Fibroblast membrane sterol kinetic domains: modulation by sterol carrier protein-2 and liver fatty acid binding protein

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Abstract The mechanism(s) of intracellular sterol trafficking among subcellular organelle membranes is not well understood. Relative contributions of vesicular, sterol carrier protein, and membrane sterol domain pathways are not resolved. A sterol kinetic assay was used to resolve multiple sterol domains in microsome (MICRO), mitochondria (MITO), and plasma (PM) membrane: exchangeable, 20–40% of total; non-exchangeable, 60–80% of total. Spontaneous sterol transfer between dissimilar donor and acceptor membranes was vectorial and depended both on acceptor and donor membrane properties. For example, sterol transfer from PM to MICRO or to MITO, or from MICRO to MITO was 3- to 5-fold slower as compared to sterol movement in the opposite direction. Sterol carrier protein-2 (SCP-2) stimulated sterol transfer in most donor/acceptor membrane combinations by decreasing exchange half-time but not domain size. SCP-2 enhanced sterol transfer selectively: PM-MICRO (12-fold); MITO-MITO, MICRO-MICRO, MICRO-PM (3-fold); PM-PM (1.4-fold); PM-MITO, MICRO-MITO (no effect). Thus, SCP-2-mediated sterol movement was vectorial and not necessarily down a membrane sterol concentration gradient. In contrast, liver fatty acid binding protein (L-FABP) revealed a modest (2-fold) stimulatory effect on sterol transfer only between PM-MITO and MICRO-MICRO. In conclusion, in vitro studies of sterol transfer among isolated subcellular membranes provided kinetic evidence for sterol domains in microsomes and mitochondria as well as plasma membranes. Furthermore, both spontaneous and protein-mediated sterol transfer appeared vectorial and selective in nature.—**Frolov, A., J. K. Woodford, E. J. Murphy, J. T. Billheimer, and F. Schroeder.** Fibroblast membrane sterol kinetic domains: modulation by sterol carrier protein-2 and liver fatty acid binding protein. *J. Lipid Res.* 1996. **37:** 1862–1874.

Supplementary key words plasma membrane • microsome • mitochondria • cholesterol transfer • fatty acid binding protein • sterol carrier protein-2 • fluorescence polarization

Cholesterol is the single most common lipid species in mammalian cells. However, cholesterol is unequally distributed not only among intracellular organelles but also within cellular membranes with plasma membrane molar ratio of cholesterol/phospholipid near 1 and intracellular membranes much lower (reviewed in refs 1–4). This cholesterol distribution is opposite to that of intracellular sterol synthetic activity (5, 6). Synthesis of steroid hormones from cholesterol occurs in the inner mitochondrial membrane which is devoid of cholesterol (7). In fact, the transport of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in steroidogenesis (8). Therefore, intracellular pathways for cholesterol trafficking must mobilize cholesterol from sites of synthesis and/or direct cholesterol to sites of utilization (2, 9–11). Intracellular cholesterol movement occurs by both vesicular and protein-mediated pathways, the relative importance and regulation of which remain unclear. This question has been examined in several ways.

Studies with intact cultured cells have been equivocal. Transfected cells expressing sterol carrier proteins or cells injected with antisense oligonucleotides clearly show an involvement of SCP-2 in intracellular sterol trafficking (12–15). In contrast, results with mutant cell lines deficient in sterol carrier proteins [Zellwegers syndrome (15–17), Niemann-Pick C (18, 19), hepatoma (20)] are neither consistent with those obtained with transfected cells nor do results with different mutant cell line models entirely agree with each other (15, 16). Part

Abbreviations: ACT, acyl-CoA:cholesterol acyltransferase; DHE, dehydroergosterol, $\Delta^{5,7,9,(11),22}$-ergostatetraen-3β-ol; HPLC, high performance liquid chromatography; L-FABP, liver fatty acid binding protein; MICRO, microsomal membranes; MITO, mitochondrial membranes; PIPES, piperazine-N,N-bis (2-ethanesulfonic acid); PM, plasma membranes; SCP-2, sterol carrier protein-2.

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of the difficulty may be the coexistence of multiple sterol trafficking pathways in cells and the fact that the gene defect in the mutant cells is either unknown or is not SCP-2 (21). Consequently, the role(s) of SCP-2 in intracellular sterol trafficking in the mutant cells is difficult to resolve.

An alternate approach has been to use isolated subcellular organellar membranes. Such studies have fewer variables and also allow examination of the role of membrane sterol domains or sterol carrier proteins. Although much is known regarding sterol transfer processes and sterol domains in model membranes (2, 9–11, 22), very little is known concerning these processes and domains in biological membranes. It was recently shown that plasma membranes have both transbilayer (1) and lateral (2, 23) cholesterol domains/pools. With the exception of erythrocyte ghosts (24, 25) and L-fibroblast cell surface membranes (2, 26, 27) there is a paucity of information on sterol domains and sterol trafficking in plasma membranes of other cell types. Even more limited is our knowledge of these processes in intracellular membranes. Likewise, mechanisms of sterol domain regulation and of sterol transfer in isolated intracellular organelle membranes are not understood.

The present objectives were to *i)* investigate the presence of sterol domains in intracellular membranes and *ii)* examine potential routes and/or regulation of intracellular sterol trafficking in vitro by cytosolic sterol binding proteins. A sterol exchange kinetic assay was used to examine relative size and kinetics of membrane sterol pools in subcellular membrane fractions, i.e., plasma membranes, microsomes, and mitochondria. In addition, the effect of cytosolic sterol carrier proteins on the size and kinetic properties of intracellular sterol domains as well as their role in vectorial sterol transfer was determined. These specialized cytosolic proteins interact with the membrane surfaces (28–32), bind sterol with micromolar affinity and molar stoichiometry 1:1 (33, 34), and modulate sterol metabolic enzymes (35–37). Extensive studies with model membranes (37–41) and more recently with L-cell fibroblast plasma membranes (2, 27) suggest that these sterol carrier proteins stimulate sterol transfer.

MATERIALS AND METHODS

Materials

Human recombinant SCP-2 (41) and rat recombinant L-FABP (42) were isolated as described. Dehydroergosterol (DHE) was prepared as reported (43, 44). Sterol standards were purchased from Steraloids, Inc. (Wilmington, NH) and used without further purification. All other chemicals were reagent grade or better.

