Plasma Membrane Rafts Complete Cholesterol Synthesis by Participating in Retrograde Movement of Precursor Sterols*

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Mammalian cells synthesize significant amounts of precursor sterols, in addition to cholesterol, at the endoplasmic reticulum (ER). The newly synthesized sterols rapidly move to the plasma membrane (PM). The mechanism by which precursor sterols move back to the ER for their enzymatic processing to cholesterol is essentially unknown. Here we performed pulse-chase experiments and showed that the C29/C30 sterols rapidly move from the PM to the ER and are converted to cholesterol. The retrograde precursor sterol transport is largely independent of the Niemann-Pick type C proteins, which play important roles in late endosomal cholesterol transport. In contrast, disrupting lipid rafts significantly retards the conversion of C29/C30 and C28 sterols to cholesterol, causing the accumulation of precursor sterols at the PM. Our results reveal a previously undisclosed function of the PM lipid rafts: they bring cholesterol biosynthesis to completion by participating in the retrograde movement of precursor sterols back to the ER.

Cholesterol is an important lipid component of biological membranes. It also serves as an obligatory precursor for the biosyntheses of steroid hormones, bile acids, and bioactive oxysterols (1). In mammals, virtually every cell of the body is capable of de novo cholesterol biosynthesis. Cholesterol biosynthesis involves successive enzymatic reactions, converting the simple, 2-carbon precursor acetyl-CoA into the 30-carbon, acyclic, apolar molecule squalene. The subsequent oxidation and cyclization of squalene yields the first sterol in the biosynthetic pathway, the 30-carbon (C30) lanosterol. The conversion of the C30 lanosterol to C27 cholesterol involves at least 18 enzymatic reactions (2) and can proceed through two pathways, with one involving desmosterol as the final precursor, and the other involving lathosterol and 7-dehydrocholesterol as the final precursors (Fig. 1). The biosynthetic enzymes responsible for converting squalene to cholesterol are all located in the endoplasmic reticulum (ER) membranes (2). Previous studies showed that, in addition to synthesizing cholesterol, mammalian cells also synthesize substantial amounts of precursor sterols (3, 4). Similar to cholesterol, the precursor sterols leave the ER and rapidly reach the plasma membrane (PM) within 30-min time (5, 6). The precursor sterols at the PM are predicted to move back to the ER to be enzymatically processed to cholesterol. This retrograde movement is an essential step to complete cholesterol biosynthesis. Little is known about the mechanism(s) of retrograde movement of sterols. In yeast Saccharomyces cerevisiae, Prinz and coworkers showed that the retrograde movement of exogenously added sterols from the PM to the ER is governed by a non-vesicular mechanism that involves ATP-binding cassette transporters (7) and oxysterol-binding protein-related proteins (8). In mammalian cells, Maxfield and coworkers showed that both vesicular and non-vesicular trafficking mechanisms operate to govern the transport of a naturally fluorescent cholesterol analog dehydroergosterol (9, 10). Earlier, based on inhibitor studies, Metherall and colleagues (11) and Field and colleagues (12) suggested that multiple drug resistance proteins may be involved in the retrograde movement of precursor sterols.

The Niemann-Pick type C1 (NPC1) protein is a multispan membrane protein containing a sterol-sensing domain, which plays an essential role in the ability of the protein to bind cholesterol (13). Studies using mutant cells that lack the NPC1 protein show that NPC1 is involved in the egress of low density lipoprotein-derived cholesterol from the late endosomes to the PM, ER, and mitochondria (14–16). In certain cell types, including macrophages, NPC1 is also involved in the post-PM trafficking of biosynthesized cholesterol, and possibly other C27 sterols, from the PM to the ER (17). On the other hand, the ability of the mutant NPC cells to convert precursor sterols, especially the methylated sterols (i.e. C28, 29, and C30 sterols), to cholesterol, is unknown.

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4 The abbreviations used are: CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; fetal bovine serum, fetal-bovine serum; PM, plasma membrane; ER, endoplasmic reticulum; TLC, thin layer chromatography; NPC, Niemann-Pick type C; sterol regulatory element-binding protein, sterol regulatory element binding protein cleavage-activating protein, sterol regulatory element-binding protein cleavage activating protein; GC-MS, gas-liquid chromatography-mass spectrometry; HCD, 2-hydroxypropyl-β-cyclodextrin; MCD, methyl-β-cyclodextrin; LE, endosome; LYS, lysosome; Hf, human fibroblast.
Lipid Rafts Support Cholesterol Biosynthesis

In this study, we monitored the intracellular fate of biosynthesized precursor sterols by feeding cells with radioactive acetate, performed pulse-chase experiments, and used several TLC systems to analyze the composition of labeled sterols. We also employed gas chromatography-mass spectrometry (GC-MS) to identify various precursor sterols in cells grown in sterol-free media. Our results show that, upon arrival at the PM, precursor sterols rapidly move back from the PM to the ER, to be enzymatically converted to cholesterol. The retrograde movement of the precursor sterols is largely independent of NPC proteins but depends on the functionality of the PM lipid rafts.

EXPERIMENTAL PROCEDURES

Materials

Various reagents and procedures were as described previously (15, 18).

