Published in final edited form as:

Arch Biochem Biophys. 2020 September 30; 691: 108518. doi:10.1016/j.abb.2020.108518.

Nanodisc scaffold peptide (NSP_r) replaces detergent by reconstituting acyl-CoA:cholesterol acyltransferase 1 into peptidiscs

Bryan Neumann¹, Kevin Chao², Catherine C. Y. Chang^{1,3}, Ta-Yuan Chang^{1,3}

¹Department of Biochemistry & Cell Biology, Geisel School of Medicine, Dartmouth College, Hanover, New Hampshire 03755, USA

²College of Medicine, SUNY Downstate Health Sciences University, Brooklyn, New York 11203, USA

Abstract

To conduct biochemical studies *in vitro*, membrane proteins (MPs) must be solubilized with detergents. While detergents are great tools, they can also inhibit the biological activity and/or perturb oligomerization of individual MPs. Nanodisc scaffold peptide (NSP_r), an amphipathic peptide analog of ApoA1, was recently shown to reconstitute detergent solubilized MPs into peptidiscs *in vitro*. Acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1), also known as sterol O-acyltransferase 1 (SOAT1), plays a key role in cellular cholesterol storage in various cell types and is a drug target to treat multiple human diseases. ACAT1 contains nine transmembrane domains (TMDs) and primarily forms a homotetramer *in vitro* and in intact cells; deletion of the N-terminal dimerization domain produces a homodimer with full retention in catalytic activity. ACAT1 is prone to inactivation by numerous detergents. Here we pursued the use of NSP_r to overcome the detergent-induced inactivation of ACAT1 by generating near detergent-free ACAT1 peptidiscs. Based on native-PAGE analysis, we showed that NSP_r reconstitutes ACAT1 into soluble peptidiscs, in which ACAT1 exists predominantly in oligomeric states greater than a homotetramer. The formation of these higher-order oligomeric states was independent of the N-terminal dimerization domain, suggesting that the oligomerization is mediated through hydrophobic interactions of multiple ACAT1 subunits. ACAT1 peptidiscs were still susceptible to heat-mediated inactivation, presumably due to the residual detergent (CHAPS) bound to ACAT1. We then conditioned ACAT1 with phosphatidylcholine (PC) to replace CHAPS prior to the formation of ACAT1 peptidiscs. The results showed, when PC was included, ACAT1 was present mainly in higher-order oligomeric states with greater enzymatic activity. With PC present, the enzymatic activity of ACAT1 peptidiscs was protected from heat-mediated inactivation. These results support the use of NSP_r to create a near detergent-free solution of ACAT1 in peptidiscs for

³Corresponding authors Ta-Yuan Chang, Ta.Yuan.Chang@Dartmouth.edu and Catherine C. Y. Chang, Catherine.C.Chang@Dartmouth.edu.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

various *in vitro* studies. Our current results also raise the possibility that, under certain conditions, ACAT1 may form higher-order oligomeric states *in vivo*.

Graphical abstract

Keywords

ACAT/SOAT; Membrane protein; Peptidisc; Detergent-free; Phospholipid; Oligomerization

1. Introduction

A significant portion of pharmaceutical drug targets are membrane proteins (MPs); MPs are notoriously difficult to study *in vitro* due to their insolubility. This difficulty is compounded with the reduced expression of MPs when compared to soluble proteins, due to the lesser volume of membranes contrasted to that of the cytosol within the cell. Once over-expressed, MPs must be solubilized from membranes which can be particularly challenging to do with conventional detergents [1]. While detergents are reliable tools to produce MP-lipid micelles, they are also well-known for inactivating enzymes, promoting aggregation, and perturbing oligomerization of MPs [2]. To MP biochemists and structural biologists, the current challenge is to express, solubilize, and purify MPs in their native conformations such that they can be used reliably for further *in vitro* studies.

Cholesterol is an essential lipid molecule and plays important structural and functional roles in various cell types. Acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1), also known as sterol O-acyltransferase 1 (SOAT1), regulates cellular cholesterol homeostasis by converting free cholesterol to cholesteryl esters. Cholesterol is partitioned in lipid bilayers, whereas the more hydrophobic cholesteryl esters coalesce into lipid droplets for storage within the cytosol. Cholesteryl esters cannot replace the function of cholesterol. Blocking ACAT1 is expected to shift cholesterol from storage such that the free, unesterified cholesterol can be utilized more efficiently by the cells. Cholesterol homeostasis is crucial, and its impairment has been implicated in various diseases. For these reasons, ACAT1 has emerged as a therapeutic target to treat multiple human diseases [3-8].

ACAT1 is a resident integral membrane protein located at the endoplasmic reticulum (ER). Its solubilization requires the use of detergents. However, several non-ionic detergents readily inactivate the enzyme [9-11]. Recently it was shown that the non-ionic detergents

Triton X-100 or octyl glucoside rapidly inactivate ACAT1 by perturbing its C-terminal dimerization domain; whereas, the less intrusive zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) gradually inactivates it without affecting its oligomeric state [12]. The same study also showed that after solubilization, substituting detergents with phosphatidylcholine (PC) protected ACAT1 from heat-mediated inactivation. Due to the labile nature of ACAT1 in detergents, it is desirable to seek alternative methods to create a homogeneous solution of ACAT1 with minimal detergents present. There are several techniques in which membrane proteins can be reconstituted into detergent-free solutions, including the use of: membrane scaffold proteins (MSPs), amphipols, styrene-maleic acid (SMA) copolymers, saposin A-based Salipro particles, and nanodisc scaffold peptide (NSP) peptidiscs. MSPs are engineered amphipathic helical proteins, initially derived from human ApoA1, which shield the hydrophobic core of lipid bilayers from aqueous solutions by encasing it in a helical protein belt to form soluble lipid nanodiscs [13]. There is an assortment of MSPs which self-assemble into nanodiscs ranging in diameter from small (6-7 nm) to large (16-17 nm) [14, 15]. They are commonly used to transfer MPs from detergent micelles into nanodiscs of desired lipid composition [16]. However, this process of self-assembly involves many factors such as detergent solubilization and purification of the target MP, selecting a suitable MSP to encapsulate both MP and lipid bilayer, mixing of MP-MSP-lipids at proper stoichiometry, and detergent removal. Amphipols are a class of amphiphilic polymers designed to keep MPs soluble in detergent-free solutions. SMA copolymers have the unique ability to solubilize cell membranes without the use of detergents [17]. This method creates SMA lipid particles (SMALPs) of various sizes which allows for MPs to be purified along with native lipids [18]. Saposins are modulators of lipid membranes within acidic lysosomes and can form lipoprotein discs *in vitro* [19]. Saposin A-based Salipro particles can be used to reconstitute detergent-solubilized MPs into desired lipids; additionally, they can directly extract MPs from crude cell membranes into Salipro particles with native lipids [20].

Nanodisc scaffold peptide (NSP) is a bihelical amphipathic peptide analog based on ApoA1. The original NSP is constructed of two 18A peptide repeats linked by a proline which were found to stabilize lipid particles, however, this peptide was not readily soluble in water [21, 22]. Duong and colleagues created a reversed sequence NSP (NSP_r) which is soluble in solution and has been shown to stabilize both α -helical and β -barrel MPs into soluble, detergent-free peptidiscs [23]. The formation of peptidiscs is spontaneous through the dilution of detergents which can be accomplished through the use of several common analytical methods including: in-gel, on-column, on-gradient and on-bead (PeptiQuick) methods [23-25].

Here, we describe the use of NSP_r to reconstitute ACAT1, a typical oligomeric integral MP, into peptidiscs and we monitor the effects of PC on the enzymatic activity and oligomerization of ACAT1 in peptidiscs. Overall, our results support the finding that detergent-free peptidiscs provide a milder and preferable environment to study ACAT1 *in vitro*.

