Cholesterol Substitution Increases the Structural Heterogeneity of Caveolae*

Received for publication, December 19, 2007, and in revised form, March 5, 2008 Published, JBC Papers in Press, March 19, 2008 DOI 10.1074/jbc.M710355200

Maurice Jansen†1, Vilja M. Pietiläinen†1, Harri Pölönen§, Laura Rasilainen§, Mirkka Koivusalo‡, Ulla Ruotsalainen‡, Eija Jokitalo‡, and Elina Ikonen‡2

From the †Institute of Biomedicine/Anatomy and the ‡Institute of Biotechnology, University of Helsinki, Helsinki 00140 and the §Institute of Signal Processing, Tampere University of Technology, Tampere 33101, Finland

Caveolin-1 binds cholesterol and caveola formation involves caveolin-1 oligomerization and cholesterol association. The role of cholesterol in caveolae has so far been addressed by methods that compromise membrane integrity and abolish caveolar invaginations. To study the importance of sterol specificity for the structure and function of caveolae, we replaced cholesterol in mammalian cells with its immediate precursor desmosterol by inhibiting 24-dehydrocholesterol reductase. Desmosterol could substitute for cholesterol in maintaining cell growth, membrane integrity, and preserving caveolar invaginations. However, in desmosterol cells the affinity of caveolin-1 for sterol and the stability of caveolin oligomers were decreased. Moreover, caveolar invaginations became more heterogeneous in dimensions and in the number of caveolin-1 molecules per caveola. Despite the altered caveolar structure, caveolar ligand uptake was only moderately inhibited. We found that in desmosterol cells, Src kinase phosphorylated Cav1 at Tyr14 more avidly than in cholesterol cells. Taken the role of Cav1 Tyr14 phosphorylation in caveolar endocytosis, this may help to preserve caveolar uptake in desmosterol cells. We conclude that a sterol C24 double bond interferes with caveolin-sterol interaction and perturbs caveolar morphology but facilitates Cav1 Src phosphorylation and allows caveolar endocytosis. More generally, substitution of cholesterol by a structurally closely related sterol provides a method to selectively modify membrane protein-sterol affinity, structure and function of cholesterol-dependent domains without compromising membrane integrity.

Caveolae are specialized plasma membrane (PM)3 microdomains enriched in cholesterol and sphingolipids (1). These

---

Experimental Procedures

Cell Culture and Antibodies—Cells were cultured as described for HeLa cells (15), HeLa Cav1-GFP cells (16), F92-99 primary human fibroblasts (17), and ts-v-Src-MDCK (temperature-sensitive viral Src expressing Madin-Darby canine kidney) cells (18), except that MDCK cells were supplemented with 5% FBS. Lipoprotein-deficient serum (LPDS) was prepared as in Ref. 19. The DHC24 reductase inhibitor 20,25-

---

flask-shape invaginations contain caveolin-1 (Cav1) as their main structural protein component (2). Caveolae are relatively immobile structures but their internalization can be stimulated by a variety of cargo, including sphingolipids (3), integrins (4), and select viruses (5). Cav1 binds cholesterol (6) and cholesterol promotes the formation of Cav1 oligomers that are required for caveola formation (7, 8). However, no detailed structural information on Cav1 membrane association is available (1). The role of cholesterol in caveolae has so far been addressed using treatments that sequester or remove cholesterol, thus compromising membrane integrity, causing the loss of invaginated caveolae and affecting many cellular processes (9, 10). Thus, functions assigned to caveola/caveolin based on cholesterol depletion must be assessed critically (9).

In this work, we studied the importance of the sterol structure for caveolae by a method circumventing the side effects of cholesterol removal or sequestration. Cells were treated with a specific inhibitor of 24-dehydrocholesterol reductase (DHC24), the enzyme converting desmosterol to cholesterol. This resulted in the accumulation of desmosterol, which differs from cholesterol by an additional double bond between carbon atoms 24 and 25 in the sterol tail. We chose this strategy as data on DHC24-deficient mice (11, 12) and on cultured cells containing desmosterol (13, 14) suggested that desmosterol accumulation may specifically interfere with the function of proteins enriched in lipid rafts/caveolae. Our results show that the minor structural difference between cholesterol and desmosterol weakens sterol-caveolin interaction, results in distorted caveolae with an altered number of Cav1 molecules per caveola, and affects the function of caveolae as endocytic vehicles.
DAC was a kind gift from Lowell A. Miller (National Wildlife Research Center, United States Department of Agriculture/APHIS/WS, Fort Collins, CO). The following Abs were used: Cav1 SC-893 (Santa Cruz) for Western blot analysis and immunofluorescence (IF) stainings, Cav1 clone 2234 (BD Transduction Laboratories) for immunoisolation, Cav1 clone C13630 (BD Transduction Laboratories) for immuno-EM, and Cav1 VIP21-N (20) for IF, TyrT<sup>14</sup>-phosphorylated Cav1 (pY14; BD Transduction Laboratories), Na<sub>K</sub>-ATPase (β1; Upstate Biotechnology), transferrin (Tfn) receptor (clone H68.4; Zymed Laboratories), α2β1 integrin (CD49b, MCA2025; Serotec), β1 integrin (102DF7 A6; from Ismo Virtanen, Institute of Biomedicine, University of Helsinki, Finland), rabbit/mouse AF568/AF488-conjugated secondary Abs (Molecular Probes), and rabbit anti-mouse IgGs (DAKO).