Cells and membrane isolation

Mouse L-cells (L aprt$^-$tk$^-$) were grown (4) in the absence or presence of DHE, a fluorescent sterol that codistributes with endogeneous sterol of L-cells, replaces in excess of 80% of L-cell endogeneous sterol, and is without effect on cellular sterol content, sterol/phospholipid ratio, phospholipid composition, or sterol-sensitive proteins (reviewed in 2). DHE (5 mg/ml, 95% nondenaturated grain ethanol) was added at a final concentration of 20 μg/ml medium (26, 27). For standard curves, medium DHE ranged from 0 to 40 μg/ml. Ethanol vehicle for DHE was held constant for each dish and did not exceed 0.25% (v/v). After 3 days growth, cells were harvested, membranes were separated (plasma membrane, microsomal, and mitochondrial fractions), and membrane purity was determined by marker enzyme activity (4) with purity near 90%. Protein concentrations were determined using the method of Lowry et al. (45).

Lipid extraction and quantitation

Mitochondrial membrane lipids were extracted in n-hexane–2-propanol 3:2 (v/v) and evaporated to dryness, redissolved in $CHCl_3$ and applied to pre-activated 2.54-cm silicic acid columns. The neutral lipid fraction was eluted with 4 column volumes of $CHCl_3$. The phospholipid fraction was eluted with 4 column volumes of MeOH and used to quantitate total lipid phosphorus (46). Neutral lipids were evaporated under N_2 and redissolved in n-hexane–2-propanol–acetic acid 98.7:1.2:0.1 (v/v/v). The sample was filtered through a 0.2-μm nylon filter (Rainin Inc., Woburn, MA), evaporated, and redissolved in a minimal volume for transfer to limited volume vials. Prior to HPLC analysis, the solvent was evaporated and the sample was redissolved in 63 μl of mobile phase. The HPLC separation used an isocratic elution profile with n-hexane–2-propanol–acetic acid 98.7:1.2:0.1 (v/v/v) as the mobile phase with the flow rate of 0.6 ml/min. The HPLC system consisted of Model 110 A HPLC Pump (Beckman Inc., Fullerton, CA), Selectosil (4.5 × 250 mm) Column (Phenomenex Inc., Torrance, CA), SPD-10 UV/VIS Spectrophotometer Detector (Shimadzu Scientific Instruments Inc., Kyoto, Japan), Data Acquisition System (Dionex Corp., Sunnyvale, CA) including UI-20 A-D Interface and PeakNet Software.

Microsomal membrane lipids were extracted from isolated membranes according to the procedure of Bligh and Dyer (47). Neutral lipids were separated from phospholipids using 2.54-cm silicic acid columns as above prior to quantitation. The phospholipid content was determined by the Ames total phosphate assay (46).

Sterol content was determined with HPLC by comparison to standard sterol solutions. The HPLC system was a Series 4 Liquid Chromatograph equipped with a LC-95 UV/VIS Spectrophotometer Detector (Perkin-Elmer Inc., Norwalk, CT), a 3390 A Reporting Integrator (Hewlett-Packard Inc., Palo Alto, CA), and C18 3U Reverse Phase Column (Alltech Assoc., Inc., Deerfield, IL). The mobile phase was MeOH–CH_3CN 70:30 (v/v) with a flow rate of 1 ml/min.

Fluorescence measurements and exchange assays

Steady-state fluorescence measurements of DHE fluorescence polarization were made with a Photon Counting Fluorimeter (ISS Instruments Inc., Champaign, IL) in the T-format with the emission monochromator removed from the optical path. The light source was a 300 W xenon arc lamp. The excitation wavelength was set at 324 with the excitation monochromator spectral slit width at 8 nm. In order to reduce an appearance of virtual artifacts due to inner filter effect, sample absorbance at 324 nm, the excitation wavelength, was kept below 0.15. Light scatter was reduced by use of dilute samples and by KV 389 low fluorescent cutoff filters (Schott Glass Technologies Inc., Duryea, PA) in the emission system.

Sterol exchange between biological membranes was measured using a fluorescence polarization method first developed for model membranes (22, 48–50). Recently the method was extended to erythrocyte and L-cell plasma membranes (25, 27). In the present investigation, donor membrane vesicles were obtained from cells grown in the presence of DHE and the exchange assay was performed basically as described earlier (25, 27). Data were corrected for residual light scatter contributions by subtracting residual fluorescence anisotropy (r), which is related to fluorescence polarization P as r = 2*P/(3 - P), of both donor and acceptor membranes from all experimental data. For each donor/acceptor combination, kinetics of DHE fluorescence polarization increase were analyzed in terms of kinetic sterol domains by using the respective standard curves as described in the Results section.

Conversion of dehydroergosterol polarization change to molecular sterol transfer in similar donor/acceptor membrane pairs

Standard curves allow conversion of dehydroergosterol DHE fluorescence polarization change, measured during intermembrane sterol exchange/transfer, into sterol molecular transfer. The standard curve analysis was first introduced and validated with model membranes by Butko et al. (50) and subsequently refined and applied to L-cell fibroblast plasma membranes (27) and erythrocyte ghosts (25). Therefore, in the present study, standard curves for L-cell microsomal and mitochondrial membranes were also constructed. In addition, these microsomal and mitochondrial membrane standard curves for molecular sterol exchange then allowed mathematical construction of separate standard curves for heterogeneous donor/acceptor pairs.

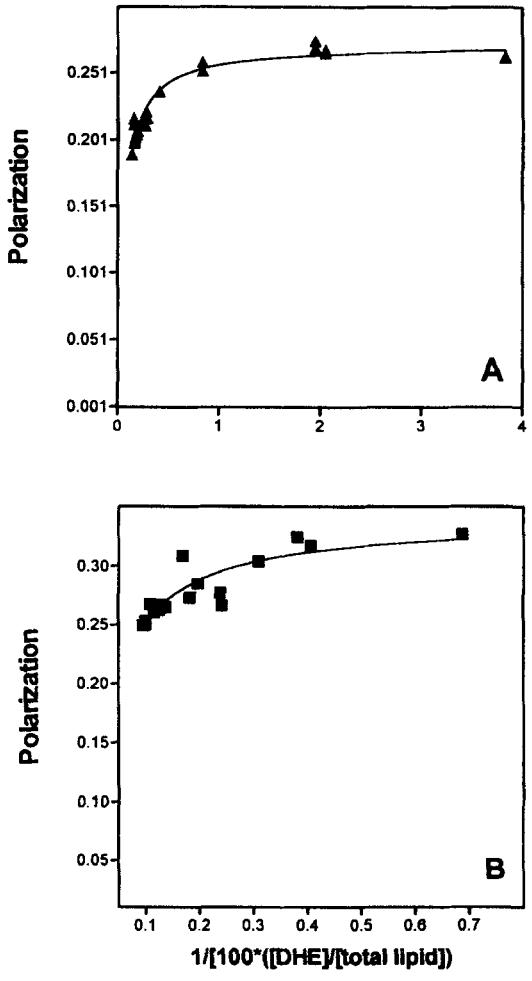

Fig. 1. Dependence of DHE fluorescence polarization on its concentration in biological membranes. The concentration of DHE in membranes was determined by HPLC of lipid extracts as described in Materials and Methods. Fluorescence polarization was measured in the T-format using the blank subtraction mode. The blank was the membrane suspension at the same concentration as the sample, but free of the fluorophore. The excitation wavelength was set at 324 nm and was selected by a monochromator (spectral slit width 8 nm). Fluorescence was observed through the KV389 low fluorescent cutoff filters. Absorbance at the excitation wavelength was ≤0.15. All measurements were made at 37°C. Panel A: fluorescence polarization of DHE in microsomes. The solid line is the best fit of experimental data to a hyperbolic function, Eq. 1 (see text for details), with the parameters $P_0 = 0.2719$ and $B = 0.0590$ ($r^2 = 0.93$). Panel B: fluorescence polarization of DHE in mitochondria. The values of the fitted parameters are $P_0 = 0.3393$ and $B = 0.0354$ ($r^2 = 0.82$).