2. Materials and Methods

2.1. Antibodies and reagents

CHAPS was from Anatrace (Maumee, OH, USA). NSP_r (sequence from N-terminus to C-terminus, FAEKFKEAVKDYFAKFWDPAAEKLKEAVKDYFAKLWD) was from Peptidisc Biotech (Vancouver, BC, CA). ProtoGel (30%) was from National Diagnostics (Atlanta, GA, USA). Amersham Protran 0.45 nitrocellulose membrane was from Cytiva (Marlborough, MA, USA). THETM DYKDDDDK (FLAG) tag mouse monoclonal antibody was from GenScript (Piscataway, NJ, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse was from Bio-Rad Laboratories (Hercules, CA, USA). Digitonin, Corning Costar Spin-X 0.22 µm centrifuge tube filters, anti-FLAG M2 affinity gel, FLAG peptide, Coomassie Brilliant Blue R-250, oleic acid, coenzyme A tri-lithium salt, sodium taurocholate, L-α-phosphatidylcholine (PC) from egg yolk, cholesterol, cholesteryl oleate, fatty acid-free bovine serum albumin, and protease inhibitor cocktail were from MilliporeSigma (St. Louis, MO, USA). HisPur Ni-NTA resin was from Thermo Fisher Scientific (Rockford, IL, USA). [³H]Oleoyl-coenzyme A (CoA) was synthesized as described previously [26]. Crude egg PC was prepared as described in [27].

2.2. Cell culture

AC29 cells, an ACAT1-deficient Chinese hamster ovary (CHO) mutant cell line [28, 29], were maintained in 50% F-12 medium (Corning, Manassas, VA, USA) and 50% DMEM medium supplemented with 10% calf serum (Atlanta Biologicals, Flowery Branch, GA, USA). HEK293 cells were maintained in DMEM medium supplemented with 10% newborn calf serum (Atlanta Biologicals, Flowery Branch, GA, USA). All cell lines were maintained at 37°C under humidified conditions and 5% CO₂.

The expression plasmid HisACAT1/FLAG was constructed as described previously [30]. In brief, an octapeptide FLAG-tag was inserted at the C-terminus of human ACAT1 cDNA containing a 6-histidine (His)-tag at the N-terminus. This fragment (HisACAT1/FLAG) was ligated into the mammalian expression vector pAG3-Zeo [31]. The expression plasmid 1-65 HisACAT1/FLAG was constructed in a similar manner, by using the HisACAT1 cDNA devoid of the first 65 amino acids [32]. HisACAT1/FLAG was purified to homogeneity as previously described [12, 30] from stably expressing HEK293 cells by promptly purifying the enzyme first with HisPur Ni-NTA resin and subsequently with anti-FLAG M2 resin. AC29 cells stably expressing HisACAT1/FLAG or 1-65 HisACAT1/FLAG were isolated as previously described [12].

2.3. In-gel reconstitution of HisACAT1/FLAG peptidiscs

The following procedure was adapted from [23]. Pure HisACAT1/FLAG (2.5 µl at ~25 ng/µl) was mixed with increasing molar ratio of NSP_r (0-0.16 µg at 40 ng/µl and diluted to a final volume of 6.5 µl with 125 mM Tris-HCl, pH 7.8). The mixtures were incubated at room temperature for 1-2 min. The clear native (CN)- polyacrylamide gel electrophoresis (PAGE) samples were supplemented with 6.5 µl of 2× native loading dye (0.2 M KCl; 0.1 M Tris-HCl, pH 6.8; 20% glycerol; 0.02% bromophenol blue) to bring the final concentration of CHAPS to 1.6 mM, which is below its 8-10 mM critical micelle concentration (CMC). The

blue-native (BN)-PAGE samples were supplemented with 6.5 μ l of 3 $\times$ native loading dye (0.3 M KCl; 0.15 M Tris-HCl, pH 6.8; 30% glycerol; 0.03% bromophenol blue) and 6.5 μ l of 0.06% Coomassie Brilliant Blue R-250 to bring the final concentration of CHAPS to 1 mM. All samples were loaded into a 4-10% or 10% native-PAGE with anode and cathode buffer (25 mM Tris; 192 mM glycine). Gels were run overnight at 12 mA before being placed into CN-PAGE gel sensitizing buffer (25 mM Tris; 192 mM glycine; 1% sodium dodecyl sulfate (SDS)) for at least 30 min at room temperature with gentle mixing on an orbital shaker. The 1% SDS, in the buffer, sensitized the proteins in the gel with negatively charged detergent which allows for protein transfer. Proteins were transferred onto nitrocellulose membrane with Towbin's buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 20% methanol; 0.01% SDS) at 350 mA for 3 h at 4°C. ACAT1 was immunoblotted by Western blot with anti-FLAG-tag antibody.

2.4. On-bead reconstitution of ACAT1 peptidiscs

This method was adapted from [12, 23, 24]. Six 15 cm plates of AC29 cells stably expressing HisACAT1/FLAG or 1-65 HisACAT1/FLAG at 95-100% confluency were used as the ACAT1 source. Media were aspirated and cell monolayers were washed twice with 9 ml of cold PBS. A 0.9 ml aliquot of Buffer A (1 M KCl; 40.6 mM (2.5%) CHAPS; 50 mM Tris-HCl, pH 7.8; 10% glycerol) with 1 $\times$ protease inhibitor was added to each plate, cells were scraped, and pooled together in a Dounce tissue grinder set with stainless steel chamber and pestle on ice. The homogenizer was plunged 60 times to homogenize cells. The lysate was transferred to a polycarbonate centrifuge tube on ice and placed in a pre-chilled 70.1 Ti rotor, and centrifuged at 39,000 rpm ($\sim 100,000 \times g$) for 1 h at 4°C. A 0.2 ml bed (0.4 ml of slurry) of anti-FLAG M2 resin was equilibrated with 0.5 ml of Buffer A in a gravity column. The lysate was loaded over the column and the column flow-through was continually reloaded at 4°C over a span of 2 h. The column was washed with 2.5 ml of Buffer B (0.5 M KCl; 8.1 mM (0.5%) CHAPS; 50 mM Tris-HCl, pH 7.8; 10% glycerol). The resin was mixed with 0.5 ml of 1 mg/ml NSP_r in 20 mM Tris-HCl, pH 7.8 for 5 min at 4°C. The NSP_r flow through was collected and stored at -20°C for further use. The column was then washed with 1 ml of Buffer C (0.2 M KCl; 50 mM Tris-HCl, pH 7.8; 10% glycerol). The resin was incubated with 0.3 ml of 300 μ g/ml FLAG peptide in Buffer E (0.1 M KCl; 50 mM Tris-HCl, pH 7.8; 10% glycerol) for 1 h at 4°C before collecting 0.1 ml aliquots of eluates into pre-chilled low adhesion microcentrifuge tubes. The previous step was repeated but incubated for 2 h at 4°C. The resin was stripped with 0.3 ml of 0.1 M glycine, pH 3 followed by collection into 30 μ l of 1 M Tris-HCl, pH 9. The regenerated resin was re-equilibrated in TBS and stored in 50% glycerol in TBS containing 0.02% sodium azide at 4°C. The eluates were stored at -80°C for further use.

2.5. Liposomal ACAT activity assay

The procedure was followed as previously described [11, 33]. This assay was routinely used to measure ACAT activity *in vitro*. In brief, 5 μ l of sample was diluted to final concentrations of 0.1 M KCl and 5 mM CHAPS with 60 μ l of preformed PC particles (11.2 mM crude egg PC; 9.3 mM taurocholate; 1.8 mM cholesterol) and 10 nmoles of [³H]oleoyl-CoA bound to BSA to deliver substrates to ACAT1. The reactions were assayed in duplicates for 30 min at 37°C.

