VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 16 July 2011
Final form accepted: 16 December 2011
Published online: 28 December 2011

Volume 17 (2012) pp 136-152
DOI: 10.2478/s11658-011-0042-8

Research article

INHIBITION OF BIOGENIC MEMBRANE FLIPPASE ACTIVITY IN RECONSTITUTED ER PROTEOLIPOSOMES IN THE PRESENCE OF LOW CHOLESTEROL LEVELS

ARCHITA RAJASEKHARAN and SATHYANARAYANA N. GUMMADI*
Department of Biotechnology, Indian Institute of Technology Madras,
Chennai 600036, India

Abstract: Biogenic membranes or self-synthesizing membranes are the site of synthesis of new lipids such as the endoplasmic reticulum (ER) in eukaryotes. Newly synthesized phospholipids (PLs) at the cytosolic leaflet of ER need to be translocated to the lumen side for membrane biogenesis and this is facilitated by a special class of lipid translocators called biogenic membrane flippase. Even though ER is the major site of cholesterol synthesis, it contains very low amounts of cholesterol, since newly synthesized cholesterol in ER is rapidly transported to other organelles and is highly enriched in plasma membrane. Thus, only low levels of cholesterol are present at the biosynthetic compartment (ER), which results in loose packing of ER lipids. We hypothesize that the prevalence of cholesterol in biogenic membranes might affect the rapid flip-flop. To validate our hypothesis, detergent solubilized ER membranes from both bovine liver and spinach leaves were reconstituted into proteoliposomes with varying mol% of cholesterol. Our results show that (i) with increase in the cholesterol/PL ratio, the half-life time of PL translocation increased, suggesting that cholesterol affects the kinetics of flipping, (ii) flipping activity was completely inhibited in proteoliposomes reconstituted with 1 mol% cholesterol, and (iii) FRAP and DSC experiments revealed that 1 mol% cholesterol did not

* Author for correspondence. e-mail: gummadi@iitm.ac.in, tel.: 91-44-22574114, fax: 91-44-22574102

Abbreviations used: DTT – dithiothreitol; ePC – egg phosphatidylcholine; ER – endoplasmic reticulum; HEPES – *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HLT1 – first half-life time; HLT2 – second half-life time; MOPS – 3-(*N*-morpholino)propanesulfonic acid; NBD-PC – 1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)aminododecanoyl}-*sn*-glycero-3-phosphocholine; PC – phosphatidylcholine; PL – phospholipid; PM – plasma membrane; PMSF – phenylmethanesulfonylfluoride; SWER – salt-washed endoplasmic reticulum; TE – Triton X-100 extract

alter the bilayer properties significantly and that flippase activity inhibition is probably mediated by interaction of cholesterol with the protein.

Key words: Biogenic membrane flippase, Cholesterol, Endoplasmic reticulum, Flip flop

INTRODUCTION

Cholesterol is an important steroid synthesized in animals and in low quantities also in plants and fungi. The importance of cholesterol for normal functioning of cell membranes may be because of its ability to alter properties of the phospholipid bilayer and (or) to interact directly with specific membrane proteins [1, 2]. It has been reported earlier that changes in erythrocyte membrane cholesterol levels result in increased phosphatidylserine (PS) externalization [3]. Plants are composed of various sterols which include stigmasterol, sitosterol, campesterol and very low levels of cholesterol. Stigmasterol, the cholesterol derivative in plants is localized in the cell membrane. Even though endoplasmic reticulum (ER) is the major site for synthesis of cholesterol and its derivatives in both plants and animals, ER contains very low amounts of sterols [4, 5]. Newly synthesized cholesterol is rapidly transported ($t_{1/2}$~10-20 min) to other organelles and highly enriched in plasma membrane (PM) [4, 6]. This accumulation of cholesterol in PM compared to other organelles is a well conserved feature of eukaryotes but is still poorly understood. Thus, cholesterol is present in very low levels at the biosynthetic compartment (ER), which is in agreement with the membrane biogenic property of ER by which it rapidly translocates newly synthesized PLs across the bilayer from cytosol to lumen. This movement of PLs is thermodynamically unfavorable because moving a charged head group across the hydrophobic bilayer usually requires metabolic energy [7, 8]. However, biogenic or self-synthesizing membranes are equipped with a special class of protein translocators called biogenic membrane flippases, which are involved in rapid flip-flop of PLs (irrespective of PL head group) and are metabolic energy independent, thereby differing from conventional ATP-dependent flippases which are localized at non-biogenic membranes such as plasma membrane (PM) [9]. Biogenic membrane flippase activity has been shown in bacterial cytoplasmic membranes, and ER of animals and plants [10-12]. We hypothesize that cholesterol affects the rapid flip-flop of PLs across the bilayer. To understand the role of cholesterol on PL flip-flop, we reconstituted detergent solubilized endoplasmic reticulum fractions isolated from both plant (spinach leaves) and animal (bovine liver) with cholesterol *in vitro* into proteoliposomes.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (ePC), 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine (DPPC), cholesterol, sodium dithionite, ULTROL-grade Triton X-100, BCA

protein estimation kit and methyl-β-cyclodextrin were obtained from Sigma; SM-2 bio-beads were obtained from Bio-Rad laboratories; 1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)aminododecanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC) were from Avanti polar lipids; HEPES, MOPS and other routine chemicals were purchased from Himedia, India.

Isolation of salt-washed ER microsomes from spinach leaves

The microsomal membrane fraction was prepared as described previously [12, 13]. Briefly, 60 g of fresh leaves were homogenized in 60 ml of buffer A [MOPS-BTP buffer (pH 7.5) and 0.33 M sucrose supplemented with 5 mM EDTA, 5 mM KCl, 5 mM DTT, 0.5 mM PMSF, 1 mM $MgCl_2$ and 0.2% (w/v) BSA]. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 270 x *g* for 5 min at 4°C to remove larger organelles. The supernatant was centrifuged at 10000 x *g* for 15 min followed by 40000 x *g* for 1 h at 4°C in a 45 Ti fixed angle rotor (Beckman Coulter). The resulting pellet was resuspended using a Dounce homogenizer in buffer A to a final volume of 20 ml and layered over a sucrose gradient (2 ml of 60% (w/w) sucrose, 3 ml of 40% (w/w) sucrose, 3 ml of 20% (w/w) sucrose). It was centrifuged in SW 41 Ti rotor at 100000 x *g* for 45 min at 4°C and the pellet between the 40-60% sucrose interface was collected. The collected fraction was resuspended in buffer A and pelleted at 100000 x *g* for 30 min. The pellet was washed 2-3 times in buffer B (10 mM HEPES/NaOH pH 7.5 and 0.2 M NaCl) and the final washed microsomal pellet was resuspended in buffer B at a protein concentration of 8-10 mg/ml. The membranes were snap frozen in liquid nitrogen and stored at -80°C for later use.