Media, Cell Lines, and Cell Culture

Medium A is Dulbecco Modified Earle’s Medium (DMEM) and Ham’s F12 at 1:1 (for Chinese hamster ovary (CHO) cells), or Dulbecco’s modified Eagle’s medium (for human fibroblasts (Hf cells)), plus 10% fetal bovine serum; Medium D is appropriate medium plus 0.2% fatty acid-free bovine serum albumin; Medium F is appropriate medium without serum. All media contain 50 units/ml penicillin and 50 μg/ml streptomycin as antibiotics. 25RA is a mutant CHO cell line that is resistant to 25-hydroxycholesterol (19) and contains a gain-of-function mutation in sterol regulatory element-binding protein cleavage-activating protein, and a premature translational termination mutation near the 3’-end of the npc1 coding sequence, producing a non-functional truncated NPC1 protein (22). The A101 clone is a CHO cell mutant lacking the NPC1 mRNA and protein; it was isolated from parental CHO cells that express the murine ecotropic retrovirus receptor (JF17 cell). CHO clones A101 and JP17 were from professors Ohno and Ninomiya (Totori University School of Medicine, Japan) (23). A normal Hf cell line (normal-1) was from Dr. Peter Pentchev (formerly National Institute of Health). A second normal Hf cell line, GM00038, was from Coriell Institute (normal-2); a mutant NPC1 human fibroblast cell line, GM03123, was also from Coriell. GM03123 contains two point mutations in the NPC1 protein: P237S and I1061T. The NPC2 human fibroblast cell line was from Dr. Yiannis Ioannou (Mount Sinai School of Medicine) (24).

Pulse-Chase Experiments with [3H]Acetate

For CHO cells, cells were seeded into 6-well plates or 100-mm dishes and grown in Medium D for 2 days as described (22). For Hfs, cells were plated and grown in Medium A to near confluency, washed twice with PBS, and grown for 2 days in Medium D. To label cells with [3H]acetate, cells were washed with pre-warmed (37 °C) PBS twice, then pulse-labeled with 20 μCi/ml [3H]acetate (20 μCi/well or 100 μCi/100-mm dish) for the time indicated at 37 °C. After pulse labeling, the medium was removed; the cells were washed twice with pre-warmed PBS and incubated with the chase media (pre-warmed Medium D or Medium B) for up to 24 h. In some experiments, the chase media were collected and extracted with chloroform/methanol (2:1, v/v) and analyzed for radiolabeled sterols. Cellular lipids were extracted with hexane/isopropanol (3:2, v/v), and cellular protein was solubilized in 0.1 n NaOH to determine protein content (25). The non-saponifiable lipids (containing the sterols) were isolated as previously described (26).

Sterol Analyses by TLC

The non-saponifiable fractions were spotted onto channeled silica TLC plates and run in methylene chloride/ethanol acetate (97:3, TLC system I) to separate C29/C30, C28, and C27 sterols (27). Lanosterol and cholesterol were added to the samples to serve as internal standards. After chromatography, the plates were briefly stained with iodine to identify the C29/C30 sterol band and the C27 sterol band. The band located between the C29/C30 and C27 sterol bands is the C28 sterol band (27). The sterol bands were scraped and counted with a liquid scintillation counter. To examine the sterol composition of the C27 sterols, the C27 sterol bands separated by TLC system I were scraped and extracted with chloroform/methanol (2:1) twice. The extracts were transferred to new glass tubes, washed once with water, and dried under nitrogen. The C27 sterols were either acetylated as described (27) or left non-acetylated. The acetylated C27 sterols were separated by TLC system II on silver nitrate impregnated plates. The plates were prepared by rapidly dipping commercially prepared silver nitrate-impregnated plates.
nated TLC plates in 10% silver nitrate solution (in acetonitrile), followed by air drying. The samples were repeatedly chromato- 
graphed (three times), each time for 1 h, using the solvent hex-
ane/benzene (80:20) (28). Non-acetylated sterol samples were 
separated using the silver nitrate-impregnated TLC plates pre-
pared as described above and chromatographed for 4 h in a 
plastic-wrap sealed glass chamber in 100% chloroform (system 
III). Plates were run with authentic cholesterol, desmosterol, 
lathosterol, and zymosterol, or their acetylated derivatives were 
spotted in parallel lanes as standards. After TLC, the standard-
prepared as described above and chromatographed for4 hi na 
the resulting 1 ml of post nuclear supernatant was placed onto a 
9-ml 30% Percoll solution and centrifuged at 200,000 × g for 30 min twice to remove the Percoll particles. 

**Sterols**

**Lipid Analyses by GC-MS**

Cellular lipids were extracted and saponified as described 
above, using nanograde organic solvents. The dried sterol sam-

dules were derivatized with 0.25 ml of Sigma-Sil-A (Sigma) at 
60 °C for 30 min. The trimethylsilyl derivatives of sterols were 

**RESULTS**

**ER-to PM Anterograde Transport of Biosynthesized Precursor Sterols**—To monitor the distribution of newly synthesized pre- 
cursors sterols inside the cells, WT CHO cells grown in Medium 
D were incubated with [3H]acetate for 1 h. The labeled cell 

determined by methods described previously (29). We also 
employed the 11% Percoll gradient centrifugation method (22) 

to isolate various crude subcellular fractions. This method effi-
ciently separated late endosomes/lysosomes from other mem-

**Cell Fractionation after Cellular Labeling with [3H]Acetic Acid**

Two different methods were used to prepare the PM and the 

**Calculations**

Data were presented as means ± S.D. unless specified in the 

**Table 1**

| Sterols          | System I | System II | System III |
|------------------|----------|-----------|------------|
| C30 sterols      | 0.53     | 0.53      | 0.42       |
| C29 sterols      | 0.53     | 0.53      | 0.42       |
| C28 sterols      | 0.42     | 0.42      | 0.42       |
| C27 sterols      | 0.35     | 0.49      | 0.49       |
| Cholesterol acetate | 0.49  | 0.49      | 0.49       |
| Desmosterol acetate | 0.18  | 0.18      | 0.18       |
| Lathosterol acetate | 0.49  | 0.49      | 0.49       |
| Zymosterol acetate | 0.18  | 0.18      | 0.18       |
| Cholesterol      | 0.42     | 0.42      | 0.42       |
| Desmosterol      | 0.33     | 0.33      | 0.33       |
| Lathosterol      | 0.60     | 0.60      | 0.60       |
| Zymosterol       | 0.42     | 0.42      | 0.42       |

**Lipid Analyses by Enzymatic Assays**

The cellular free cholesterol and choline-containing phospho-
lipids were determined as described before (29).