Membrane DHE fluorescence polarization, P, is a nonlinear function of membrane DHE concentration (25, 27, 50):

$$P = P_0 * C / (B + C) \qquad \textit{Eq. 1}$$

where P is the measured fluorescence polarization, P_0 is the DHE fluorescence polarization at infinite dilution in membranes, C is the DHE membrane concentration, and B is a constant. To construct molecular sterol transfer standard curves for microsomal and mitochondrial sterol exchange, DHE fluorescence polarization was corrected for membrane DHE content, measured by HPLC of lipid sterols of the same membranes. As shown (**Fig. 1**), DHE fluorescence polarization in microsomal (panel A) and mitochondrial (panel B) membranes decreased with increasing DHE membrane concentration, consistent with Weber's theory of concentration dependent fluorescence depolarization (51). These results were also in agreement with the earlier model membrane (50) and other plasma membrane data (25, 27). The experimental data best fit a hyperbolic equation (Eq. 1) with $0.82 < r^2 < 0.93$. The use of another analytical function, i.e., polynomial, exponential, etc., yielded unacceptable fits as shown by the F test (Graphpad Prizm Software). The hyperbolic fit of the experimental data to Eq. 1, yielded the parameters $P_0 = 0.2719$, $B = 0.0590$, and $r^2 = 0.93$ (microsomes); $P_0 = 0.3393$, $B = 0.0354$, and $r^2 = 0.82$ (mitochondria). The respective values for plasma membranes were $P_0 = 0.3366$, $B = 0.0576$, and $r^2 = 0.95$ (27). Microsomes had the lowest P_0 value as compared to plasma membranes and mitochondria, suggesting that microsomal DHE rotational motions are least restricted among the three membrane fractions examined.

Equation 1 is derived for samples containing the same donor and acceptor membrane type. As sterol transfer from donor to acceptor membranes proceeds, DHE fluorescence polarization signal from acceptor vesicles must also be taken into account. The DHE fraction remaining in donor (X_d) and acceptor (X_a) is defined as:

$$X_d = C_d / C_t \qquad \textit{Eq. 2}$$

$$X_a = 1 - X_d = 1 - (C_d / C_t) \qquad \textit{Eq. 3}$$

where C_t and C_d were the initial (before the addition of an acceptor) and current DHE concentrations in the donor membrane, respectively. The relation between C_d and DHE concentration in the acceptor membranes (C_a) was described as following:

$$C_T = C_d + K * C_a \qquad \textit{Eq. 4}$$

where C_T is the initial DHE concentration in donor membranes (determined by lipid extraction and HPLC analysis), and K is the acceptor/donor molar ratio (in our experiments, K = 10). Further, DHE fluorescence polarization measured after the addition of an acceptor, originated both from the donor and the acceptor membranes. Because fluorescence anisotropy, but not fluorescence polarization, is an additive parameter, polarization (P) was converted into fluorescence anisotropy (r) using the expression: $r = (2*P)/(3 - P)$. Then, the calculated anisotropy of both donor and acceptor vesicles were presented as the linear combination:

$$r = f_d * r_d + f_a * r_a \qquad \textit{Eq. 5}$$

where r_d and r_a were the DHE fluorescence anisotropies in donor and acceptor membranes, respectively; f_d and f_a were their corresponding fractions (50, 52).

Conversion of DHE polarization change to molecular sterol transfer in dissimilar donor/acceptor membrane pairs

Substitution of f_d in Eq. 5 by X_d, allowed derivation of the DHE anisotropy in a mixed donor-acceptor pair:

$$r = r_0 * \{ X_d / (1 + X_d * D) + (1 - X_d) / [1 + 10 * (1 - X_d) / D] \} \qquad \textit{Eq. 6}$$

where r was the measured fluorescence anisotropy, and r_0 was the fluorescence anisotropy at an infinite dilution of DHE in membranes. The constant D in Eq. 6 was related to constant B as:

$$D = Z * B * [1 + (r_0 / 2)] \qquad \textit{Eq. 7}$$

where Z is the molar percent of DHE in the total membrane lipid. Parameter Z, in its turn, was calculated on the basis of Eq. 8:

$$Z = (P_0 - P) / P * B \qquad \textit{Eq. 8}$$

where P was the measured DHE fluorescence polarization in the donor membranes in the absence of an acceptor, P_0 and B parameters were derived from Eq. 1.

The first term in Eq. 6 represented the DHE fluorescence anisotropy of the donor component, while the second term described the acceptor fraction for the resultant fluorescence anisotropy in the similar donor-acceptor combinations (e.g., PM-PM, MICRO-MICRO, MITO-MITO). Standard curves for dissimilar donor-acceptor pairs were derived on the basis of Eq. 6 with the respective combinations of D values in its two terms. For example, for the PM-MICRO standard curve Eq. 6 was rewritten as:

$$r = r_0 * \{ X_d / (1 + X_d * D_{PM}) + (1 - X_d) / [1 + 10 * (1 - X_d) / D_{MICRO}] \} \qquad \textit{Eq. 9}$$

Then, fluorescence anisotropy was determined for X_d of 0 to 1 and converted to polarization via the expres-

sion: $P = 3*r/(2 + r)$. The generated standard curves for the similar donor-acceptor pairs are presented in Fig. 3. Standard curves for heterogeneous donor-acceptor pairs are not shown.

Calculated DHE fluorescence polarization dependencies on the remaining DHE fraction in the donor membranes for all possible donor-acceptor combinations were computer best fit to polynomial Eq. 10 in order to obtain b_n values.

$$P = \sum b_n x^n \qquad \textit{Eq. 10}$$

For the different donor-acceptor pairs the number of terms in the Eq. 10 required to give a good fit varied from 2 to 4. The respective data are presented in the following sections.