Isolation of salt-washed ER microsomes from bovine liver

ER microsomes were isolated from bovine liver as described previously [11, 14, 15]. Briefly, 25 g of bovine liver was homogenized in 100 ml of buffer A [10 mM HEPES/NaOH (pH 7.5) and 0.25 M sucrose] in a homogenizer until homogeneous to the eye. The homogenate was centrifuged at 630 x *g* for 10 min at 4°C in a table-top centrifuge. The supernatant was centrifuged at 11000 x *g* for 10 min at 4°C. The previous step was repeated and to the supernatant PMSF and CsCl was added to a final concentration of 0.5 mM and 15 mM respectively. 50 ml was transferred to polycarbonate ultracentrifuge tubes and carefully ~15 ml of sucrose cushion (1.3 M sucrose, 10 mM HEPES/NaOH pH 7.5, 15 mM CsCl) were added under the supernatant. Total microsomes were isolated by centrifugation at 150000 x *g* at 4°C for 2.5 h. The pellet was rinsed with buffer A and resuspended in the same using a Dounce homogenizer. It was then washed with low salt buffer [10 mM HEPES/NaOH pH 7.5, 40 mM $Mg(CH_3COO)_2$] followed by high salt buffer (50 mM HEPES/NaOH pH 7.5, 1 M CH_3CO_2K) and centrifuged at 100000 x *g* for 45 min at 4°C in a 100 Ti rotor. The pellet was resuspended in buffer A, snap frozen in liquid nitrogen and stored at -80°C for later use.

Preparation of Triton X-100 extract (TE) from salt-washed ER microsomes
Salt-washed ER microsomes were prepared as described above. TE was prepared by adding 250 µl of either bovine or spinach salt-washed ER microsomes with equal volume of 2x reconstitution buffer [10 mM HEPES/ NaOH pH 7.5 buffer containing 100 mM NaCl and 1% (w/v) Triton X-10]. The sample was mixed and kept at 4°C with end-over-end rocking for 45 min. It was then ultracentrifuged in an MLA 130 rotor at 175000 x *g* for 30 min at 4°C. The supernatant, Triton extract was carefully removed and used directly for reconstitution at a protein concentration of ~ 3 mg/ml.

Reconstitution of liposomes and proteoliposomes
Liposomes and proteoliposomes were prepared as described previously [13, 16]. 4.5 µmol egg PC (ePC) and 0.3 mol% of NBD-PC was dried under a stream of nitrogen and solubilized in 10 mM HEPES/NaOH pH 7.5 containing 100 mM NaCl and 1% (w/v) Triton X-100. To study the effect of cholesterol on flipping, different amounts of cholesterol were added along with ePC. For preparation of proteoliposomes, TE prepared either using bovine liver ER or spinach ER was added to solubilized lipid samples with known protein concentration. Pre-treated SM2 bio-beads were used to remove Triton X-100 to form liposomes or proteoliposomes. To study the effect of spinach and bovine ER lipid extracts on flippase activity, the lipids were extracted by Bligh-Dyer method as described previously [17]. ePC vesicles were supplemented with bovine and spinach ER lipid extracts corresponding to 100 µl TE respectively. The reconstituted liposomes and proteoliposomes were collected by ultracentrifugation in an MLA 130 rotor at 230000 x *g* for 45 min at 4°C and washed 3-4 times with 10 mM HEPES/NaOH pH 7.5 and 100 mM NaCl to remove background fluorescence due to non-reconstituted lipids and proteins. The protein/phospholipid ratio (PPR) of proteoliposome samples was determined as described previously [17, 18]. The vesicles were resuspended in 1 ml of the same buffer and passed ~10 times through a 0.1 µm polycarbonate membrane filter using a lipid extruder to make vesicles of uniform size (100 nm).

Assay for flippase activity using dithionite reduction
The assay was performed using a Perkin Elmer LS–5S Fluorescence spectrophotometer as described earlier [12]. A total of 20 µl of NBD-PC labeled liposomes and proteoliposomes was added to 1.98 ml of 10 mM HEPES/NaOH pH 7.5 and 100 mM NaCl in a fluorescence cuvette. Time-dependent fluorescence (excitation at 470 nm and emission at 530 nm) was monitored with constant low-speed stirring at 22°C. After the fluorescence intensity stabilized (~100 s), sodium dithionite (1 M prepared freshly in Tris base pH 10) was added to 2 mM final concentration and decrease in fluorescence was measured for ~ 400 s. The initial fluorescence (F_0) was taken as the average value of fluorescence intensity of the first plateau; the fluorescence after dithionite reduction (F_t) was taken as the average value of the second plateau. The % of NBD-PC reduced (P_{red}) upon

dithionite addition was calculated by normalizing the F_0 to 1.0 and F_t as the fraction of F_0. The activity of flippase in a proteoliposome preparation was taken as the difference between the percent reduced in the sample and the percent reduced in a liposome sample (control). The flippase activity with vesicles containing cholesterol was calculated as the percentage of untreated control proteoliposomes. Briefly,

$$P_{red} = \left(1 - \frac{F_t}{F_0}\right) x100$$

$$\text{Flippase activity } (A) = [P_{red,proteoliposomes} - P_{red,liposome}]$$

The kinetics of flipping was studied by fitting the data to one phase and two phase exponential decay for liposomes and proteoliposomes respectively. Calculation of half-life time was performed by using the equation

$$F(t) = F_0 - [A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)]$$

where, F(t) is the fluorescence as a function of time and F_0 is the fluorescence intensity at time, t = 0 s (i.e. initial fluorescence of the vesicles), k_1 and k_2 are the rate constants for the first (fast) and second (slow) phases respectively. A_1 and A_2 are the amplitudes of the fast and slow phases respectively.