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(Fig. 2A) show that fractions 1–3 contain ~75% of the total PM signal. Fractions 4 and 5 contain ~90% of the total HMG-CoA reductase signal. The labeled sterols present in fractions 1–10 were extracted and analyzed. The results show that the majority (>70%) of newly synthesized C29/C30, C27, and C28 sterols were present in fractions 1–3; <25% were present in fractions 4 and 5 (Fig. 2A). Cholesterol mass analysis showed that its distribution was similar to that of newly synthesized sterols; roughly 70% of cholesterol was present in fractions 1–3, whereas <25% was present fractions 4 and 5 (data not shown). In Method II, the analysis showed that the PM-rich fraction contained 60% of the total cell surface biotinylated protein signals, the IM-rich fraction contained 80% of the total HMG-CoA reductase signal (data not shown). The results of the labeled sterol distribution analysis (Fig. 2B) show that >60% of the newly synthesized C29/C30, C27, and C28 sterols were present in the PM fraction, whereas ~25% was present in the IM fraction. Small but significant amounts of 3H-labeled biosynthetic precursor sterols were recovered in the cytosol fraction. Cholesterol mass analysis showed that it was mainly recovered in the PM fraction as expected (Fig. 2B, white bar). These results show that the second method tended to underestimate the proportion of labeled sterols present in the PM fraction by ~10–12% compared with the first method. We sought to test the findings described in Fig. 2 (A and B) by using another approach. Cycloextrin is a water-soluble molecule that has high affinity for sterols. We and others had previously shown that when 2-hydroxypropyl-β-cyclodextrin (HCD) is added to the medium of intact CHO cells for 10 min or less, it efficiently removes cell surface cholesterol; in contrast, under the same condition, cholesterol sequestered in the internal membrane compartment(s) is very resistant to extraction by HCD (15). We pulse-labeled the parental 25RA CHO cells and the NPC1-deficient mutant CT43 cells with [3H]acetate for 1 h, then exposed these cells to HCD for 10 min. Labeled lipids in cells and in the media were extracted and analyzed by TLC. The results show that, in both cell types, >35% of the total newly synthesized C27 sterols were accessible to HCD (Fig. 2C). These results, together with Fig. 2 (A and B), suggest that after 1 h of synthesis, most of the biosynthesized C27 sterols is located at the PM. The results in Fig. 2C show that newly synthesized C28 and C29/C30 sterols were also extractable by HCD, although less so than C27 sterols, suggesting that HCD may bind with less affinity toward C28 and C29/C30 sterols than C27 sterols. It is possible that the additional methyl groups present in steroid ring A (the 4,4-methyl moieties) and/or present in the junction between rings C/D (the 14-α-methyl moiety) may hinder the binding between HCD and the sterol molecule. However, we cannot rule out the possibility that the C28 and C29/C30 sterols may reside in a microdomain of the PM different from where the C27 sterols reside. The results presented in Fig. 2C do imply that after 1 h of synthesis, a substantial amount of the biosynthesized sterols is located at or near the PM to be

FIGURE 2. Presence of biosynthetic precursor sterols at the PM. A, on day 0, WT CHO cells were plated in triplicate at 1 × 10⁶ cells per 100-mm dish and grown in Medium A (8 ml/dish). On day 1, the medium was switched to Medium D, and the cells were grown for another 2 days. On day 3, the cells were radiolabeled with [3H]acetate acid (20 μCi/ml, 5 ml/dish) for 1 h in Medium F at 37 °C. Cell homogenate was subjected to the 30% Percoll gradient centrifugation analysis (Method I) as described under “Experimental Procedures.” Afterward, lipids were extracted from each fraction, and the non-saponifiable lipids were analyzed by the TLC system I to identify radiolabeled biosynthetic precursor sterols. The distribution of biotinylated proteins and HMG-CoA reductase in each fraction were analyzed as described under “Experimental Procedures.” Data were reported as % of total in each sterol species as indicated. The results shown are averages of two experiments; the error bars indicate the sizes of difference between the average values. B, WT CHO cells were radiolabeled as described above. Cell homogenates were subjected to subcellular fractionation Method II to prepare the plasma membrane (PM), intracellular membranes (IM), and cytosol (CS) fractions (described under “Experimental Procedures”). Afterward, lipids were extracted from each fraction, the non-saponifiable lipids were analyzed by the TLC system I to determine [3H]sterols. Mass of free cholesterol (FC) was determined by the colorimetric assay as described under “Experimental Procedures.” Data are reported as % total in each lipid species as indicated. The results shown are averages ± S.D. of the biosynthesized sterols is located at or near the PM to be

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extractable to HCD. These results also show that the availability of various newly synthesized precursor sterols to HCD was almost identical between the 25RA and the CT43 cells, indicating that the movement of C28 sterols and C29/C30 sterols to the cell surface was independent of a functional NPC1 protein.