**Immunofluorescence Microscopy and Western Blotting**—IF stainings and filipin permeabilization were performed as in Ref. 17. When used, saponin (0.05%) (Sigma) was present in all steps of IF stainings. Images of Cav1 and filipin were acquired with an inverted Olympus IX-71 microscope equipped with a ×60 water objective (NA 1.2), and deconvolved using the blind-deconvolution wizard of Huygens® Essential before processing figures in ImagePro Plus 5.1. For anti-Cav1 Western blotting, cells or trichloroacetic acid-precipitated gradient fractions were lysed in Laemmli sample buffer and boiled unless noted otherwise. For phospho-Tyr immunoblotting, cells were scraped in boiling 1% SDS, 10 mM Tris-HCl, pH 7.4, 1 mM Na<sub>3</sub>VO<sub>4</sub>, including a protease inhibitor cocktail. The homogenate was transferred to a SW40 tube and centrifuged at 100,000 × g for 5 min and incubated for 10 min on ice in 240 μl of lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 10% sucrose, and 1% Triton X-100, CLAP). The lysate was then mixed with 480 μl of 60% Optiprep™ (Nycomed-Pharma, Oslo, Norway) and overlaid with step gradients of 35, 30, 25, 20, and 0% Optiprep™ in lysis buffer. After centrifugation for 4 h at 40,000 × g at 4 °C with a SW60Ti rotor, fractions were collected and used for protein or lipid determinations. The detergent-resistant membranes (DRM) fractions of ts-v-S-MDCK cells were isolated as above except that Na<sub>2</sub>VO<sub>4</sub> was present in all buffers and cells were homogenized by passing for 10 times through a 18-gauge needle before mixing with 60% Optiprep™.

**Immunosolation of Caveolar Membranes**—Adipocytes were prepared from epididymal fat of male Wistar rats according to Ref. 28. The fat pads were minced in Krebs-Ringer HEPES buffer, pH 7.4, containing 5 mM d-glucose, 3% BSA, 20 mM NaHCO<sub>3</sub>, 118 mM NaCl, 1.25 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM sodium pyruvate, and 10 mM HEPES. Adipose tissue fragments were digested in the same buffer in the presence of collagenase (1 mg/ml; c5138, Sigma) with gentle shaking for ~1 h at 37 °C and the cell suspension filtered through a 200-μm nylon mesh and washed three times in Krebs buffer. The cells were cultured for 2 days in Dulbecco’s modified Eagle’s medium containing 10% LPDS, 10 mM 20,25-DAC, [<sup>3</sup>H]desmosterol (1 nmol, 1.6 Ci/mmol), and [<sup>14</sup>C]cholesterol (20 nmol, 0.05 Ci/mmol), after which the cell-associated dissociations per minute (dpm) were similar for both labels. Caveolar membranes were immunosolated essentially as described in Refs. 29 and 30. Briefly, cells resuspended in 0.25 M sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA were broken by 10 strokes with a glass Teflon Dounce and the material centrifuged for 15 min at 16,000 × g. The pellet was resuspended in 2 ml of 500 mM Na<sub>2</sub>CO<sub>3</sub>, pH 11, including CLAP, and sonicated three times for 20 s at full power using a MSE Soniprep 150 sonicator. The homogenate was transferred to a SW40 tube and adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose in 20 mM Tris, pH 7.5, 2 mM EDTA and CLAP. The sample was overlaid with 4 ml of 35 and 5% sucrose in the same buffer. After centrifugation (39,000 × g for 24 h at 4 °C with an SW40 rotor) the caveolae-enriched membrane fraction at the 5–35% sucrose interphase (fraction 3) was collected. This fraction was divided into two and incubated overnight with or without Cav1 Ab (clone 2234), followed by a 1-h incubation with Dynal beads (Dynal Biotech) linked to anti-mouse IgG and washed according to the manufacturer’s instructions. Control pull-down without Cav1 antibodies contained ~10% of [<sup>3</sup>H] and [<sup>14</sup>C] radioactivity compared with the specific pull-down and was subtracted. The pull-down contained on average 1.6% [<sup>14</sup>C]cholesterol and 0.9% [<sup>3</sup>H]desmosterol found in fraction 3.