Fluorescence recovery after photobleaching (FRAP) analysis for lateral diffusion of lipids in presence and absence of cholesterol

FRAP analysis was performed using a Zeiss LSM 710 confocal microscope using Zen 2009 version software. Fluorescent labeled (NBD-PC) giant vesicles were prepared as described earlier [19, 20]. Briefly, 1 mg/ml ePC with 0.1 mol% NBD-PC were taken in a glass vial and dried under nitrogen followed by drying in a vacuum for 3 h to remove all chloroform. 1 ml of buffer (100 mM sucrose, 50 mM KCl) preheated to 60°C was added along the walls of the tube. It was incubated overnight at 60°C and stored at 4°C. Bovine and spinach proteoliposomes were prepared as described in the previous section and integrated into giant liposomes by freeze thawing 3-4 times. The vesicles were diluted and immobilized on a glass cover slip and placed under the objective lens of a laser scanning confocal fluorescence microscope. The argon ion laser was set at 2% power and samples were excited using the 488 nm line of the laser. The objective used was Plan-Apochromat 10x/0.45 M27. In order to obtain a sufficient speed and suitable signal to noise ratio, image size was set to 512 pixels and speed to 0.79 μs/pixel. The zoom factor was set to 14.9. For bleaching, a region of interest (ROI) was defined and centered on position x = 56.84 μm, y = 56.84 μm, and adjacent to it a control region also was set. At zoom 14.9, the ROI was 2 μm in diameter. Before bleaching ~ 7-10 images were recorded to define initial fluorescence, after which it was scanned for 30 seconds. The recovery due to lateral diffusion is calculated using

$$D = r_o^2 \gamma / 4T_{1/2}$$

where, D is the diffusion coefficient; r_o is the $1/e^2$ radius of the Gaussian profile laser beam used; γ is a parameter that depends on the extent of photobleaching (1.0-1.2); $T_{1/2}$ is the time required for 50% recovery.

Differential scanning calorimetry (DSC)

DSC measurements for liposomes and proteoliposomes generated using ePC in the absence and presence of 1 mol% cholesterol were monitored using MDSC Q200 (TA instruments) as described earlier [21]. ePC liposomes and proteoliposomes were prepared as described above. Briefly, 4.5 µmol of ePC with 1 mol% cholesterol concentration were dried under a stream of nitrogen. The lipid mixture was hydrated in 1 ml of buffer (10 mM HEPES/NaOH pH 7.5, 100 mM NaCl) at room temperature. The vesicles were pelleted at 230000 x *g* for 20 min at 4°C. ~2 mg wet weight of the pelleted sample was taken for calorimetric measurement. All scans were conducted between -60°C to 100°C in nitrogen atmosphere. The scans were repeated twice to ensure that cholesterol was properly equilibrated in the lipid mixture. The heating rate in all cases was 10°C/min.

Cyclodextrin treatment

Numerous reports have shown that exposing cells to β cyclodextrin results in solubilization and removal of cellular cholesterol [22, 23]. To check if flippase activity is restored after cholesterol removal, we treated bovine ER proteoliposomes reconstituted with 1 mol% cholesterol with 5 mM methyl-β-cyclodextrin. The sample was incubated at 37°C for 1 h. Proteoliposomes were collected by centrifugation and resuspended in fresh assay buffer. After 2 washes the sample was checked for flippase activity as described earlier.

RESULTS

Flippase activity measurement by dithionite reduction

The measurement of flippase activity is based upon the amount of fluorescent PLs available for quenching by dithionite, a membrane impermeable reagent, at 22ºC on the outer leaflet (Fig. 1). In the case of liposomes or proteoliposomes which do not have flippases, all the PLs on the outer leaflet were quenched upon addition of dithionite and yielded ~50-55% quenching, whereas inner leaflet PLs were protected from dithionite (Fig. 2, trace a). Time-dependent fluorescence quenching of liposomes follows single phase exponential decay kinetics as reported earlier [10]. Liposomes when reconstituted with ePC and lipids extracted from bovine liver ER and spinach leaf ER showed no flippase activity (Fig. 2A, trace b and c). When proteoliposomes were reconstituted with detergent solubilized ER membrane fractions containing flippase activity, two-phase exponential decay kinetics were observed (Fig. 2B and 2C, trace b). Similarly, proteoliposomes reconstituted with ePC along with extracted lipids from bovine and spinach ER did not alter the flippase activity or kinetics of flipping (Fig. 2B, 2C trace c) and was same as the control proteoliposomes

prepared from pure ePC (Fig. 2B and 2C, trace b). With increase in TE (protein) concentration used for reconstitution, percentage quenching increased suggesting that flipping is protein mediated (Fig. 2D and 2E trace b and c). A sharp decrease in fluorescence to ~ 50-55% was observed upon dithionite addition, followed by a slow decrease in fluorescence. The sharp decrease in fluorescence is due to quenching of labeled lipids on the outer leaflet, whereas the slower decrease in fluorescence is due to flipping of inner labeled PLs to the outer leaflet, which subsequently gets quenched by dithionite [12, 24]. However, upon addition of 0.1% (w/v) Triton X-100 to liposomes, ~ 100% quenching was observed, indicating that the membrane permeability was disturbed, leaving all the PLs accessible to dithionite for quenching (data not shown).

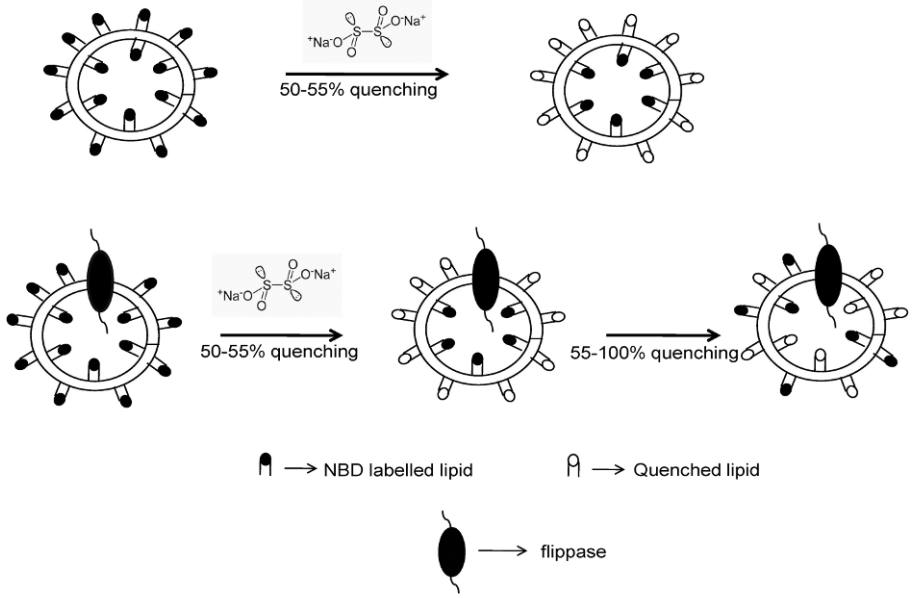

Fig. 1. Schematic representation of flippase assay by dithionite quenching. Reconstituted liposomes and proteoliposomes without flippase when treated with sodium dithionite (a membrane impermeable quenching reagent) quenches half ~50-55% the NBD-labeled PLs. In vesicles reconstituted with a functional flippase capable of bi-directional PL translocation there is a second phase of slow quenching of NBD-labeled lipids to ~55-100% in addition to initial rapid quenching.