If sterol transfer between donor and acceptor membranes follows multiexponential kinetics:

$$X_d = \left[\sum f_i \exp(-k_i * t)\right] + F \qquad \textit{Eq. 11}$$

Eq. 10 can be rewritten as:

$$P = \sum b_n \left[\sum f_i \exp(-k_i * t) + F\right]_n^n \qquad \textit{Eq. 12}$$

where f_i represented the exchangeable sterol fraction (domain) in the donor membrane and k_i was the respective rate constant. The parameter F, therefore, depicted the sterol fraction that is unavailable for transfer. The experimental data on kinetic of DHE fluorescence polarization change due to sterol molecular transfer from donor to acceptor membranes were fit to Eq. 12. The number of exponential components in Eq. 12 were varied from 1 to 3. The "goodness" of fit was judged by minimal χ^2 value, the fitting error for each parameter, and by the distribution of residuals.

The above algorithm was applied to all donor-acceptor pairs. The single exponential model:

$$X_d = f_1 \exp(-k*t) + f_2 \qquad \textit{Eq. 13}$$

$$P = \sum b_n \left[f_1 \exp(-k*t) + f_2\right]^n \qquad \textit{Eq. 14}$$

consistent with one exchangeable (f_1) and one nonexchangeable (f_2) sterol domain in the donor vesicles, best fit the kinetics of sterol transfer/exchange in both homo- and heterogeneous donor-acceptor pairs. In the case of sterol exchange between plasma membranes (PM-PM), the two exponential model, with two exchangeable and one nonexchangeable domains, yielded as good a fit as a single exponential model. As the size of the first exchangeable domain was calculated to be very small, only 2–4% of the total membrane sterol, preference was given to the model with only one exchangeable domain. For consistency with the terminology of previous publications in this field (2, 22, 25–27), the velocity of sterol transfer was described by its half-time, i.e., time for transfer of 50% sterol from donor membrane exchangeable domain to acceptor vesicles. The half-time ($t_{1/2}$) is related to the rate constant k as: $t_{1/2} = (\ln 0.5)/k$.

RESULTS

Plasma membranes as sterol donors

As shown in **Fig. 2A** (curve 1), the fluorescence polarization of DHE in donor plasma membranes (PM) was stable in the absence of acceptor plasma membranes during the time of assay. Addition of 10-fold excess of acceptor PM to donor PM at zero time induced rapid increase of DHE fluorescence polarization (Fig. 2A, curve 2), reflecting spontaneous sterol molecular exchange between plasma membranes. The observed DHE fluorescence polarization change was analyzed in terms of kinetic sterol domains using the respective standard curve (**Fig. 3**) and Eq. 14 as depicted above. Molecular sterol exchange was best described by a single exponential function, yielding an exchangeable membrane sterol fraction of about 0.4 with half-time $t_{1/2}$ = 137 min (**Table 1**). Addition of SCP-2 did not affect the size of the exchangeable sterol domain, but significantly decreased its half-time, ~1.5-fold (Table 1). In contrast to SCP-2, the presence of L-FABP led to a moderate, ~1.3-fold, increase of the size of the exchangeable domain without altering respective $t_{1/2}$ value (Table 1). Thus, both cytosolic sterol binding proteins affected sterol molecular exchange between plasma membranes, albeit via different mechanism(s). In summary, these findings for PM-PM exchanges extend those reported earlier with fractional polarization changes (27) to examination of molecular sterol domains. This allows comparison of molecular sterol transfer in dissimilar donor/acceptor exchanges.

Because of differences in physico-chemical characteristics of subcellular membrane fractions [PM, microsomes (MICRO), and mitochondria (MITO)], the resultant DHE fluorescence polarization signal from the dissimilar (PM-MICRO, PM-MITO) donor-acceptor combinations did not allow the use of the same standard curve designed for homogeneous (e.g., PM-PM) exchange. Therefore, separate standard curves were derived according to Eq. 9 (see Methods) for each type of sterol transfer with identical donors (PM-MICRO, PM-MITO). The standard curves for PM-MICRO and PM-MITO heterogeneous donor-acceptor pairs were quite different as compared to PM-PM and fit different polynomial functions (see Table 1 legend). These standard curves allowed kinetic analysis of sterol transfer between PM donors and MICRO or MITO acceptors according to Eq. 14. Application of this kinetic analysis to sterol

molecular transfer from PM donor to MICRO or MITO acceptor showed that substitution of PM acceptor by MICRO or MITO significantly altered PM donor sterol

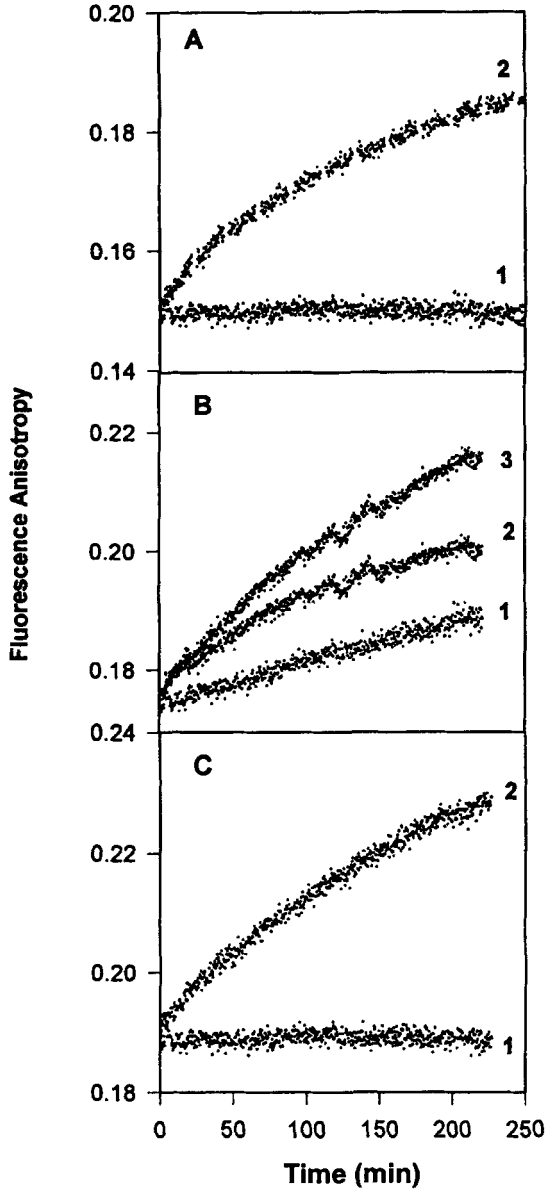

Fig. 2. Sterol exchange between subcellular membranes. Panel A: fluorescence polarization of DHE as a function of time in the donor plasma membranes (7 μg protein/ml) only (curve 1) and after addition of acceptor plasma membranes (70 μg protein/ml) to the donor membranes (7 μg protein/ml) at the time zero (curve 2). Panel B: the kinetics of DHE fluorescence polarization change in microsomal donor membranes alone (7 μg protein/ml, curve 1) and immediately after addition of acceptor microsomal membranes (70 μg protein/ml) to a donor membrane suspension (7 μg protein/ml, curve 3). Experimental data were corrected as described in Methods, yielding the resultant curve 2 (see text for more details on the correction procedure). Panel C: kinetic of DHE fluorescence polarization in the solution of the mitochondrial donor membranes (7 μg protein/ml, curve 1) and upon addition of 70 μg protein/ml mitochondrial acceptor membranes to the donor vesicles (7 μg protein/ml, curve 2).

