Sterol Carrier Protein-2 Overexpression Enhances Sterol Cycling and Inhibits Cholesterol Ester Synthesis and High Density Lipoprotein Cholesterol Secretion*

Charles L. Baum, Erica J. Reschly, Apurba K. Gayen, Margaret E. Groh, and Kevin Schadick

From the Department of Medicine, Clinical Nutrition Research Unit and Section of Gastroenterology, University of Chicago, Chicago, Illinois 60637

Recent data indicate that sterol carrier protein-2 (SCP-2) functions in the rapid movement of newly synthesized cholesterol to the plasma membrane (Puglielli, L., Rigotti, A., Greco, A. V., Santos, M. J., and Nervi, F. (1995) J. Biol. Chem. 270, 18723–18726). In order to further characterize the cellular function of SCP-2, we transfected McA-RH7777 rat hepatoma cells with a pre-SCP-2 cDNA expression construct. In stable transfec-
tants, pre-SCP-2 processing resulted in an 8-fold increase in peroxisomal levels of SCP-2. SCP-2 overexpression increased the rates of newly synthesized cholesterol transfer to the plasma membrane and plasma membrane cholesterol internalization by 4-fold. There was no effect of SCP-2 overexpression on the microosomal levels of acyl-CoA:cholesterol acyltransferase and neutral cholesterol ester (CE) hydrolase; however, in the intact cell, CE synthesis and mass were reduced by 50%. SCP-2 overexpression also reduced high density lipoprotein-cholesterol secretion and apoA-I gene expression by 70% and doubled the rate of plasma membrane desmo
ersterol conversion to cholesterol. We conclude that SCP-2 overexpression enhances the rate of cholesterol cycling, which reduces the availability of cholesterol for CE synthesis and alters the activity of a cellular cholesterol pool involved in regulating apoA-I-mediated high density lipoprotein cholesterol secretion. The net result of these changes in cholesterol metabolism is a 48% increase in plasma membrane cholesterol content, the implications of which are discussed.

Cellular free cholesterol is predominantly located in the plasma membrane (reviewed in Ref. 1). Cellular cholesterol content, however, is determined by the concerted action of intracellular enzymes and regulatory proteins as follows: ACAT1 which catalyzes the synthesis of CE, sterol regulatory element binding proteins, which regulate the transcription of a number of genes involved in cholesterol metabolism (2, 3), and various cell type-specific metabolic reactions, e.g. lipoprotein secretion, steroidogenesis, and bile acid synthesis. Since cholesterol is highly insoluble in an aqueous environment, it has been postulated that sterol carrier protein-2 (SCP-2) regulates the movement and thus the availability of cholesterol for different cellular processes (4–6). The evidence supporting this contention was initially derived in large part from studies demonstrating that the addition of purified SCP-2 stimulated the in vitro conversion of sterol intermediates to cholesterol (7) and cholesterol to 7α-hydroxycholesterol (8) steroid hormones (9–13) and cholesterol ester (14). More recent studies indicate that SCP-2 gene expression is regulated by changes in cellular cholesterol content (15–17); however, in these studies, a direct role in cholesterol trafficking and esterification was not demonstrated. The strongest support for a role in cellular cholesterol metabolism comes from studies on the role of SCP-2 in steroidogenesis. SCP-2 gene expression is coordinately regulated with steroid hormone synthesis (18, 19); moreover, Yamamoto et al. (20) have demonstrated that the delivery of cholesterol to mitochondria for steroidogenesis is enhanced by SCP-2 overexpression, and Chanderbahn et al. (21) have shown that steroidogenesis is inhibited by intracellular delivery of anti-SCP-2 antibody.

Although it was originally thought that SCP-2 acted as a soluble cytosolic sterol carrier protein, it is now clear that SCP-2 is a peroxisomal protein (22–26). In peroxisome-deficient cells, derived from patients with Zellweger’s syndrome, pre-SCP-2 is not processed to SCP-2 and is rapidly degraded (27). Furthermore, peroxisome-deficient cell lines have a reduced capacity for cholesterol biosynthesis (28, 29) and alterations in cholesterol metabolism (30, 31); however, it is currently unclear if any of these abnormalities can be attributed to SCP-2 deficiency. In this regard, Puglielli et al. (32) recently demonstrated that Zellweger’s fibroblasts have a delay in the rate of nascent cholesterol transfer to the plasma membrane and that this defect can be reproduced in normal fibroblasts by antisense SCP-2 oligonucleotide treatment. In these studies, it was also noted that antisense oligonucleotide treatment resulted in a decrease in the level of another SCP-2 gene product, referred to as SCPx (33). Although a recent report seems to indicate that it is more likely that SCPx functions as a peroxisomal 3-oxoacyl-coenzyme A thiolase (34), our laboratory and others have demonstrated that SCPx has in vitro sterol transfer activity (34, 35).

