Media contained 0.2% BSA and either HDL_3 (1 mg protein/ml) or PC-SUV (1 mg/ml). In some experiments, media also contained 0.1% ethanol, a control for drug additions to other incubations in the same experiment (e.g., Table 3).

[b]Each value is an average obtained by combining data from the indicated number of experiments. In each experiment, each type of incubation was performed in triplicate. To calculate fractional efflux of whole-cell cholesterol, initial cell [^{14}C]FC was used as the divisor. To calculate fractional efflux of lysosomal cholesterol, the 4-h production of [^{3}H]FC was used as the divisor. Within an experiment, SD values (not given in table) were typically about 5% of average values. Between experiments, the variation in efflux for a given cell and acceptor combination was 17% or less.

[c]Each value is the mean ± 1 SD of the values obtained using either of the two acceptors. Normalization was done experiment-by-experiment and then the normalized values were averaged.

[d]Normal human skin fibroblasts.

[e]Zellweger skin fibroblasts.

[f]Significantly different from GM3468A ($P < 0.05$) as determined by the one-tailed Wilcoxon signed rank test for pairs.

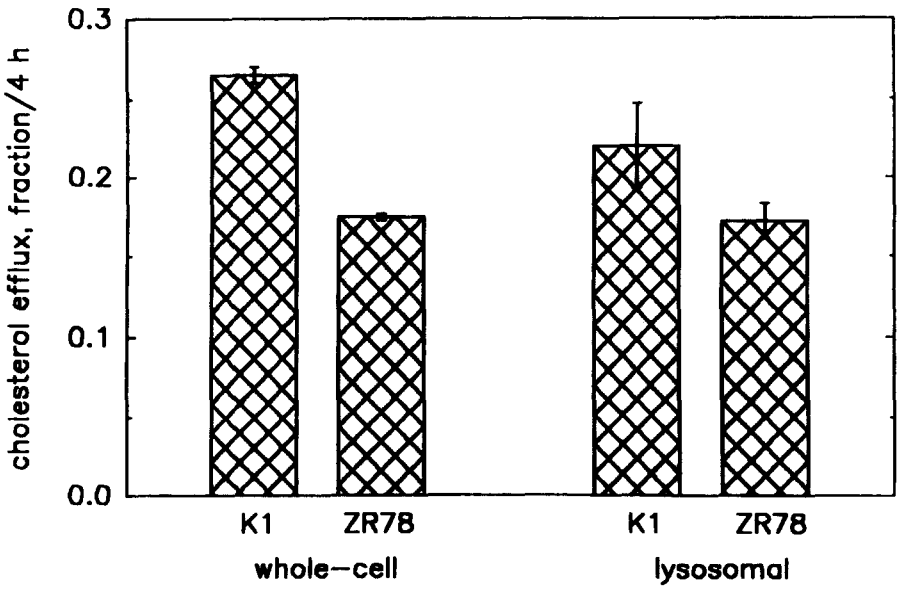

Fig. 3. Efflux of whole-cell and lysosomal cholesterol from CHO-K1 cells and CHO-ZR78 cells to HDL_3. Incubation conditions were as described in Fig. 2. In CHO-K1 cells, initial cell protein was 80 ± 6 μg/well and initial [^{14}C]FC was 8197 ± 254 cpm/well. The 4-h production of lysosomal [^{3}H]FC was 1213 ± 180 cpm/well. In CHO-ZR78 cells the initial cell protein was 148 ± 5 μg/well and initial [^{14}C]FC was 11967 ± 326 cpm/well. The 4-h production of [^{3}H]FC was 2514 ± 336 cpm/well.

cholesterol from cells. **Table 2** provides data obtained with Niemann-Pick C fibroblasts, showing that the fractional efflux of whole-cell cholesterol from these cells to HDL_3 was ca. 126% of normal and the fractional efflux of lysosomal cholesterol was 55% of normal, resulting in a normalized efflux of lysosomal cholesterol that was 44% of normal. **Table 3** shows that the addition of 1 μg/ml of U18666A to HDL_3 efflux medium did not have a significant effect on the efflux of whole-cell cholesterol from either normal fibroblasts or CHO-K1 cells, but reduced the fractional efflux of lysosomal cholesterol from these two cell types of cells by 60% and 47%, respectively. These results show that the present methods were adequate to demonstrate genuine defects in the transport of lysosomal cholesterol to the plasma membrane.