The kinetics of liposome quenching follow single exponential decay kinetics with a half-life (HLT1) of ~ 0.3 min. In the case of proteoliposomes generated from both bovine liver ER and spinach ER, the kinetics showed double exponential decay. The half-life of first phase decay varied between 0.25 and 0.3 min, which is consistent with values obtained for pure liposomes. This suggests that quenching of fluorescence on the outer leaflet is independent of protein content in proteoliposomes [24]. However, the half-life of the second phase is higher than the first phase half-life for both bovine liver and spinach ER

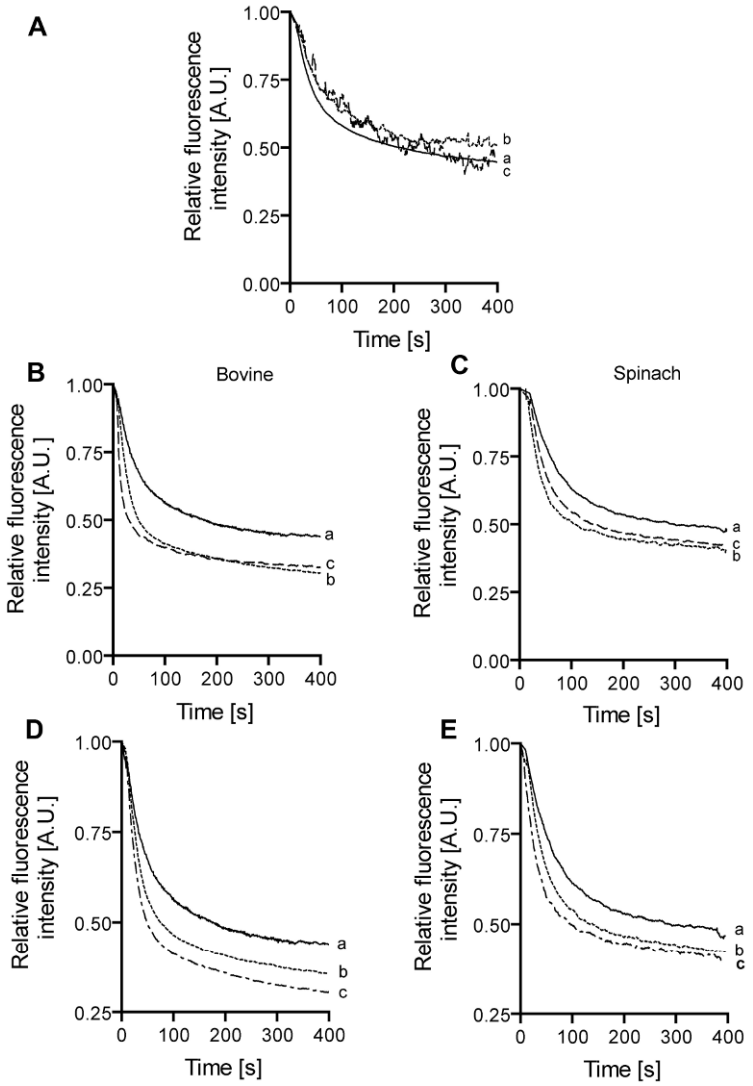

Fig. 2. Effect of bovine and spinach lipid extract and varying TE on flippase activity. A – ePC liposomes reconstituted with bovine and spinach ER lipid extract corresponding to 100 µl TE (trace a – liposome, trace b – bovine ER lipid extract, trace c – spinach ER lipid extract). B and C – Flippase activity with bovine ER lipid extract or spinach ER lipid extract (trace a – liposome, trace b – bovine ER and spinach ER proteoliposomes respectively, trace c – bovine ER and spinach ER proteoliposomes reconstituted with extracted lipids respectively). D and E – Flippase activity increases with increase in amount of TE (trace a – liposome, trace b – proteoliposome reconstituted with 10 µl bovine TE and 75 µl spinach TE, trace c – proteoliposome reconstituted with 25 µl bovine TE and 100 µl spinach TE respectively). All the experiments were performed at least thrice and the values reported are means with ± 1% to ± 8% standard deviation.

membranes. The half-time of PC translocation was 2 and 6 min for proteoliposomes generated from bovine liver and spinach ER respectively (Tab. 1). This implies that second phase quenching is slower because of transbilayer movement of PLs from the inner leaflet to the outer leaflet. The results obtained in this study are consistent with the half-life time reported earlier by several researchers [10-12].

Tab. 1. Kinetics of NBD-PC flip-flop in liposomes and proteoliposomes generated from detergent extract of bovine liver and spinach leaf ER membranes with various mol% of cholesterol.

Condition	% Quenching	HLT2 (min)
Liposomes	55	-
Proteoliposomes[a] (bovine liver ER)	67	2.0 ± 0.1
Proteoliposomes + 0.1 mol % cholesterol	65	2.3 ± 0.1
Proteoliposomes + 0.3 mol % cholesterol	65	2.4 ± 0.2
Proteoliposomes + 0.5 mol % cholesterol	64	3.3 ± 0.1
Proteoliposomes + 0.8 mol % cholesterol	45	-
Proteoliposomes + 0.9 mol % cholesterol	47	-
Proteoliposomes + 1.0 mol % cholesterol	50	-
Proteoliposomes (spinach ER)	60	5.9 ± 0.1
Proteoliposomes + 0.5 mol % cholesterol	58	5.8 ± 0.1
Proteoliposomes + 0.8 mol % cholesterol	57	9.0 ± 0.6
Proteoliposomes + 1.0 mol % cholesterol	54	-

[a] Proteoliposomes were generated with PPR ratio ~40-50 mg/mmol. HLT2: half-life time of the exponential decay of the fluorescence present in the inner leaflet. - fitted to mono-exponential kinetics. HLT1: half-life time of the exponential decay of the fluorescence present in the outer leaflet was calculated to be 0.3 ± 0.05 min for all the cases. The experiments were performed thrice and values are reported as mean with standard deviation.

Effect of cholesterol on flippase activity

In proteoliposomes reconstituted using TE extract of bovine and spinach ER with cholesterol, incorporation of up to 0.5 mol% cholesterol did not result in any loss of flippase activity while 1 mol% showed extensive inhibition (Fig. 3A and 3B, trace b, c and d). The second half-life time (HLT2) of translocation increased from 2 to 3.3 min with increase in cholesterol content up to 0.5 mol%, whereas the % NBD quenching was not affected suggesting that the kinetics of PL flipping is inhibited with increase in cholesterol content (Tab. 1). With further increase in cholesterol, the extent of NBD quenching drops to ~ 50% which is almost equal to that of quenching values obtained for liposome.

Effect of cholesterol on membrane fluidity measured by FRAP and DSC

It is known that the presence of cholesterol alters the membrane fluidity. To check if 1 mol% of cholesterol had any significant effect on the membrane fluidity, we performed FRAP and DSC experiments using vesicles with the same bilayer composition as used for flippase activity measurements.