The results presented in Fig. 2, together with previous studies (5), show that immediately after biosynthesis, the majority of precursor sterols are transported from the ER to the PM. Thus, one or more PM-to-ER retrograde sterol movement systems are needed to bring precursor sterols arriving at the PM back to the ER, where all the post-squalene cholesterol biosynthetic enzymes reside, for their eventual conversion to cholesterol.

Role of NPC Proteins in the PM-to-ER Retrograde Transport of Biosynthesized Precursor Sterols—Because the NPC proteins play an important role in endosomal cholesterol transport, we examined whether the NPC-dependent sterol transport system is involved in the retrograde transport of newly synthesized precursor sterols. It is known that all the enzymes responsible for converting C29/C30 or C28 sterols to C27 sterols are located at the ER. This allowed us to use the conversions of C29/C30 or C28 sterols to C27 sterols as a biological assay to examine the retrograde movement of biosynthesized precursor sterols. Cells were pulse-labeled with [3H]acetate for 1 h, followed by chasing in the absence of the label for up to 6 h. At each time point, cellular sterols were analyzed by TLC systems I, II, and III. In unchased 25RA cells, roughly 58% of the label was in C27 sterols, with 15 and 27% of the label in C28 sterols and C29/C30 sterols, respectively (Table 2). The CT43 cells exhibited a somewhat different sterol distribution pattern than that of the 25RA cells, with ~43% in C29/C30 sterols, 16% in C28 sterols, and 42% of the label in C27 sterols (Table 2). We further analyzed the composition of the labeled C27 sterols by using TLC systems II and III. This analysis illustrated that the 25RA and CT43 cells exhibit very similar profiles for cholesterol and its C27 precursor sterols (Table 2), with ~50% of the total [3H]acetate in cholesterol, 10–12% in lathosterol, 15% in zymosterol, and 23–26% in desmosterol. Data from the chase experiments show that, in both cell types, the amount of labeled C29/C30 and C28 precursor sterols significantly decreased (by >2- to 3-fold), whereas the amount of labeled C27 sterols significantly increased (Fig. 3, A–C). Furthermore, the rates of production of C29/30 or C28 sterols, as well as the rate of consumption of the C27 sterols, were very similar in both the 25RA and CT43 cells. The labeled C27 sterols were further analyzed by TLC systems I, II, and III as described under “Experimental Procedures.” The results are plotted as % labeled sterols remaining at 2, 4, or 6 h of chase, relative to the values determined at 0-h chase time point (100%). The results shown are averages ± S.D.

FIGURE 3. The appearance or disappearance of various biosynthetic precursor sterols with time in parental and mutant NPC cells. A–C, parental (25RA) and NPC1 mutant CHO cells (CT43); D–F, normal human fibroblasts (NL-I and NL-2) and fibroblasts from NPC patients (NPC1 and NPC2). The CHO cells were plated in triplicate in 6-well dishes as described in Fig. 2B in Medium A. On day 1, the medium was switched to Medium D, and the cells were further grown for 2 days. The Hfs plated in triplicates in 6-well dishes were grown to near confluent stage were incubated in Medium D (1 ml/well) for 2 days before labeling with [3H]acetate. Cells were pulse-labeled with [3H]acetate (20 μCi/1 ml well) in Medium D (A–C) or Medium F (D–F) for 1 h. The cells were then harvested (0 h) or chased in Medium D (A–C) at 1 ml/well or in Medium F (D–F) at 1 ml/well for the indicated time. Afterward, cellular lipids were extracted, saponified, and analyzed by TLC systems I, II, and III as described under “Experimental Procedures.” The results are presented as % of the total labeled sterols remaining at 0, 2, 4, or 6 h of chase, relative to the values determined at 0-h chase time point (100%). The results shown are averages ± S.D.

### Table 2: Distribution of endogenously synthesized sterols in 25RA and CT43 CHO cells at 0- or 6-h chase times

| Sterol         | Chase | 25RA       | CT43       |
|---------------|-------|------------|------------|
| C29/C30 sterols | 0     | 27.3 ± 5.8 | 42.1 ± 5.0 (1.5)* |
| C28 sterols    | 0     | 5.1 ± 0.6  | 10.4 ± 1.8 (2.0) |
| C27 sterols    | 6     | 48.4 ± 0.4 | 9.8 ± 0.6 (2.0)  |
| Desmosterol   | 0     | 580 ± 6.1  | 41.6 ± 4.0 (0.7) |
| Lathosterol   | 6     | 901 ± 2.3  | 79.8 ± 5.1 (0.9) |
| Zymosterol    | 0     | 150 ± 2.0  | 15.2 ± 2.2 (1.0) |
| Cholesterol   | 6     | 261 ± 1.7  | 22.6 ± 1.9 (0.9) |

* Values in parentheses are ratios of values in CT43 cells versus values in 25RA cells.

### Notes:

A–C: Values reported (means ± S.D.) are % of total labeled sterols. They are derived from the results reported in Fig. 3. A–C.