2.6. On-bead purification and reconstitution of 1-65 HisACAT1/FLAG peptidiscs

The following method was adapted from [12, 33]. Six 15 cm plates of AC29 cells stably expressing 1-65 HisACAT1/FLAG at 95-100% confluency were used as the ACAT1 source. Cells were lysed as previously described in section 2.4. A 0.1 ml bed (0.2 ml of slurry) of anti FLAG M2 resin was equilibrated with 0.5 ml of Buffer A in a Corning Costar Spin-X 0.22 μ m centrifuge tube filter. The resin was added to the lysate in a 15 ml conical tube and gently mixed for 2 h at 4°C on an orbital shaker. Resin was centrifuged at $500 \times g$ for 5 min at 4°C and the supernatant was collected. The resin was washed with 3 ml of Buffer B and was centrifuged at $500 \times g$ for 5 min at 4°C. The supernatant was collected, and the resin was mixed with Buffer B to a total volume of 0.4 ml. The resin/Buffer B slurry was transferred as 100 μ l aliquots into four individual Spin-X centrifuge tube filters. The four spin filters were labeled by which eluant 1-65 HisACAT1/FLAG was to be eluted with: Buffer B (CHAPS), Buffer D (0.5 M KCl; 16.3 μ M digitonin; 50 mM Tris-HCl, pH 7.8; 10% glycerol) or 0.1 M KCl with or without NSP_r. The following centrifugation steps were all conducted at $3000 \times g$ for 10-60 s at 4°C. One of the 0.1 M KCl spin filters was pelleted and mixed with 0.1 ml of 1 mg/ml NSP_r in 20 mM Tris-HCl, pH 7.8 for 5 min at 4°C. Each resin was pelleted and washed with 0.25 ml of their respective eluant buffer.

1-65 HisACAT1/FLAG was eluted with 0.1 ml of 300 μ g/ml FLAG peptide in the respective eluant buffer and incubated for 2 h at 4°C before collecting the eluate into a pre-chilled low adhesion microcentrifuge tube. The resin was stripped with 0.1 ml of 0.1 M glycine, pH 3 followed by collection into 10 μ l of 1 M Tris-HCl, pH 9. The resin was regenerated as previously described in section 2.4 and the eluates were stored at -80°C for further use.

2.7. Conditioning of 1-65 HisACAT1/FLAG with detergents or with phosphatidylcholine on-bead prior to peptidisc formation

The following methods were adapted from section 2.6. A 0.1 ml bed (0.2 ml of slurry) of anti-FLAG M2 resin was bound with the cell lysate of six 15 cm plates of AC29 cells stably expressing 1-65 HisACAT1/FLAG and split into four separate Spin-X centrifuge tube filters. Two spin filters were washed with 0.25 ml of Buffer B and the other two were washed with 0.25 ml of PC particles (9.3 mM taurocholate; 11.2 mM crude egg PC). One spin filter from each condition was pelleted and mixed with 0.1 ml of 1 mg/ml NSP_r in 20 mM Tris-HCl, pH 7.8 for 5 min at 4°C. The NSP_r treated resins were washed with 0.25 ml of Buffer C. The individual spin filters were incubated with 0.1 ml of 300 μ g/ml FLAG peptide in their respective eluant buffers or Buffer E for 2 h at 4°C before collection.

In a similar experiment, a 75 μ l bed (0.15 ml of slurry) of anti-FLAG M2 resin was bound with the cell lysate of six 15 cm plates of AC29 cells stably expressing 1-65 HisACAT1/FLAG and split into three separate Spin-X centrifuge tube filters. The individual spin filters were conditioned with 0.25 ml of Buffer B, Buffer D, or PC particles for 5 min at 4°C. All three spin filters were mixed by loading 0.1 ml of 1 mg/ml NSP_r in 20 mM Tris-HCl, pH 7.8 for 5 min at 4°C. Afterwards, they were washed with 0.25 ml of Buffer C. The individual spin filters were incubated with 0.1 ml of 300 μ g/ml FLAG peptide in Buffer E for 2 h at 4°C before collection.

In a larger experiment, a 0.2 ml bed (0.4 ml of slurry) of anti-FLAG M2 resin was bound with the cell lysate of twelve 15 cm plates of AC29 cells stably expressing 1-65 HisACAT1/FLAG and split into seven separate Spin-X centrifuge tube filters. The seven resins were pelleted and the individual spin filters were conditioned for 5 min at 4°C with 0.1 ml of the following: CHAPS (Buffer B); CHAPS, crude egg PC (9.3 mM CHAPS; 11.2 mM crude egg PC); CHAPS, pure egg PC (9.3 mM CHAPS; 11.2 mM pure egg PC); Tauro (9.3 mM taurocholate); Tauro, crude egg PC (9.3 mM taurocholate; 11.2 mM crude egg PC); Tauro, crude egg PC, CHOL (9.3 mM taurocholate; 11.2 mM crude egg PC; 1.8 mM cholesterol); or Tauro, pure egg PC (9.3 mM taurocholate; 11.2 mM pure egg PC). Each resin was pelleted and mixed with 0.1 ml of 1 mg/ml NSP_r in 20 mM Tris-HCl, pH 7.8 for 5 min at 4°C. Following NSP_r exposure, each filter was washed with 0.25 ml of Buffer C. The individual spin filters were incubated with 0.1 ml of 300 µg/ml FLAG peptide in Buffer E for 2 h at 4°C before collection. Conditioned ACAT1 peptidisc eluates were loaded onto 10% SDS-PAGE to evaluate the efficacy of purification.

2.8. Heat-mediated inactivation of conditioned 1-65 HisACAT1/FLAG peptidiscs

This method was adapted from [12]. In brief, each conditioned ACAT1 peptidisc eluate, prepared as described in section 2.7, was split into 12 µl aliquots using low adhesion microcentrifuge tubes. These tubes were placed in a 37°C water bath for a desired amount of time before being halted by placing on ice. The enzymatic activity of heat-treated 1-65 HisACAT1/FLAG peptidiscs were measured by using the liposomal ACAT activity assay.

2.9. Statistical analysis

Results were reported as the mean ± standard deviation (SD), unless noted otherwise. GraphPad Prism 5 software was used for plotting data and statistical analysis. Statistical comparisons for data were performed using Student's t-tests for unpaired comparisons and were considered statistically significant if $p < 0.05$.

3. Results

3.1. In-gel reconstitution of HisACAT1/FLAG into peptidiscs stabilizes the enzyme in oligomeric states with sizes greater than a tetramer

When solubilized in the zwitterionic detergent CHAPS, ACAT1 can be purified to homogeneity with full retention in enzymatic activity [11], and the enzyme behaves as a homotetrameric protein [12, 34]. ACAT1 contains two dimerization motifs with one motif located near the N-terminus. Deletion of the N-terminal dimerization domain (by removal of the first 65 amino acids) converts ACAT1 to a dimer with full catalytic activity; therefore, ACAT1 is a two-fold dimer [32]. Here, to assess the effects of peptidiscs on ACAT1, we performed the in-gel reconstitution method [23] by loading the purified ACAT1, with a N-terminal 6×His-tag and a C-terminal FLAG-tag (HisACAT1/FLAG), and NSP_r together in a PAGE gel without detergents. Using this method, the reconstitution of MPs into peptidiscs occurs as the sample migrates through the gel in a detergent-free state. We analyzed the results by using both clear native (CN)-PAGE, which separates proteins by its charge-to-mass ratio, and blue native (BN)-PAGE, which shifts the charge of proteins due to the addition of negatively charged Coomassie Brilliant Blue R-250.