domain structure. Indeed, the size of exchangeable domain for both the PM-MICRO and PM-MITO donor-acceptor pair combination decreased by almost 2-fold (Table 1). In fact, the size of the exchangeable sterol domain in the latter experiments was very similar to that observed in the acceptor membranes MICRO-MICRO and MITO-MITO. In contrast, the effect of dissimilar acceptors on $t_{1/2}$ value was less prominent. Only PM-MITO, but not PM-MICRO donor-acceptor combination revealed an alteration in $t_{1/2}$ value. The former increased by approximately 2-fold. In summary, these results showed that the rate of sterol desorption from the donor membrane was not necessarily the rate-limiting step in spontaneous sterol transfer. Exchangeable sterol domain size in dissimilar donor/acceptor exchanges appeared to be highly dependent on exchangeable domain size in the acceptor, rather than donor membrane. Spontaneous sterol transfer from PM, MICRO, and MITO was vectorial in nature as based on half-times.

The effect of SCP-2 on sterol domains and vectorial sterol transfer in dissimilar donor/acceptor exchanges was examined. SCP-2 dramatically increased sterol transfer from PM to MICRO as shown by raw fluorescence polarization data increase (**Fig. 4A**). Analysis in terms of molecular sterol exchange showed that SCP-2 decreased $t_{1/2}$ 12-fold, without changing sterol domain size (Table 1). In contrast, SCP-2 effects on sterol transfer in the reverse direction from MICRO to PM shown by raw fluorescence polarization increase were much less (Fig. 4B). Analysis in terms of molecular sterol exchange demonstrated only a 1.8-fold decrease in $t_{1/2}$ and no effect on sterol domain size (Table 1). This was consistent with the stimulatory effect of SCP-2 being vectorial in nature. L-FABP did not significantly affect either the $t_{1/2}$ of sterol transfer or the domain size between PM-MITO and MITO-PM (Table 1).

Microsomal membranes as donors

In contrast to MITO and PM donor membranes, DHE polarization in microsomal donors (MICRO) slowly decreased in the absence of MICRO acceptor, Fig. 2B (curve 1). Thus, all experimental data on MICRO-MICRO sterol molecular transfer were corrected for this changing baseline (Fig. 2B, curve 2), as described in Methods. Molecular sterol transfer data were obtained by fitting this curve to Eq. 14 using b_n values obtained from standard curve (Fig. 3B). As shown in **Table 2,** MICRO-MICRO exchange showed one exchangeable sterol domain (f_1 ~0.4), of similar size as in PM membranes (Table 1) and 2 times larger than in MITO membranes (**Table 3**). The MICRO-MICRO exchange half-time, $t_{1/2}$ ~ 130 min, was similar to that of PM

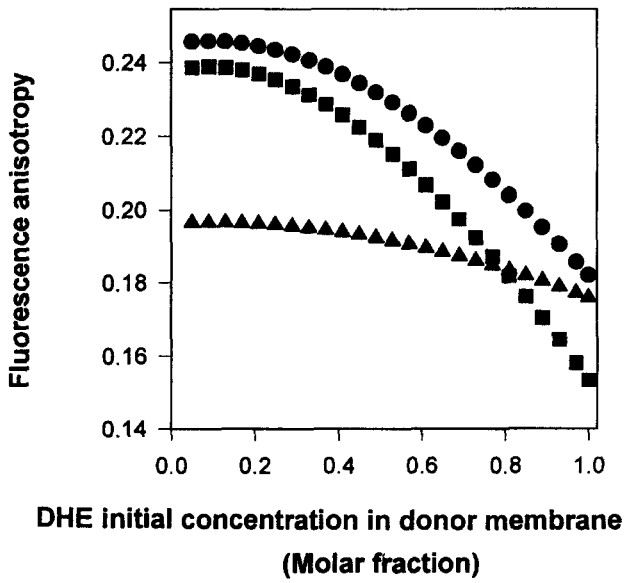

Fig. 3. Standard curves for sterol exchange between subcellular membranes. Standard curves for sterol exchange assay calculated by using Eq. 6 (see Materials and Methods for details) for plasma membranes–plasma membranes (squares), microsomes–microsomes (triangles), and mitochondria–mitochondria donor/acceptor combinations (circles).

membranes ($t_{1/2}$ ~ 130 min, Table 1), but significantly (~2.5-fold) longer, as compared to sterol exchange between MITO membranes ($t_{1/2}$ ~50 min, Table 3). Both SCP-2 and L-FABP stimulated MICRO-MICRO sterol transfer without altering domain structure. SCP-2 and L-FABP decreased the half-time of MICRO-MICRO exchange ~3-fold and 1.7-fold, respectively (Table 2).

Replacement of MICRO acceptor in the similar MICRO-MICRO donor acceptor pair by PM lowered the fraction of exchangeable sterol domain in donor vesicles 2-fold (Table 2). This significant decrease in the fraction of membrane sterol being available for transfer/exchange was accompanied by a 3-fold decreased $t_{1/2}$ (Table 2). The latter showed faster spontaneous sterol movement from microsomes to plasma membranes versus homogeneous MICRO-MICRO sterol exchange. However, the molecular basis for this observation is not known at this time. Concomitantly, neither sterol domain structure nor the exchange/transfer half-times were statistically altered when MICRO acceptor was substituted by MITO (Table 2).

SCP-2 stimulated sterol movement from MICRO to PM (3-fold decreased $t_{1/2}$) without altering sterol domain structure, exhibiting the same efficiency as for the homogeneous MICRO-MICRO donor-acceptor pair (Table 2). In the MICRO-MITO donor-acceptor combination SCP-2 did not significantly affect either sterol domain structure or the $t_{1/2}$ value (Table 3). L-FABP under the same conditions was ineffective in altering sterol exchange kinetics or domain size with either MICRO-PM or MICRO-MITO dissimilar donor-acceptor pairs.