To test whether the results observed in the 25RA/CT43 CHO cells were applicable to other cell types, we performed the same pulse-chase experiments in two strains of normal Hfs, one mutant NPC1 Hf, and one mutant NPC2 Hf. The results indicate (Fig. 3, D–F, and Table 3) that at 0-h chase, in normal Hf, 57–59% of the labeled sterols were C27 sterols, whereas 25–27 and 15% of the labeled sterols were C29/C30 and C28 sterols, respectively. In mutant NPC1 and NPC2 Hfs, 39–43% of the labeled sterols were C27 sterols, whereas 36–44% and 17–21% of the labeled sterols were C29/C30 and C28 sterols, respectively. Thus, similarly to the 25RA/CT43 CHO cell system, modest increases in the newly synthesized C29/C30 and C28 sterols (~1.5- or 1.2-fold, respectively) were seen in the mutant NPC cells. When chased up to 6 h, both the normal and mutant NPC Hfs demonstrated similar rates of increase in labeled C27 sterols (Fig. 3F), and similar rates of decreases in the labeled C29/C30 sterols (Fig. 3D). The decreases in C28 sterols were slightly slower in the mutant NPC Hfs (Fig. 3E). At 6-h chase time, mutant NPC Hfs showed 2.5- and 1.9-fold increases in the residual labeled C29/C30 and C28 sterols (Table 3); these results are similar to the findings described in the 25RA/CT43 CHO cell system (Table 2).

The data presented in Table 2 show that, in both the CHO cell system and the HF system, parental and mutant NPC cells...
Sterol content in parental and mutant NPC1 CHO cells

Cells as indicated were grown in Medium D for 2 days. Cellular sterols were analyzed by GC-MS as described under "Experimental Procedures." Results shown are means ± S.D. of three experiments in 25RA, CT43, and CT43/npc1 cells, and means ± difference of two experiments in JP17 and A101 cells.

| Sterol                     | 25RA    | CT43    | CT43/npc1 | JP17    | A101    |
|---------------------------|---------|---------|-----------|---------|---------|
|                           | ng/mg cell protein |         |           |         |         |
| Lanosterol                | 33 ± 8  | 126 ± 43 (3.8) | 69 ± 10 (0.5) | 293 ± 112 | 667 ± 100 (2.2) |
| Mu dimethylsterol         | 31 ± 10 | 562 ± 248 (18.1) | 102 ± 6 (0.2) | 185 ± 10 | 328 ± 14 (1.7) |
| Mu monomethylsterol       | ND      | 95 ± 24 | 62 ± 4 (0.7) | ND      | ND      |
| Zymosterol                | 151 ± 63 | 240 ± 37 (1.6) | 209 ± 42 (0.9) | 142 ± 64 | 379 ± 46 (2.7) |
| Δ7,24-Sterol              | ND      | 28 ± 7  | 124 ± 19 (4.4) | 303 ± 9  | 859 ± 94 (2.8) |
| Desmosterol               | 385 ± 48 | 536 ± 113 (1.4) | 425 ± 67 (0.8) | 4,677 ± 13 | 6,318 ± 135 (1.4) |
| Labosterol                | 50 ± 5  | 193 ± 78 (3.4) | 78 ± 16 (0.4) | ND      | ND      |
| Cholesterol               | 25,436 ± 4,996 | 19,820 ± 1,706 (0.8) | 17,669 ± 1,942 (0.9) | 23,669 ± 295 | 34,961 ± 450 (1.5) |
| Total C28/29/30           | 215     | 1,023 (4.8) | 442 (0.4) | 620     | 1,374 (2.2) |
| Total precursors          | 650     | 1,780 (2.7) | 1,069 (0.6) | 5,599   | 8,550 (1.5) |
| Total sterol              | 25,086  | 21,600 (0.8) | 18,678 (0.9) | 29,268  | 43,511 (1.5) |

*Values in parentheses are -fold change comparing values between CT43 cells and CT43/npc1 cells.
*The -fold change comparing values between CT43/npc1 cells and CT43 cells.
*Values in parentheses are -fold change comparing values between A101 cells and JP17 cells.
*Values in parentheses are ratios of values in NPC human fibroblast cells.
*ND, not detectable.
*Mu, mono-unsaturated.
*Du, di-unsaturated.
*Values reported (means ± S.D.) are % of total labeled sterols. They were derived from the results reported in Fig. 3, D–F.
that an NPC-dependent endocytic pathway plays only a minor role in distributing various precursor sterols present in the LE/LYS to the ER for further processing.

**Role of Lipid Rafts in the PM-to-ER Retrogade Movement of Biosynthesized Precursor Sterols**—To further pursue the nature of the rapid, retrograde sterol movement independent of NPC proteins, we focused on an internalization process that involves lipid raft/caveolae. Lipid rafts are cholesterol-rich, sphingolipid-rich microdomains located mainly in the PM that play important roles in the internalizations of various biological molecules (32–34). It has been shown that newly synthesized sterols are rapidly transported from the site of their synthesis (ER) to the lipid rafts (5, 30). We used the conversion of biosynthetically labeled C29/C30 and C28 sterols to C27 sterols as an assay to test whether lipid rafts also play a role in the retrograde movement of biosynthesized precursor sterols from the PM to the ER. We treated WT CHO cells grown in Medium D with methyl-β-cyclodextrin (MCD) at different concentrations (0–10 mM) for 30 min to disrupt lipid rafts, then performed pulse-chase experiments with [3H]acetate. The results show that, after 1-h chase, in cells not treated with MCD, the labeled C29/C30 and C28 sterols decreased while the labeled C27 sterols increased; this demonstrates the rapid conversion of C29/C30 and C28 sterol to C27 sterols as expected (Fig. 4, A–C, lanes 1 and 2). However, in cells treated with MCD, retarded conversions of C29/C30 and C28 sterols to C27 sterols were observed (Fig. 4, A–C, lanes 3–6). The retardation of conversion depended on MCD concentration. Significant inhibition of precursor sterol conversion was observed at as low as 1 mM MCD (Fig. 4, A–C, lane 4), indicating that the retrograde movement is highly sensitive to cholesterol depletion. A 10 mM MCD treat-