The results showed that without adding NSP_r, the apparent molecular weight of HisACAT1/FLAG is ~250 kDa (ACAT1₄) (Fig. 1A, B; lane 0). This value is consistent with the apparent molecular weight of HisACAT1/FLAG when estimated by sucrose density gradient centrifugation in 1 M KCl and 8.1 mM CHAPS [12, 34]. Addition of NSP_r resulted in the conversion of HisACAT1/FLAG into two multimeric states, with sizes both greater than its usual homotetrameric state (Fig. 1A; lanes 5-40). The first state appears to be very large in size and never enters the 4% stacking gel; its intensity decreases with increasing concentrations of NSP_r (Fig. 1A, B; lanes 5-40). Whereas another state appears to be a large oligomer (ACAT1_n) which does not enter the 10% resolving gel; this state accumulates with increasing concentrations of NSP_r (Fig. 1A; lanes 5-40). Next, to better resolve these multimeric states, replicate samples were loaded into a gradient 4-10% CN-PAGE; this approach revealed that ACAT1_n enters the resolving gel and appears as a smear, suggesting that NSP_r convert HisACAT1/FLAG into a variety of oligomers above 250 kDa (Fig. 1B; lanes 5-40). This result implies NSP_r reconstitutes HisACAT1/FLAG from CHAPS micelles into soluble peptidiscs such that ACAT1 is present as variable sized oligomers.

3.2. The conversion of ACAT1 into higher-order oligomeric states by peptidiscs is independent of the N-terminal dimerization domain within ACAT1

Full-length HisACAT1/FLAG contains two dimerization domains, one in the N-terminal dimerization domain and another near the C-terminus [12, 32, 35]. We used the on-bead method [23, 24] to examine the oligomerization states of the full-length ACAT1 and the dimeric ACAT1 in peptidiscs. The on-bead method utilizes affinity chromatography to expose tagged MP, while bound to beads, to NSP_r. We used anti-FLAG M2 resin to capture HisACAT1/FLAG or 1-65 HisACAT1/FLAG onto the beads. Once NSP_r is added, detergent is reduced by washing the beads with detergent-free buffer. The washing step promotes the reconstitution of target MPs into peptidiscs.

The results showed that when pure HisACAT1/FLAG was reconstituted into peptidiscs by the on-bead method, the enzyme was primarily present in the ACAT1_n state (Fig. 1A, B; lanes on-bead). To determine whether the N-terminal dimerization domain is involved in the formation of these higher-order oligomers, we next prepared partially purified HisACAT1/FLAG or 1-65 HisACAT1/FLAG peptidiscs by using the on-bead method (described in section 2.4). The results showed that both HisACAT1/FLAG and 1-65 HisACAT1/FLAG were stabilized as tetramers when reconstituted into peptidiscs via the on-bead method (Fig. 2A; lanes 1-6). This result demonstrates that oligomerization induced by peptidiscs formation is independent of the N-terminal dimerization domain, suggesting that the oligomerization of ACAT1 induced by peptidiscs is dependent on the hydrophobic interactions between transmembrane domains (TMDs) of ACAT1 subunits. With the in-gel method, pure HisACAT1/FLAG was found to form oligomers larger than a tetramer. This discrepancy with the on-bead method may be a result of the varied concentration of CHAPS present in the different enzyme sources or the effectiveness of the methods to dilute detergent prior to peptidisc formation which may modify ACAT1 oligomerization.

When present in the crude cell lysates, both the full-length HisACAT1/FLAG and the 1-65 HisACAT1/FLAG preparations are enzymatically active (Fig. 2B). Surprisingly, we found

that while the size of the HisACAT1/FLAG peptidiscs were tetrameric they were enzymatically inactive, unlike the 1-65 HisACAT1/FLAG peptidiscs (Fig. 2B). Further analysis shows that the HisACAT1/FLAG in peptidiscs was present mostly as a fragment of ~30 kDa in SDS-PAGE (Fig. 2C). These results suggest that for unknown reason(s), during the purification process, the full-length HisACAT1/FLAG is more susceptible to proteolytic degradation than 1-65 HisACAT1/FLAG and provides a plausible explanation as to why the HisACAT1/FLAG peptidiscs were enzymatically inactive. In contrast, the partially purified 1-65 HisACAT1/FLAG peptidiscs were found to retain enzymatic activity and remained soluble after $100,000 \times g$ centrifugation (Fig. 2D). For the remainder of this study, we utilize 1-65 HisACAT1/FLAG as the enzyme source to further investigate the effects of NSP_r on ACAT1 peptidisc formation.

3.3. ACAT1 solubilized in digitonin or reconstituted in peptidiscs were more enzymatically active than when solubilized in CHAPS

Next, we wanted to evaluate how reconstitution of 1-65 HisACAT1/FLAG in peptidiscs compares to conventional purification methods with mild detergents (CHAPS or digitonin [CMC 0.5 mM, 0.061%]). We used the on-bead method to address this question. Cell lysates prepared from AC29 cells stably expressing 1-65 HisACAT1/FLAG were bound to anti-FLAG M2 resin; this resin was split equally into four fractions and each fraction was treated with different eluants: CHAPS (0.5 M KCl; 8.1 mM (0.5%) CHAPS), digitonin (0.5 M KCl; 16.3 μ M (0.002%) digitonin), 0.1 M KCl or 0.1 M KCl with exposure to 1 mg/ml NSP_r. After elution, the protein content and enzymatic activities of 1-65 HisACAT1/FLAG eluates were analyzed. This procedure is outlined in Fig. 3A.

The results showed that, when CHAPS, digitonin, or NSP_r were included, 1-65 HisACAT1/FLAG could be eluted from the column, whereas low salt (0.1 M KCl) alone was incapable of eluting 1-65 HisACAT1/FLAG from the column (Fig. 3B, D). These results suggest that without detergent or peptidiscs, 1-65 HisACAT1/FLAG is insoluble in aqueous condition. CHAPS, digitonin and NSP_r 1-65 HisACAT1/FLAG conferred similar enzymatic activities (Fig. 3C). However, when accounted for how much 1-65 HisACAT1/FLAG protein was present in the eluate of each condition, as determined from the Western signal of Fig. 3D, ACAT1 solubilized by digitonin or reconstituted in peptidiscs conferred more enzymatic activity per unit of ACAT1 as compared to that of CHAPS eluate (Fig. 3E). This also implies that NSP_r can be used to stabilize ACAT1 in detergent-free solutions and when reconstituted into peptidiscs ACAT1 is more active per unit of enzyme as compared to ACAT1 solubilized with CHAPS.

3.4. The detergents or phospholipids present prior to peptidisc formation affects 1-65 HisACAT1/FLAG oligomeric state

With the on-bead method, MPs bound to resin can be conditioned with various detergents or phospholipids prior to the formation of peptidiscs. Here we describe the use of this method to provide a detergent-free environment to promote the study of phosphatidylcholine (PC) association to 1-65 HisACAT1/FLAG. Cell lysates of AC29 cells stably expressing 1-65 HisACAT1/FLAG were bound to anti-FLAG M2 resin and conditioned with either CHAPS (0.5 M KCl; 8.1 mM CHAPS), or digitonin (0.5 M KCl; 16.3 μ M digitonin) or PC particles

(9.3 mM taurocholate; 11.2 mM crude egg PC) prior to exposure with NSP_r. As expected, the results showed that in the presence of PC without NSP_r 1-65 HisACAT1/FLAG was insoluble, whereas CHAPS produced the characteristic tetramer (Fig. 4A; lane 1). Upon the addition of NSP_r, CHAPS stabilized 1-65 HisACAT1/FLAG into oligomers larger than a tetramer (Fig. 4A; lane 2). In a similar experiment, we used digitonin to condition 1-65 HisACAT1/FLAG prior to peptidisc reconstitution; the result showed that digitonin produced similar but slightly different effects than CHAPS on ACAT1 oligomerization (Fig. 4B; lanes 3-4). When conditioned with PC, peptidiscs stabilized 1-65 HisACAT1/FLAG into two states, an oligomeric state with size(s) greater than that of CHAPS or digitonin, and a large aggregate (Fig. 4A; lanes 3-4, B; lanes 5-6). The yield of soluble 1-65 HisACAT1/FLAG varied among the various conditions employed, with CHAPS peptidiscs stabilizing the most soluble enzyme (Fig. 4C). Overall, these results led us to conclude that PC can readily substitute detergents (CHAPS) bound to ACAT1 before the enzyme is reconstituted into peptidiscs.