TABLE 2. Efflux of whole-cell and lysosomal cholesterol from normal and Niemann-Pick C fibroblasts to HDL_3[a]

Cell	Whole-Cell FC (Fraction Released/4 h)	Lysosomal FC (Fraction Released/4 h)
GM3468A[b]	0.11 ± 0.003	0.10 ± 0.004
GM03123[c]	0.13 ± 0.01	0.057 ± 0.002

[a]Medium contained 1 mg protein/ml of HDL_3, 0.2% BSA, and 0.1% ethanol. Ethanol was present in the medium as a control for other incubations in this experiment, which involved treatment of cells with compound U18666A (see Table 3).

[b]Normal human skin fibroblasts. Data and calculations are those for GM3468A (control treatment) described in Table 3.

[c]Niemann-Pick C skin fibroblasts. Fractional efflux was calculated as described in Table 1 (note *b*). Initial [^{14}C]FC in cells was 4337 ± 78 cpm/well. Total [^{3}H]FC production after 4 h was 1917 ± 114 cpm/well.

In the above fibroblast comparisons, only one normal cell line (GM3468A) was used. To determine whether this cell line was representative of other normal fibroblasts, we performed efflux comparisons between GM3468A cells and two other normal human fibroblast lines (GM0970A and GM0041B). As shown in **Table 4,** the values for normalized efflux of lysosomal cholesterol from GM0970A

TABLE 3. Effects of Upjohn compound U18666A on efflux of whole-cell and lysosomal cholesterol from CHO-K1 cells and from normal fibroblasts to HDL_3[a]

Cell	Treatment	Whole-Cell FC (Fraction Released/4 h)	Lysosomal FC (Fraction Released/4 h)
GM3468A[b]	control	0.11 ± 0.003	0.10 ± 0.004
GM3468A	U18666A	0.12 ± 0.01	0.04 ± 0.003
CHO-K1[c]	control	0.26 ± 0.01	0.22 ± 0.03
CHO-K1	U18666A	0.27 ± 0.01	0.12 ± 0.01

[a]HDL_3 concentration was 1 mg protein/ml. Media also contained 0.2% BSA. When used, U18666A concentration was 1 μg/ml and was added to media from an ethanolic stock solution of 1 mg/ml (final ethanol concentration = 0.1%). This concentration of ethanol was added to control medium in the experiment with fibroblasts. Ethanol was not added to control medium in the experiment with CHO-K1 cells.

[b]Normal human skin fibroblasts. Fractional efflux was calculated as described in Table 1 (note *b*). The initial [^{14}C]FC content of cells was 6524 ± 259 cpm/well. The 4-h production of [^{3}H]FC was 1583 ± 89 cpm/well. These parameters differed by no more than 10% between control and U18666A-treated cells.

[c]Wild type CHO cells. Fractional efflux was calculated as described in Table 1 (note *b*). Initial [^{14}C]FC was 8195 ± 226 cpm/well. Total [^{3}H]FC production after 4 h was 1262 ± 130 cpm/well. These parameters differed by no more than 10% between control and U18666A-treated cells.

TABLE 4. Efflux of whole-cell and lysosomal cholesterol from three different normal human fibroblast lines and Niemann-Pick C fibroblasts

Cell	Cholesterol Efflux, Fraction/4 h[a]		
	Whole-Cell (^{14}C)	Lysosomal (^{3}H)	Normalized ($^{3}H/^{14}C$)
GM3468A (Normal)	0.15 ± 0.003	0.072 ± 0.004	0.47 ± 0.03
GM0970A (Normal)	0.21 ± 0.01	0.073 ± 0.005	0.35 ± 0.03[b]
GM0041B (Normal)	0.16 ± 0.001	0.069 ± 0.011	0.43 ± 0.07[c]
GM03123 (NP-C)	0.14 ± 0.01	0.040 ± 0.003	0.29 ± 0.02[d]

[a]The extracellular acceptor was HDL_3 (1 mg protein/ml). Conditions were as described in Table 1. Data are from a single experiment with three replicate wells for each cell type.
[b]Significantly different from GM3468A ($P < 0.05$) by unpaired t test.
[c]Not significantly different from GM3468A.
[d]Significantly different from each of the three normal fibroblasts.

and GM0041B cells were 74% and 91%, respectively, of the GM3468A value. The difference between GM0970A and GM3468A was significant and similar to the difference observed between GM3468A and Zellweger cells (Table 1). These results suggest that the "normal" range of variation in the efflux of lysosomal cholesterol is large and probably includes the values obtained with Zellweger fibroblasts. Thus it is unlikely that the small apparent deficit in the normalized efflux of lysosomal cholesterol from Zellweger cells (Table 1) represents a significant impairment in the delivery of lysosomal cholesterol to the plasma membrane.