Sterol Replacement Alters Caveolae

Dilp1 Determinations—For sterol analyses, lipids were extracted (21) and the free sterol fraction isolated by thin-layer chromatography (petroleum ether/diethyl ether/acetic acid, 60:40:1) was further analyzed by silver ion high performance liquid chromatography (UV detection (22). For total sterol determination, lipid extracts were analyzed by the Amplex red sterol determination kit (Molecular Probes). For quantification of PC, SM, PE, and PS, lipids were extracted (23) and spiked with the following mixture of internal standards dissolved in chloroform/methanol (1:2), di14:1-PC, di20:1-PC, di22:1-PC, 17:0-SM, 23:0-SM, 25:0-SM, di14:1-PE, di20:1-PE, di22:1-PE, di14:1-PS, di20:1-PS, and di22:1-PS. The concentrations of phospholipid standards were determined as described (24, 25). Extracts were evaporated under nitrogen and dissolved in chloroform/methanol, 1:2, containing 10 mM ammonium acetate. Electrospray ionization mass spectrometry analysis was carried out with a Quattro Micro Triple quadropole mass spectrometer (Micromass, Manchester, UK). Lipids were analyzed in the positive ion mode, PC and SM giving their characteristic parent m/z 184, PE neutral loss of 141, and PS neutral loss of 185. Data analysis was performed with the LIMSA software (26).

N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium (TMA-DPH) Anisotropy—The PM fluidity of whole living cells was analyzed by TMA-DPH anisotropy according to Ref. 27. Briefly, trypsinized HeLa cells were resuspended in phosphate-buffered saline to a dilution of 2 million cells/ml. 100 μl of cell suspension was mixed with 300 μl of 1 μM TMA-DPH solution. Polarized fluorescence was measured and anisotropy calculated. Cell death was found to be <5% as determined by trypan blue exclusion after anisotropy measurements.
Sterol Replacement Alters Caveolae

The relative amount of [3H]desmosterol compared with [14C]cholesterol in the same pull-down was determined.

Intensity Analysis of Cav1-GFP Structures—Total internal reflection fluorescence microscopy (TIRFM) images were acquired using an Olympus IX-71 microscope with a PLAPON TIRFM ×60/1.45 objective and deconvolved using the blind-deconvolution wizard of Huygens® Essential. Cells were kept at 37 °C in CO2-independent medium (Invitrogen). Cav1-GFP containing structures were automatically detected by defining potential dot regions (31) and then finding pixels with a higher intensity than their 8-pixel neighborhood. Each dot was assumed to approximate a three-dimensional normal distribution, with pixel intensity considered as the third dimension. Total dot intensity was defined by the volume under this multivariate normal distribution. If two or more dots overlapped, they were treated as a mixture distribution of multivariate normal distributions. Each dot had six parameters (location x, location y, covariance matrix with three parameters and volume) that were estimated by a genetic algorithm (32). Dot intensities per cell were plotted in a histogram. Peaks in the intensity histogram were considered to represent different pools of caveolar structures (33). The histograms were cropped, leaving only the first three peaks, and a mixture distribution of three univariate Gaussian distributions was fitted with the algorithm (32). The distance between the first and the third peak in each histogram was normalized by multiplication to convert the data from different cells and experiments to the same scale.

Quantification Cav1-GFP Mobility—Cav1-GFP mobility was analyzed using the same principle as in Ref. 34. HeLa Cav1-GFP cells were imaged by TIRFM using 67 × 67-nm sampling and an exposure time of 500 ms. Per condition at least 20 separate 1-min movies were acquired containing 4 frames per minute. Images were deconvolved as described above and Pearson correlation was calculated for succeeding frames (15-s interval).

Endocytosis Assays—The internalization of BODIPY-lactosylceramide (LacCer, d-erythro isomer) was analyzed essentially as in Ref. 3. Briefly, fibroblasts were incubated with 5 μM BODIPY-FL5-LacCer-BSA complex in Earle’s minimal essential medium for 30 min at 0 °C and, after imaging for labeling efficiency, incubated for 5 min at 37 °C. Unintialized label was removed from the PM by washing the cells 6 × 10 min with 5% BSA in Earle’s minimal essential medium at 0 °C. Images were acquired using an inverted Olympus IX-71 microscope equipped with a ×60 water objective (N.A. 1.2), 1 pixel = 232 × 232 nm. Fluorescence intensity per cellular area was determined after background subtraction using a rolling ball radius of 5 pixels (Image J). Samples with a similar labeling efficiency were used for quantification. To analyze Tf or dextran uptake, HeLa cells were incubated with 50 μg/ml of AF568-Tfn (Molecular Probes) for 30 min at 4 °C followed by 5 min at 37 °C, or with 1 mg/ml of AF488-dextran (10,000 M₉; Molecular Probes) for 20 min at 37 °C, prior to acid-stripping as described (35). Control samples treated similarly but without 37 °C internalization were included for both dextran and Tfn uptake assays. Imaging and fluorescence intensity analysis were performed as described for BODIPY-LacCer. For integrin cross-linking, HeLa Cav1-GFP cells were incubated with primary and secondary Abs as described (4). The colocalization between α2β1 inte-
Sterol Replacement Alters Caveolae