Therefore, in order to further examine the cellular role of SCP-2, in the context of intact peroxisomes and without confounding changes in SCPx, we prepared stable transfectants of McA-RH7777 rat hepatoma cells that overexpress SCP-2. Our studies demonstrate that cellular levels of SCP-2 determine the
rate of bi-directional cholesterol movement, between the plasma membrane and intracellular cholesterol pools, and the availability of cholesterol for CE synthesis and HDL cholesterol secretion.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1H]Acetic acid (sodium salt, 5.3 Ci/mmol), [14C]cholesterol (54 mCi/mmol), [3H]cholesterol (51 Ci/mmol), [3H]cholesterol oleate (66 Ci/mmol), [3H]oleic acid (14 Ci/mmol), [3H]oleoyl coenzyme A (53 mCi/mmol), and 38-Protein Labeling Mix (43.5 TBq/mmol) were purchased from DuPont NEN. [3H]Desmosterol was prepared by detoxifying Mca-RH7777 cells with [3H]Acetate as described previously (36). Cholesterol oxidase (EC 1.1.3.6; *Brevibacterium sp.*) was from Sigma. Cholesterol and 25-hydroxycholesterol were from Steraloids (Wilton, NH). Zymosterol was purified from Mca-RH7777 cells lipid extracts as described previously (37). All other sterols were from Sigma. Compound 58–035 was provided by Dr. John Heider (Sandoz Inc., East Hanover, NJ). Oxidized steroid standards were prepared by treatment with cholesterol oxidase as described previously (37). HPLC grade solvents were from Fisher. DNA restriction and modification enzymes were purchased from New England BioLabs. All other chemicals were of reagent grade or better.

**Plasmid Construction**—A full-length cDNA of rat pre-SCP-2 (33) was processed by Dr. Udo Seedorf (Institut für Arterioskleroseforschung an der Universität Münster, Münster D-4400, Germany). The EcoRI-digested 800-base pair cDNA containing the pre-SCP-2 coding region extending from base 72 to 503 was cloned into the pCB6+ vector producing a plasmid referred to as pPre-SCP-2. pCB6+ (provided by Dr. V. Sukhatme, Department of Medicine, Beth Israel Hospital, Boston, MA) was developed from pCMV4 by inserting EcoRI, KpnI, NotI, ClaI, and EcoRV sites between the BgII and HindIII sites of the polylinker and cloning the neomycin resistance gene into the BclI sites at 1951 and 3290 (39). The cloned cDNA fragment is under the transcriptional control of the immediate early gene of the human cytomegalovirus promoter, and the vector contains polyadenylation signals and the ampicillin resistance gene. pPre-SCP-2 plasmids were isolated and sequenced to ascertain that no mutations were introduced during replication.

**Cell Culture and Transfection**—Mca-RH7777 rat hepatoma cells (ATCC CRL 1601) were grown in DMEM (Life Technologies, Inc.) and supplemented with 10% FBS (Life Technologies, Inc.) or 5% lipoprotein-deficient fetal bovine serum (unless otherwise indicated), 1% penicillin supplemented with 10% FBS (Life Technologies, Inc.) or 5% lipoprotein-deficient fetal bovine serum (unless otherwise indicated), and 10% NEAA, 1% sodium pyruvate, and 5% lipofectin (ATCC CRL 1601) were grown in DMEM (Life Technologies, Inc.) and supplemented with 10% FBS (Life Technologies, Inc.) or 5% lipoprotein-deficient fetal bovine serum (unless otherwise indicated), and 10% NEAA, 1% sodium pyruvate, and 5% lipofectin (ATCC CRL 1601). All transfections were performed by electroporation in serum-deficient medium supplemented with 10% FBS (unless otherwise indicated).

**SDS-Gel Electrophoresis and Immunoblotting Techniques**—Transfected cells were harvested by scraping into a protease inhibitor mixture consisting of 20 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 100 μg/ml leupeptin, and 5 mM EDTA and passed through a 26-gauge needle. Protein concentrations were measured using the Bio-Rad protein assay kit (45). Samples (100 μg) were separated by 12.5% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes (Immobilon, Millipore) as described previously (41). A polyclonal rabbit anti-rat SCP-2 antisera was incubated with membranes as described previously and detected with an anti-rabbit horseradish peroxidase-labeled secondary antibody-catalyzed chemiluminescence reaction (Amersham Corp.) (41). Protein bands were quantitated by scanning laser densitometry.

**Immunoelectron Microscopy**—Immunoelectron microscopy was used to demonstrate the subcellular location of SCP-2 and was performed by Dr. John Lewis (MICROMED microscopy facility, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC) as described (44). Briefly, samples for ImmunoGold analysis were embedded in LR white acrylic resin. Sections with thickness in the range of 70–100 nm were collected on copper grids and stained with anti-SCP-2 antisera (2 μg/ml). A mixture of secondary anti sera containing equal parts of goat anti-mouse antibodies conjugated to a 15-nm gold bead and goat anti-rabbit antibodies conjugated to a 30-nm gold bead were used. Representative micrographs were taken, and a morphometric analysis was performed to determine the number of 30-nm gold beads (specific immunoreactivity) and 15-nm gold beads (nonspecific background immunoreactivity) in peroxisomes and extraperoxisomal areas.

**Lipid Analysis**—Unless otherwise stated, lipids were extracted (45) and saponified using ethanol KOH as described previously (41). [14C]Cholesterol was added prior to lipid extraction in order to determine recovery, which typically averaged 80–85%.

