Chapter 12

Monitoring Sterol Uptake, Acetylation, and Export in Yeast

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Summary

Sterols are essential lipid components of eukaryotic membranes. They are synthesized in the endoplasmatic reticulum (ER) from where they are efficiently transported to the plasma membrane, which harbors ~90% of the free sterol pool of the cell. The molecular mechanisms that govern this lipid transport, however, are not well characterized and are challenging to analyze. Saccharomyces cerevisiae offers the opportunity to circumvent some of the technical limitations associated with studying this forward transport of sterols from the ER to the plasma membrane, because the organism can also take up sterols from the environment, incorporate them into the plasma membrane and transport them back to the ER, where the free sterol is converted to steryl esters. This reverse sterol transport, however, occurs only under anaerobic conditions, where the cells become sterol auxotroph, or in mutant cells that cannot synthesize heme. The reverse sterol transport pathway, however, is more amenable to experimental studies, because arrival of the sterol in the ER membrane can be monitored unambiguously by following the formation of steryl esters. Apart from sterol acylation, we have recently described a reversible sterol acetylation cycle that is operating in the lumen of the ER. Acetylation occurs on both cholesterol and pregnenolone, a steroid precursor, and serves as a signal for export of the acetylated sterols into the culture media. The time-dependent appearance of acetylated sterols in the culture supernatant thus provides a new means to monitor the forward transport of chemically modified sterols out of the ER.

Key words: Sterol, Cholesterol, Steroids, Pregnenolone, Sterol Acetylation, Lipid, Transport, Membranes, Yeast, Saccharomyces cerevisiae

1. Introduction

Sterols are essential lipid components of eukaryotic membranes that determine many membrane properties and are required for polar sorting events during vesicle transport in animal, plant, and fungal cells (1, 2). Changes in sterol levels in membranes can have profound effects on signal transduction pathways, and on...
the trafficking of membrane proteins and lipids. Eukaryotic cells synthesize sterols in the ER from where they need to be efficiently transported to the plasma membrane, which harbors ~90% of the free sterol pool of the cell. This transport process is facilitated by vesicular as well as non-vesicular components (3). This forward transport of sterols, however, is challenging to study because arrival of the newly synthesized sterol at the plasma membrane in not accompanied by any covalent modification of the lipid. This is frequently being circumvented by analyzing radio-labeled cyclodextrin extractable material from the intact plasma membrane (4–6).

Animal cells take up exogenous sterols through receptor-mediated endocytosis of low-density lipoproteins (LDL). Once delivered to late endosome or lysosome, LDL-derived cholesteryl esters are hydrolyzed, and the free cholesterol is rapidly cycled back to the plasma membrane and/or the ER for re-esterification (7). This back transport pathway of sterols from the plasma membrane/endosome to the ER is technically more robust to analyze as arrival of the sterol at the ER is accompanied by a covalent modification of the lipid, i.e. acylation (8, 9).

Fungal cells synthesize ergosterol instead of cholesterol as their main sterol and many aspects of sterol homeostasis are conserved between yeast and human (10–12). Under aerobic conditions, ergosterol is synthesized in the ER membrane and is greatly enriched at the yeast plasma membrane (13). Under these conditions, cells do not take up exogenous sterols, a phenomenon known as “aerobic sterol exclusion.” Under anaerobic growth conditions or in mutants that lack heme, however, S. cerevisiae becomes auxotrophic for sterols and unsaturated fatty acids, because the synthesis of these lipids requires molecular oxygen (14). Under anaerobic conditions, cells thus induce a sterol uptake pathway to enable growth. The fact that S. cerevisiae is a facultative anaerobic organism that displays robust growth when appropriately supplemented indicates that lipid uptake is efficient. Anaerobic or heme-deficient conditions thus allow to investigate the reverse transport of sterols from the plasma membrane to the ER. Arrival of the radio-labeled sterol that is typically supplied to the media of these sterol uptake competent cells is monitored by the time-dependent formation of steryl esters, because the enzymes that convert free sterols into steryl esters, the acyl-CoA:sterol acyltransferases, are located in the ER membrane (7, 15–17).

