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Mechanistic studies of sterol carrier protein-2 effects on L-cell fibroblast plasma membrane sterol domains

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The factors which regulate intermembrane sterol domains and exchange in biomembranes are not well understood. A new fluorescent sterol exchange assay allowed correlation of changes in polarization to sterol transfer. Analysis of spontaneous sterol exchange between L-cell plasma membranes indicated two exchangeable and one very slowly or nonexchangeable sterol domain. The exchangeable domains exhibited halftimes of 23 and 140 min with fractional contributions of 5 and 30%, respectively. Sterol carrier protein-2 (SCP-2) enhanced sterol exchange between L-cell plasma membranes and altered sterol domain size in a concentration dependent manner. Previous model membrane studies indicate that SCP-2 alters sterol domains and exchange through interaction with anionic phospholipids. In contrast to these observations, the ionic shielding agents KCl, low pH, or neomycin were either totally or partially ineffective inhibitors of SCP-2 action in L-cell plasma membrane exchanges. Thus the mechanism of SCP-2 in sterol transfer appears to be less charge dependent in L-cell plasma membranes than in model membranes. The cholesterol lowering drug probucol was also capable of altering the sterol exchange kinetics.

Introduction

Approximately 90% of cellular cholesterol resides in the plasma membrane in a non-random intramembrane distribution (for review, see Refs. 1,2). Structural sterol domains exist in membranes, i.e., cholesterol-rich and cholesterol-poor regions [1–3]. Microvillar and canalicular plasma membrane fractions, for example, are cholesterol rich while basolateral and serosal plasma membrane fractions from the same cells are cholesterol poor (for review, see Ref. 1). In the case of intestinal, hepatic, and renal epithelial cells, these macrodomains are easily separable by sedimentation techniques.

On a molecular scale, the distribution of cholesterol can be described by both transbilayer [2,4] and lateral [1] cholesterol domains. Sterol exchange kinetics and structural data are consistent with multiple cholesterol domains in plasma membranes of mycoplasma [5], cultured cells [1,6–8], and erythrocytes [9,10] as well as in model membrane vesicles [1,11–18]. At least three cholesterol domains have been resolved and defined kinetically by their halftime of exchange to be rapidly, slowly, or very slowly exchangeable [1]. Some earlier studies reported the existence of only one kinetic cholesterol pool in both biological [19,20] and model [21,22] membrane systems. However, in these published studies, there were at most two time points reported per hour. Thus, discrimination between the

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Abbreviations: LDL, low density lipoprotein; SCP-2, sterol carrier protein-2; MDP, *N*-α-succinyl-Leu-Leu-Glu-Lys-Leu-Leu-Glu-Lys-Leu-Lys-NH_2; FBS, fetal bovine serum; BOP, benzotriazo-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; HF, hydrogen fluoride; DIEA, *N*,*N*-diisopropylethylamine; TFA, trifluoroacetic acid; Ac-SCP-2_{13-24}amide, CH_3CO-Phe-Lys-Glu-Ile-Glu-Lys-Lys-Leu-Glu-Glu-Glu-Gly-NH_2; Boc, *t*-butyloxycarbonyl; DMF, *N*,*N*-dimethylformamide; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FBS, fetal bovine serum; Pipes, 1,4-piperazinediethanesulfonic acid; $^1t_{1/2}$, halftime of sterol exchange from the rapidly exchangeable sterol domain; $^2t_{1/2}$, halftime of sterol exchange from the slowly exchangeable sterol domain; f_1, rapidly exchangeable sterol domain; f_2, slowly exchangeable sterol domain; f_3, nonexchangeable sterol domain.

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small rapidly exchangeable and larger slowly exchangeable pools would not be possible. In some of the above studies [6,8,19–21], all the cholesterol was found to be exchangeable, while in others, a portion was nonexchangeable [1,9,11–15,17,18]. Kan and Bittman [23] recently proposed that this discrepancy may be due to the length of the experiment. They found that the calculated size of the nonexchangeable pool in sphingomyelin bilayers decreased 63% when 7.6 days data were analyzed vs. 0.7 days. Biological membrane samples are subject to natural degradation which does not allow for such long measurements.