3.5. 1-65 HisACAT1/FLAG peptidiscs conditioned with PC were more enzymatically active and more resistant to heat-mediated inactivation

Thus far, we determined that PC affects the oligomeric state of the ACAT1 peptidiscs and we speculate that PC may also affect the enzymatic activity of the ACAT1 present in peptidiscs. We have found that crude egg PC, prepared in our lab, consistently provides higher ACAT enzymatic activity than commercially purchased pure L- α -PC from egg yolk. The pure egg PC is 99% pure, whereas the crude egg PC contains 5-10% phosphatidylethanolamine (PE), as determined by thin-layer chromatography (data not shown). Our results are consistent with the previous finding by Mathur & Spector [10], who showed that PE included in PC based liposomes at 33% mole percent increased the activity of solubilized ACAT enzyme preparations by 12 or 20%. To test this possibility, we used the on-bead method [23, 24] and conditioned 1-65 HisACAT1/FLAG with detergent or with PC (present in various formulations as indicated in Fig. 5A), prior to the formation of peptidiscs. Once formed, the ACAT1 peptidiscs were eluted from anti-FLAG M2 resin with FLAG peptide in Buffer E (0.1 M KCl; 50 mM Tris-HCl, pH 7.8; 10% glycerol); the enzymatic activity was measured by using the liposomal ACAT activity assay. This procedure is outlined in Fig. 5A.

Regardless of the PC used to condition 1-65 HisACAT1/FLAG prior to peptidisc formation, ACAT1 formed higher-order oligomers than when conditioned with CHAPS or taurocholate (Fig. 5B). The results showed that conditioning of 1-65 HisACAT1/FLAG with PC reduced overall yield of the ACAT1 protein (Fig. 5C), presumably due to an increase in the formation of large ACAT1 aggregates on beads prior to reconstitution of ACAT1 into peptidiscs. On the other hand, even though PC-conditioned 1-65 HisACAT1/FLAG yielded less protein, the relative ACAT unit per Western signal present in PC-conditioned peptidiscs was significantly higher than that present in detergent-conditioned (CHAPS or taurocholate) ACAT1 peptidiscs (Fig. 5D).

We have previously reported that when present in preformed PC particles (9.3 mM taurocholate; 11.2 mM crude egg PC), the enzymatic activity of HisACAT1/FLAG is more

resistant to heat-mediated inactivation than that of enzyme present in CHAPS or Triton X-100 micelles [12]. Here we performed similar experiments to assess the rate of heat-mediated enzymatic inactivation of 1-65 HisACAT1/FLAG peptidiscs conditioned with various formulations of PC particles versus those conditioned with detergent (CHAPS or taurocholate) micelles. The results showed that detergent-conditioned 1-65 HisACAT1/FLAG peptidiscs were more susceptible to heat-mediated inactivation than when conditioned with PC (Fig. 6). Analyses of Fig. 6 show that the half-time of decay in ACAT activity is increased with PC-conditioned 1-65 HisACAT1/FLAG peptidiscs as compared to those conditioned with detergent. This suggests that when placed in peptidiscs, significant interactions between PC and ACAT1 occur which can be readily demonstrated through the protection of ACAT activity from heat.

4. Discussion

NSP_r is an efficient reagent to reconstitute MPs into detergent-free peptidiscs. The formation of peptidiscs is spontaneous upon dilution of detergents which can be accomplished through several common analytical methods. Due to the disruptive nature of detergents, it is difficult to assess how phospholipids associate with MPs. Here, we report the use of NSP_r to reconstitute ACAT1 into peptidiscs. Our results show that with minimal detergents bound to ACAT1, NSP_r stabilizes the enzyme into oligomers larger than a tetramer (Fig. 1). The formation of higher-order oligomers was found to be independent of the ACAT1 N-terminal dimerization domain, suggesting that this oligomerization involves the hydrophobic association of several TMDs present in multiple ACAT1 subunits (Fig. 2A). Reconstituted ACAT1 peptidiscs confer higher enzymatic activity than that of detergent solubilized ACAT1 (Fig. 3E). We then used the method of on-bead reconstitution of ACAT1 peptidiscs to study how PC affects ACAT1 enzymatic activity and oligomerization. When conditioned with PC, ACAT1 in peptidiscs is found to be in a multimeric state, with sizes even greater than the ACAT1 oligomers present in detergent-conditioned peptidiscs (Fig. 4). PC-conditioned ACAT1 peptidiscs were more enzymatically active compared to detergent-conditioned peptidiscs (Fig. 5D) and PC provides protection of the enzyme from heat-mediated inactivation (Fig. 6). Overall, we conclude that NSP_r is effective in reconstituting ACAT1 into near detergent-free peptidisc complexes for various biochemical studies. Here we define “near detergent-free” ACAT1 as being soluble in solution without detergent through stabilization by NSP_r to form peptidiscs, although some detergents, which are highly associated with ACAT1, cannot be readily substituted by NSP_r.

Future experiments could evaluate the size of ACAT1 peptidiscs by size exclusion chromatography (SEC), dynamic light scattering (DLS), or transmission electron microscopy (TEM). SEC could separate the various oligomers by molecular weight; however, the larger oligomers may elute in the void volume. DLS measures fluctuations in scattered light intensity due to diffusing particles which can be used to determine the mean particle diameter and/or the molecular weight of isolated oligomers. Negative staining of ACAT1 peptidiscs could be used to directly visualize the oligomers by TEM, whereas single-particle cryo-electron microscopy (cryo-EM) could be used to reconstruct 3D density maps to elucidate oligomeric binding interfaces. Previous studies showed that when solubilized by the detergent CHAPS, ACAT1 exists largely as a homotetramer *in vitro* [12,

34]. In addition, when the chemical cross-linker dithiobis(succinimidyl) propionate (DSP) was used in intact cells (High Five or CHO cells), the results showed that ACAT1 exists mainly as a homotetramer [34]. However, the same study showed that with increased DSP concentrations, the tetrameric form was reduced, suggesting that in intact cells, a certain portion of ACAT1 may exist in oligomeric states greater than a homotetramer. Here we show that PC propagates ACAT1 to form higher-order oligomers *in vitro*. A previous *in vitro* study showed that when microsomal extracts of Ehrlich ascites cells were treated with phospholipid liposomes, the apparent density of the active ACAT fraction became larger [10]. Together, these results raise the possibility that under certain conditions ACAT1 could form multimeric forms, with size(s) larger than the tetrameric state, with altered enzymatic activity. In the future, techniques such as radiation inactivation [36, 37], fluorescence fluctuation spectroscopy of fluorescent protein tags [38], or X-ray fluorescence microscopy to visualize 3D images of lanthanide-binding tags [39] can be used to determine if larger oligomeric states of ACAT1 exist in intact cells. The higher-order ACAT1 oligomers captured by peptidiscs might be an artifact induced by reducing detergent/delipidation. Further investigations are required to assess if higher oligomeric states of ACAT1 are physiologically relevant.

Three recent studies [40–42] have reported the high-resolution structure of ACAT1 by cryo-EM. These studies show that ACAT1 is a two-fold dimer; the dimerization occurs through the hydrophobic interface of TMD1 of one protomer and TMDs 6 and 9 of the adjacent protomer. When inserted into nanodiscs, without supplemented phospholipids, or glycodiosgenin micelles, the ACAT1 dimer-of-dimers formed through interactions between the N-terminal dimerization domain, as well as a hydrophobic interface involving TMDs 2, 5, 6 and an intracellular helix of two protomers of opposing dimers [40, 42].