Apart from this well characterized sterol acylation and deacylation cycle, we have recently described a novel covalent sterol modification: the sterol acetylation and deacetylation cycle (18). Sterol acetylation requires the acetyltransferase ATE2, whereas deacetylation requires SAT1, a membrane-anchored deacetylase. Both enzymes are located
in the ER membrane with their active site facing the ER lumen (18). Exogenous cholesterol is subject to acetylation and deacetylation and cholesterol acetate accumulates only if cells are deleted for the deacetylase, SAT1. Lack of SAT1, however, results in the secretion of acetylated sterols into the culture media (18). Monitoring the appearance of sterol acetate in the culture medium thus can serve as a novel readout for the forward transport of sterols from the ER to the extracellular space. Similar to cholesterol, the steroid precursor pregnenolone is also subject to Atf2-dependent acetylation, but unlike cholesterol acetate, pregnenolone acetate is not deacetylated by Say1. As a consequence, pregnenolone acetate is rapidly excreted by the cells and accumulates in the culture supernatant. Monitoring the appearance of acetylated pregnenolone thus provides a novel tool to investigate the cellular uptake of steroids as well as the molecular basis for the excretion of acetylated steroids.

This paper discusses the uptake and secretion of radio-labeled sterols by yeast S. cerevisiae. Radio-labeled lipids are typically provided to heme-deficient, i.e., uptake competent cells, for some time and then extracted again from whole cell lysates as well as from the culture media. The metabolically modified lipids are then separated by ascending thin layer chromatography (TLC) using plates coated with silica gel (60 Å pore size). This technique of lipid separation is simple, versatile, and highly sensitive with the flexibility to be used both quantitatively and qualitatively (19). After TLC separation, radio-labeled lipids are detected and quantified using either a radio TLC analyzer or by exposing the TLC plate to a phosphorimager screen or an X-ray film.

2. Materials

2.1. Equipment

1. Tabletop centrifuge with appropriate adaptors (Eppendorf, Hamburg, Germany).
2. TLC chamber with cover and Whatman filter paper (GE Healthcare, Piscataway, NJ).
3. Incubator, shaker, and water bath.
5. Liquid scintillation counter (Tri-Carb 2100TR, Perkin-Elmer, Waltham, MA) and scintillation cocktail (Zinsser Analytic, Frankfurt, Germany).
6. Phosphorimager (Bio-Rad, Hercules, CA).
7. Radio TLC-linear analyzer (Berthold Technologies, Bad Wildbad, Germany).
2.2. Reagents and Supplies

1. Yeast growth media. Rich media, YEPD: 1% (w/v) yeast extract, 2% (w/v) bactopeptide, 2% (w/v) glucose. Synthetic media, SC-LEU: 0.67% (w/v) nitrogen base without amino acids, 2% (w/v) glucose, 0.07% (w/v) amino acid drop out mix without leucine (20). Heme-deficient cells must be grown either with lipid supplementation (5 mg/ml Tween 80, which substitutes for unsaturated fatty acids, and 20 µg/ml cholesterol or ergosterol) or in the presence of aminolevulinic acid (ALA, 10 µg/ml), which bypasses the hemeIΔ deficiency (17).

2. Glass beads: 0.25–0.3 mm diameter (Braun Melsungen, Melsungen, Germany).

3. Silica gel 60 TLC plates (Merck, Darmstadt, Germany).

4. Solvent system for neutral lipid separation by TLC: petroleum ether/diethyl ether/glacial acetic acid (70:30:2, v/v/v). The organic solvents, i.e., chloroform and methanol, are of analytical grade. All solvent manipulations are to be carried out in a ventilated hood while wearing protective clothing, because chloroform is listed as harmful, and methanol is toxic (see Note 1).

5. Falcon tubes, 50 ml (BD Biosciences, San Jose, CA).

6. Eppendorf safe-lock tubes, 1.5 and 2 ml (Eppendorf, Hamburg, Germany).

7. 0.5% tergitol solution (Sigma-Aldrich, St. Louis, MO).

8. Tween 80 (Carl Roth, Karlsruhe, Germany).

9. Cholesterol 99.9% pure (Sigma-Aldrich, St. Louis, MO).

10. [4-¹⁴C]-cholesterol (0.1 mCi/ml; American Radiolabeled Chemicals, Inc. St. Louis, MO).

11. [9,10-³H]-palmitic acid (10 mCi/ml; American Radiolabeled Chemicals, Inc.).

12. [7-³H]-pregnenolone (1 mCi/ml; American Radiolabeled Chemicals, Inc.).

3. Methods

3.1. [¹⁴C]-Cholesterol Uptake

1. Grow heme-deficient yeast cells (hemeIΔ) in a falcon tube containing 20 ml of SC-LEU supplemented with aminolevulinic acid at 24°C overnight.