Although the factors regulating cholesterol domain size and exchange rate in the plasma membrane are not known, they are expected to have relevance in both reverse cholesterol transport [24] and intracellular cholesterol homeostasis [25]. Active research to identify these factors includes the study of lipid binding proteins such as sterol carrier protein-2. This intracellular protein both binds sterols [26] and modulates sterol domains in phospholipid/cholesterol model membranes [1,26]. However, the physiological function of SCP-2 remains unclear.

It was shown previously in model membranes that the presence of negatively charged phospholipids enhanced SCP-2 mediated sterol transfer [27]. On the basis of charge shielding studies, it was concluded that direct interaction of the SCP-2 ($pI = 8.6$ [27]) with the anionic phospholipids occurred in model membranes [28]. The present investigation uses a sterol exchange assay adapted from model membranes [11,12,15,29] to examine the mechanism by which SCP-2 alters L-cell plasma membrane lateral sterol exchange.

Materials and Methods

Materials. SCP-2 was isolated and characterized as previously described [28]. Dehydroergosterol was synthesized and purified per the methods of Fischer et al. [30,31]. MDP peptide and probucol were generous gifts of Marion Merrell Dow Research Laboratories (Cincinnati, OH). FBS was purchased from GIBCO BRL (Grand Island, NY). Neomycin was from Sigma (St. Louis, MO). *p*-Methylbenzhydrylamine resin and protected amino acids were from Peptides International (Louisville, KY). Additional amino acids were from Bachem (Torrence, CA). BOP was from Advanced Chemtech (Louisville, KY); HF was from Curtis Matheson Scientific, (Cincinnati, OH); DIEA and ninhydrin were from Aldrich (Milwaukee, WI); and both TFA and HPLC grade acetonitrile were from Fisher Scientific (Pittsburgh, PA). All other chemicals were of reagent grade or better.

Peptide synthesis and characterization. Synthesis of Ac-SCP-2_{13-24}amide was carried out via standard solid phase methods [29] on a Vega Coupler 1000 (Bachem, Torrence, CA). Boc protection of the N^{α}-amine groups was used throughout. Side chain protection was as follows: Lys, 2,6-dichlorobenzyloxycarbonyl; Glu, *O*-cyclohexyl. Coupling of amino acids was performed in DMF using 3 equivalents protected amino acid, 3 equivalents BOP reagent, and 9 equivalents DIEA. Each coupling step was monitored using ninhydrin [32]. Liquid HF was used to deprotect the completed peptide and cleave it from the resin. The HF was evaporated in vacuo and the peptide extracted with glacial acetic acid prior to lyophilization.

The crude material was purified by semi-preparative HPLC [ISCO 2350 HPLC (ISCO, Lincoln NE) equipped with a C_{18} reverse-phase column (Vydac 218TP1010, Hesperia, CA) and an ISCO V4 uv-vis detector] using a linear gradient from 0–40% CH_3CN (containing 0.1% TFA) in 1 h, flow rate 4 ml/min. Analytical HPLC of the major component isolated was carried out using a Vydac C_{18} reverse-phase column (model 218TP104) with the same elution gradient as above at a flow rate of 1 ml/min. Amino acid analysis of Ac-SCP-2_{13-24}amide was performed with a Picotag gas phase amino acid sequencing apparatus (Millipore, Bedford, MA)

Incorporation of dehydroergosterol into L-cells. Mouse L-cells (L $aprt^{-}tK^{-}$ from Dr. David Chaplin, Washington University, St. Louis, MO) were grown in Higuchi medium supplemented with 10% FBS as described earlier [33]. Immediately after subculture (1:3 dilution), stock dehydroergosterol (4 mg/ml, 95% nondenatured grain ethanol) was added to the medium at a final concentration of 10 μg/ml medium [34]. The final concentration of ethanol did not exceed 0.5% (v/v). After 3 days culture, the cells were harvested.

Plasma membrane isolation and characterization. Plasma membranes were isolated from cells (cultured either with or without dehydroergosterol) using sucrose gradient centrifugation [33]. Purification of the plasma membrane was determined by the marker enzyme Na^{+},K^{+}-ATPase whose specific activity increased 6.5-fold in the plasma membrane fraction vs. the crude homogenate. The plasma membrane lipids were extracted and fractionated into neutral lipids and phospholipids [33]. Reverse phase HPLC (Perkin Elmer Series 4 Liquid Chromatograph equipped with a LC-95 uv/vis Spectrophotometer Detector and a C_{18} reverse phase column) of the neutral fraction resolved cholesterol and dehydroergosterol [30,31]. Supplementation with dehydroergosterol did not alter the plasma membrane sterol/phospholipid ratio, phospholipid composition, or phospholipid fatty-acyl composition [34].