Cellular sterol mass, sterol secretion, and total sterol synthesis were determined as follows. On day 0, cells were plated at a density of 2 × 10⁶ cells per 100-mm dish in medium with 10% FBS. On day 1, cell monolayers were rinsed with PBS and changed to fresh medium containing 5% LPDS. On day 2, plates to be used for sterol synthesis were pulsed with 100 μCi of [3H]Acetate and incubated for 2 h. At the end of 2 h, all cells and medium were harvested, and lipid extracts were prepared and saponified. Samples were analyzed by HPLC essentially as described (37) with the following modifications. Reversed-phase HPLC analysis was carried out using a Varian 5000 LC system with a Hewlett-Packard model 3390A programmable integrator connected to a Phenomenex IB-Sil 5-μm C18 reversed phase column (4.6 × 250 mm). The mobile phase consisted of acetonitrile/water (99.1, v/v) at a flow rate of 1.5 ml/min, and the effluent was monitored at 205 nm. In this system zymosterol, cholesterol, 7-dehydrocholesterol, and 5α,6α-cholesten-3β-ol peaks routinely accounted for 90–95% of the total radioactivity. Lipid mass was calculated from the A<sub>205</sub> of lipid (zymosterol, 0.5 pg/μg; desmosterol, 0.5 pg/μg; lanosterol, 0.5 pg/μg; cholesterol, 1.4 pg/μg; squalene, 0.1 pg/μg). The limits of detection were experimentally determined to be 0.01–0.1 μg sterol/mg protein.

**Microsomal Assay of the Conversion of [3H]Desmosterol to [3H]Cholesterol**—Microsomes (100 μg) were prepared and incubated at 37 °C for 1 h with 1 mM NADPH and 0.5 μCi of [3H]desmosterol as described (46). Incubations were terminated by chilling on ice, and cells and medium were harvested and lipid extracts prepared and saponified. The conversion of [3H]desmosterol to [3H]cholesterol was determined by HPLC analysis and liquid scintillation counting.

**Pulse-Chase Analysis of Cholesterol Biosynthesis**—Cells (∼10⁶) were incubated in 10 ml of medium containing 5% LPDS and [3H]acetate (100 μCi) at 37 °C. After 1 h, the medium was removed and fresh chase medium containing 10 mM sodium acetate was added as described previously (42). At the indicated times, cells were extensively rinsed with PBS, dissociated with trypsin, extracted, and analyzed by HPLC for the radioactivity in cholesterol and sterol intermediates.

![Sterol Carrier Protein-2 Enhances Cholesterol Trafficking](https://example.com/figure1.png)

**TABLE I**

| Sterol                          | Retention time (min) |
|--------------------------------|---------------------|
| Desmosterol/zymosterol          | 142                 |
| 7-Dehydrocholesterol            | 167                 |
| Lanosterol                      | 18.9                |
| Cholesterol/lathosterol         | 21.6                |
| Squalene                        | 25.6                |
| Dihydrolanosterol               | 29.1                |
| Oxidized zymosterol             | 10.5                |
| Oxidized desmosterol            | 11.0                |
| Oxidized 7-dehydrocholesterol   | 12.6                |
| Oxidized lanosterol             | 15.1                |
| Oxidized cholesterol (cholastane) | 16.2              |

**TABLE II**

Separation of squalene and sterols by HPLC

| Compound                  | Retention time (min) |
|---------------------------|---------------------|
| Cholesterol/lathosterol   | 21.6                |
| Squalene                  | 25.6                |
| Dihydrolanosterol         | 29.1                |
| Oxidized zymosterol       | 10.5                |
| Oxidized desmosterol      | 11.0                |
| Oxidized 7-dehydrocholesterol | 12.6            |
| Oxidized lanosterol       | 15.1                |
| Oxidized cholesterol      | 16.2                |

**Notes:** Values are means ± SEM of at least three experiments. Values for squalene and sterol intermediates were corrected for 90% recovery of the injected radioactivity.
**Sterol Carrier Protein-2 Enhances Cholesterol Trafficking**

**Measurement of Cholesterol Transfer to and from the Plasma Membrane**—The rate of newly synthesized cholesterol transfer to the plasma membrane was determined essentially as described (36). Briefly, on day 1, 1 × 10^6 cells were plated in media containing 10% FBS. On day 2, the medium was changed to medium with 5% LPDS. On day 3, cells were incubated with radiolabeled LDL (100 μg/ml), and cholesterol bands, visualized by exposure to iodine, were quantitated by scintillation counting. Microsomal ACAT activity was assayed as described previously (56). Neutral CE hydrolysis activity was assayed in cell homogenates as described previously (57).

**Analysis of Lipoprotein Secretion and Apolipoprotein Gene Expression**—To analyze the effect of SCP-2 overexpression on lipoprotein secretion of radiolabeled LDL, high-density lipoprotein (HDL), and lipoprotein-free samples. Every three samples were combined, and cholesterol bands, visualized by exposure to iodine, were scraped into scintillation vials and counted.

The effect of SCP-2 overexpression on apolipoprotein A-I (apoA-I) and E (apoE) secretion was determined by immunoprecipitation of [35S]methionine-labeled apolipoproteins (58). Briefly, 4 × 10^6 cells in 100-mm dishes were incubated with 100 μCi/ml [35S]Protein labeling mix and 40 mM methionine in methionine-free DMEM with 1% LPDS for 18 h. The medium was harvested and cellular debris removed by centrifugation. Equal amounts of trichloroacetic acid-precipitable counts (250,000 cpm) were immunoprecipitated with anti-rat apoA-I and apoE antisera (52), followed by separation on a 12.5% SDS-PAGE and autoradiography.