Mitochondria membranes as donors

In the absence of acceptor mitochondria (MITO), DHE fluorescence polarization in donor MITO membranes was stable, Fig. 2C (curve 1). Addition of 10-fold excess of MITO acceptor resulted in rapid increase of

TABLE 1. Kinetic parameters of spontaneous and protein-mediated sterol exchange between plasma membranes-donors and different acceptors

Donor-Acceptor	Protein	f_1	f_2	$t_{1/2}$ (min)
PM-PM	none	0.47 ± 0.03	0.53 ± 0.03	137 ± 5
	SCP-2	0.56 ± 0.11	0.44 ± 0.11	99 ± 0[a]
	L-FABP	0.61 ± 0.04[a]	0.39 ± 0.04[a]	158 ± 11
PM-MICRO	none	0.23 ± 0.03[c]	0.77 ± 0.03[c]	128 ± 6[c]
	SCP-2	0.21 ± 0.02[c]	0.79 ± 0.04[c]	11 ± 8[a]
	L-FABP	0.31 ± 0.02[b]	0.69 ± 0.02[b]	117 ± 15
PM-MITO	none	0.20 ± 0.03[c]	0.80 ± 0.03[c]	253 ± 47
	SCP-2	0.24 ± 0.04[c]	0.76 ± 0.04[c]	149 ± 61
	L-FABP	0.11 ± 0.04[c]	0.89 ± 0.04[c]	122 ± 55[b]

The kinetics of sterol transfer within different donor-acceptor pairs were analyzed on the basis of the observed DHE fluorescence polarization change by using the following standard curves (for more details see Methods). PM-PM: $P = b_0 + b_2*X_d^2$, $b_0 = 0.3155$; $b_2 = -0.131$. PM-MICRO: $P = b_0 + b_1*X_d + b_2*X_d^2 + b_3*X_d^3$, $b_0 = 0.2574$; $b_1 = 0.085$; $b_2 = -0.1680$; $b_3 = 0.0392$. PM-MITO: $P = b_0 + b^1*X_d + b_2*X_d^2 + b_3*X_d^3$, $b_0 = 0.3188$; $b_1 = 0.0057$; $b_2 = -0.2286$; $b_3 = 0.0610$. Values represent the mean ± SE (n = 3–5). All measurements were performed at 37°C.

[a]Represents $P < 0.05$ as compared to no protein.

[b]Refers to $P < 0.1$ as compared to no protein.

[c]Refers to $P < 0.05$ as compared to PM-PM.

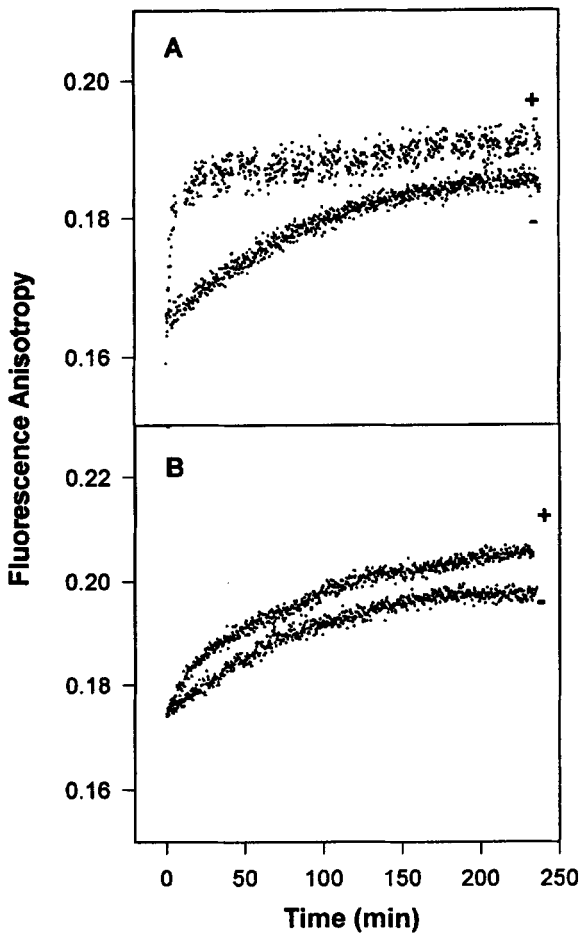

Fig. 4. Selectivity of SCP-2 effect on sterol transfer between intracellular membranes. Panel A: effect of SCP-2 on sterol transfer from donor plasma membranes to microsome acceptor membranes: (-) without SCP-2; (+) in the presence of 1.5 μM SCP-2. Panel B: stimulatory effect of SCP-2 on the reverse sterol transfer from donor microsomes to plasma membranes acceptor: (-) without SCP-2; (+) in the presence of 1.5 μM SCP-2 (for more details see Materials and Methods).

fluorescence polarization (Fig. 2C, curve 2), reflecting intermembrane sterol movement. Kinetic analysis of this molecular sterol transfer, based on Eq. 14 and the respective standard curve (Fig. 2C), showed the existence of a single exchangeable (f_1 ~0.2) domain with corresponding $t_{1/2}$ ~ 50 min (Table 3). Neither SCP-2 nor L-FABP altered the MITO kinetic domain structure. However, SCP-2 enhanced sterol movement, approximately 3-fold (Table 3).

As shown in Table 3, replacement of MITO acceptor in the similar MITO-MITO donor-acceptor membrane pair combination by PM or MICRO acceptor membranes altered neither the MITO sterol domain structure nor the $t_{1/2}$ values.

SCP-2 stimulated sterol transfer 2- to 3-fold in all donor-acceptor combinations wherein MITO served as donors without altering sterol domain structure (Table 3). L-FABP under the same conditions did not significantly affect the kinetics of sterol transfer in any of the above cases (Table 3).

DISCUSSION

The results in this report help to clarify some of the issues arising from divergent conclusions concerning the effect of intracellular sterol binding proteins on intracellular sterol trafficking in transfected versus mutant cell lines as pointed out earlier. Clearly, SCP-2 does stimulate sterol trafficking between endoplasmic reticulum (microsomes) and plasma membranes in vitro. These findings substantiate those with intact transfected cells (8, 12–15). Interestingly, the SCP-2-mediated enhancement of sterol transfer from MICRO-PM (3.3-fold decreased half-time, Table 2) in vitro compares favorably with the approximately 4-fold decrease in rate of sterol movement from endoplasmic reticulum to plasma membranes observed in intact normal human fibroblasts transfected with SCP-2 antisense oligonucleotides (15). Equally important is the observation made herein that SCP-2 stimulates sterol transfer from endoplasmic reticulum (microsomes) to plasma membranes by decreasing the half-time of exchange but without altering sterol domain size. Taken together with the finding that SCP-2 does increase exchangeable sterol domain size in microsomes (MICRO-MICRO, Table 2), but not plasma membranes (PM-PM, Table 1), it is suggested that the acceptor plasma membrane sterol domain size defines the size of the exchangeable sterol domain in sterol transfer from microsomes to plasma membrane in vitro.