It may be noted in Table 4 that the normalized efflux of lysosomal cholesterol from GM3468A cells (0.47) was unusually low in comparison to earlier experiments (e.g., Table 1). The reason for this low value and the similar low values obtained with the other normal fibroblasts is unknown. However, it should also be noted that this experiment included measurements of efflux from Niemann-Pick C fibroblasts (GM03123), and that for each of the three normal fibroblasts, the normalized efflux of lysosomal cholesterol was significantly greater than that from the Niemann-Pick C cells. Thus, despite the low values for efflux of lysosomal cholesterol obtained in this experiment, significant relative differences between cell types (as reported in Table 2) were maintained.

LDL-induced esterification of cholesterol and cholesterol transport to the RER

To examine the ability of LDL-derived cholesterol to stimulate cellular cholesterol esterification, fibroblasts (GM3468A, GM04340, and GM03123) were prelabeled for 2 days with [^{14}C]cholesterol under the same conditions used in the efflux studies (which minimized esterification), and then incubated for 8 h at 37°C with either BSA alone or BSA plus 100 μg protein/ml of native human LDL. Cellular [^{14}C]cholesteryl ester was quantified at the beginning and at the end of the 8-h incubations. The results (**Table 5**) show that LDL stimulated an approximate tenfold increase in cholesterol esterification in both the normal fibroblasts (GM3468A) and the Zellweger fibroblasts (GM04340), but did not cause any significant esterification in Niemann-Pick C fibroblasts (GM03123). The latter result was expected and is consistent with previous work showing that cholesterol esterification and

TABLE 5. LDL-induced esterification of whole-cell cholesterol in normal, Zellweger, and Niemann-Pick C (NP-C) fibroblasts

Cell	Incubation Time	LDL	Cholesterol Esterification (% of Total [^{14}C]Cholesterol)
	h	*μg protein/ml*	
GM3468A	0		0.6 ± 0.02
(Normal)	8	0	0.5 ± 0.1
	8	100	5.1 ± 0.5
GM04340	0		0.5 ± 0.1
(Zellweger)	8	0	0.5 ± 0.1
	8	100	5.1 ± 0.5
GM03123	0		0.3 ± 0.1
(NP-C)	8	0	0.3 ± 0.1
	8	100	0.4 ± 0.1

Fibroblasts were labeled for 2 days with [^{14}C]cholesterol and then incubated for 8 h at 37°C with 0.2% BSA and either 0 or 100 μg protein/ml of native human LDL. Cells were analyzed for esterified [^{14}C]cholesterol content at the beginning and at the end of the 8-h incubation. Esterified cholesterol content is expressed as the percentage of total [^{14}C]cholesterol. Initial cell protein was 64 ± 3, 48 ± 2, and 31 ± 1 μg/well in GM3468A, GM04340, and GM03123 cells, respectively. Initial total [^{14}C]cholesterol was 7518 ± 264, 6406 ± 190, and 4351 ± 206 cpm/well, respectively.

other metabolic responses to LDL occur sluggishly in Niemann-Pick C cells (23). The lack of responsiveness in these cells served as a negative control in the present situation. A pattern of results very similar to that in Table 5 was also obtained when esterification was monitored in the same three cell types by the incorporation of exogenous [^{3}H]oleic acid into cholesteryl ester (data not shown).