Apolipoprotein transcript abundance was determined in total RNA extracted as described (59). RNA was determined to be intact by following separation by 1% agarose-formaldehyde gel electrophoresis of 20-μg aliquots. Northern blots were probed with using nitrocellulose membranes and were hybridized to rat apoA-I and E cDNAs as described (52). The apoA-I cDNA (60) was provided by Dr. Jeffrey Gordon (Washington University, St. Louis, MO), and the apoE cDNA (61) was provided by Dr. John Taylor (Gladstone Foundation, San Francisco, CA). mRNA abundance was calculated by quantitative scanning densitometry.

**Data Analysis**—Data were analyzed by a paired t test or one-way analysis of variance and Dunnett’s test for multiple comparisons (Minitab). Kinetic modeling was performed by computer using the SAAM II software for Windows (SAAM, Institute, University of Washington).

**RESULTS**

**Establishment of Stable Pre-SCP-2 Transfectants**—After transfection with pPre-SCP-2, or the empty NEO vector, and selection with G418, multiple colonies were obtained and screened for expression by Western blotting. In SCP-2 overexpressing cells (SCPmed and SCPhigh cells), the level of pre-SCP-2 was <25% of the total SCP-2 immunoreactivity, and the level of SCP-2 was increased ~10-fold compared with NEO cells (Fig. 1). The level of SCP-2 in pre-SCP-2, however, was within the physiologic range found in normal rat liver (compare lanes 3 and 4 with lane 5). SCP-2 overexpression had no effect on SCPx levels or the previously recognized 30- and 35-kDa immunologically cross-reactive isoforms of SCP-2 secretion.

**Immunolocalization of SCP-2**—SCP-2 overexpression resulted in a ratio of SCP-2 immunostaining (large gold beads) to nonspecific immunostaining (small gold beads) in peroxisomes that averaged 8 ± 1.4 with a range of 3–18 (Fig. 2B). In NEO cells, peroxisomal SCP-2 immunostaining was infrequent, and...
Sterol Carrier Protein-2 Enhances Cholesterol Trafficking

**Fig. 1.** Immunoblot analysis of SCP-2 levels in wild-type and transfected McA-RH7777 cells and rat liver. Samples of total cell lysate and rat liver homogenate (50 μg each) were fractionated by 12.5% SDS-PAGE and electrophoretically transferred to Immobilon P. Blots were immunostained with an anti-rat SCP-2 antisera and detected as described under “Experimental Procedures.” 1st lane, wild-type McA-RH7777 cells; 2nd lane, vector only transfectants (NEO); 3rd lane, pre-SCP-2 transfectant (SCPhigh); 4th lane, pre-SCP-2 transfectant (SCPmed); and 5th lane, rat liver homogenate.

**Fig. 2.** Immunoelectron microscopy of NEO and SCPhigh transfectants. A and B are representative micrographs of cells immunostained with a goat anti-mouse antibody conjugated to a 15-nm gold bead (nonspecific probe) and anti-SCP-2 antibody counterstained with a goat anti-rabbit antibody conjugated to a 30-nm gold bead (specific probe). Large arrows identify anti-SCP-2 immunoreactivity in peroxisomes. Small arrows identify nonspecific immunoreactivity. Mitochondria are identified by M. The micrographs were taken at a magnification of ~20,000 ×.

at most the ratio of SCP-2 to nonspecific immunostaining was 2 (Fig. 2A). In extraperoxisomal areas, there was a ratio of SCP-2 immunoreactivity to nonspecific immunoreactivity of 1.6 ± 0.6 in NEO cells (n = 9) and 2.1 ± 0.7 in SCPhigh cells (n = 15), p > 0.05.

**Effect of SCP-2 Overexpression on Sterol Synthesis and Mass—**Total sterol synthesis was reduced by 12%, from 156,200 ± 3010 in NEO cells to 137,400 ± 4100 dpm/h/mg protein in SCPhigh cells. Cellular cholesterol mass, however, was 38 and 46% higher in SCPhigh and SCPhigh cells, in parallel with 80, 67, and 53% reductions in the levels of lanosterol, zymosterol, and desmosterol, respectively, compared with levels in NEO cells (Table II). There was no difference in the sterol content between wild-type McA-RH7777 cells and NEO transfectants (data not shown). Squalene content was unaffected by SCP-2 overexpression and was 0.5 ± 0.2 μg/mg protein. The lower levels of cellular desmosterol, in SCPhigh cells, could not be explained by an increase in the microsomal enzyme activity responsible for converting desmosterol to cholesterol (data not shown). In order to determine the effect of SCP-2 overexpression on cholesterol distribution, NEO and SCPhigh cells were fixed and treated with cholesterol oxidase. Consistent with previous reports (37) and in both cell lines, 90% of cholesterol and 80% of desmosterol were oxidized by cholesterol oxidase. These findings indicate that the plasma membrane contains the majority of cellular sterol and that the changes in sterol mass, induced by SCP-2 overexpression, predominantly affect plasma membrane sterol pools.