The results present several additional new findings characterizing intracellular membrane sterol domains as well as spontaneous and protein mediated sterol trafficking in vitro. The following observations were based on the kinetics of sterol transfer between similar and dissimilar cellular subfractions, such as plasma membranes, microsomes, and mitochondria.

First, analysis of DHE fluorescence polarization change during sterol molecular transfer/exchange between donor and acceptor allowed resolution of multiple sterol kinetic domains in donor subcellular membranes (Tables 1–3). This relatively simple model of discrete pools or domains, rather than a distribution of pools or domains, is based on both model and biological membrane data and has been chosen for a number of reasons. One reason is that in both model (48) and biological (2) membranes DHE lifetimes can either be fit by nonlinear least squares analysis (sum of discrete exponentials) or by Lorentzian distributional (distribution of lifetimes around centers of distribution) analysis with only small differences in Chi^2 or "goodness of fit"

TABLE 2. Kinetic parameters of spontaneous and protein-mediated sterol exchange between microsomal membranes-donors and different acceptors

Donor-Acceptor	Protein	f_1	f_2	$t_{1/2}$ (min)
MICRO-MICRO	none	0.41 ± 0.03	0.59 ± 0.03	131 ± 12
	SCP-2	0.58 ± 0.04[a]	0.42 ± 0.04[a]	38 ± 5[a]
	L-FABP	0.44 ± 0.07	0.56 ± 0.07	78 ± 4[a]
MICRO-PM	none	0.22 ± 0.04[b]	0.78 ± 0.04[b]	49 ± 3[b]
	SCP-2	0.18 ± 0.03[b]	0.72 ± 0.03[b]	15 ± 1[a]
	L-FABP	0.23 ± 0.05[b]	0.77 ± 0.05[b]	49 ± 3
MICRO-MITO	none	0.50 ± 0.20	0.50 ± 0.20	105 ± 18
	SCP-2	0.31 ± 0.09[b]	0.69 ± 0.09[b]	62 ± 13
	L-FABP	0.19 ± 0.05[b]	0.81 ± 0.05[b]	107 ± 24

The kinetics of sterol transfer within different donor-acceptor pairs were analyzed on the basis of the observed DHE fluorescence polarization change by using the following standard curves (for more details see Methods). MICRO-MICRO: $P = b_0 + b_1*X_d + b_2*X_d^2$, $b_0 = 0.2685$; $b_1 = 0.0043$; $b_2 = -0.0300$ MICRO-PM: $P = b_0 + b_1*X_d + b_2*X_d^2$, $b_0 = 0.3331$; $b_1 = -0.057$; $b_2 = -0.0340$. MICRO-MITO: $P = b_0 + b_1*X_d + b_2*X_d^2$, $b_0 = 0.3359$; $b_1 = -0.061$; $b_2 = -0.044$. Values represent the mean ± SE (n = 3–5). All measurements were performed at 37°C.

[a]Represents $P < 0.05$ as compared to no protein.

[b]Refers to $P < 0.05$ as compared to MICRO-MICRO.

values. In both cases, the best fits of the data were represented by either two discrete components or by two distributions of components wherein the centers of distribution in the latter analysis were literally identical to those obtained by discrete analysis. More important, the fractional contributions due to each discrete DHE lifetime component were essentially the same as the fractional contributions of the two distributions of DHE lifetimes. Because the polarization exchange assay differs substantially from the lifetime analysis type of assay, obtaining a distributional analysis of anisotropies is not straightforward. Therefore, the more simplistic model has been applied herein. Another reason for using the discrete rather than the distributional model is that the membrane vesicles used herein for both donors and acceptors are much larger than those for model membranes. In model membranes, vesicle size can substantially alter exchange kinetics (53). Thus, if sterol domains are differentially distributed according to vesicle size, this could result in a distribution of donor model membrane DHE anisotropies. In contrast, the much larger plasma membrane vesicles used herein can be separated into subfractions that do not differ in lipid composition (54). Thus, all types of sterol domains may coexist in each donor biomembrane vesicle. Finally, the data in the literature with both model and biological membranes indicate that the sterol domain size is not significantly affected by changing the ratio of donor:acceptor over a 30-fold range in biomembrane (25) or a 20-fold range in model membranes (55). In the present work as well as previously, a donor:acceptor ratio of 1:10 was chosen to minimize back exchange from the acceptors, thereby facilitating calculations. In conclusion, we suggest that the model described herein probably provides average sterol domain sizes whose values would be expected to be similar to the centers of distributional sizes if the latter type of analysis were applicable.

Second, the sterol domain sizes of subcellular membranes differed significantly. Spontaneous sterol movement between similar donor-acceptor pairs, i.e., PM-PM, MITO-MITO, and MICRO-MICRO, was characterized by the presence of two sterol kinetic domains: one exchangeable, f_1 = 0.2–0.4, and one nonexchangeable, f_2 = 0.6–0.8 (Tables 1–3). This observation strongly indicates that not all membrane sterol is available for transfer/exchange. A similar large nonexchangeable sterol domain (f_2 ~0.7) was also reported to be present in model membranes (2, 22, 50), erythrocyte ghosts (25, 56–58), and L-cell fibroblast plasma membranes (26, 27). While sterol fractions resolved on the basis of exchange-dependent DHE fluorescence polarization change represent kinetic and not structural domains, evidence in the literature indicates that sterol kinetic and structural domains may be related. The steady-state and time-resolved fluorescence anisotropy experiments on model membranes with incorporated fluorescent sterols (DHE and cholestatrienol) demonstrated the existence of several sterol fractions in the lipid bilayer (49, 59–63). The size and number of those fractions are in a good agreement with membrane cholesterol phase diagram studies employing other methods (review in 2). In addition, multiple fluorescence lifetimes of DHE and cholestatrienol in liposomes (2, 59, 60) are consistent with a non-uniform distribution of sterol molecules in model membranes. In organic solvents, these fluorescent sterols display a single lifetime.

Third, the parameters of sterol transfer revealed that the domain fractions and their half-times are affected not only by the type of donor membranes, but also by the type of acceptor. This tendency can be clearly seen when the PM acceptor in the PM-PM donor-acceptor pair was replaced by microsome acceptor (Table 1), or when MICRO acceptor in MICRO-MICRO combination was substituted by plasma membranes (Table 2). The first replacement demonstrated significant (2-fold) decrease in the fraction of the exchangeable domain without alteration of $t_{1/2}$ value (Table 1), while the second replacement was accompanied by 2-fold reduction in the exchangeable domain fraction and 3-fold decline in the respective $t_{1/2}$ value (Table 2). Thus, the commonly held assumption (based on model membranes studies) that the desorption of sterol molecules from a donor membrane, followed by their passive diffusion through the aqueous phase, is the rate-limiting step in sterol transfer process (24, 64) is not generally applicable to subcellular organelle membranes. Thus, acceptor membrane properties as well as desorption rate can play a significant role in this process. In this regard, some recent evidence in model membranes has shown a similar dependence of sterol and phospholipid transfer on the nature of the acceptor (25, 59, 64).