2. Harvest 5 OD₆₀₀ units of cells by centrifugation at 2,000 rpm for 5 min and wash the cells twice with SC-LEU media.

3. Resuspend the cells in 10 ml SC-LEU media supplemented with cholesterol and Tween 80.
4. Add [\(^{14}\)C]-cholesterol (0.025 μCi/ml) to the culture (see Note 2).

5. Let the culture grow at 24°C overnight or to the time point required.

6. Next morning control the OD\(_{600}\), and take the volume necessary to obtain 30 OD units of cells.

7. Centrifuge the cells at 2,000 rpm for 5 min. Pour off the supernatant and discard the radio-labeled waste.

8. Wash the cells twice with 5 ml of 0.5% tergitol solution, centrifuge each time at 2,000 rpm for 7 min. Pour off the supernatant and discard the radio-labeled waste.

9. Prepare a 1:100 dilution of \([^{3}\text{H}]\)-palmitic acid (in ethanol) to be added as internal standard to control the efficacy of lipid extraction.

10. Add 600 μl of chloroform/methanol (1:1, v/v) to the cell pellet and then transfer it to a 2 ml safe-lock tube containing 300 μl of glass beads. Add 1 μl of the diluted \([^{3}\text{H}]\)-palmitic acid as internal standard.

11. Freeze the samples at -20°C.

1. Thaw the cell pellet in a water bath set to 24°C. Freeze the samples again in liquid nitrogen for 1 min. Repeat this freeze–thaw cycle once more.

2. Break cells in a Precellys 24, cell homogenizer at 5,000 rpm, 4°C, using three cycles of homogenization for 30 s interrupted by a pause of 30 s each. Alternatively, the cells can be homogenized on a Vortex set at top speed for 30 min at 4°C.

3. Centrifuge samples at 13,000 rpm for 5 min and transfer the supernatant to a new 2 ml Eppendorf safe-lock tube.

4. Wash the glass beads with 300 μl chloroform/methanol (1:1, v/v), centrifuge at 13,000 rpm for 5 min and pool the supernatant. Repeat this wash step twice.

5. Centrifuge the samples at 13,000 rpm for 5 min and transfer the supernatant to a new tube. Avoid carry over of glass beads.

6. Take 5 μl of the lipid extract and mix with 2 ml of scintillation cocktail and vortex briefly for 20 s.

7. Count radioactivity of the lipid samples by liquid scintillation counter and remove a sample volume corresponding to 20,000 cpm. Dry this lipid sample under a stream of nitrogen gas.

8. Resuspend the dried lipids in 20 μl of chloroform/methanol (1:1, v/v) and spot the sample on a TLC plate (see Note 3). Wash the tube again with 20 μl chloroform/methanol (1:1, v/v) and place onto the first spot on the TLC.
9. Let the TLC plate dry at room temperature for 5–10 min. Saturate the TLC chamber with the solvent system, petroleum ether/diethyl ether/glacial acetic acid (70:30:2, v/v/v) for 40–60 min prior to run (see Note 4). Separate neutral lipids for 30 min at room temperature (see Note 5).

10. After separation is complete, the TLC plate is removed from the chamber and left to dry in a ventilated hood for at least 1–2 h. The radio-labeled lipid classes (free cholesterol, cholesterol esters, and triacylglycerols) are then quantified by scanning using a radio TLC analyzer (see Note 6). Alternatively, the TLC plate can be exposed to phosphorimaging plate for 24 h and then be analyzed, using a phosphorimager (Bio-Rad) (see Notes 7 and 8).

3.2. \(^{14}\)C-Cholesterol Export

1. Grow heme-deficient yeast strains in a lipid supplemented medium at 24°C overnight.

2. Next day harvest 10 OD\(_{600}\) units of cells in a tabletop centrifuge at 4,000 rpm for 5 min. Pour off the supernatant and discard.

3. Wash the cells twice with SC-LEU media.

4. Resuspend the cell in 10 ml of SC-LEU media supplemented with cholesterol and Tween 80. Add \(^{14}\)C-cholesterol (0.025 \(\mu\)Ci/ml) and grow cells overnight at 24°C to let them take \(^{14}\)C-cholesterol.

5. Next day pellet the cells at 4,000 rpm for 5 min. Pour off the supernatant and discard the radio-labeled waste.

6. Wash the cells twice with SC-LEU media. Remove supernatant and discard the radio-labeled waste.

7. Resuspend the cell pellet in 10 ml of SC-LEU media supplemented with cold cholesterol (20 \(\mu\)g/ml) and Tween 80 (5 mg/ml).