In order to directly monitor the esterification of LDL-derived cholesterol and thereby determine whether SCP_2 is needed for the transport of lysosomal cholesterol to the RER (the intracellular site of ACAT), we used a somewhat different experimental strategy that involved pulsing cells at 15°C with reconstituted LDL containing [^{3}H]cholesteryl butyrate and then chasing the cells for 8 h at 37°C in medium containing BSA (0.2%) and unlabeled oleic acid (32 μM), with or without native LDL (100 μg protein/ml). As noted in Materials and Methods, free cholesterol, cholesteryl butyrate, and cholesteryl oleate can be separated from each other by a simple thin-layer chromatographic method. Thus, it was possible to monitor the lysosomal hydrolysis of the LDL-derived cholesteryl butyrate and the ACAT-mediated re-esterification of the resulting free cholesterol to form cholesteryl oleate during the 8-h chase period. The results are shown in **Fig. 4.** The top panel (Fig. 4A) illustrates in detail the data obtained with normal fibroblasts (GM3468A), and shows that [^{3}H]cholesteryl butyrate in LDL was readily hydrolyzed in cells during the chase period, that this hydrolysis was inhibited by chloroquine, that a small fraction of the resulting LDL-derived free cholesterol was re-esterified to a form co-migrating with cholesteryl oleate when the chase medium contained LDL, and that this re-esterification was prevented by addition of the specific