In order to further characterize the reason for the reduced levels of sterol intermediates in SCPhigh cells, we examined the kinetics of lanosterol, zymosterol, and desmosterol conversion to cholesterol by acetate pulse-chase combined with the cholesterol oxidase method. At the initial time point, in both NEO and SCPhigh cells, the sum of labeled lanosterol, zymosterol, and zymostenone was less than 10% of total labeled sterol and fell to undetectable levels by 4 h (data not shown). Desmostenone, on the other hand, was detected in both cell lines up to the last time point (Fig. 3), indicating that the conversion of desmosterol to cholesterol is the rate-limiting step in sterol intermediate processing in these cells. In both NEO and SCPhigh cells, there was a strict precursor product relationship between plasma membrane desmosterol and total cholesterol (cholesterol + cholestenone). SCP-2 overexpression facilitated this reaction, and after 6 h of acetate chase, over 90% of cellular desmosterol was converted to cholesterol, as compared with only 46% in NEO cells. A nonlinear regression analysis of the desmostenone disappearance data demonstrated that for both cell lines, the kinetics of plasma membrane desmosterol to cholesterol conversion best fit a two-compartment model. In NEO cells, 20% of plasma membrane desmosterol was in a rapidly turning over pool (t1/2 ~36 min), and 80% was in a slower pool with a t1/2 ~14 h. SCP-2 overexpression increased the proportion of desmosterol in the fast pool to 57% (t1/2 ~28 min) and doubled the rate of desmosterol flux through the slow pool (t1/2 ~7 h).

**Effect of SCP-2 Overexpression on Sterol Trafficking—**The rate of transfer of newly synthesized desmosterol and cholesterol to the plasma membrane was examined using cholesterol oxidase. The rate of appearance of labeled desmosterol in the plasma membrane was unchanged by SCP-2 overexpression and reached the steady state proportion of 80% by 120 min with a t1/2 ~42 min (Fig. 4A). The transfer of nascent cholesterol to the plasma membrane, however, was significantly increased by SCP-2 overexpression (Fig. 4B). In NEO cells, it took 120 min before the percentage of newly synthesized cholesterol in the plasma membrane reached 90% or steady state levels (t1/2 ~32 min). In contrast, in SCPhigh cells, the appearance of newly synthesized cholesterol in the plasma membrane reached 90% or steady state by 45 min (t1/2 ~8 min). This 4-fold increase in transfer rate could not be explained by an increase in the amount of labeled sterol secreted into the media (in both NEO and SCPhigh cells, <1% of the total labeled cholesterol and desmosterol was secreted into the media at 2 h).

In order to determine if SCP-2 overexpression altered the rate of plasma membrane cholesterol internalization, studies were performed on cells loaded with exogenously administered [3H]cholesterol followed by cholesterol oxidase treatment at the indicated times (Fig. 5). The rate of appearance of [3H]cholesterol within the cell was significantly increased by SCP-2 overexpression and reached steady state levels at 2–4 h with a t1/2 ~1 h. In NEO cells, however, it took between 8 and 16 h to reach steady state with a t1/2 ~4.1 h that was 4-fold slower. The difference in the rate of cholesterol influx could not be ex-
Sterol Carrier Protein-2 Enhances Cholesterol Trafficking

Effect of SCP<sub>2</sub> expression on cellular sterol levels

Table II

| Transfected cell line | Lanosterol | Zymosterol | Desmosterol | Cholesterol | μg/mg protein |
|-----------------------|------------|------------|-------------|-------------|---------------|
| NEO                   | 0.6 ± 0.1  | 0.3 ± 0.1  | 6.2 ± 0.3   | 11.2 ± 0.2  |
| SCP<sub>med</sub>     | 0.1 ± 0.1* | 0.1 ± 0.1* | 2.8 ± 0.2*  | 15.5 ± 0.2* |
| SCP<sub>high</sub>    | 0.1 ± 0.1* | 0.1 ± 0.1* | 2.9 ± 0.2*  | 16.4 ± 0.3* |

*p < 0.05 compared with NEO cells.

Fig. 3. Pulse-chase study of cholesterol synthesis. NEO cells (dashed lines) and SCP<sub>high</sub> cells (solid lines) were incubated in medium containing 5% LPDS for 24 h. Fresh medium was added containing 100 μCi of [3H]acetate in ethanol (<1% final), and the cells were incubated for 1 h at 37°C. At time 0, the medium was removed and fresh medium containing 10 mM sodium acetate was added followed by continued incubation for the times indicated. Cells were cholesterol oxidase-treated, and lipid extracts were analyzed by HPLC and assayed for radioactivity as described under “Experimental Procedures.” Open circles represent desmosterol and closed circles represent cholesterol + cholestene. Each point is the mean of triplicate determinations. Standard deviations are not shown and were ≤10% of the mean for all points.

Fig. 4. Time course of transfer of newly synthesized desmosterol and cholesterol to the plasma membrane. NEO cells (open circles) and SCP<sub>high</sub> cells (closed circles) were incubated in medium containing 5% LPDS for 24 h. At time 0, 100 μCi of [3H]acetate in ethanol (<1% final) was added to each dish, and after incubation for the times indicated, cells were cholesterol oxidase-treated, and lipid extracts were analyzed by HPLC as described under “Experimental Procedures.” Results are the mean ± S.E. of triplicate determinations, and for some time points the error bars are smaller than the symbols. A illustrates the time course of the percentage of cellular [3H]desmosterol that is susceptible to cholesterol oxidase. B illustrates the time course of the percentage of cellular [3H]cholesterol that is susceptible to cholesterol oxidase. *p < 0.05 compared with NEO cells.
manner and plateaued at a 25-hydroxycholesterol dose of 4
SCPhigh cells, CE synthesis increased in a dose-dependent
cholesterol at the doses indicated (Fig. 7). In both NEO and
and NEO cells. Cells were preincubated for 24 h in medium
the effect of 25-hydroxycholesterol on CE synthesis in SCPhigh
cells, respectively. Cells were preincubated for 24 h in medium
5% LPDS, and after incubation for the times indicated, cells were
cholesterol oxidase-treated and lipid extracts were analyzed by TLC as
described under “Experimental Procedures.” The ordinate represents
the time course of changes in the ratio of cellular [3H]cholesterol to
[3H]cholestenone. Results are the mean ± S.E. of triplicate determinations,
and for some time points the error bars are smaller than the
symbols. *p < 0.05 compared with NEO cells.

which was 90 ± 12 and 84 ± 6 pmol/h/mg protein in NEO and
SCPhigh cells, respectively.