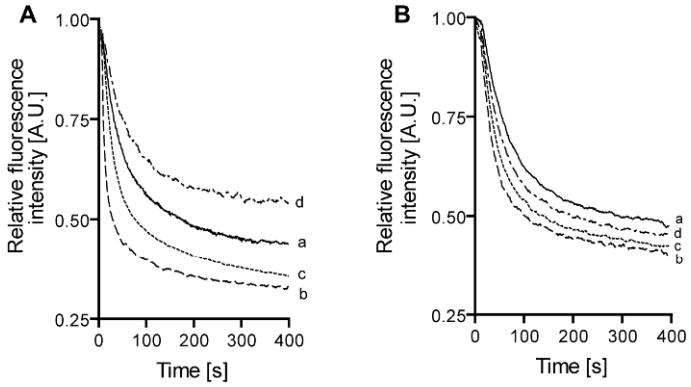

Fig. 3. Effect of cholesterol on flippase activity. (A) Inhibition of flippase activity in proteoliposomes generated from TE of bovine liver ER microsomes. Cholesterol concentrations of 0.3, 0.5 and 1 mol % were used (traces b, c and d respectively, liposomes – trace a). (B) Inhibition of flippase activity in proteoliposomes generated from TE of spinach leaf ER microsomes. The cholesterol content used in the reconstitution was 0.5, 0.8 and 1 mol % (traces b, c and d respectively). All the experiments were performed at least thrice and the values reported are means with ± 1% to ± 10% standard deviation.

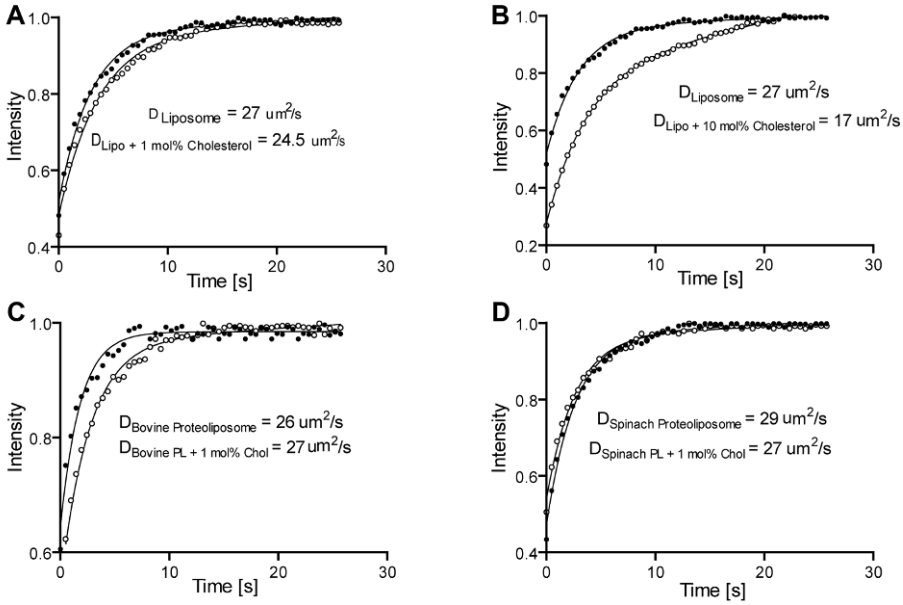

Fig. 4. FRAP recovery plot. Averaged FRAP in (A) giant ePC liposomes in presence (unfilled circles) and absence of 1 mol% cholesterol (black filled circles) with $t_{1/2}$ value calculated to be 2 ± 0.5 s for both. Samples with cholesterol are represented as black filled circles and in absence are shown as unfilled circles; (B) giant ePC liposomes in presence ($t_{1/2}$ 4 ± 0.4 s) and absence of 10 mol% cholesterol; (C) giant bovine proteoliposomes in presence and absence of 1 mol% cholesterol with $t_{1/2}$ value 2 ± 0.6 s for both; (D) giant spinach proteoliposomes in presence and absence of 1 mol% cholesterol with $t_{1/2}$ value 2 ± 0.4 s for both. In all, $t_{1/2}$ values were similar implying that lateral diffusion was not altered at 1 mol% concentration.

Fluorescence recovery after photo bleaching (FRAP). FRAP analysis with giant ePC liposomes in the absence and presence of 1 mol% cholesterol exhibited a similar fluorescence recovery pattern with lateral diffusion rates of 27 $\mu m^2/s$ and 24.5 $\mu m^2/s$ respectively as there was negligible difference in $t_{1/2}$ (Fig. 4A). Liposome reconstituted with 10 mol% cholesterol was also analyzed and the diffusion rate was found to be 17 $\mu m^2/s$ (Fig. 4B). Giant proteoliposomes were also prepared with ePC in the presence and absence of 1 mol% cholesterol and FRAP analysis revealed diffusion rates for bovine proteoliposomes to be 27 $\mu m^2/s$ and 26 $\mu m^2/s$ respectively (Fig. 4C) and in spinach proteoliposomes they were calculated to be 27 $\mu m^2/s$ and 29 $\mu m^2/s$ respectively (Fig. 4D).

Differential scanning calorimetry (DSC). ePC liposomes and proteoliposomes generated from bovine liver and spinach ER were pelleted and analyzed by DSC. The phase transition endothermic peaks for liposome, proteoliposomes from bovine liver and spinach ER were -0.50°C, 0.30°C, -0.42°C respectively (Fig 5). These values are slightly higher than the values obtained for pure ePC (Fig. 5). In the presence of cholesterol the phase transition temperature increased to 0.08°C in liposome and to 0.02°C in the case of spinach proteoliposome. FRAP and DSC results revealed that presence of 1 mol% cholesterol did not alter the membrane fluidity.