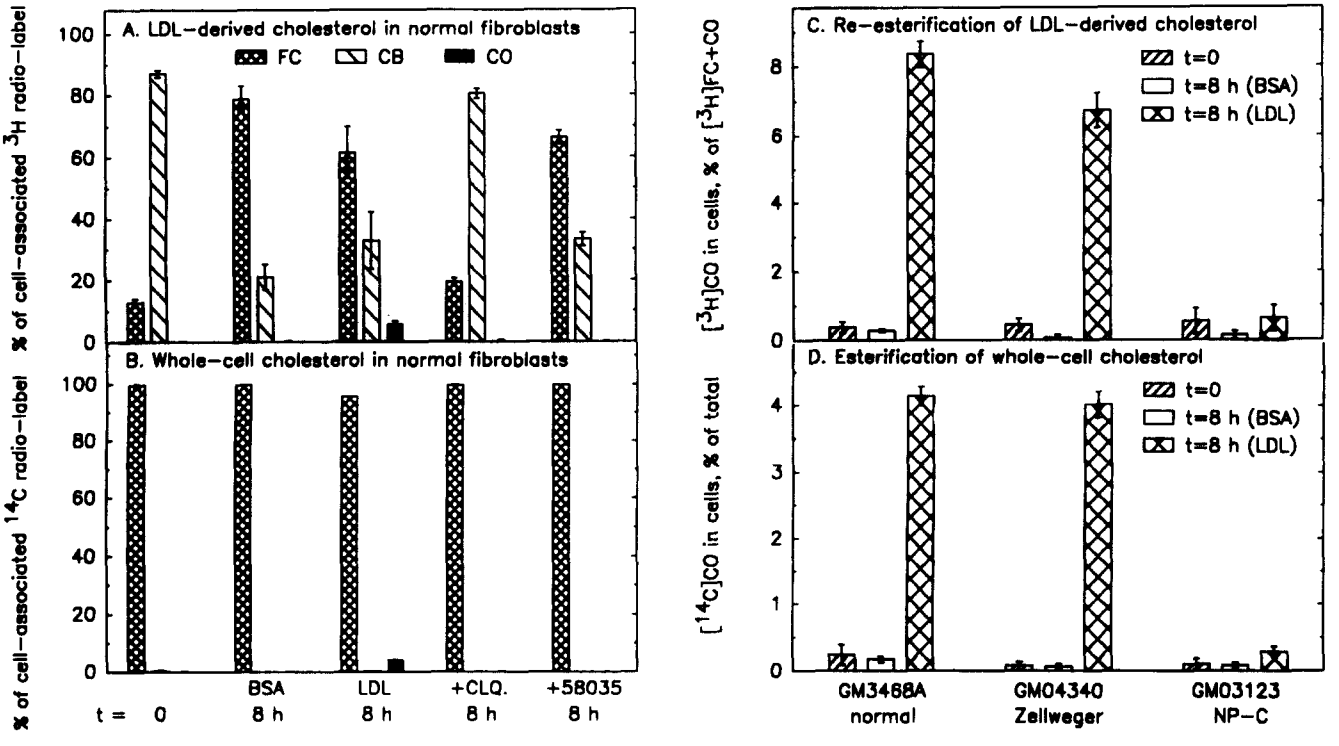

Fig. 4. LDL-induced esterification of whole-cell and lysosomal cholesterol in fibroblasts. Normal (GM3468A), Zellweger (GM04340), and Niemann-Pick C (GM03123) fibroblasts were labeled for 2 days with [^{14}C]cholesterol as in Table 5, then incubated for 5 h at 15°C with 10 μg protein/ml of r[^{3}H-CB]LDL, rinsed extensively at 4°C, pre-equilibrated for 30 min at 4°C with or without drug additions (see below), and then incubated (chased) for 8 h at 37°C in media containing BSA (0.2%) and unlabeled oleic acid (32 μM), with either 0 or 100 μg protein/ml of native human LDL. In some cases the LDL-containing chase medium also contained 100 μM chloroquine (+CLQ) or 1 μg/ml of Sandoz compound 58035 (+58035, dispersed with 0.5% dimethylsulfoxide). Cell lipids were obtained at the beginning and at the end of the 8-h chase incubations and analyzed by thin-layer chromatography for the distributions of ^{14}C and ^{3}H among free cholesterol (FC), cholesteryl butyrate (CB), and cholesteryl oleate (CO). Results are expressed as the percentage of each radiolabel in the indicated chemical form. Panels A and B show the complete data obtained with normal (GM3468A) fibroblasts. In several instances, the quantity of radiolabel in cholesteryl butyrate or cholesteryl oleate was very small and not discernible on these graphs. Panels C and D show the formation of labeled cholesteryl oleate in the three fibroblast lines, omitting the results from incubations involving chloroquine or compound 58035. In panel C, the percentage of [^{3}H]cholesteryl oleate at t = 0 was calculated by normalization to the total hydrolysis of [^{3}H]cholesteryl butyrate after 8 h. In this way bar height also provides a relative indication of the absolute cpm in cholesteryl oleate. For the incubations not involving any drug additions (panels C and D), the initial cell protein was 103 ± 3, 63 ± 3, and 33 ± 1 μg/well in GM3468A, GM04340, and GM03123 cells, respectively. The initial total [^{14}C]cholesterol in cells was 9282 ± 350, 6196 ± 333, and 3564 ± 193 cpm/well, respectively. The initial total [^{3}H]cholesterol in cells was 4712 ± 615, 6464 ± 813, and 4404 ± 340 cpm/well, respectively.

ACAT inhibitor, Sandoz compound 58035 (24), to the medium. Fig. 4B shows corresponding data for whole-cell [^{14}C]cholesterol that had been exchanged into the cells prior to the LDL pulse; these data reproduce the observations of Table 5 and, in addition, show that free cholesterol and cholesteryl oleate did not significantly contaminate the cholesteryl butyrate thin-layer fraction obtained in these analyses. Using Zellweger and Niemann-Pick C fibroblasts, results very similar to those in Figs. 4A and 4B were obtained, except that in the Niemann-Pick C cells, LDL did not stimulate esterification of either whole-cell or lysosomal cholesterol. The data in the top two panels of Fig. 4 validate the use of r[^{3}H-CB]LDL in combination with the thin-layer method described in Materials and Methods as a procedure for monitoring the ACAT-mediated esterification of LDL-derived cholesterol.

The data in panels C and D of Fig. 4 are from the same experiment and show the extent of ACAT-mediated esterification of LDL-derived [^{3}H]cholesterol (Fig. 4C) and of whole-cell [^{14}C]cholesterol (Fig. 4D) in normal (GM3468A), Zellweger (GM04340), and Niemann-Pick C (GM03123) fibroblasts. For whole-cell cholesterol, LDL induced approximately 4% esterification in both the normal and Zellweger cells, whereas there was virtually no stimulation of esterification in the Niemann-Pick C cells (Fig. 4D). Essentially the same qualitative pattern was apparent for LDL-derived cholesterol: very similar re-esterification (7–8%) in the normal and Zellweger cells, and negligible re-esterification in the Niemann-Pick C cells (Fig. 4C). The results in Table 5 and Fig. 4 thus show that Zellweger fibroblasts, despite their lack of SCP_2, exhibited essentially normal ACAT-mediated esterification of both whole-cell and lysosomal cholesterol when stimulated to form esters by LDL.