FIGURE 1. Inhibition of DHCR24 results in the replacement of cholesterol by desmosterol without gross alterations in major phospholipids in HeLa cells. A, cells were grown for 8 days in LPDS with and without 20,25-DAC. Sterols were extracted and analyzed using Ag⁺-HPLC. Shown are the chromatograms of the sterol extracts and a sterol standard mixture. B, percentage of desmosterol of total sterols in cells grown for up to 8 days in LPDS or FBS ± 20,25-DAC. C, quantification of major membrane lipids in cells grown for 8 days in LPDS or FBS ± 10 nM 20,25-DAC (± S.E.; PC, PE, and PS, n = 3; sterol, n = 5). D, quantification of phospholipid molecular species in cells grown as in C. The most abundant species constituting at least 70% of each phospholipid class are shown, the numbering indicates the total number of carbon atoms and unsaturations in the acyl chains (± S.E.; PC, PE, and PS n = 3; SM, n = 2). * indicates a significant difference compared with non-treated control (p < 0.05).

FIGURE 2. Sterol exchange does not change cellular sterol or Cav1 distribution. A, epifluorescence images of HeLa cells stained with filipin (unesterified sterols) and anti-Cav1 Abs after filipin or saponin permeabilization. Bar, 10 μm. B, sucrose density gradient fractions were analyzed for sterol levels and Cav1 distribution by Western blotting. Fractions containing Na,K-ATPase immunoreactivity (PM marker) are indicated. C, quantification of major membrane lipids in cells grown for 8 days in LPDS with and without 20,25-DAC. The most abundant species constituting at least 70% of each phospholipid class are shown, the numbering indicates the total number of carbon atoms and unsaturations in the acyl chains (± S.E.; PC, PE, and PS n = 3; SM, n = 2). * indicates a significant difference compared with non-treated control (p < 0.05).

Sterol C24 Double Bond Reduces Affinity for Caveolae—To analyze the association of cholesterol and desmosterol with caveolar membranes, primary rat adipocytes were labeled for 2 days with both [³H]desmosterol and [¹⁴C]cholesterol in the presence of 10 nM 20,25-DAC (to prevent the conversion of [³H]desmosterol). Cells were then disrupted by sonication and the caveolea-enriched membrane fraction isolated without detergents as described (Fig. 3A) (29, 30). Caveolar membranes were further immunosolated using Cav1 Abs as described in Ref. 30. We found that both [³H]desmosterol and [¹⁴C]cholesterol specifically precipitated with Cav1 (Fig. 3B). However, the amount of co-isolated [³H]desmosterol was 35% lower than that of [¹⁴C]cholesterol (Fig. 3B), indicating a lower affinity of desmosterol than cholesterol for caveolar membranes.