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**TABLE 5** Percentage of individual [3H]sterols present in the LE/LYS-rich fractions in various CHO cells as indicated

| Sterols     | 25RA | CT-43 | JP17 | A101 |
|-------------|------|-------|------|------|
| C29/C30 sterols | 16.7 | 26.7 (1.6) | 23.8 | 40.2 (1.7) |
| C28 sterols  | 12.9 | 27.5 (2.1) | 22.4 | 30.2 (1.3) |
| Desmosterol  | 13.4 | 30.0 (2.2) | 19.0 | 66.4 (3.5) |
| Cholesterol  | 19.4 | 22.5 (1.2) | 16.3 | 31.6 (1.9) |
| Total sterol  | 19.1 | 23.0 (1.2) | 17.1 | 37.7 (2.2) |

a Values in parentheses are ratios of the value in CT43 cells versus that in 25RA cells.

b Values in parentheses are ratios of the value in JP17 cells versus that in A101 cells.

Results reported are one of two experiments with similar results.

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**TABLE 6** Percentage of individual [3H]sterols present in the LE/LYS-rich fractions in various HF cells as indicated

| Sterols     | Normal-1 | Normal-2 | NPC1 | NPC2 |
|-------------|-----------|-----------|------|------|
| C29/C30 sterols | 30.2 | 34.5 | 47.1 (1.5) | 52.9 (1.6) |
| C28 sterols  | 28.8 | 25.1 | 43.5 (1.6) | 52.0 (1.9) |
| C27 sterols  | 30.1 | 25.0 | 35.8 (1.3) | 46.4 (1.7) |
| Total sterol  | 30.1 | 25.0 | 36.2 (1.3) | 46.7 (1.7) |

a Values in parentheses are the average value in NPC 1 or NPC2 cells versus the average value of normal-1 and normal-2 cells. Results reported are from one of two experiments with similar results.

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**FIGURE 4. Effects of disrupting lipid rafts on the conversion of biosynthetic precursor sterols.** A–C, WT CHO cells plated as described in Fig. 2 were grown in Medium D in 6-well dishes. On day 3, the cells were washed and treated with or without various concentration of MCD as indicated in Medium F (1 ml/well) for 30 min at 37 °C. Afterward, cells were washed with prewarmed PBS, pulse-labeled with [3H]acetate in Medium F (20 μCi/ml/well) for 45 min at 37 °C, and harvested right away (no chase), or chased for 1 h in Medium F then harvested. Cellular lipids were extracted and were analyzed by the TLC system I to determine the counts in C29/C30 sterols (A), C28 sterols (B), and C27 sterols (C). The results shown are average ± S.D. Similar results were obtained in three independent experiments. Relative total sterol synthesis rates in various conditions, reported as % of value found in the control cells, are as follows: 0 mM MCD with no chase, 97.4 ± 7.5; 0.1 mM MCD, 122.7 ± 16.3; 1 mM MCD, 111.5 ± 9.2; 2.5 mM MCD, 127.5 ± 3.9; 10 mM MCD, 110.9 ± 13.5. The value in the control cells (non-MCD treated cells with 1-h chase) was −9 × 10^4 dpm/cell of protein. D–F, WT CHO cells were set up as described above. On day 3, the cells were not treated, or treated with chlorpromazine (CPZ), genistein (Gen), nystatin (Nys), or MCD at the indicated concentration in Medium F for 30 min at 37 °C. The cells were pulse-labeled with [3H]acetate for 45 min in medium F at 37 °C as described above and harvested (no chase) or chased for 1 h in Medium F then harvested. Except for MCD, the agent as described were included throughout the pulse and chase periods. Cellular sterols were analyzed by the TLC system I to determine counts in C29/C30 sterols (D), C28 sterols (E), and C27 sterols (F). The results shown are averaged ± S.D. and represent one of two experiments with similar results. Total sterol synthesis rates under various conditions were as follows (% of value found in the control cells): No chase, 97.3 ± 5.5; 5 μM CPZ, 74.5 ± 5.9; 10 μM CPZ, 74.1 ± 3.8; 50 μM Gen, 122.8 ± 12.9; 100 μM Gen, 91.9 ± 4.2; 25 μM Nys, 69.5 ± 3.3; 50 μM Nys, 34.9 ± 0.5; and 10 μM MCD, 116.1 ± 4.1. The value found in the control cells (non-treated cells with 1-h chase) was −7 × 10^4 cpm/mg of cell protein. Values statistically different from the control value are indicated by asterisks: *, p < 0.001; **, p < 0.01; and ***, p < 0.05.
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As noted earlier in the text, the cholesterol homeostasis model emphasizes the role of cholesterol in lipid rafts. In the current work, we show that cholesterol biosynthesis is regulated by lipid raft functions.

We next tested the effects of several other agents also known to disrupt lipid raft functions, on the retrograde movement of biosynthesized precursor sterols. The agents that we chose to use here have all been shown to disrupt lipid raft functions in CHO cells (35). The results of these experiments (Fig. 4, D–F) demonstrate that genistein, a protein-tyrosine kinase inhibitor (36, 37), or nystatin, an agent that disrupts lipid rafts by interacting with cholesterol in membranes (38), also significantly retarded the conversion of C29/C30 and C28 sterols to C27 sterols. The effects of genistein and MCD are more specific, because they did not cause significant alteration in total sterol synthesis rate; in contrast, nystatin at higher concentration significantly inhibited total sterol synthesis rate (rate values were reported in the Fig. 4 legend). Additional results show that chlorpromazine, an agent known to inhibit clathrin-dependent endocytosis (39), only slightly inhibited the conversion of C29/C30 sterols to C27 sterols, suggesting that clathrin-dependent endocytic internalization may play a minor role in the retrograde movement of precursor sterols to the ER.