DISCUSSION

The efflux comparisons between normal and SCP_2-deficient cells (Figs. 2 and 3; Table 1) suggest that mature SCP_2 is not essential for the efficient transport of cholesterol from lysosomes to the plasma membrane. The comparisons in which LDL-induced cholesterol esterification was examined (Table 5; Fig. 4) suggest that SCP_2 is not required for the delivery of lysosomal cholesterol to the RER (the site of ACAT), for ACAT to be activated by LDL, or for ACAT to function efficiently. The present results thus indicate that SCP_2 is not needed for some of the major aspects of the trafficking and metabolism of LDL-derived lysosomal cholesterol in mammalian cells.

Van Heusden et al. (25) recently reported that SCP_2-deficient CHO cells were essentially normal with respect to uptake and degradation of LDL and the release of the resulting free cholesterol to medium containing LDL. In addition, it was found that these cells exhibited normal down-regulation of cholesterol synthesis in response to LDL uptake. Where similar functional comparisons have been performed, the present results are consistent with those of van Heusden et al. (25). Together, the results of these two studies suggest the general conclusion that mature SCP_2 is not required for the efficient trafficking of LDL-derived cholesterol in cells or for the metabolic signaling events that follow LDL uptake and degradation.

A potential objection to studies that utilize efflux to monitor the delivery of intracellular sterol to the plasma membrane arises from results suggesting that in most cells the processes of cholesteryl ester hydrolysis in lysosomes and of sterol efflux from the plasma membrane are slow in comparison to the rate of sterol transport from lysosomes to the plasma membrane (12, 26). Thus, the latter process may not always be rate-limiting for the efflux of LDL-derived cholesterol, and under some circumstances it may be difficult to detect changes in sterol transport from lysosomes to the plasma membrane by monitoring efflux from intact cells. Recognizing this concern, we performed the experiments shown in Tables 2 and 3, which demonstrated that the efflux procedures used in the present studies were adequate to demonstrate the effects of both a metabolic disease (Niemann-Pick C) and a drug (U18666A) that are well-established to reduce the transport of lysosomal cholesterol to the plasma membrane (21, 22). Although these data do not establish the minimal deficit in intracellular sterol transport that is detectable by efflux protocols, they do indicate strongly that intracellular transport can be rate-limiting for efflux of lysosomal cholesterol and that the presently used efflux approach is a valid means of demonstrating reductions in this transport.

In several experiments we observed a small reduction in the normalized efflux of lysosomal cholesterol from Zellweger fibroblasts in comparison to normal fibroblasts (Fig. 2 and Table 1). In only some cases was this difference statistically significant (Results). Additionally, in comparisons between three normal human fibroblast lines (Table 4), we found considerable variation in the normalized efflux of lysosomal cholesterol, suggesting that the efflux of lysosomal cholesterol from the Zellweger cells probably falls within the normal range. These results illustrate the difficulty of consistently demonstrating any significant difference between normal and Zellweger cells in the efflux of lysosomal cholesterol, and indicate that there is unlikely to be any functionally significant impairment in the transport of lysosomal cholesterol to the plasma membrane in the Zellweger cells.

In some cases we observed that efflux of plasma membrane cholesterol was elevated in Zellweger fibroblasts in comparison to normal fibroblasts (Table 1). This difference may be attributable to alterations in plasma membrane polar lipid composition that result from lack of peroxisomes. These alterations are likely to include reduced plasmalogen content and increased content of

phosphatidylethanolamine and very long chain fatty acyl groups in membrane polar lipids (19).

The presence of high molecular weight proteins that react with anti-SCP_2 antibodies in both normal and peroxisome-deficient cells (Fig. 1) has been reported previously (7, 8, 18). The various high molecular weight homologues of SCP_2 are thought to be translation products of a family of mRNAs, each of which encodes the complete SCP_2 sequence plus an additional amount of peptide N-terminal to the SCP_2 sequence (18, 27–29). The mature 13-kDa form of SCP_2 appears to be generated post-translationally from the 15-kDa pro-form (18, 30). The absence of SCP_2 in Zellweger cells is thought to be due to rapid degradation of either the mature protein or its 15-kDa precursor (18). Of the various high molecular weight homologues of SCP_2, only the 15-kDa form has been reported to have lipid transfer activity (20).