Contrary to cholesterol cells, Cav1 was largely excluded from the DRM fraction in desmosterol cells (Fig. 3B; HeLa cells; similar results were obtained from primary fibroblasts, not shown). The decrease in Cav1 detergent resistance was fully restored when cells were grown in the presence of the drug and FBS (data not shown), indicating that 20,25-DAC did not act unrelated to the sterol exchange. The fraction of desmosterol partitioning into DRMs was also lower than that of cholesterol (23 ± 3 versus 37 ± 1%; mean ± S.D., n = 3), in accordance with earlier results (13). Cav1 has been described to form SDS-resistant oligomers (38). When the stability of Cav1 oligomers was analyzed by Western blotting, we found that 18 ± 8% of Cav1 immunoreactivity remained as heat-resistant oligomers in desmosterol cells as compared with 43 ± 6% in cholesterol cells (Fig. 3D; primary fibroblasts, a similar tendency was observed in HeLa cells, not shown). Thus, the detergent resistance of Cav1 and the stability of Cav1 oligomers were decreased in desmosterol cells. These differences could result from a relative increase in the Golgi pool of Cav1 (8). However, as no apparent Golgi accumulation of Cav1 was observed in HeLa Cav1-GFP cells (Fig. 7A), it seems likely that the decreased detergent resistance and oligomer stability of Cav1 result from the lower affinity of desmosterol for caveolae.
Sterol Exchange Alters Caveolar Structure—When HeLa Cav1-GFP cells were analyzed by TIRFM (Fig. 4A), we found the number of caveolar structures to be closely similar in desmosterol and cholesterol cells, as identified by GFP fluorescent dots (77/11006 and 72/11006 dots per 100/9262 m2, respectively; mean ± S.E. n = 14 cells). Using this cell line, PM caveolae were shown to be composed of unit size building blocks containing 144 Cav1 molecules (33): structures composed of 1 unit were characterized as individual caveolae and structures of multiple units as grape-like caveolar clusters. To analyze the effect of sterol exchange on Cav1 composition of caveolae, we developed an automated method for the identification and intensity quantification of Cav1-GFP structures in the TIRF plane (Fig. 4B). Using this method, we found that the distribution of caveolar structures to quantal units was less evident in desmosterol cells (Fig. 4C). Although separate distributions of fluorescence intensity were observed, their internal variation was larger, as indicated by increased peak width. The average width of the first peak, representing individual caveolae, was ~30% larger in desmosterol cells (Fig. 4C). This can be explained by a higher variation in the number of Cav1-GFP molecules per individual caveola. By immunoelectron microscopy, Cav1 Abs decorated uncoated PM invaginations. However, both in fibroblasts (Fig. 5A) and HeLa Cav1-GFP cells (not shown), the sterol exchange altered caveolar morphology. In desmosterol cells, uncoated PM invaginations with small openings (20–45 nm) were less...
abundant (Fig. 5A), and there was an increase in the average width and depth of the invaginations (Table 1). The most striking difference in desmosterol cells was an increased variance in all the measured dimensions (Table 1). This difference was not observed upon culturing the cells in the presence of FBS and 20,25-DAC, indicating that the drug was not directly responsible for the altered morphology. These results imply that the additional double bond in desmosterol results in a higher variation in the morphology and Cav1 composition of individual caveolae and suggest that cholesterol controls caveolar uniformity.

Sterol Exchange Impairs Caveolar Uptake but Facilitates Cav1 Src Phosphorylation—When the mobility of Cav1-GFP structures in HeLa cells was analyzed by TIRFM using a similar method as in Ref. 34, no difference in either basal or vanadate-stimulated caveolar mobility was observed between desmosterol and cholesterol cells (Fig. 6A). Also the intracellular mobility of Cav1-GFP vesicles was similar as assessed by fluorescence recovery after photobleaching.4 To study the effect of sterol exchange on caveolar endocytosis, we analyzed the internalization of BODIPY-LacCer (3). Fibroblasts were labeled with BODIPY-LacCer at 0 °C for 30 min and the label internalized for 5 min at 37 °C. Uninternalized label was removed from the PM by BSA back-exchange and the remaining intensity quantified. We found that desmosterol cells showed a moderate but significant reduction in BODIPY-LacCer uptake compared with cholesterol cells (Fig. 6B). In comparison, no inhibition in the internalization of the fluid-phase tracer dextran or Tfn, a marker of clathrin-mediated endocytosis, was observed in desmosterol cells (Fig. 6C). Together, these data suggest that desmosterol replacement did not perturb the steady-state or vanadate-stimulated mobility of caveolae but selectively affected caveolar lipid uptake.

When caveolar internalization of Ab-clustered integrins was analyzed as in Ref. 4 we found that in desmosterol cells, the caveolar localization of clustered integrins was slightly reduced, as judged by colocalization of the α2β1 integrin with Cav1-GFP (Fig. 7A). Integrin cross-linking is known to induce Cav1 phosphorylation at Tyr14, which is required for caveolar endocytosis (39). However, we found that in desmosterol cells, Cav1 phosphorylation upon integrin cross-linking was not attenuated but was in fact more robust (Fig. 7B). To address the mechanism underlying the increased Cav1 phosphorylation in desmosterol cells, we studied the effect of sterol exchange on the activity of Src kinase using an inducible MDCK cell line overexpressing Src (18). We found that both the basal Cav1 Tyr14 phosphorylation and in particular, the Src-induced increase in this phosphorylation were more pronounced in desmosterol cells (Fig. 8). In the same cells, the overall degree of Tyr phosphorylation in basal or Src-induced conditions was not significantly increased (Fig. 8). These results indicate that desmosterol replacement selectively facilitated Cav1 phosphorylation by Src and imply that the inhibition of caveolar uptake was not due to impaired Cav1 Tyr14 phosphorylation.