Fourth, spontaneous sterol transfer in heterogeneous donor-acceptor pairs is vectorial in nature, i.e., sterol movement in one direction may proceed much faster than in the opposite direction. Indeed, as shown in Tables 1–3, exchange half-times for spontaneous sterol transfer from PM donor to MICRO or MITO acceptor, and from MICRO donor to MITO acceptor are 3- to 5-fold slower as compared to sterol trafficking in the opposite direction.

Finally, perhaps the most significant finding made in this investigation is that SCP-2 is an effective and diverse stimulator of sterol trafficking among a variety of intracellular membranes in vitro. The protein at a concentration of 1.5 μM [within the range of that of present in normal tissues, 1–50 μM (65, 66)] potentiated sterol movement both in similar and dissimilar donor-acceptor pairs (Tables 1–3). SCP-2 was the most effective in accelerating sterol transfer (12-fold decrease in $t_{1/2}$) in the PM-MICRO donor-acceptor pair (Table 1). In contrast, SCP-2 was much less effective accelerating sterol transfer from MICRO to PM, 3- versus 12-fold decrease in $t_{1/2}$ (Tables 1 and 2). These data would suggest that the kinetics of SCP-2-mediated sterol transfer between PM and MICRO are unlikely to account for the enrichment of cholesterol in the plasma membrane. Another striking example for the vectorial nature of this SCP-2 stimulatory effect can be seen in the contrasting data obtained with the PM-MITO and MICRO-MITO donor-acceptor pairs. The SCP-2 did not significantly stimulate sterol movement from PM to MITO and from MICRO to MITO (Tables 1 and 2), but prominently accelerated sterol flux in the reversed direction, by 3- and 2-fold, respectively (Tables 1 and 3). Furthermore, the SCP-2-enhanced sterol transfer in the latter examples appeared to be opposite to the cholesterol gradient. As plasma membranes have more cholesterol than microsomes, which in turn have more cholesterol than mitochondria,

TABLE 3. Kinetic parameters of spontaneous and protein-mediated sterol exchange between mitochondrial membranes-donors and different acceptors

Donor-Acceptor	Protein	f_1	f_2	$t_{1/2}$ (min)
MITO-MITO	none	0.16 ± 0.05	0.84 ± 0.05	51 ± 9
	SCP-2	0.10 ± 0.01	0.90 ± 0.01	16 ± 1[a]
	L-FABP	0.11 ± 0.02	0.89 ± 0.02	43 ± 7
MITO-PM	none	0.45 ± 0.05[c]	0.55 ± 0.05[c]	188 ± 38[c]
	SCP-2	0.33 ± 0.05[c]	0.67 ± 0.05[c]	70 ± 9[b,c]
	L-FABP	0.35 ± 0.05[c]	0.65 ± 0.02[c]	160 ± 28[c]
MITO-MICRO	none	0.87 ± 0.13[c]	0.13 ± 0.13[c]	200 ± 73[d]
	SCP-2	0.78 ± 0.02[c]	0.22 ± 0.02[c]	75 ± 14[c]
	L-FABP	0.34 ± 0.02[c]	0.66 ± 0.04[c]	146 ± 43[d]

The kinetics of sterol transfer for different donor-acceptor pairs were analyzed on the basis of the observed DHE fluorescence polarization change and standard curves (see Methods). MITO-MITO: $P = b_0 + b_1*Xd + b_2*X_d^2$, $b_0 = 0.3309$; $b_1 = 0.0069$; $b_2 = -0.074$. MITO-PM: $P = b_0 + b_1*X_d + b_2*X_d^2$, $b0 = 0.3141$; $b_1 = 0.0238$; $b_2 = -0.1121$. MITO-MICRO: $P = b_0 + b_1*X_d + b_2*X_d^2 + b_3*X_d^3$, $b_0 = 0.2539$; $b_1 = 0.0983$; $b_2 = -0.1422$; $b_3 = 0.0280$. Values represent the mean ± SEM (n = 3–7). All measurements were performed at 37°C.

[a]Represents $P < 0.05$ as compared to no protein.

[b]Refers to $P < 0.1$ as compared to no protein.

[c]Represents $P < 0.05$ as compared to MITO-MITO.

[d]Refers to $P < 0.1$ as compared to MITO-MITO.

this observation suggests that SCP-2 may not play a rate-limiting role in transfer of cholesterol from extramitochondrial sources to mitochondria.

Unlike SCP-2, L-FABP under the same conditions only modestly (2-fold) stimulated sterol transfer in select donor-acceptor combinations (e.g., PM-MITO and MICRO-MICRO, Tables 1 and 2), and was without significant effect in other combinations. It is of interest that L-FABP was without effect when mitochondria were donors of fluorescent sterol (Table 3). The results on stimulation of sterol movement by SCP-2 and L-FABP in vitro could lead to the erroneous conclusion that the latter protein has little physiological effect regarding regulation of sterol trafficking in vivo. It must be recognized, however, that SCP-2 and L-FABP are characterized by very different intracellular concentrations and subcellullar compartmentalization. L-FABP concentration in liver and intestine is up to 63-fold higher than that of SCP-2 (66). In addition, L-FABP is mainly cytosolic (1, 23, 66), while SCP-2 is in cytosol, peroxisomes, and/or mitochondria (6).

In most cases, SCP-2 and L-FABP enhanced sterol transfer by decreasing the half-time values without alteration in the sterol domain fractions (Tables 1–3). This observation is consistent with a mode of SCP-2 and L-FABP action whereby the proteins increase the rate of sterol transfer by increasing the rate of sterol desorption from the donor membrane (28, 64, 67, 68) without major sterol redistribution within a bilayer. However, it appears that SCP-2 and L-FABP can also stimulate sterol transfer via sterol rearrangements among the kinetic domains. The latter was observed when SCP-2 and L-FABP increased the fraction of exchangeable domains in PM-PM (1.3-fold), MICRO-MICRO (1.4-fold), and PM-MICRO (1.3-fold) donor acceptor pairs, respectively, while not affecting $t_{1/2}$ values (Tables 1 and 2). The phenomenon of sterol redistribution among the kinetic domains induced by SCP-2 was observed earlier in model membranes (40).

In summary, the results presented in this report extend our knowledge on the existence and structure of sterol kinetic domains in subcellular membranes and potential modulation of intracellular sterol trafficking by SCP-2 and L-FABP.

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