8. Let the cells grow at 24°C overnight, or for the time required, to allow export of \(^{14}\)C-cholesterol or acetylated \(^{14}\)C-cholesterol into the culture medium.

9. Next morning pellet the cells at 4,000 rpm for 5 min and keep aside the culture supernatant in a 50 ml falcon tube.

10. Wash the cells with 5 ml 0.5% tergitol and centrifuge at 2,000 rpm for 5 min. Pour off the supernatant and discard the radio-labeled waste.

11. Resuspend the cells in 400 \(\mu\)l of chloroform/methanol (1:1, v/v) and transfer them into a 2 ml safe-lock tube containing 200 \(\mu\)l glass beads.

12. Freeze the cell pellet (−20°C).

13. Lipid extraction from whole cells and TLC analysis of radio-labeled lipids is performed as described in Section 3.1.1.
3.2.1. Lipid Extraction from The Culture Supernatant

1. Add 30 ml of chloroform/methanol (1:1, v/v) to the 10 ml culture supernatant in a 50 ml falcon tube.
2. Vortex well for 2–3 min.
3. Centrifuge at 4,000 rpm for 10 min to separate organic and aqueous phases.
4. Carefully transfer the lower organic phase with a 5 ml pipette into a new 50 ml falcon tube (avoid the aqueous phase as well as the interphase).
5. Pour off the aqueous phase and discard the radio-labeled waste.
6. Let the tube containing the organic phase stand for 1 h at room temperature with caps open, and aspirate carefully the aqueous layer formed on the top (if any).
7. Count 5 μl of the organic phase by liquid scintillation counter and aliquot sample volumes corresponding to 10,000 cpm (i.e., same counts for the lipids extracted from the cell pellet) in 2 ml tube and dry under a stream of nitrogen gas.
8. Load samples on TLC, separate and quantify lipids as described in Section 3.1.1.

3.3. [3H]-Pregneneolone Uptake and Secretion

1. Grow yeast in a suitable medium (e.g., YEPD) overnight at 24°C.
2. Control the optical density and harvest 5 OD₆₀₀ units.
3. Pellet the cells at 1,500 rpm for 5 min in a tabletop centrifuge.
4. Resuspend the cells in 5 ml fresh YEPD media, and add 1 μCi/ml [3H]-pregnenolone.
5. Incubate the cells for 2 h with shaking at 24°C.
6. Check the optical density, pellet the cells at 4,000 rpm for 5 min. Keep aside the culture supernatant in a 50 ml falcon tube at 4°C.
7. Wash the cell pellet with 1 ml YEPD, centrifuge at 4,000 rpm for 5 min and transfer the supernatant to the culture supernatant that was kept aside.
8. Resuspend the cells in 400 μl of chloroform/methanol (1:1, v/v) and transfer into a 2 ml safe-lock tube containing 200 μl glass beads.
9. Freeze the cells at -20°C.
10. Continue with lipid extraction from the whole cells as described under Section 3.1.1.
11. Dry down the whole lipid extract and analyze by TLC as described under Section 3.1.1.
12. Extract lipids from the culture supernatant once with 20 ml chloroform/methanol (1:1, v/v), vortex well for 2–3 min.

13. Centrifuge at 4,000 rpm in a tabletop centrifuge for 5 min to separate the organic and aqueous phases.

14. Carefully transfer the lower organic phase with a 5 ml pipette into a new 50 ml falcon tube (avoid the aqueous phase as well as the interphase).

15. Pour off the supernatant and discard the radio-labeled waste.

16. Dry down the organic phase under a stream of nitrogen gas and analyze and quantify the lipids after TLC separation as described in Section 3.1.1.

3.4. Results

1. Cholesterol uptake under the conditions described in Section 3.1 is time-dependent and the internalized cholesterol is efficiently back transported to the ER membrane where it is converted to steryl esters (Fig. 1). This lipid uptake pathway is affected in a number of sterol uptake and transport mutants that were isolated in a genome-wide screen for mutants that fail to grow under anaerobic conditions (12, 17).

Fig. 1. Uptake and esterification of [14C]-cholesterol in heme-deficient cells. hem1Δ cells were grown in the presence of [14C]-cholesterol and Tween 80 at 24°C. At the indicated time points, equal OD units of cells were harvested, lipids were extracted and analyzed by TLC. FC, free cholesterol; STE, steryl esters.
Fig. 2. Acetylated sterols are secreted. Heme-deficient wild-type and $say1\Delta$-mutant cells were labeled with [14C]-cholesterol for 16 h, diluted into fresh media containing cold cholesterol, and cultivated for 16 h. Lipids were extracted from the cell pellet (I) and from the culture media (E) and analyzed by TLC. FC, free cholesterol; CA, cholesterol acetate; STE, steryl esters.