Fluorescence polarization measurement. Dehydroergosterol polarization was measured at 37°C in the T-format with an SLM 4800 Spectrofluorometer (SLM-Aminco, Urbana, IL) equipped with a 450 watt Xe-arc lamp. Excitation was at 325 nm. As shown

previously, photobleaching did not occur under these conditions [17]. Sample absorbance at 325 nm was maintained below 0.15 in order to preclude inner filter effects. Light scattering was reduced through use of GG-375 emission cutoff filters (Janos Technology, Townshend, VT).

Sterol exchange assay. Donor plasma membranes (up to 14 mol% of total lipid as dehydroergosterol) were diluted in Pipes (10 mM, pH 7.4) to 3.5 μg protein/ml. Where indicated, effector proteins, drugs, or peptides were added at this stage. Acceptor plasma membrane (35 mol% of total lipid as cholesterol) was added to give a final concentration of 38.5 μg protein/ml. The polarization was taken continuously and averaged every 20 s for four hours.

Initial rates were determined from the first five minutes and first one minute of exchange for spontaneous and SCP-2 mediated exchange, respectively. Light scatter contributions from the acceptor membranes were subtracted from the entire curve prior to computer analysis as described in the following sections.

Dehydroergosterol standard curve for plasma membrane sterol exchange assays. Plasma membranes were isolated from L-cells supplemented with increasing dehydroergosterol concentration. The fluorescence polarization of the membranes was measured as described above. The decrease of polarization in L-cell plasma membrane with increasing mole fraction dehydroergosterol was non-linear (Fig. 1) in agreement with earlier model membrane studies [11,12,27,35,36]. Radiationless energy transfer among fluorophores causes a hyperbolic dependency of polarization on fluorophore concentration [37]. The polarization data in Fig. 1 display the same pattern yielding a hyperbolic fit, Eqn. 1, with the parameters $P_o = 0.3366$ and $B = 0.0576$ ($r^2 = 0.946$)

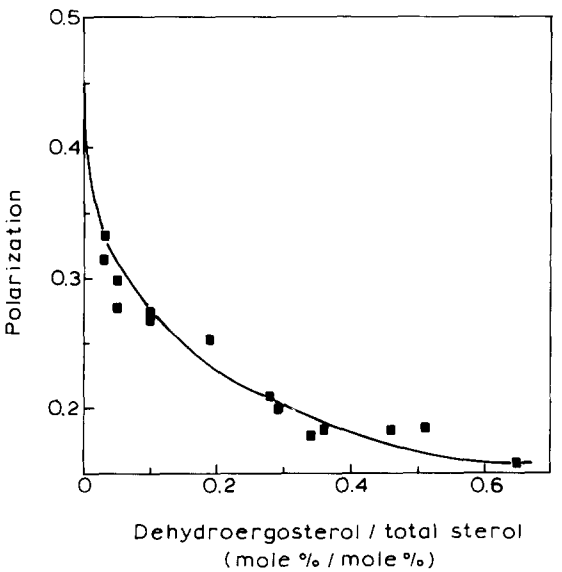

Fig. 1. Polarization of dehydroergosterol as a function of the fluorophore concentration in plasma membranes. The data are best fit by a hyperbolic curve, Eqn. 1, with the parameters $P_o = 0.3366$ and $B = 0.0576$. The total sterol content of the membranes was 35 mol%.

$$P = P_o C/(B + C) \quad (1)$$

where P is polarization and C is concentration. Thus, procedures for determining sterol molecular exchange from non-linear polarization data were adapted from model membrane systems [35] to L-cell plasma membranes.