In order to determine if the effect of SCP-2 on CE synthesis
could be reversed by treatment with oxysterol, we examined the
effect of 25-hydroxycholesterol on CE synthesis in SCPhigh
and NEO cells. Cells were preincubated for 24 h in medium
with 5% LPDS, followed by treatment for 5 h with 25-hydroxy-
cholesterol at the doses indicated (Fig. 7). In both NEO and
SCPhigh cells, CE synthesis increased in a dose-dependent
manner and plateaued at a 25-hydroxycholesterol dose of 4
μg/ml. At the 4 μg/ml dose of 25-hydroxycholesterol, CE syn-
thesis was 2-fold higher in SCPhigh cells. The incorporation of
[3H]oleate into triglyceride was unaffected by SCP-2 overexpres-
sion as well as 25-hydroxycholesterol treatment.

Effect of SCP-2 Overexpression on Sterol and Lipoprotein
Cholesterol Secretion—Incubation of cells in lipid-free medium
for 24 h and assay of medium sterol levels revealed that SCP-2
overexpression reduced the appearance of desmosterol by 85%
and cholesterol by 59 and 72% in SCPmed and SCPhigh cells,
respectively (Table IV). In order to determine if the reduction in
cholesterol appearance in the medium was due to a reduction in
lipoprotein cholesterol secretion, we labeled cells with [3H]ac-
etate and [14C]cholesterol and separated secreted lipoproteins by
FPLC. Of the total labeled cholesterol secreted, >95% was
unesterified. The major lipoprotein species found in the me-
dium of both NEO and SCPhigh cells eluted with the same
mobility as HDL (fractions 33–50) and contained >95% of the
labeled cholesterol. SCP-2 overexpression reduced the secre-
tion of [3H]cholesterol by 70% (Fig. 8A) and [14C]cholesterol by
52% (Fig. 8B). The 70% reduction in the secretion of newly
synthesized HDL cholesterol parallels the 72% reduction in
cellular cholesterol loss (see Table IV).

The final series of experiments examined the effect of SCP-2
overexpression on the gene expression of apolipoproteins (apo)
A-1 and E. The secretion of [35S]methionine-labeled apoA-I and
apoE was examined in the medium from wild-type McA-

Fig. 5. Time course of transfer of plasma membrane choles-
terol to the cholesterol oxidase-insensitive pool. NEO cells (open
circles) and SCPhigh cells (closed circles) were incubated in medium
containing 5% LPDS for 24 h, followed by incubation in unsupple-
mented DMEM with 0.05% bovine serum albumin and 5 μCi of [3H]cho-
lesterol. After 30 min, the medium was changed to fresh medium with
5% LPDS, and after incubation for the times indicated, cells were
cholesterol oxidase-treated and lipid extracts were analyzed by TLC as
described under “Experimental Procedures.” The ordinate represents
the percentage of cellular [3H]cholesterol that is susceptible to cholesterol
oxidase. Each point is the mean of triplicate determinations. Standard
deviations are not shown and were ≤10% of the mean for all points.

RH7777, NEO, SCPmed, and SCPhigh cells. SCP-2 overexpres-
sion reduced the secretion of apoA-I and E by 70 and 23%,
respectively (Fig. 9A). This decrease was due to a 72 and 50%
decrease in apoA-I and E transcript levels (Fig. 9B). Thus, the
magnitude of the changes in HDL cholesterol and apoA-I se-
cretion, observed in these studies, correlates with the decrease
in total cholesterol mass secreted by SCP-2 transfectants (see
Table IV and Fig. 8).

DISCUSSION

The transfer of nascent cholesterol to the plasma membrane
is a rapid (62) energy (63) and SCP-2 (32) -dependent reaction
that occurs against a large cholesterol gradient. The current
studies, taken together with data from Puglielli et al. (32),
indicate that the rate of this reaction is regulated and propor-
tional to the peroxisomal level of SCP-2. Moreover, in SCPhigh
cells the rate of cholesterol internalization paralleled the rate
of cholesterol transfer to the plasma membrane, suggesting
that the latter event determines the rate of bi-directional cho-
lesterol flux. Lange et al. (64) has demonstrated that ACAT
activity is substrate-dependent and limited by the supply of
cholesterol from the plasma membrane. At steady state, how-
ever, only a fraction of the cholesterol that is internalized
from the plasma membrane is converted to CE, whereas the remain-
der is rapidly transported back to the plasma membrane (64).
Thus, the bi-directional movement of cholesterol represents a
continuous cycle that may have regulatory significance for
ACAT action. In this regard, SCP-2 overexpression reduced CE
synthesis, regardless of whether cells were cholesterol-de-
prived or repleted with LDL in the medium. In hepatoma cells,
exogenous and endogenously synthesized sources of cholesterol
are equally available for esterification, indicating that choles-
terol molecules from all sources rapidly mix at some point prior
to ACAT action (65). Although the methodology used, in the
current study, to determine the rate of cholesterol transfer to
the plasma membrane measures newly synthesized cholesterol
movement, the effect of SCP-2 overexpression on CE synthesis
implies that SCP-2 enhances the trafficking of intracellular
cholesterol, regardless of the source. Since lysosomal derived
cholesterol transfers to the plasma membrane prior to ACAT