A possible discrepancy with previous findings was the detection of the 15-kDa pro-form of SCP_2 in CHO cells (Fig. 1). In previous characterizations of CHO-K1 and CHO-ZR78 cells, the detection of this protein was not reported (8). Although the reason for this discrepancy is not clear, it is noteworthy that the accumulation of pro-SCP_2 to a significant level is unusual. As shown by data in Fig. 1, it did not occur in fibroblasts. Likewise, pro-SCP_2 was not detectable in Zellweger liver tissue (7) or in a variety of normal rat tissues that showed clear expression of mature SCP_2 (4). Studies on SCP_2 biosynthesis have indicated that pro-SCP_2 is short-lived in both normal and Zellweger fibroblasts, preventing any significant accumulation of this protein (18). Thus, the detection of pro-SCP_2 in CHO cells was unexpected and is difficult to explain.

Results of the present study suggest that SCP_2 is not required for the efficient movement of cholesterol from lysosomes to the plasma membrane and the RER. Therefore, it appears that other mechanisms are responsible for these aspects of intracellular sterol trafficking. The feasible alternative mechanisms include unmediated diffusion between membranes, vesicular transport, and mediation by proteins other than SCP_2.

We are grateful to Dr. Christian R. H. Raetz (Merck, Sharp, and Dohme) for providing cultures of CHO-K1 and CHO-ZR78 cells, to Dr. John Heider (Sandoz Corporation) for the gift of compound 58035, and to the Upjohn Company for the gift of compound U18666A. Christine E. Ackerman provided excellent technical assistance. We also thank Drs. Jane M. Glick, Michael C. Phillips, and George H. Rothblat (Medical College of Pennsylvania) for helpful discussions. This work was supported in part by NIH Program Project Grant HL22633.

Manuscript received 18 December 1992, in revised form 27 September 1993, and in re-revised form 10 November 1993.

REFERENCES

1. Urbani, L., and R. D. Simoni. 1990. Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane. *J. Biol. Chem.* **265:** 1919–1923.
2. Liscum, L., and N. K. Dahl. 1992. Intracellular cholesterol transport. *J. Lipid Res.* **33:** 1239–1254.
3. Tsuneoka, M., A. Yamamoto, Y. Fujiki, and Y. Tashiro. 1988. Nonspecific lipid transfer protein (sterol carrier protein-2) is located in rat liver peroxisomes. *J. Biochem.* **104:** 560–564.
4. Van Heusden, G. P. H., K. Bos, and K. W. A. Wirtz. 1990. The occurrence of soluble and membrane-bound nonspecific lipid transfer protein (sterol carrier protein 2) in rat tissues. *Biochim. Biophys. Acta.* **1046:** 315–321.
5. Vahouny, G. V., R. Chanderbhan, A. Kharroubi, B. J. Noland, A. Pastuszyn, and T. J. Scallen. 1987. Sterol carrier and lipid transfer proteins. *Adv. Lipid Res.* **22:** 83–113.
6. Billheimer, J. T., and M. P. Reinhart. 1990. Intracellular trafficking of sterols. *In* Subcellular Biochemistry. H. J. Hilderson, editor. Plenum Publishing, New York. 301–332.
7. Van Amerongen, A., J. B. Helms, T. P. van der Krift, R. B. H. Schutgens, and K. W. A. Wirtz. 1987. Purification of nonspecific lipid transfer protein (sterol carrier protein 2) from human liver and its deficiency in livers from patients with cerebro-hepato-renal (Zellweger) syndrome. *Biochim. Biophys. Acta.* **919:** 149–155.
8. Van Heusden, G. P. H., K. Bos, C. R. H. Raetz, and K. W. A. Wirtz. 1990. Chinese hamster ovary cells deficient in peroxisomes lack the nonspecific lipid transfer protein (sterol carrier protein 2). *J. Biol. Chem.* **265:** 4105–4110.
9. Santos, M. J., T. Imanaka, H. Shio, and P. B. Lazarow. 1988. Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. *J. Biol. Chem.* **263:** 10502–10509.
10. Johnson, W. J., M. J. Bamberger, R. A. Latta, P. E. Rapp, M. C. Phillips, and G. H. Rothblat. 1986. The bidirectional flux of cholesterol between cells and lipoproteins. Effects of phospholipid depletion of high density lipoprotein. *J. Biol. Chem.* **261:** 5766–5776.
11. Johnson, W. J., F. H. Mahlberg, G. K. Chacko, M. C. Phillips, and G. H. Rothblat. 1988. The influence of cell and lipoprotein cholesterol contents on the flux of cholesterol between fibroblasts and high density lipoprotein. *J. Biol. Chem.* **263:** 14099–14106.
12. Johnson, W. J., G. K. Chacko, M. C. Phillips, and G. H. Rothblat. 1990. The efflux of lysosomal cholesterol from cells. *J. Biol. Chem.* **265:** 5546–5553.
13. Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 1–68.
14. Krieger, M., M. S. Brown, J. R. Faust, and J. L. Goldstein. 1978. Replacement of endogenous cholesteryl esters of low density lipoprotein with exogenous cholesteryl linoleate. *J. Biol. Chem.* **253:** 4093–4101.
15. Barenholz, Y., D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson, and F. D. Carlson. 1977. A simple method for the preparation of homogeneous phospholipid vesicles. *Biochemistry.* **16:** 2806–2810.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London).* **227:** 680–685.
17. Roff, C. F., A. Pastuszn, J. F. Strauss III, J. T. Billheimer,