The data in Fig. 1 and Eqn. 1 described the special case obtained when donor plasma membrane vesicles are the only fluorescent species present. In an exchange assay, polarization contributions from dehydroergosterol which has transferred from donor to acceptor must also be accounted for. The relative concentration of fluorescent dehydroergosterol present in the donor (χ_d) and acceptor (χ_a) plasma membranes at any time are given as

$$\chi_d = C_d/C_t \quad (2)$$

$$\chi_a = 1 - \chi_d = 1 - (C_d/C_t) \quad (3)$$

where C_t and C_d represent the initial and current dehydroergosterol concentrations in the donor plasma membrane, respectively. Application of Eqn. 4 to Eqn. 5 for many values of χ_d describes the polarization dependence for a sample containing both donor and acceptor [35]

$$P = 3r/(2 + r) \quad (4)$$

$$r = r_o\{\chi_d/(1 + D\chi_d) + (1 - \chi_d)/[1 + D(1 - \chi_d)/10]\} \quad (5)$$

where r is anisotropy, and r_o is anisotropy at infinite dilution. D is related to the constant B in Eqn. 1 as described in Eqn. 6

$$D = Z \cdot B[1 + (r_o/2)] \quad (6)$$

where Z is the mol% of total lipid as dehydroergosterol.

In the model membrane studies [35], the vesicles were prepared such that Z was constant at 35 mol%. Analysis of preliminary plasma membrane data in a review article [1] was based on a standard curve assuming $Z = 35$ mol%, i.e., all the donor membrane sterol was dehydroergosterol. This assumption was not correct. In the present investigation, the dehydroergosterol content ranged from 7–14 mol%. The standard curve method has been refined to take into account the variation of initial dehydroergosterol concentration in the donors.

A polynomial equation was computer fit to the data points calculated using Eqns. 4–6. Shown in Fig. 2 are

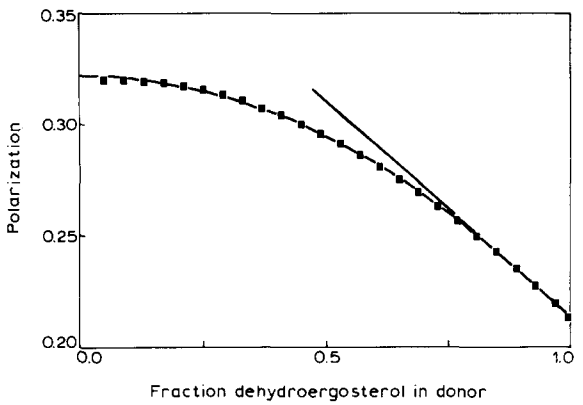

Fig. 2. Polarization in the donor/acceptor mixture as a function of relative concentration, χ_d, of dehydroergosterol in donor plasma membranes. The points were calculated using Eqns. 7–9 with an initial dehydroergosterol concentration of 10 mol% total lipid in the donor. A computer fit of the data yielded Eqn. 7 with the parameters $a = 0.1090$ and $c = 0.3219$. The straight line depicts the near linearity of the polarization change in the region $\chi_d = 0.8$–1.0, which would be at the initial stages of an exchange assay. This figure differs from that of Fig. 1 in that Fig. 1 describes a homogenous population of membranes, while this figure shows the polarization of a donor/acceptor mixture as a function of dehydroergosterol content in donor.

the data points and curve determined for the case where the initial dehydroergosterol concentration in the donor was 10 mol% of the total lipid. The curve was described by Eqn. 7 with the parameters $a = 0.1090$ and $c = 0.3219$ ($r^2 = 0.999$).

$$P = -a\chi_d^2 + c \tag{7}$$

The straight line in Fig. 2 showed that the polarization changed linearly from 0.8–1.0 χ_d. Thus, in an exchange assay, the initial 0–20% of dehydroergosterol transfer was directly proportional to the polarization change. The parameters a and c were calculated for each initial dehydroergosterol concentration and the appropriate values were used in the computer fit of Eqn. 9 below.

Computer analysis of exchange data. Earlier results from this laboratory using model membrane systems suggested that sterol exchanges with bi-exponential kinetics [11,12,35] described by the following equation:

$$\chi_d = A_2 \cdot \exp(-k_{E_2} t) + B_2 \cdot \exp(-k_{E_2} t) + C_2 \tag{8}$$