Fig. 6. Time course of transfer of lipoprotein-derived choles-
terol to the plasma membrane. NEO cells (open circles) and SCPhigh
cells (closed circles) were pulsed with radiolabeled LDL containing
[3H]cholesteryl oleate, followed by a chase with unlabeled LDL. At the
times indicated, cells were cholesterol oxidase-treated, and lipid ex-
tracts were analyzed by HPLC and assayed for radioactivity as de-
scribed under “Experimental Procedures.” The ordinate represents
the percentage of cellular [3H]cholesterol that is susceptible to cholesterol
oxidase. Each point is the mean of triplicate determinations. Standard
deviations are not shown and were ≤10% of the mean for all points.
action (51, 66), and SCP-2 overexpression had no effect on this transfer reaction, it appears that LDL-derived cholesterol must complete a cycle before it becomes substrate for SCP-2 action. The inverse relationship between SCP-2 levels and CE synthesis, observed in the current studies, has been previously reported (67). Furthermore, Hirai et al. (16) have recently studied the time course of changes in SCP-2 gene expression and CE synthesis during macrophage foam cell formation and found that, initially, CE synthesis increased and then declined as levels of SCP-2 protein increased in parallel with free cholesterol levels. In the absence of effects of SCP-2 overexpression on the in vitro activity of ACAT and neutral CE hydrolase, we propose that SCP-2 inhibits CE synthesis by increasing the rate of cholesterol cycling, which prevents cholesterol from equilibrating with ACAT, and by shifting the distribution of cholesterol from the cell interior to the plasma membrane. Moreover, the transfer action of SCP-2 secondarily increases plasma membrane cholesterol capacity, which functions to decrease the mass of cycled cholesterol. The distribution of cellular cholesterol has important implications for the regulation of sterol-dependent genes that respond to changes in putative pools of regulatory sterol. In this regard, current knowledge suggests that the rate of apoA-I production is the main determinant of HDL production (68); hence, the decrease in apoA-I gene expression induced by SCP-2 overexpression decreases HDL cholesterol secretion. Changes in the gene expression of apoA-I and E have been correlated with changes in cellular cholesterol content (52, 69–71), suggesting that SCP-2 overexpression alters the expression of these genes by reducing CE

### Table III

**Effect of SCP-2 expression on cholesterol esterification**

| Transfected cell line | Medium | Cholesterol Mass | Cholesterol ester synthesis | Triglyceride synthesis |
|-----------------------|--------|------------------|----------------------------|-----------------------|
|                       |        | Free (µg/mg protein) | Ester (nmol/h/mg protein) |                       |
| NEO                   | LPDS   | 12.5 ± 0.4       | 3.2 ± 0.7                   | 0.6 ± 0.06            | 1.8 ± 0.2            |
|                       | LDL    | 35.7 ± 1.9       | 10.7 ± 1.5                  | 1.6 ± 0.01            | 6.4 ± 0.3            |
| SCP-high              | LPDS   | 17.6 ± 0.6*      | 1.5 ± 0.1*                  | 0.3 ± 0.03*           | 1.8 ± 0.2            |
|                       | LDL    | 37.4 ± 1.7       | 3.2 ± 1.8*                  | 0.7 ± 0.07*           | 6.3 ± 0.3            |

* p < 0.05 compared with NEO cells in the same medium conditions.

### Table IV

**Effect of SCP-2 expression on sterol secretion**

Cells were plated and grown as described in the legend to Table II in medium with 5% LPDS. After 24 h, the medium was collected and lipids extracted. Medium sterol levels were assayed by HPLC as described under “Experimental Procedures.” Values represent the means ± S.E. of triplicate determinations.

| Transfected cell line | Desmosterol (total) (µg) | Cholesterol (µg) |
|-----------------------|---------------------------|------------------|
| NEO                   | 0.7 ± 0.1                 | 3.9 ± 0.4        |
| SCP-med               | 0.1 ± 0.1*                | 1.6 ± 0.1*       |
| SCP-high              | 0.1 ± 0.1*                | 1.1 ± 0.2*       |

* p < 0.05 compared with NEO cells.

---

**FIG. 7. Effect of 25-hydroxycholesterol on CE synthesis.** NEO cells (open circles) and SCP-high cells (closed circles) were incubated for 24 h in medium with 5% LPDS, followed by a 5-h treatment with 25-hydroxycholesterol at the indicated doses. CE synthesis was determined as described under “Experimental Procedures.” Results are the mean ± S.E. of triplicate determinations. *p < 0.05 compared with NEO cells.

**FIG. 8. Distribution of labeled cholesterol in medium lipoproteins.** NEO cells (open circles) and SCP-high cells (closed circles) were labeled 24 h with [3H]acetate and [14C]cholesterol in medium with 1% LPDS. At the end of the incubation, the medium was collected and analyzed by PPLC on a Superose 6 column (see “Experimental Procedures”). Eluted fractions were extracted and analyzed by thin layer chromatography, and labeled cholesterol bands were scraped and assayed by liquid scintillation counting. A illustrates the elution profile of lipoproteins labeled with endogenously synthesized cholesterol. B illustrates the elution profile of lipoproteins labeled with exogenous [14C]cholesterol.