M. T. Vanier, R. O. Brady, T. J. Scallen, and P. G. Pentchev. 1992. Deficiencies in sex-related expression and levels of two hepatic sterol carrier proteins in a murine model of Niemann-Pick Type C disease. *J. Biol. Chem.* **267:** 15902–15908.

18. Suzuki, Y., S. Yamaguchi, T. Orii, M. Tsuneoka, and Y. Tashiro. 1990. Nonspecific lipid transfer protein (sterol carrier protein-2) defective in patients with deficient peroxisomes. *Cell Struct. Funct.* **15:** 301–308.
19. Lazarow, P. B., and H. W. Moser. 1989. Disorders of peroxisome biogenesis. *In* The Metabolic Basis of Inherited Disease. 6th ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Information Services Company, New York. 1479–1509.
20. Matsuura, J. E., H. J. George, N. Ramachandran, J. G. Alvarez, J. E. Strauss III, and J. T. Billheimer. 1993. Expression of the mature and the pro-form of human sterol carrier protein 2 in *E. coli* alters bacterial lipids. *Biochemistry.* **32:** 567–579.
21. Liscum, L., R. M. Ruggiero, and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick C fibroblasts. *J. Cell Biol.* **108:** 1625–1636.
22. Liscum, L., and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one. *J. Biol. Chem.* **264:** 11796–11806.
23. Pentchev, P. G., H. S. Kruth, M. E. Comly, J. D. Butler, M. T. Vanier, D. A. Wenger, and S. Patel. 1986. Type C Niemann-Pick disease. A parallel loss of regulatory responses in both the uptake and esterification of low density lipoprotein-derived cholesterol in cultured fibroblasts. *J. Biol. Chem.* **261:** 16775–16780.
24. 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.
25. Van Heusden, G. P. H., J. R. C. M. van Beckhoven, R. Thieringer, C. R. H. Raetz, and K. W. A. Wirtz. 1992. Increased cholesterol synthesis in Chinese hamster ovary cells deficient in peroxisomes. *Biochim. Biophys. Acta.* **1126:** 81–87.
26. Brasaemle, D. L., and A. D. Attie. 1990. Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts. *J. Lipid Res.* **31:** 103–112.
27. Mori, T., T. Tsukamoto, H. Mori, Y. Tashiro, and Y. Fujiki. 1991. Molecular cloning and deduced amino acid sequence of nonspecific lipid transfer protein (sterol carrier protein 2) of rat liver: a higher molecular mass (60 kDa) protein contains the primary sequence of nonspecific lipid transfer protein as its C-terminal part. *Proc. Natl. Acad. Sci. USA.* **88:** 4338–4342.
28. He, Z., R. Yamamoto, E. E. Furth, L. J. Schantz, S. L. Naylor, H. George, J. T. Billheimer, and J. F. Strauss III. 1991. cDNAs encoding members of a family of proteins related to human sterol carrier protein 2 and assignment of the gene to human chromosome 1 p21→pter. *DNA Cell Biol.* **10:** 559–569.
29. Seedorf, U., and G. Assman. 1991. Cloning, expression, and nucleotide sequence of rat liver sterol carrier protein 2 cDNAs. *J. Biol. Chem.* **266:** 630–636.
30. Fujiki, Y., M. Tsuneoka, and Y. Tashiro. 1989. Biosynthesis of nonspecific lipid transfer protein (sterol carrier protein 2) on free polyribosomes as a larger precursor in rat liver. *J. Biochem.* **106:** 1126–1131.