Dimeric ACAT1 is the smallest functional unit to confer enzymatic activity [12, 40, 42] and it has been suggested the presumed sterol substrate transmembrane (T) tunnel of two protomers are blocked in tetrameric ACAT1 [41]. These observations supports the hypothesis just two protomers of tetrameric ACAT1 are enzymatically active which could help explain the reduced enzymatic capacity of tetrameric compared to dimeric ACAT1 [32]. As a result of our current study, we hypothesize that the bilayer fluid phase of ER membranes may affect ACAT1 oligomerization and its enzymatic activity. Previous work reported that in macrophages grown in normal medium, ACAT1 resides in medium-density ER membranes and has low ACAT enzyme activity, whereas in macrophages grown in cholesterol-loaded conditions, ACAT1 resides in lower-density ER-derived vesicles with a 3-fold increase in ACAT enzymatic activity than the medium-density membrane fractions [43]. It is possible that cholesterol loading may affect the bilayer fluid phase of ER membranes and in turn affect the oligomeric state and the enzymatic activity of ACAT1 as a way to subcompartmentalize cholesteryl ester synthesis in the ER membrane bilayer.

Based on our current study, the addition of PC in the samples used for cryo-EM could further induce the two-fold ACAT1 dimer into higher-order oligomers. This concept could be applicable to other membrane-bound O-acyltransferase (MBOAT) family members, such as the dimeric diacylglycerol O-acyltransferase 1 (DGAT1) [44, 45]. Our current study

suggests that it will be important to investigate how phospholipids regulate the oligomeric state of ACAT1 and possibly affect its enzymatic properties *in vitro* and *in vivo*.

Acknowledgements

We thank members of the Chang lab for helpful discussion during the course of this work and Dr. Andreia Verissimo for suggesting the use of peptidiscs. We also thank Dr. Franck Duong Van Hoa of The University of British Columbia for providing NSP_T and the detailed methods for the reconstitution of membrane proteins into peptidiscs.

Funding

This work was supported by NIH/NIGMS COBRE award P20-GM113132 to BioMT- Institute for Biomolecular Targeting and NIH grant RO1 AG063544 to T.Y. Chang and C.C.Y. Chang.

Abbreviations

ACAT	acyl-coenzyme A:cholesterol acyltransferase
BN	PAGE, blue native polyacrylamide gel electrophoresis
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary cells
CMC	critical micelle concentration
CN-PAGE	clear native polyacrylamide gel electrophoresis
CoA	coenzyme A
Cryo-EM	cryogenic electron microscopy
DGAT1	diacylglycerol O-acyltransferase 1
ER	endoplasmic reticulum
MBOAT	membrane-bound O-acyltransferase
MP	membrane protein
MSP	membrane scaffold protein
NSP	nanodisc scaffold peptide
PC	phosphatidylcholine
PE	phosphatidylethanolamine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOAT	sterol O-acyltransferase
SMA	styrene-maleic acid
SMALP	SMA lipid particles

TMD transmembrane domain

5. References

- [1]. Garavito RM, Ferguson-Miller S, Detergents as tools in membrane biochemistry, *The Journal of biological chemistry* 276(35) (2001) 32403–6. [PubMed: 11432878]
- [2]. Bowie JU, Stabilizing membrane proteins, *Current opinion in structural biology* 11(4) (2001) 397–402. [PubMed: 11495729]
- [3]. Chang TY, Li BL, Chang CC, Urano Y, Acyl-coenzyme A:cholesterol acyltransferases, *American journal of physiology. Endocrinology and metabolism* 297(1) (2009) E1–9. [PubMed: 19141679]
- [4]. Murphy SR, Chang CC, Dogbevia G, Bryleva EY, Bowen Z, Hasan MT, Chang TY, Acat1 knockdown gene therapy decreases amyloid-beta in a mouse model of Alzheimer's disease, *Molecular therapy : the journal of the American Society of Gene Therapy* 21(8) (2013) 1497–506. [PubMed: 23774792]
- [5]. Yue S, Li J, Lee SY, Lee HJ, Shao T, Song B, Cheng L, Masterson TA, Liu X, Ratliff TL, Cheng JX, Cholesteryl ester accumulation induced by PTEN loss and PI3K/AKT activation underlies human prostate cancer aggressiveness, *Cell metabolism* 19(3) (2014) 393–406. [PubMed: 24606897]
- [6]. Geng F, Cheng X, Wu X, Yoo JY, Cheng C, Guo JY, Mo X, Ru P, Hurwitz B, Kim SH, Otero J, Puduvalli V, Lefai E, Ma J, Nakano I, Horbinski C, Kaur B, Chakravarti A, Guo D, Inhibition of SOAT1 Suppresses Glioblastoma Growth via Blocking SREBP-1-Mediated Lipogenesis, *Clinical cancer research : an official journal of the American Association for Cancer Research* 22(21) (2016) 5337–5348. [PubMed: 27281560]
- [7]. Huang LH, Melton EM, Li H, Sohn P, Jung D, Tsai CY, Ma T, Sano H, Ha H, Friedline RH, Kim JK, Usherwood E, Chang CCY, Chang TY, Myeloid-specific Acat1 ablation attenuates inflammatory responses in macrophages, improves insulin sensitivity, and suppresses diet-induced obesity, *American journal of physiology. Endocrinology and metabolism* 315(3) (2018) E340–E356. [PubMed: 29533741]
- [8]. Melton EM, Li H, Benson J, Sohn P, Huang LH, Song BL, Li BL, Chang CCY, Chang TY, Myeloid Acat1/Soat1 KO attenuates pro-inflammatory responses in macrophages and protects against atherosclerosis in a model of advanced lesions, *The Journal of biological chemistry* 294(43) (2019) 15836–15849. [PubMed: 31495784]
- [9]. Kaduce TL, Schmidt RW, Spector AA, Acylcoenzyme A:cholesterol acyltransferase activity: solubilization and reconstitution in liposomes, *Biochemical and biophysical research communications* 81(2) (1978) 462–8. [PubMed: 666765]
- [10]. Mathur SN, Spector AA, Effect of liposome composition on the activity of detergent-solubilized acylcoenzyme A: cholesterol acyltransferase, *Journal of lipid research* 23(5) (1982) 692–701. [PubMed: 6126513]
- [11]. Chang CC, Lee CY, Chang ET, Cruz JC, Levesque MC, Chang TY, Recombinant acyl-CoA:cholesterol acyltransferase-1 (ACAT-1) purified to essential homogeneity utilizes cholesterol in mixed micelles or in vesicles in a highly cooperative manner, *The Journal of biological chemistry* 273(52) (1998) 35132–41. [PubMed: 9857049]
- [12]. Neumann B, Chang CCY, Chang TY, Triton X-100 or octyl glucoside inactivates acyl-CoA:cholesterol acyltransferase 1 by dissociating it from a two-fold dimer to a two-fold monomer, *Archives of biochemistry and biophysics* 671 (2019) 103–110. [PubMed: 31251920]
- [13]. Denisov IG, Grinkova YV, Lazarides AA, Sligar SG, Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size, *Journal of the American Chemical Society* 126(11) (2004) 3477–87. [PubMed: 15025475]
- [14]. Ritchie TK, Grinkova YV, Bayburt TH, Denisov IG, Zolnerciks JK, Atkins WM, Sligar SG, Chapter 11 - Reconstitution of membrane proteins in phospholipid bilayer nanodiscs, *Methods in enzymology* 464 (2009) 211–31. [PubMed: 19903557]
- [15]. Grinkova YV, Denisov IG, Sligar SG, Engineering extended membrane scaffold proteins for self-assembly of soluble nanoscale lipid bilayers, *Protein engineering, design & selection : PEDS* 23(11) (2010) 843–8.