**DISCUSSION**

In this study, we exchanged cellular cholesterol for desmosterol by inhibiting DHCR24, to study the effects of sterol replacement on Cav1 and caveolae. Because other post-lanosterol enzymes of cholesterol biosynthesis can operate on substrates containing the C24 double bond, the enzyme inhibition

| Condition       | Depth  | Width   | Opening |
|-----------------|--------|---------|---------|
| Cholesterol     | 142 ± 27 a | 79 ± 26 c | 60 ± 22 |
| Desmosterol     | 165 ± 45 a,b | 95 ± 40 a,b | 69 ± 38 |
| FBS + 20,25-DAC | 103 ± 30 | 68 ± 15 | 57 ± 22 |

* Significance increase in average compared with "FBS + 20,25-DAC"; p < 0.01.
* Significant increase in average compared with "cholesterol" and "FBS + 20,25-DAC"; p < 0.0025.
* Significant increase in variance compared with "cholesterol" and "FBS + 20,25-DAC"; p < 0.0025, as analyzed by F test.

4 V. Pietiäinen, unpublished observation.
Sterol Replacement Alters Caveolae

A

Cav1-GFP/ cross-linked α2 integrin

0 min surface

15 min intracellular

cholesterol desmosterol

Person's correlation

0.00 0.15

0 5 15 min

cholesterol desmosterol

B

α-PY14-Cav1 (22-25 kDa)

no abs α2-cross-linked

α-PY14-Cav1 (22-25 kDa)

WB signal (a.u.)

40.5°C 35.0°C

α-Cav1 (22 kDa)

α-PY14-Cav1 (22-25 kDa)

α-PY (28-250 kDa)

cholesterol desmosterol

40.5°C 35.0°C

FIGURE 7. Sterol exchange results in reduced caveolar localization of cross-linked integrins but increased Cav1 Tyr14 phosphorylation. A, colocalization of cross-linked α2-integrin molecules with Cav1-GFP was followed in HeLa Cav1-GFP cells. Merged confocal images (colocalization: white) of Ab-clustered α2-integrins (anti-α2 Ab; red) in HeLa Cav1-GFP cells (Cav1; green) at 0 min (upper panel) and 15 min (lower panel) at 37 °C. Colocalization of α2β1 integrin with Cav1-GFP was analyzed by Pearson correlation from confocal sections (± S.E.; n > 20 images; *, p < 0.02). B, Cav1 Tyr14 phosphorylation after integrin cross-linking was determined by Western blotting from 20 μg of cellular protein, followed by quantification of signal intensity (± S.E.; n = 3; *, p = 0.05).

FIGURE 8. Sterol exchange facilitates Cav1 phosphorylation at Tyr14 by Src. ts-v-Src-MDCK cells were incubated in non-permissive (40.5 °C, inactive v-Src) or permissive (35 °C, active v-Src) temperature and cell lysates immunoblotted with anti-Cav1, anti-phospho-Cav1 (PY14-Cav1), and anti-phospho-Tyr (PY) Abs (20 μg of cellular protein). Intensities were normalized against the average intensity of cholesterol sample at 40.5 °C (± S.E.; n = 3; *, p < 0.05; **, p < 0.0005).

lead to the accumulation of the penultimate precursor of cholesterol, desmosterol (40). After the treatment, the levels of total sterols and major phospholipids remained similar. We cannot exclude the possibility that minor changes in other lipids might contribute to the observed effects. Nevertheless, considering the extent of sterol replacement and the close association of Cav1 with cholesterol, this is unlikely to play a major role. The sterol exchange was found to occur reproducibly and without toxic effects in all the cell types studied, i.e. primary fibroblasts, HeLa, and MDCK cells. Primary human fibroblasts were used for electron microscopy and BODIPY-LacCer uptake assays because of their abundance of caveolae, HeLa Cav1-GFP cells to study dynamic caveolar processes in living cells, and MDCK cells with an inducible Src expression to address Cav1 modification by phosphorylation.

First, we found that the sterol C24 double bond reduced the ability of the sterol to associate with Cav1. This was based on the preferential co-precipitation of cholesterol with Cav1 from cells into which differently radiolabeled cholesterol and desmosterol were simultaneously introduced. It should be noted, however, that this result does not allow us to make conclusions about the overall sterol content of caveolae in the two conditions. This would require large scale preparation of immunosolated caveolae from 20,25-DAC-treated versus untreated animals, for example. The C24 double bond gives rise to additional stress in the sterol tail, favors tilting of the molecule in the bilayer (13), and may thereby sterically hinder interactions with Cav1. The reduced sterol affinity may, in turn, explain the reduced association of Cav1 with DRMs as well as the decreased stability of caveolin oligomers.