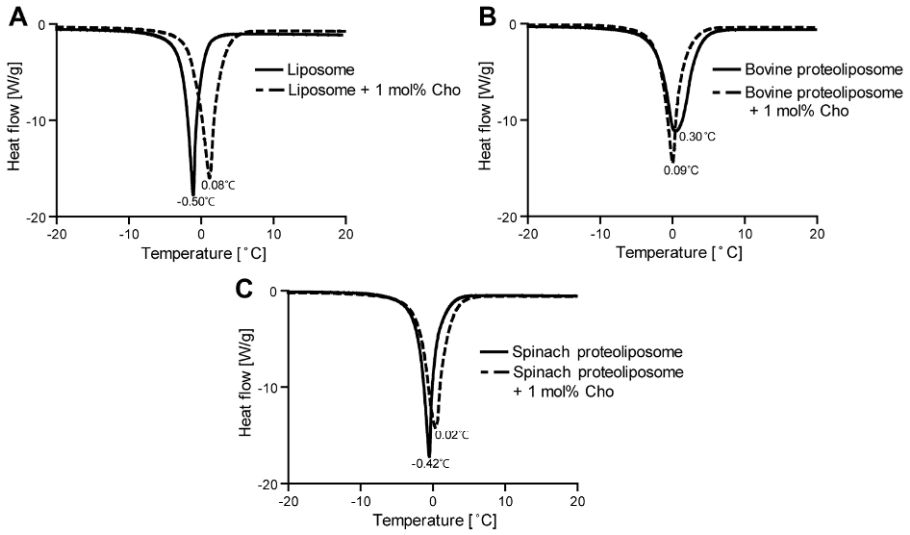

Fig. 5. DSC for ePC liposomes with varying cholesterol concentrations. DSC endotherms for liposomes and proteoliposomes reconstituted with (1 mol%) and without cholesterol. A – Liposomes, B – proteoliposomes generated from detergent extract of bovine liver ER microsomes, C – proteoliposomes generated from detergent extract of spinach leaf ER microsomes. About ~ 2 mg wet weight of the pelleted vesicles in presence and absence of 1 mol% cholesterol was scanned between -60°C and 100 in nitrogen atmosphere. The scans were repeated thrice to ensure that cholesterol was properly equilibrated in the lipid mixture and heating rate was set at 10°C/min. All samples showed a single endothermic peak.

Cyclodextrin treatment

In order to remove bound cholesterol from proteoliposomes reconstituted with ePC and 1 mol% cholesterol, we treated proteoliposomes with methyl-β-cyclodextrin and checked whether biogenic membrane flippase activity was restored. The results clearly showed that upon cholesterol removal using 5 mM cyclodextrin, ~ 91% of the biogenic membrane flippase activity in proteoliposomes was restored (Fig. 6, trace d).

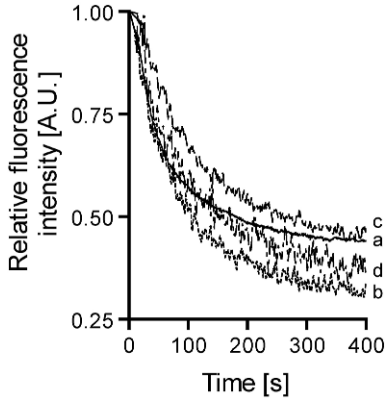

Fig. 6. Cyclodextrin treatment of bovine proteoliposomes reconstituted with 1 mol% cholesterol and cholesterol localization study: Flippase activity after 5 mM of methyl-β-cyclodextrin treatment (trace a - liposome, b - bovine proteoliposome, c - bovine proteoliposome reconstituted with 1 mol% cholesterol, d - cyclodextrin treatment of bovine proteoliposome treated with 1 mol% cholesterol).

DISCUSSION

We hypothesized that the presence of cholesterol affects the PL flip-flop activity of biogenic membrane flippase, which in turn affects the membrane biogenesis. The ER fractions were isolated on a sucrose density gradient. The isolated fractions were assayed for CCR activity, which was enriched in the final fraction compared to the crude. Assays were also performed to check contaminants. Chlorophyll estimation and ATPase assay were performed to check for chloroplast and plasma membrane contamination, which was found to be negligible (data not shown). To prove our hypothesis, we reconstituted detergent solubilized ER membranes from both bovine liver and spinach leaves with varying amounts of cholesterol at a constant protein to PL ratio (~40-50 mg/mmol). The vesicles were washed and assayed for flippase activity as mentioned previously. It has been reported earlier that in lipid bilayers a number of factors influence the translocation of PLs such as lipid chain length and saturation, bilayer physical state and the membrane composition [25, 26]. Therefore, bovine and spinach ER lipids were extracted and lipids corresponding to 100 µl TE were reconstituted into liposomes and proteoliposomes. The presence of extracted lipids did not alter the quenching of PLs on the outer leaflet (~55%) for liposomes (Fig. 2A) and the kinetic data were fitted to a single exponential

decay function. Similarly, in the case of proteoliposomes, the addition of extracted lipids altered neither the % NBD quenching nor the kinetics of flipping (Fig. 2B and 2C). These results clearly indicated that the presence of lipid extract from bovine liver and spinach ER does not have any influence on the assay and flippase activity. A study similar to ours showed that lipid extract of rat liver ER when reconstituted did not have any effect on flippase activity [9]. In the absence of protein (liposomes), there was ~55% NBD quenching and HLT1 was calculated to be 0.3 ± 0.05 min (Tab. 1), which is due to the rapid quenching of NBD-PC on the outer leaflet. Proteoliposomes reconstituted with TE of bovine ER showed % NBD quenching of 65-67% (Fig. 2D, trace b) with rapid HLT1 of 0.3 ± 0.05 min, similar to liposome, and HLT2 of 2 min (Tab. 1). The HLT1 and HLT2 values obtained were similar to the data reported earlier. The half-life time of flipping has been reported to be ~ 3-4 min for rat liver ER [11], ~ 1 min for bacterial cytoplasmic membranes [10] and ~ 1 min for spinach ER [12]. The activity was confirmed to be protein mediated and in our earlier study we also confirmed the sensitivity to chemical modification. ER flippase activity (NBD-PC) was affected by ~60% after DEPC and DTNB treatment, ~50% after PG and ~10% after AEBSF [12].

Cholesterol accounts for only 3-10% of total sterols in plant membranes [27]. Also, they do not accumulate at the ER in steady state. They are rapidly transported to the PM via the Golgi [28, 29]. Not much is yet known about how sterols are transported through the plant secretory pathway and also the effect of cholesterol on flippase activity [30]. Bovine and spinach proteoliposomes were reconstituted with increasing cholesterol concentration. With increase in cholesterol content in reconstituted vesicles up to 0.5 mol%, the extent of NBD quenching was not affected (Fig. 3A, trace b and Tab. 1). Interestingly, HLT2 of translocation increased from 2 to 3.3 min with increase in cholesterol content up to 0.5 mol%, whereas the % NBD quenching was not affected to a large extent. This suggests that increase in cholesterol content inhibits the kinetics of PL flipping, which can be observed by the difference in quenching rate (Fig. 3A, trace b and d). With further increase in cholesterol, the extent of NBD quenching drops to ~50% which is almost equal to quenching values obtained for liposome. To rule out the possibility that the presence of cholesterol might interfere with the quenching process, liposomes were generated with and without 1 mol% cholesterol and assays were performed. The extent of NBD quenching is not affected by the presence of cholesterol and the % NBD quenching was ~50-55%, where the kinetic data fit to a single exponential decay function (data not shown). To check if flippase activity from other eukaryotes also has a similar effect, we isolated ER from plants (spinach leaves) and reconstituted it into proteoliposomes with varying concentrations of cholesterol. The results obtained with spinach ER match those obtained from bovine liver ER microsomes (Fig. 3B, Tab. 1). Flippase activity was completely inhibited when reconstituted with 1 mol% cholesterol. Proteoliposomes with 5 and 10 mol% cholesterol were also reconstituted and assayed, which showed complete inhibition of activity (data

not shown). Also, the HLT2 of flipping increased with increase in cholesterol content (Tab. 1). These results clearly indicate that cholesterol beyond 1 mol% completely inhibits the flipping process in eukaryotes and is in good agreement with the fact that ER maintains very low levels of cholesterol even though synthesis occurs at ER [4, 31].