- [16]. Denisov IG, Sligar SG, Nanodiscs for structural and functional studies of membrane proteins, *Nature structural & molecular biology* 23(6) (2016) 481–6.
- [17]. Knowles TJ, Finka R, Smith C, Lin YP, Dafforn T, Overduin M, Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer, *Journal of the American Chemical Society* 131(22) (2009) 7484–5. [PubMed: 19449872]
- [18]. Lee SC, Knowles TJ, Postis VL, Jamshad M, Parslow RA, Lin YP, Goldman A, Sridhar P, Overduin M, Muench SP, Dafforn TR, A method for detergent-free isolation of membrane proteins in their local lipid environment, *Nature protocols* 11(7) (2016) 1149–62. [PubMed: 27254461]
- [19]. Popovic K, Holyoake J, Pomes R, Prive GG, Structure of saposin A lipoprotein discs, *Proceedings of the National Academy of Sciences of the United States of America* 109(8) (2012) 2908–12. [PubMed: 22308394]
- [20]. Frauenfeld J, Loving R, Armache JP, Sonnen AF, Guettou F, Moberg P, Zhu L, Jegerschold C, Flayhan A, Briggs JA, Garoff H, Low C, Cheng Y, Nordlund P, A saposin-lipoprotein nanoparticle system for membrane proteins, *Nature methods* 13(4) (2016) 345–51. [PubMed: 26950744]
- [21]. Chung BH, Anatharamaiah GM, Brouillette CG, Nishida T, Segrest JP, Studies of synthetic peptide analogs of the amphipathic helix. Correlation of structure with function, *The Journal of biological chemistry* 260(18) (1985) 10256–62. [PubMed: 4019511]
- [22]. Kariyazono H, Nadai R, Miyajima R, Takechi-Haraya Y, Baba T, Shigenaga A, Okuhira K, Otaka A, Saito H, Formation of stable nanodiscs by bihelical apolipoprotein A-I mimetic peptide, *Journal of peptide science : an official publication of the European Peptide Society* 22(2) (2016) 116–22. [PubMed: 26780967]
- [23]. Carlson ML, Young JW, Zhao Z, Fabre L, Jun D, Li J, Li J, Dhupar HS, Wason I, Mills AT, Beatty JT, Klassen JS, Rouiller I, Duong F, The Peptidisc, a simple method for stabilizing membrane proteins in detergent-free solution, *eLife* 7 (2018).
- [24]. Saville JW, Troman L, Duong Van Hoa F, PeptiQuick, a One-Step Incorporation of Membrane Proteins into Biotinylated Peptidiscs for Streamlined Protein Binding Assays, *Journal of visualized experiments : JoVE* (153) (2019).
- [25]. Angiulli G, Dhupar HS, Suzuki H, Wason IS, Duong Van Hoa F, Walz T, New approach for membrane protein reconstitution into peptidiscs and basis for their adaptability to different proteins, *eLife* 9 (2020).
- [26]. Bishop JE, Hajra AK, A method for the chemical synthesis of 14C-labeled fatty acyl coenzyme A's of high specific activity, *Analytical biochemistry* 106(2) (1980) 344–50. [PubMed: 7447002]
- [27]. Singleton WS, Gray MS, Brown ML, White JL, Chromatographically Homogeneous Lecithin from Egg Phospholipids, *Journal of the American Oil Chemists' Society* 42 (1965) 53–6. [PubMed: 14228472]
- [28]. Cadigan KM, Heider JG, Chang TY, Isolation and characterization of Chinese hamster ovary cell mutants deficient in acyl-coenzyme A:cholesterol acyltransferase activity, *The Journal of biological chemistry* 263(1) (1988) 274–82. [PubMed: 3335499]
- [29]. Chang CC, Chen J, Thomas MA, Cheng D, Del Priore VA, Newton RS, Pape ME, Chang TY, Regulation and immunolocalization of acyl-coenzyme A: cholesterol acyltransferase in mammalian cells as studied with specific antibodies, *The Journal of biological chemistry* 270(49) (1995) 29532–40. [PubMed: 7493995]
- [30]. Chang CC, Miyazaki A, Dong R, Kheirollah A, Yu C, Geng Y, Higgs HN, Chang TY, Purification of recombinant acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1) from H293 cells and binding studies between the enzyme and substrates using difference intrinsic fluorescence spectroscopy, *Biochemistry* 49(46) (2010) 9957–63. [PubMed: 20964445]
- [31]. Ikeuchi T, Dolios G, Kim SH, Wang R, Sisodia SS, Familial Alzheimer disease-linked presenilin 1 variants enhance production of both Abeta 1-40 and Abeta 1-42 peptides that are only partially sensitive to a potent aspartyl protease transition state inhibitor of “gamma-secretase”, *The Journal of biological chemistry* 278(9) (2003) 7010–8. [PubMed: 12493731]

- [32]. Yu C, Zhang Y, Lu X, Chen J, Chang CC, Chang TY, Role of the N-terminal hydrophilic domain of acyl-coenzyme A:cholesterol acyltransferase 1 on the enzyme's quaternary structure and catalytic efficiency, *Biochemistry* 41(11) (2002) 3762–9. [PubMed: 11888294]
- [33]. Neumann B, Chang CCY, Chang TY, A simple method to disrupt and restore subunit interaction of acyl-CoA:cholesterol acyltransferase 1, *MethodsX* 6 (2019) 2242–2247. [PubMed: 31667125]
- [34]. Yu C, Chen J, Lin S, Liu J, Chang CC, Chang TY, Human acyl-CoA:cholesterol acyltransferase-1 is a homotetrameric enzyme in intact cells and in vitro, *The Journal of biological chemistry* 274(51) (1999) 36139–45. [PubMed: 10593897]
- [35]. Guo ZY, Chang CC, Chang TY, Functionality of the seventh and eighth transmembrane domains of acyl-coenzyme A:cholesterol acyltransferase 1, *Biochemistry* 46(35) (2007) 10063–71. [PubMed: 17691824]
- [36]. Chamberlain BK, Berenski CJ, Jung CY, Fleischer S, Determination of the oligomeric structure of the Ca²⁺ pump protein in canine cardiac sarcoplasmic reticulum membranes using radiation inactivation analysis, *The Journal of biological chemistry* 258(19) (1983) 11997–2001. [PubMed: 6225783]
- [37]. Verkman AS, Skorecki K, Ausiello DA, Radiation inactivation of oligomeric enzyme systems: theoretical considerations, *Proceedings of the National Academy of Sciences of the United States of America* 81(1) (1984) 150–4. [PubMed: 6582472]
- [38]. Dunsing V, Luckner M, Zuhlke B, Petazzi RA, Herrmann A, Chiantia S, Optimal fluorescent protein tags for quantifying protein oligomerization in living cells, *Scientific reports* 8(1) (2018) 10634. [PubMed: 30006597]
- [39]. Victor TW, O'Toole KH, Easthon LM, Ge M, Smith RJ, Huang X, Yan H, Chu YS, Chen S, Gursoy D, Ralle M, Imperiali B, Allen KN, Miller LM, Lanthanide-Binding Tags for 3D X-ray Imaging of Proteins in Cells at Nanoscale Resolution, *Journal of the American Chemical Society* 142(5) (2020) 2145–2149. [PubMed: 31923358]
- [40]. Qian H, Zhao X, Yan R, Yao X, Gao S, Sun X, Du X, Yang H, Wong CCL, Yan N, Structural basis for catalysis and substrate specificity of human ACAT1, *Nature* (2020).
- [41]. Long T, Sun Y, Hassan A, Qi X, Li X, Structure of nevanimibe-bound tetrameric human ACAT1, *Nature* (2020).
- [42]. Guan C, Niu Y, Chen S-C, Kang Y, Wu J-X, Nishi K, Chang CCY, Chang T-Y, Luo T, Chen L, Structural insights into the inhibition mechanism of human sterol O-acyltransferase 1 by a competitive inhibitor, *Nature communications* 11(1) (2020) 2478.
- [43]. Sakashita N, Chang CC, Lei X, Fujiwara Y, Takeya M, Chang TY, Cholesterol loading in macrophages stimulates formation of ER-derived vesicles with elevated ACAT1 activity, *Journal of lipid research* 51(6) (2010) 1263–72. [PubMed: 20460577]
- [44]. Wang L, Qian H, Nian Y, Han Y, Ren Z, Zhang H, Hu L, Prasad BVV, Laganowsky A, Yan N, Zhou M, Structure and mechanism of human diacylglycerol O-acyltransferase 1, *Nature* (2020).
- [45]. Sui X, Wang K, Gluchowski NL, Elliott SD, Liao M, Walther TC, Farese RV, Structure and catalytic mechanism of a human triacylglycerol-synthesis enzyme, *Nature* (2020).