Second, we noted that caveolae in desmosterol cells had altered morphology. They were less uniform in dimensions than in cholesterol cells as measured by electron microscopy, and in particular the opening of the invaginations was increased. Moreover, by analyzing the fluorescence intensity of Cav1-GFP structures in HeLa cells we found that the distribution of fluorescence into quantal units was less evident in desmosterol cells. This suggests that the number of Cav1 molecules per individual caveola was more variable (33), agreeing well with our electron microscopic observation of invaginations with less uniform dimensions. Hindrance of sterol-caveolin interaction may partially account for the altered shape of caveolae in desmosterol cells. Given that Cav1 is directly involved in bending the membrane (41), the larger variation in Cav1 molecules per caveola may also contribute.

Third, we found caveolar ligand uptake to be inhibited by −25%, as assessed by endocytosis of BODIPY-LacCer. This lipid analogue is predominantly taken up by a caveolin-related mechanism, as judged by 80–90% inhibition of BODIPY-LacCer endocytosis in Cav1-depleted cells (42). Simian virus 40 (SV40) enters host cells via caveolae (43) although this is not its exclusive entry route (44). We therefore also studied whether the sterol exchange affects SV40 infectivity, and found cholesterol and desmosterol cells to be similarly infected as judged by T-antigen immunostaining. Moreover, integrins are recycled by a caveolin/lipid raft-dependent mechanism (45, 46) and we found reduced co-localization of α2β1 integrin with Cav1, suggestive of impaired caveolar uptake in desmosterol cells. However, also in this case the difference to cholesterol cells was minor. Considering the marked changes in caveolin-
sterol association and caveolar morphology, the observed moderate effects on caveolar endocytosis were unexpected. We therefore analyzed the phosphorylation of Cav1 Tyr14, which is the substrate for Src (47) and a key signal mediator that is induced by integrin cross-linking and required for caveolar endocytosis (39). The results from HeLa and MDCK cells indicated that the amount of phospho-Tyr14-Cav1 was markedly increased in desmosterol cells, potentially helping to preserve caveolar endocytosis. The mechanism of increased Cav1 Tyr14 phosphorylation is not known but could for instance involve reduced feedback inhibition of Src via C-terminal Src kinase (48, 49).

Because cholesterol removal or chelation abolishes caveolar invaginations (2), the inhibition of desmosterol-cholesterol conversion allowed us to pinpoint, for the first time, parameters relying specifically on the structure of cholesterol in invaginated caveole. Some additional features that differed from those observed with cholesterol removal were Cav1-GFP mobility and Src activation, as cholesterol removal was found to increase Cav1-GFP mobility (50) and attenuate Src activation (51). These differences may be due to non-caveolar effects of cholesterol removal that are difficult to control (9, 10). Notably, although the effects of sterol exchange on caveolar functions were subtle, our data suggest that chitin-coated pit endocytosis was not perturbed, contrary to cholesterol depletion (52). Thus, substitution of cholesterol by a closely related sterol appears as a suitable strategy for more selective modification of membrane protein-sterol affinity and sterol-dependent domains. This approach is analogous to the one taken in yeast, using genetic strategies (53). The more distant sterol precursors of cholesterol are likely to yield more pronounced effects on sterol-protein/lipid affinities but will also more severely perturb the overall membrane architecture. Therefore, the sterol(s) of choice should depend on the sterol interaction and cell system of interest.

Acknowledgments—We acknowledge Anna Uro and Birgitta Rantalainen for expert technical assistance, Mika Hukkanen, Mikko Liljestro¨m, Lucas Pelkmans from ETH Zu¨rich, Switzerland for HeLa Cav1-GFP cells, and Ismo Virtanen for integrin antibodies.