It has been shown that cholesterol in synthetic liposomes affects membrane properties. To check if membrane fluidity was affected in the presence of cholesterol, commonly used biophysical approaches were explored using vesicles prepared with ePC. It has been shown by fluorescence resonance after photo bleaching (FRAP) that cholesterol > 10 mol% in synthetic liposomes affects lateral diffusion [32]. ePC giant liposomes and proteoliposomes were prepared with and without 1 mol% cholesterol and FRAP analysis showed similar recovery time, ~ 2 ± 0.8 s, whereas vesicles incorporated with 10 mol% cholesterol showed slower recovery time, ~ 4 ± 0.4 s (Fig. 4). This clearly showed that lateral diffusion of PL is not affected in the presence of 1 mol% cholesterol. Several studies have shown that cholesterol incorporation of 20-25 mol% in a bilayer membrane broadens and eventually eliminates the cooperative gel to liquid phase transition temperature and decreases the permeability of the bilayer. Also it has been reported that sterols with lengths greater than the mean thickness of the host PC bilayer will preferentially stabilize the gel phase of the PC bilayer, in turn increasing the transition temperature [33]. The results clearly indicated that addition of 1 mol% cholesterol was very less to affect membrane fluidity as there was no decrease in lateral diffusion or increase in phase transition temperature which would have resulted in membrane rigidity. Many studies have shown that cyclodextrin solubilizes membrane cholesterol [34]. To check if cholesterol was affecting flippase activity by interaction with biogenic membrane flippases, we treated bovine proteoliposomes (1 mol% cholesterol) with 5 mM methyl-β-cyclodextrin. Flippase activity was measured after cyclodextrin treatment, which showed ~91% recovery of flippase activity.

Several models have been proposed for the flippase mechanism, one of which includes the formation of a hydrophilic pore through which the head group passes while the hydrophobic chain passes along the lipid bilayer [35, 36]. The critical importance of cholesterol to normal functioning of cell membranes may lie in its ability to both alter fundamental properties of the phospholipid bilayer and to interact directly with specific membrane proteins. We hypothesize that cholesterol directly interacts with biogenic membrane proteins and inhibits activity. Studies have showed that 22-NBD-cholesterol was bound with high affinity (nM) by intracellular lipid binding proteins (SCP-2, ADRP) and was bound to these proteins with orientation similar to cholesterol [37-42]. Biogenic membrane flippase which is involved in lipid flip-flop might have similar interaction with cholesterol. This could be the reason for complete inhibition of flippase activity.

Our results showed that flipping activity is inhibited in both animals and plants when reconstituted into proteoliposomes in the presence of 1 mol% cholesterol.

These results provide more insight in understanding membrane biogenesis and the reason why cholesterol is not localized in larger amounts at its site of synthesis.

Acknowledgements. This work was supported by a research grant from the Department of Biotechnology, Government of India. We thank Mr. Surya (Biotechnology department, IITM) and Mr. Narayanan (Chemistry department, IITM) for their technical assistance with FRAP analysis and DSC respectively.

REFERENCES

1. Albert, A.D. and Boesze-Battaglia, K. The role of cholesterol in rod outer segment membranes. **Prog. Lipid Res.** 44 (2005) 99-124.
2. Yeagle, P.L. Modulation of membrane function by cholesterol. **Biochemie** 73 (1991) 1303-1310.
3. López-Revuelta, A., Sánchez-Gallego, J.I., García-Montero, C.A., Hernández-Hernández, A.,Sánchez-Yagüe, J. and Llanillo, M. Membrane cholesterol in the regulation of aminophospholipid asymmetry and phagocytosis in oxidized erythrocytes. **Free. Radic. Biol. Med.** 42 (2007) 1106-1118.
4. Lange, Y., Echevarria, F. and Steck, T.L. Movement of zymosterol, a precursor of cholesterol, among three membranes in human fibroblast. **J. Biol. Chem.** 266 (1991) 21439-21443.
5. Ridsdale, A., Denis, M., Gougeon, P.Y., Ngsee, J.K., Presley, J.F. and Zha, X. Cholesterol is required for efficient endoplasmic reticulum-to-Golgi transport of secretory membrane proteins. **Mol. Biol. Cell.** 17 (2006) 1593-1605.
6. DeGrella, R.F. and Simonia, R.D. Intracellular transport of cholesterol to the plasma membrane. **J. Biol. Chem.** 257 (1982) 14256-14262.
7. Kornberg, R.D. and McConnell, H.M. Inside-outside transitions of phospholipids in vesicle membranes. **Biochemistry** 10 (1971) 1111-1120.
8. Gummadi, S.N. and Kumar, K.S. The mystery of phospholipid flip-flop in biogenic membranes. **Cell. Mol. Biol. Lett.** 10 (2005) 101-121.
9. Menon, A.K. Flippases. **Trends Cell. Biol.** 5 (1995) 355-360.
10. Hrafnsdo′ttir, S. and Menon, A.K. Reconstitution and partial characterization of phospholipid flippase activity from detergent extracts of *Bacillus subtilis* cell membrane. **J. Bacteriol.** 182 (2000) 4198-4206.
11. Gummadi, S.N. and Menon, A.K. Transbilayer movement of dipalmitoylphosphatidylcholine in proteoliposomes reconstituted from detergent extracts of endoplasmic reticulum: kinetics of transbilayer transport mediated by a single flippase and identification of protein fractions enriched in flippase activity. **J. Biol. Chem.** 277 (2002) 25337-25343.
12. Sahu, S.K. and Gummadi, S.N. Flippase activity in proteoliposomes reconstituted with *Spinacea oleracea* endoplasmic reticulum membrane proteins: evidence of biogenic membrane flippase in plants. **Biochemistry** 47 (2008) 10481-10490.