Highlights

- Nanodisc scaffold peptide form ACAT1 peptidiscs in a near detergent-free state.
- ACAT1 in peptidiscs remain soluble and form homo-oligomers.
- Residual detergents in ACAT1 peptidiscs slowly inactivate ACAT1.
- Phosphatidylcholine causes ACAT1 in peptidiscs to form higher-order oligomers.
- Phosphatidylcholine protects ACAT1 in peptidiscs from heat-mediated inactivation.

Fig. 1. HisACAT1/FLAG reconstituted into peptidiscs via the in-gel method stabilizes the enzyme in higher-order oligomers.

For this and following figures, Western blots were probed with anti-FLAG-tag antibody for ACAT1 detection. NSP_r and pure HisACAT1/FLAG were mixed at the indicated molar ratio before loading onto (A) 10% Clear native (CN) and Blue native (BN)-PAGE or (B) 4-10% gradient CN-PAGE. On-bead HisACAT1/FLAG peptidiscs were eluted from anti-FLAG M2 resin. HisACAT1/FLAG purified to apparent homogeneity was prepared according to [12, 30]. The enzyme preparation was stored at -80°C until usage.

Fig. 2. HisACAT1/FLAG partially purified in peptidiscs were cleaved and enzymatically inactive, whereas 1-65 HisACAT1/FLAG partially purified in peptidiscs were uncleaved and enzymatically active.

This experiment was conducted according to the on-bead reconstitution of ACAT1 peptidiscs method described in section 2.4. **(A)** Western blot of 4-10% gradient CN-PAGE of HisACAT1/FLAG and 1-65 HisACAT1/FLAG peptidiscs. **(B)** The liposomal ACAT activity of HisACAT1/FLAG and 1-65 HisACAT1/FLAG peptidiscs is reported as mean $\pm$ standard deviation (SD). **(C)** Western blot of 10% SDS-PAGE of HisACAT1/FLAG and 1-65 HisACAT1/FLAG peptidiscs displaying cleavage of HisACAT1/FLAG to a ~30 kDa fragment. **(D)** Comparison of liposomal ACAT activity of 1-65 HisACAT1/FLAG peptidiscs before (marked as input) and after spinning at $100,000 \times g$ for 30 min at 4°C (marked as soluble) reported as mean $\pm$ SD. ns = not significant

Fig. 3. Purification of 1-65 HisACAT1/FLAG with or without NSP_r.

The on-bead purification of 1-65 HisACAT1/FLAG was conducted as described in section 2.6 and depicted in (A). The 'X' denotes the purification of no detectable ACAT1 protein. The composition of various anti-FLAG M2 resin eluants are as indicated. Anti-FLAG-tag antibody was used for all Western blot analyses. (B) Western blot of 4-10% gradient CN-PAGE of 1-65 HisACAT1/FLAG with various eluants. (C) Liposomal ACAT activity of 1-65 HisACAT1/FLAG with various eluants reported as mean $\pm$ SD (n = 3). (D) Western blot of 10% SDS-PAGE of 1-65 HisACAT1/FLAG with various eluants. (E) Relative liposomal ACAT activity as compared to Western signal from (D) reported as mean $\pm$ SD (n = 3). ns = not significant; * p = 0.05; ** p = 0.01; *** p = 0.0001

Fig. 4. Conditioning of 1-65 HisACAT1/FLAG with CHAPS, or digitonin, or phosphatidylcholine prior to peptidisc reconstitution.

Procedure for on-bead conditioning and reconstitution were described in section 2.7. The conditioning buffers are indicated at the top of the gels. (A) Western blot of 4-10% gradient CN-PAGE loaded with the column eluates. The eluates contained 1-65 HisACAT1/FLAG in CHAPS (0.5 M KCl; 8.1 mM CHAPS), or in PC particles (9.3 mM taurocholate; 11.2 mM crude egg PC), or in 0.1 M KCl with exposure to 1 mg/ml NSP_r were as indicated. Western blot of (B) 4-10% gradient CN-PAGE or (C) 10% SDS-PAGE loaded with the on-bead reconstitution of 1-65 HisACAT1/FLAG peptidiscs in CHAPS, or digitonin (0.5 M KCl; 16.3 μM digitonin), or PC particles prior to the addition of NSP_r (E = eluate, S = strip).

Fig. 5. Conditioning of 1-65 HisACAT1/FLAG with detergent micelles (CHAPS or taurocholate), or with several formulations of phosphatidylcholine particles, prior to peptidisc reconstitution.

The composition of conditioning buffers are as follows: CHAPS (0.5 M KCl, 8.1 mM CHAPS); CHAPS, crude PC (9.3 mM CHAPS; 11.2 mM crude egg PC); CHAPS, pure PC (9.3 mM CHAPS; 11.2 mM pure egg PC); Tauro (9.3 mM taurocholate); Tauro, crude PC (9.3 mM taurocholate; 11.2 mM crude egg PC); Tauro, crude PC, CHOL (9.3 mM taurocholate; 11.2 mM crude egg PC; 1.8 mM cholesterol); or Tauro, pure PC (9.3 mM taurocholate; 11.2 mM pure egg PC). (A) Diagram of the experiment where the 'X' denotes no protection from heat-mediated inactivation and the 'check mark' denotes protection from heat-mediated inactivation. Representative Western blots of (B) 4-10% CN-PAGE or (C) 10% SDS-PAGE of differentially conditioned 1-65 HisACAT1/FLAG peptidiscs (1x = 2.5 μ l of sample, 2x = 5 μ l of sample). (D) Comparison of relative ACAT units to Western blot signal of ACAT1 peptidiscs conditioned with various reagents as indicated (n = 3). Reported as mean $\pm$ SD. ** p = 0.01; *** p = 0.0001

Fig. 6. Comparison of the heat-mediated enzymatic inactivation of 1-65 HisACAT1/FLAG when present in detergent-conditioned or in phosphatidylcholine-conditioned peptidiscs.

The procedure for conducting heat-mediated inactivation of ACAT1 at 37°C was described in section 2.8. Time course of heat-mediated inactivation of 1-65 HisACAT1/FLAG peptidiscs conditioned with CHAPS, or taurocholate, or PC particles ($n = 3$). Non-linear regression models are reported as $\log(\text{inhibitor})$ vs. normalized response with variable slope as determined by GraphPad Prism 5. The half-time of ACAT enzymatic activity decay ($T_{1/2}$) of the differentially conditioned 1-65 HisACAT1/FLAG peptidiscs at 37°C are reported as $IC_{50} \pm 95\%$ confidence interval (CI). ($n = 3$).