REFERENCES

9. Parton, R. G., and Simons, K. (2007) Nat. Rev. Mol. Cell Biol. 8, 185–194
10. Smart, E. J., and Anderson, R. G. (2002) Methods Enzymol. 353, 131–139
11. Lu, X., Kambe, F., Cao, X., Yoshida, T., Ohmori, S., Murakami, K., Kaji, T., Ishii, T., Zadworny, D., and Seo, H. (2006) Endocrinology 147, 3123–3132
12. Mirza, R., Hayasaka, S., Takagishi, Y., Kambe, F., Ohmori, S., Maki, K., Yamamoto, M., Murakami, K., Kaji, T., Zadworny, D., Murata, Y., and Seo, H. (2006) J. Investig. Dermatol. 126, 638–647
13. Vainio, S., Jansen, M., Koivusalo, M., Rog, T., Karttunen, M., Vattulainen, I., and Ilonen, E. (2006) J. Biol. Chem. 281, 348–355
14. Cramer, A., Biondi, E., Kuehnil, K., Lutjohann, D., Thelen, K. M., Parga, S., Dotti, C. G., Nitsch, R. M., Lede, M. D., and Mohajer, M. H. (2006) EMBO J. 25, 432–443
15. Blom, T. S., Linder, M. D., Snow, K., Pihko, H., Hess, M. W., Jokitalo, E., Veckman, V., Syvånen, A. C., and Ilonen, E. (2000) Curr. Biol. 10, 95–98
16. Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M., and Birchmeier, W. (1993) J. Cell Biol. 120, 757–766
17. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) Methods Enzymol. 98, 241–260
18. Bartlett, E. M., and Lewis, D. H. (1970) Anal. Biochem. 36, 159–167
19. Naoi, M., Lee, Y. C., and Rosenman, S. (1974) Anal. Biochem. 58, 571–577
20. Haimi, P., Uphoff, A., Hermansson, M., and Somerharju, P. (2006) Anal. Chem. 78, 8324–8331
21. Kuhry, J. G., Duportail, G., Bronner, C., and Lausriott, G. (1985) Biochim. Biophys. Acta 845, 60–67
22. Rodbell, M. (1964) J. Biol. Chem. 239, 375–380
23. Ortegren, U., Karlsson, M., Blazic, N., Blomqvist, M., Nystrom, F. H., Gustavsson, J., Fredman, P., and Stralfors, P. (2004) Eur. J. Biochem. 271, 2028–2036
24. Oh, P., and Schnitzer, J. E. (1999) J. Biol. Chem. 274, 23144–23154
25. Otsu, N. (1979) IEEE Trans. Systems Man Cybernetics 9, 62–66
26. Tohka, J., Krestyaninkov, E., Dinov, I. D., MacKenzie Graham, A., Shat-Duck, W. W., and Togawa, A. W. (2007) IEEE Trans. Med. Imaging 26, 696–711
27. Pelkmans, L., and Zerial, M. (2005) Nature 436, 128–133
28. Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L., and Helenius, A. (2005) J. Cell Biol. 170, 769–779
29. Singh, R. D., Holicky, E. L., Cheng, Z. J., Kim, S. Y., Wheatley, C. L., Marks, D. L., Bittman, R., and Pagano, R. E. (2007) J. Cell Biol. 176, 895–901
30. Salonen, A., Vasiljeva, L., Blazic, N., Blomqvist, M., Nystrom, F. H., Gustavsson, J., Fredman, P., and Stralfors, P. (2004) Eur. J. Biochem. 271, 2028–2036
31. Parton, R. G., Hanzal-Bayer, M., and Hancock, J. F. (2006) J. Cell Sci. 119, 787–796
32. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992) Cell 68, 673–682
33. Singh, R. D., Puri, V., Valiyaveettil, J. T., Marks, D. L., Bittman, R., and Pagano, R. E. (2003) Mol. Biol. Cell 14, 3254–3265
34. Upla, P., Marjomaki, V., Kankaanpaa, P., Ivaska, J., Hyypia, T., Van Der Goot, F. G., and Heino, J. (2004) Mol. Biol. Cell 15, 625–636
35. Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001) Nat. Cell Biol. 3, 473–483
36. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., and Simons, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10339–10343
37. Monier, S., Dietzen, D. J., Hastings, W. R., Lublin, D. M., and Kurzchalia, T. V. (1996) FEBS Lett. 388, 143–149
38. Pol, A., Martin, S., Fernandez, M. A., Ingelmo-Torres, M., Ferguson, C., Enrich, C., and Parton, R. G. (2005) Mol. Biol. Cell 16, 2091–2105

Sterol Replacement Alters Caveolae.
Sterol Replacement Alters Caveolae

Schwartz, M. A. (2007) Nat. Cell Biol. 9, 1381–1391
46. del Pozo, M. A., Balasubramanian, N., Alderson, N. B., Kiosses, W. B., Grande-Garcia, A., Anderson, R. G., and Schwartz, M. A. (2005) Nat. Cell Biol. 7, 901–908
47. Glenney, J. R., Jr., and Zokas, L. (1989) J. Cell Biol. 108, 2401–2408
48. Cao, H., Courchesne, W. E., and Mastick, C. C. (2002) J. Biol. Chem. 277, 8771–8774
49. Radel, C., and Rizzo, V. (2005) Am. J. Physiol. 288, H936–H945
50. Thomsen, P., Roepstorff, K., Stahlhut, M., and van Deurs, B. (2002) Mol. Biol. Cell 13, 238–250
51. Radel, C., Carlile-Klusacek, M., and Rizzo, V. (2007) Biochem. Biophys. Res. Commun. 358, 626–631
52. Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H., and McGraw, T. E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6775–6780
53. Heese-Peck, A., Pichler, H., Zanolari, B., Watanabe, R., Daum, G., and Riezman, H. (2002) Mol. Biol. Cell 13, 2664–2680