We sought to find out in which compartment(s) the biosynthesized precursor sterols accumulate, when their conversion to cholesterol is retarded by lipid raft disruption. To address this question, we treated WT cells with or without CD, prepared the PM, IM, and cytosolic fraction by using Method II (described under “Experimental Procedures”), and performed labeled sterol composition analysis in these fractions. The results of the control experiment (Fig. 5A) show that, in the whole cell homogenates, as expected, the MCD treatment significantly increased the % of labeled C29/C30 sterols, and significantly decreased the % labeled C27 sterols. Additional analysis illustrates that in MCD-treated cells, most of the labeled C27, C29, and C29/C30 sterols accumulated in the PM fractions (Fig. 5, B–E). We used the PM:IM/CS ratio to estimate the proportion of labeled sterols in the PM. As shown in Fig. 5E, in control cells, the value for the PM:IM/CS ratio for various labeled sterols averaged 1.2; in MCD-treated cells, this value increased to 2.0. Thus, MCD treatment increased the proportion of various labeled sterols present in the PM by almost 70%. As noted earlier in the text, the fractionation procedure employed in this experiment (Method II) tended to underestimate the % labeled sterols in the PM fraction by 10–12%. However, the degree of imprecision of this method would only modestly modify the magnitudes of increase reported here. Thus, we conclude that the lipid raft disruption by MCD caused various biosynthesized sterols, including C29/C30, C28, and C27, to accumulate at the PM.

We next asked whether the effect of MCD on retrograde movement of precursor sterols might be reversible by cholesterol repletion in the medium. To test this possibility, we treated the WT CHO cells with MCD at a relatively low dose (1 mM) and incubated the MCD-treated cells without or with cholesterol, or with the cholesterol analogue epico-prostanol, then examined the conversion of the biosynthesized C29/C30, and C28 precursor sterols to C27 sterols. The results (Fig. 6) show that adding cholesterol reduced the labeled C29/C30 sterols (compare lane 3 to lane 2 in A) and increased the labeled C27 sterols (compare lane 3 to lane 2 in C). In contrast, adding an equal amount of epico-prostanol, a hydrophobic stereoisomer of cholesterol, did not produce the same rescue effect as did cholesterol. The results of a control experiment show that, without MCD treatment, cholesterol added to the medium did not significantly alter the labeled C29/C30 and C27 sterol distribution (Fig. 6, A and C; compare lane 5 to lane 1), whereas epico-prostanol added slightly inhibited the conversion of C29/C30 sterols to C27 sterols (Fig. 6, A and C; comparing lane 6 to lane 1; p value = 0.039 for C29/C30 sterols). The changes of C28 sterol levels under various treatments were too small to be informative (Fig. 6B). The inhibitory effect of epico-prostanol reported here can be rationalized as follows: in model membrane studies, cholesterol and a few other related sterols such as epicholesterol and 25-hydroxycholesterol, are shown to induce a domain that is enriched in cholesterol and saturated lipids. Other sterols, such as coprostanol, androstanol, and 4-choles-teneone, inhibit the domain formation (40). Thus, epico-prostanol, a sterol closely related to coprostanol, may act by inhibiting lipid raft formation in intact cells. Overall, these results indicate that a cholesterol-based PM lipid raft domain plays an important role in the retrograde movement of biosynthesized C29/C30 sterols from the PM to the ER. Fig. 7 is a model for intracellular sterol trafficking based on our findings. It is an extension of a previous model (1) and focuses on the fate of biosynthetic precursor sterols described in the current work.
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**DISCUSSION**

In the current study, we performed labeled acetate pulse experiments to monitor the fate of biosynthesized precursor sterols and showed that, upon synthesis at the ER, the majority of the labeled C29/C30, C28, and C27 sterols rapidly move to the PM independently of NPC1 (Fig. 2). We then performed chase experiments to monitor the disappearance of C29/C30 sterols with time (Fig. 2, A and D), and demonstrated that the retrograde movement of endogenously synthesized C29/C30 sterols occurs with a half time of <60 min in a manner largely independent of NPC1/NPC2 proteins. Interestingly, Pentchev and colleagues (41) had previously reported that the labeled C27 sterol desmosterol added exogenously to mouse fibroblasts is converted to cholesterol inside the cells in a manner independent of the NPC1 mutation. We then showed that treating cells with MCD, genistein, or nystatin severely inhibits the conversion of C29/C30 precursor sterols to C27 sterols. These agents disrupt the function of lipid rafts, but they do so via different modes of action. MCD efficiently extracts cholesterol from the cholesterol-rich domain in the PM, nystatin binds tightly to cholesterol, whereas genistein inhibits lipid raft function by acting as a tyrosine kinase inhibitor. We next showed that the MCD treatment led to retention of the labeled C29/C30 and C28 sterols at the PM (Fig. 5); the inhibitory effect of MCD can be reversed by cholesterol repletion (Fig. 6). These results led us to conclude that lipid rafts at the PM play a crucial role in bringing cholesterol biosynthesis to completion. They do so by participating in the retrograde movement of biosynthesized C29/C30 and C28 precursor sterols, bringing these sterols back from the PM to the ER so they can be enzymatically processed to cholesterol. As indicated earlier, close analysis of results presented in Fig. 3 (E and F) revealed that, when compared with WT Hfs, slightly slower rates of C28 sterol disappearance and C27 sterol appearance occurred in the NPC1−/− Hfs. This finding can be rationalized by the earlier finding reported by Garver and colleagues (42), who showed that the concentration of cholesterol in the PM caveolae isolated from NPC1−/− fibroblasts was significantly decreased compared with that of the WT fibroblasts. Thus, in the mutant NPC Hfs, the PM lipid raft function in mediating the retrograde movement of the precursor sterols may be slightly compromised.