---

* Sterol Carrier Protein-2 Enhances Cholesterol Trafficking
Sterol Carrier Protein-2 Enhances Cholesterol Trafficking

stores or the distribution of free cholesterol within the cell.

The mechanism by which SCP-2 acts from within peroxisomes to regulate cholesterol transfer to the plasma membrane is unclear; however, insight into this process can be gleaned from ultrastructural studies of peroxisomes and biochemical studies of cholesterol trafficking. Previous studies have demonstrated an inverse relationship between peroxisomal SCP-2 content and peroxisomal levels of free cholesterol (67). This observation suggests that reduced SCP-2 results in slower clearance of intracellular cholesterol and peroxisomal cholesterol accumulation. Peroxisomal cholesterol may arise from peroxisomal cholesterol biosynthesis (28, 29) or it may come from adjacent lipid droplets or vesicles (26), derived from intracellular cholesterol is transferred to the plasma membrane (47, 72). Under these conditions, the branching reticular structure of peroxisomes may allow the plasma membrane to act as a more rapidly exchanging plasma membrane domain. The effect of SCP-2 on desmosterol to cholesterol conversion does not result from a change in the level of microsomal 24-reductase activity, or the rate of desmosterol transfer to the plasma membrane but instead reflects an enhanced rate of desmosterol internalization, which is consistent with the observed shift from a slowly exchanging domain to a more rapidly exchanging plasma membrane domain.

In conclusion, increasing evidence indicates that SCP-2 gene expression is regulated in a tissue-specific manner (41) and in response to a growing number of stimuli (15–18, 41, 79). The current studies suggest that changes in SCP-2 gene expression may have a significant role in the regulation of sterol metabolism through changes in the rate of cholesterol cycling and distribution. In this regard, the role of SCP-2 in bile acid synthesis and biliary cholesterol enrichment needs to be examined, particularly in view of recent data demonstrating a strong correlation between hepatic SCP-2 levels, free cholesterol content, and the incidence of gallstones (80). SCP-2-mediated changes in plasma membrane cholesterol content may also be important in the regulation of integral membrane protein function (81, 82), the regulation of cholesterol supply for plasma membrane production during cell growth (41), and HDL-mediated reverse cholesterol transport.

Acknowledgments—We are grateful to Drs. Skui Krisans and Yvonne Lange for helpful discussions and to Barbara Dixon for her typing and graphical skills. The HPLC used for these studies was a generous gift from Dr. Angelo Scanu.

REFERENCES

1. Lange, Y. (1992) J. Lipid Res. 33, 315–321
2. Wang, X., Sato, R., Brown, K. H., and Goldstein, J. L. (1994) Cell 77, 53–62
3. Gasic, G. P. (1994) Cell 77, 17–19
4. Pastuszyn, A., Noland, B. J., Bazan, J. F., Fletterick, R. J., and Scallen, T. J. (1987) J. Biol. Chem. 262, 12319–12327
5. Kesav, S., McLaughlin, J., and Scallen, T. J. (1992) Biochem. Soc. Trans. 20, 818–824
6. Billheimer, J. T., and Reinhardt, M. P. (1990) in Subcellular Biochemistry (Hilderson, H. J., ed) pp. 301–331, Plenum Publishing Corp., New York
7. Vahouny, G. V., Chandlerhahn, R., and Kharroubi, A. (1985) Chem. Phys. Lipids 38, 1–225
8. Selman, H., Diven, W., Eirk, M., Noland, B. J., Chandlerhahn, R., and Scallen, T. J. (1986) Biochem. J. 230, 19–24
9. Chandlerhahn, R., Noland, B. J., Scallen, T. J., and Vahouny, G. V. (1982) J. Biol. Chem. 257, 8928–8934
10. van Noot, M., Rommerts, F. F. G. van Amersingen, A., and Wirtz, K. W. A. (1988) Biochem. Biophys. Res. Commun. 154, 60–65
11. Vahouny, G. V., Chandlerhahn, R., Noland, B. J., Irwin, D., Dennis, P., Lambeth, J. D., and Scallen, T. J. (1983) J. Biol. Chem. 258, 11731–11737
12. McNamaro, B. C., and Jefcoate, C. R. (1989) Arch. Biochem. Biophys. 275, 53–62
13. Chandlerhahn, R., Tanaka, T., Strauss, J. F., Irwin, D., Noland, B. J., Scallen, T. J., and Vahouny, G. V. (1983) Biochem. Biophys. Res. Commun. 117, 702–709
14. Gavey, K. L., Noland, B. J., and Scallen, T. J. (1981) J. Biol. Chem. 256, 2995–2999
15. Groh, M., Flaviano, A., Kansal, S., and Baum, C. L. (1993) FASEB J. 7, A566
16. Hirai, A., Kino, T., Tokina, K., Tahara, K., Tamura, Y., and Yoshida, S. (1994) J. Clin. Invest. 94, 2215–2223
17. Kruemer, R., Pomerantz, K. B., Kesav, S., Scallen, T. J., and Hajjar, D. P. (1995) J. Lipid Res. 36, 2630–2638
18. Billheimer, J. T., Strehl, L. I., Davis, G. L., Strauss, J. F., III, and Davis, L. G. (1996) DNA Cell Biol. 15, 159–165