13. Lord, J.M., Kiagawva, T. and Beevers, H. Intracellular distribution of enzymes of the cytidine diphosphate choline pathway in castor bean endosperm. **Proc. Nat. Acad. Sci. U.S.A.** 69 (1972) 2429-2432.
14. Backer, J.M. and Dawidowicz, E.A. Reconstitution of a phospholipid flippase from rat liver microsomes. **Nature** 327 (1987) 341-343.
15. Menon, A.K., Watkins, W.E. and Hrafnsdo´ttir, S. Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmic reticulum. **Curr Biol.** 10 (2000) 241-252.
16. Lèvy, D., Bluzat, A., Seigneuret, M. and Rigaud, J.L. A systematic study of liposome and proteoliposomes reconstitution involving Bio-Bead mediated Triton X-100 removal. **Biochim. Biophys. Acta** 1025 (1990) 179-190.
17. Bligh, E.G. and Dyer, W.J. A rapid method of total lipid extraction and purification. **Can. J. Med. Sci.** 37 (1959) 911-917.
18. Kaplan, R.S. and Pedersen, P.L. Sensitive protein assay in the presence of high levels of lipid. **Methods Enzymol.** 172 (1989) 393-399.
19. Akashi, K., Miyata, H., Itoh, H. and Kinosita, K. Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope. **Biophys. J.** 71 (1996) 3242-3250.
20. Dimova1, R., Aranda, S., Bezlyepkina, N., Nikolov, V., Riske, K.A. and Lipowsky, R. A practical guide to giant vesicles - Probing the membrane nanoregime via optical microscopy. **J. Phys. Condens. Matter.** 18 (2006) S1151-S1176.
21. Taylor, K.M.G. and Morris, R.M. Thermal analysis of phase transition behaviour in liposomes. **Thermochim. Acta** 248 (1994) 289-301.
22. Yancey, P.G., Rodrigueza, W.V., Kilsdonk, E.P.C., Stoudt, G.W., Johnson, W.J., Phillips, M.C. and Rothblat, G.H. Cellular cholesterol efflux mediated by cyclodextrins. **J. Biol. Chem.** 271 (1996) 16026-16034.
23. Eckford, P.D.W. and Sharom, F.J. Interaction of the P-glycoprotein multidrug efflux pump with cholesterol: effects on ATPase activity, drug binding and transport. **Biochemistry** 47 (2008) 13686-13698.
24. Chang, Q., Gummadi, S.N. and Menon, A.K. Chemical modification identifies two population of glycerophospholipid flippase in rat liver ER. **Biochemistry** 43 (2004) 10710-10718.
25. Bishop, R.W. and Bell, R.M. Assembly of the endoplasmic reticulum phospholipid bilayer: the phosphatidylcholine transporter. **Cell** 42 (1985) 51-60.
26. Vigh, L., Escriba, P.V., Sonnleitner, A., Sonnleitner, M., Piotto, S., Marseca, M., Horvath, I. and Harwood J.L. The significance of lipid composition for membrane activity: New concepts and ways of assessing function. **Prog. Lipid Res.** 44 (2005) 303-344.
27. Betts, H. and Moore, I. Plant cell polarity: The Ins-and-outs of sterol transport. **Curr. Biol.** 13 (2003) 781-783.
28. Moreau, P., Hartmann, M.A., Perret, A.M., Sturbois-Balcerzak, B. and Cassagne C. Transport of sterols to the plasma membrane of leek seedlings. **Plant. Physiol.** 117 (1998) 931-937.

29. Grebe, M., Xu, J., Möbius, W., Ueda, T., Nakano, A., Geuze, H.J., Rook, M.B. and Scheres, B. *Arabidopsis* sterol endocytosis involves actin mediated trafficking via ARA6-positive early endosomes. **Curr. Biol.** 13 (2003) 1378-1387.
30. Boutte, Y. And Grebe, M. Cellular processes relying on sterol function in plants. **Curr. Opin. Plant Biol.** 12 (2009) 705-713.
31. Meer, G.V., Voelker, D.R. and Feigenson, G.W. Membrane lipids: where they are and how they behave. **Nat. Rev. Mol. Cell. Biol.** 9 (2008) 112-124.
32. Smith, M.L. and McConnell, H.M. Pattern photobleaching of fluorescent lipid vesicles using polarized laser light. **Biophys. J.** 3 (1981) 139-146.
33. McMullen, T.P.W., Vilcheze, C., McElhaney, R.N. and Bittman, R. Differential scanning calorimetric study of the effect of sterol side chain length and structure on dipalmitoylphosphatidylcholine thermotropic phase behaviour. **Biophys. J.** 69 (1995) 169-176.
34. Zidovetzki, R. and Levitan, I. Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. **Biochim. Biophys. Acta** 1768 (2007) 1311-1324.
35. Tieleman, D.P. and Marrink, S.J. Lipids out of equilibrium: energetics of desorption and pore mediated flip-flop. **J. Am. Chem. Soc.** 128 (2006) 12462-12467.
36. Gurtovenko, A.A. and Vattulainen, I. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. **J. Phys. Chem. B.** 111 (2007) 13554-13559.
37. Schroeder, F., Frolov, A., Schoer, J., Gallegos, A., Atshaves, B.P., Stolowich, N.J., Scott, A.I. and Kier, A.B. **Intracellular sterol binding proteins, cholesterol transport and membrane domains, in intracellular cholesterol trafficking** (Chang, T.Y. and Freeman, D.A., Eds), 1998, Kluwer Academic Publishers, Boston.
38. Stolowich, N., Frolov, A., Petrescu, A.D., Scott, A.I., Billheimeri, J.T. and Schroeder, F. Holo-sterol carrier protein-2. **J. Biol. Chem.** 274 (1999) 35425-35433.
39. Schroeder, F., Frolov, A., Starodub, O., Atshaves, B.B., Russelli, W., Petrescu, A., Huang, H., Gallegos, A.M., McIntosh, A., Tahotna, D., Russelli, D.H., Billheimer, J.T., Baum, C.L. and Kier, A.B. Pro-sterol carrier protein-2. **J. Biol. Chem.** 275 (2000) 25547-25555.
40. Avdulov, N.A., Chochina, S.V., Igbavboa, U., Warden, C.S., Schroeder, F. and Wood, W.G. Lipid binding to sterol carrier protein-2 is inhibited by ethanol. **Biochim. Biophys. Acta.** 1437 (1999) 37-45.
41. Serrero, G., Frolov, A., Schroeder, F., Tanaka, K. and Gelhaar, L. Adipose differentiation related protein: expression, purification of recombinant protein in Escherichia coli and characterization of its fatty acid binding properties. **Biochim. Biophys. Acta.** 1488 (2000) 245-254.
42. Atshaves, B.P., Starodub, O., McIntosh, A., Petrescu, A., Roths, J.B., Kier, A.B. and Schroeder, F. Sterol carrier protein-2 alters high density lipoprotein-mediated cholesterol efflux. **J. Biol. Chem.** 275 (2000) 36852-36861.