2. Formation of cholesterol acetate is barely detectable in wild-type cells, but accumulates in cells lacking the sterol deacetylase ($say1\Delta$) (Fig. 2). Discrimination between intra- and extracellular lipids reveals that $say1\Delta$ mutant cells excrete sterol acetate. Excretion of this lipid is selective for acetylated sterols because long-chain steryl esters are not detectable in the culture supernatant of wild-type or $say1\Delta$ mutant cells. The identity of the modified sterol that accumulates in $say1\Delta$ mutant cells was independently confirmed by mass spectrometry (18).

3. Radio-labeled pregnenolone is rapidly acetylated and excreted from wild-type and deacetylase ($say1\Delta$) mutant cells (Fig. 3). The fact that acetylation-deficient ($atf2\Delta$) mutant cells accumulate radio-labeled long-chain ester intracellularly indicates that acetylation is required for export of the modified pregnenolone and that a failure to acetylate results in the intracellular accumulation of this steroid precursor in form of long-chain esters. The identity of these long-chain pregnenolone esters is based on the fact that formation of this product is abolished in cells lacking the two acyl-CoA:sterol acyltransferases, ARE1 and ARE2 (17).
Fig. 3. Export of steroids is controlled by sterol acetylation/deacetylation. Atf2 is required for acetylation and secretion of pregnenalone. Wild-type, say1Δ, and att2Δ mutant cells were labeled with [3H]-pregnenalone; lipids were extracted from the culture media (extracellular), and the cell pellet (intracellular), and analyzed by TLC. [3H]-pregnenalone was loaded as standard (std). Preg, free pregnenalone; PA, pregnenalone acetate; AcP, acylated pregnenalone.

4. Notes

1. The organic solvents used are toxic or even carcinogenic. Chloroform/methanol mixture will rapidly leach the skin lipids from hands. Further contact with the solvents will give rise to irritation. It is therefore advisable to handle all the solvents with care. If mixtures of chloroform/methanol spill over a body part, that region should immediately be rinsed with cold running water to minimize the burning sensation, which could last for about 10 min.

2. All radioactive mixes should be handled with utmost care and precautions, always wearing protective gloves. If there is a spill of radioactive mix over any part of the body, that region should be immediately rinsed with plenty of running cold water and then seeking the advice of a physician.

3. At a given humidity, the amount of water adsorbed by the silica gel increases as pore size decreases. The water content of the silica gel increases the polarity of the adsorbent and hence its chromatographic properties. For good separations, the water content of the silica gel on TLC plates therefore must be carefully controlled. To remove the water, the silica gel is “activated” by heating the plates immediately before use for 10 min at temperature above 100°C.

4. Only the purest solvents are used in the development of the plates, and the component solvents should be thoroughly...
mixed. When solvent systems containing large proportions of polar solvents such as methanol are employed, the chambers should be lined with filter paper to help saturate the atmosphere. However, with nonpolar solvents such as petroleum ether or diethyl ether, the lining of chambers is not necessary.

5. The time taken for a TLC plate to develop depends on the ambient temperature and the solvent system employed. For example, with a solvent system to separate nonpolar lipids, a standard 20 × 20 cm plate will be developed fully in approximately 30 min at room temperature.

6. Although lipid classes can be identified by reference to published \( R_f \) values, the application of commercially available lipid standards, either as mixtures or individually, alongside the lipid being analyzed, greatly aids in the identification of the components present in the lipid sample. Within any laboratory, the \( R_f \) values, of lipid classes in a given solvent system are not always constant owing to day-to-day variations in temperature, humidity, and perhaps even the batch of plates used. By routinely analyzing lipid standards alongside samples, such variations can be taken into account.

7. For the TLC plates to be exposed to \([^{32}P]\)- or \([^3H]\)-phosphorimager screens, it is important to dry the plates very well, or otherwise the acetic acid used in the solvent system will stick to the screens and contaminate them.

8. To analyze the radioactive lipid bands by phosphorimaging, an exposure time of 24 h is usually adequate. Care should be taken to avoid exposure of the screen to light before scanning.

Acknowledgments

This research was supported by the Swiss National Science Foundation (grants PP00A3-110450 and 3100A0-20650).

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