How the PM lipid rafts mediate the retrograde movement of endogenously synthesized sterols is unknown at present. It seems plausible that at least two different mechanisms may be involved. The first is a lipid raft-originated, non-clathrin-mediated vesicular trafficking pathway (43). In particular, the SV-40 virus can utilize caveolae-dependent endocytosis to enter cells and is transported to the ER through “caveosomes,” which are vesicles that do not contain markers for endosomes, lysosomes, ER, Golgi, or clathrin-coated vesicles. Such a vesicular trafficking pathway may be involved in the delivery of biosynthesized precursor sterols from the PM to the ER. A second mechanism may involve non-vesicular sterol transport (44) and may involve the participations of soluble proteins, such as the StAR (steroidogenic acute regulatory protein)-related proteins (45), and the oxysterol-binding protein-related proteins, which have high affinities for sterols and/or for other lipid species (8, 46). The multiple-drug resistance proteins are members of the P-glycoprotein ATPase-binding cassette transport family and have been implicated in the retrograde movement of sterols (11, 12). The PM lipid raft may act in concert with the multiple drug resistance protein(s) to mediate the movement of the precursor sterols back to the ER. In yeast, the...
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In the current work, we showed that various biosynthesized sterols, including the C29/C30 sterols, desmosterol, and cholesterol, all exhibited elevated levels of accumulations within the LE/LYS compartment of mutant NPC cells. However, the accumulation is only modest, and it took 8 h or longer to detect the sterol accumulation in the LE/LYS. Based on these results, we conclude that the NPC-dependent sterol transport mechanism is not the primary mechanism for the rapid retrograde movement of biosynthesized sterols. How the biosynthesized sterols move to the LE/LYS is currently unknown; our current results showed that chlorpromazine slightly inhibited the conversion of C29/C30 sterols to C27 sterols, suggesting that a clathrin-dependent endocytic mechanism may be involved in internalizing some of the biosynthesized sterols to the LE/LYS, in a manner similar to the internalization of low density lipoprotein-derived cholesterol. The inhibitory effects of the precursor sterol conversion by lipid raft disruptions were twice as efficient as the effect caused by using the clathrin pathway inhibitor. We speculate that, after arriving at the PM, 70–80% of the precursor sterols may be internalized by a lipid raft-dependent process, whereas the remaining 20–30% may be transported to the ER through a clathrin-dependent transport pathway. Further investigations are needed to resolve this issue.

Are PM lipid rafts also involved in the retrograde movement of cholesterol? Cholesterol is an excellent substrate for the enzyme acyl-CoA:cholesterol acyltransferase 1, a resident enzyme in the ER (1); however, C29/C30 precursor sterols are very poor substrates for acyl-CoA:cholesterol acyltransferase (47). We and others had taken advantage of the acyl-CoA:cholesterol acyltransferase substrate specificity and employed the % esterification of labeled cholesterol by acyl-CoA:cholesterol acyltransferase 1 as an assay to monitor the retrograde movement of cholesterol. The results showed that, in macrophages, an NPC-dependent mechanism plays a significant role, whereas in fibroblasts or in hepatocytes, one or more NPC-independent mechanisms participate significantly in cholesterol esterification (17). We also noted that, in the various cell types we examined, esterification of cholesterol delivered exogenously occurs in several hours, whereas the conversion of biosynthesized C29/C30 sterols to C27 sterols occurs within 1 h. Thus, whether the NPC-independent mechanism for the retrograde movement of cholesterol shares the same machinery for the retrograde movement of biosynthetic precursor sterols cannot be determined at present.

Why should cells produce precursor sterols in addition to cholesterol? Recent studies have shown that certain biosynthesized precursor sterols have specific biological functions that cannot be replaced by cholesterol. For example, the C30 precursor sterols lanosterol and dihydrolanosterol, but not cholesterol or other C27 sterols, promote the ubiquitination of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, resulting in down-regulation of reductase enzyme activity (48). The C27 sterol desmosterol, or the C29 sterol 4,4-dimethylsterol, but not lanosterol, acts as a ligand for the liver X receptors (49, 50) that transcriptionally up-regulate multiple genes involved in lipid metabolism. Thus, it is tempting to speculate that the PM lipid raft may be involved in affecting the rate of sterol biosynthesis, and/or the activity of the liver X receptor. In addition to their normal physiological functions, the abnormal build-up of various biosynthetic precursor sterols is involved in pathophysiology. In humans, several malformation syndromes, including RSH/Smith-Lemli-Opitz syndrome, desmosterolosis, and X-linked dominant chondrodysplasia punctata type 2, are due to genetic deficiencies in various enzymes involved in the late stages of the cholesterol biosynthetic pathway, causing abnormal accumulation of various precursor sterols in various tissues of these patients (51). Treating rodents with various teratogens causes abnormal build-up of lanosterol and other precursor sterols and produces phenotypic abnormalities that mimic the malformation syndromes found in humans (52). These teratogens include certain plant alkaloids such as cyclopamine and jervine, which resemble cholesterol in structure and are believed to act as inhibitors of enzyme(s)
involved in the catalytic conversion of precursor sterols to cholesterol (52). Based on our current finding, it would be interesting to test whether some of the teratogens act by inhibiting the function of the PM lipid raft, in addition to blocking enzymes in distal stage of the cholesterol biosynthetic pathway.

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