When Eqn. 8 is inserted into Eqn. 7, we obtain:

$$P = -a[A_2 \cdot \exp(-2k_{E_1} t) + 2A_2B_2 \cdot \exp(-k_{E_1} t - k_{E_2} t) + B_2 \cdot \exp(-2k_{E_2} t) + 2A_2C_2 \exp(-k_{E_1} t) + 2B_2C_2 \exp(-k_{E_2} t)] + c \tag{9}$$

where A_2, B_2, and C_2 represent the two exchangeable and the nonexchangeable sterol fractions, respectively, k_{E_1} and k_{E_2} represent the corresponding rate constants of the exchangeable fractions, e = 2.718, and the constants a and c are those from Eqn. 7. Eqn. 9 was used in the fitting of the experimental data. Although the small rapidly exchangeable pool in control exchanges could be combined with the slowly exchangeable pool to give a statistically acceptable one-exponential fit, the one-exponential equation was not used for two reasons. First, increasing the size of the rapidly exchangeable pool by a modest 3.9% (by addition of SCP-2) resulted in distinctly non-random residuals for a one exponential fit. Second, two-exponential analysis of exchange data from microsomal membranes collapses to yield only one exchangeable pool (unpublished data). Thus the rapidly exchangeable pool calculated in plasma membranes is significant.

Results

Exchange assay polarization curves

The polarization curves depicted in Fig. 3 are representative of typical exchange data obtained in the absence and presence of SCP-2. The line at the bottom of the figure, curve A, was obtained in the absence of acceptor vesicles and demonstrates the stability of the donors over the time course of the experiments. While the curves shown in Fig. 3 are consistent with those shown in a preliminary report [1], the standard curve

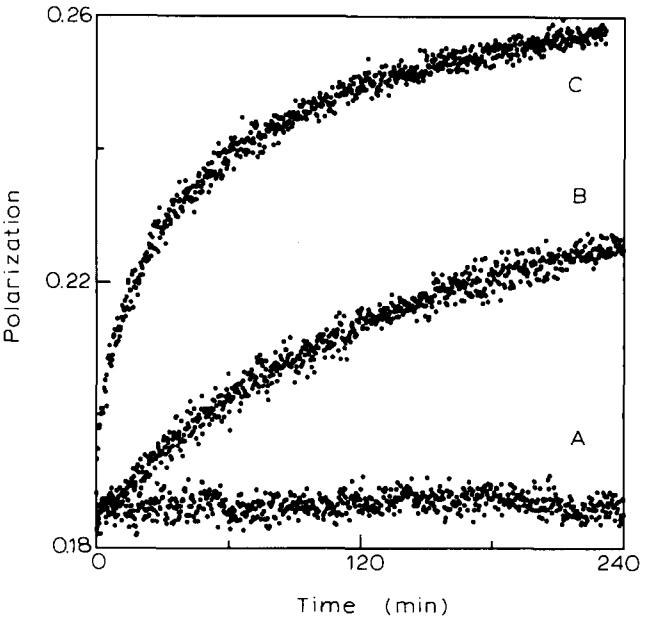

Fig. 3. Polarization as a function of time upon addition of 70 μg acceptor plasma membranes (100% of sterol as cholesterol) to 7 μg donor plasma membranes (up to 40% of sterol as dehydroergosterol). Curve B is spontaneous exchange while curve C was obtained with 1.5 μM SCP-2 present in the assay medium. The bottom curve (A) is polarization of 7 μg donor plus 1.5 μM SCP-2. All exchanges were performed at 37°C in 10 mM Pipes (pH 7.4).

analysis was refined herein as described in Materials and Methods. Additional experiments are also reported which were designed to further probe the mechanism of SCP-2 action.

SCP-2 mediated alterations of sterol domains in L-cell plasma membranes

The refined computer analysis resolved the complex kinetics of the exchange into three kinetic domains: rapidly, slowly and very slowly (or nonexchangeable) exchangeable domains. Consistent with a previous preliminary analysis, the exchange halftimes for spontaneous sterol exchange were 23.0 ± 2.1 min and 140 ± 9 min for the rapidly (${}^1t_{1/2}$) and slowly (${}^2t_{1/2}$) exchangeable domains, respectively (Table I). The very slowly exchangeable domain was regarded as nonexchangeable as its exchange halftime was not resolvable in the time frame of these experiments. The fraction of sterol represented by the two exchangeable domains was $4.7 \pm 0.4\%$ and $30.5 \pm 1.7\%$ for the rapidly exchangeable (f_1) and slowly exchangeable (f_2) domains, respectively. The size of the nonexchangeable domain (f_3) was $64.8 \pm 1.8\%$ (Table I). Due to the refinement in the standard curve analysis, the size of the nonexchangeable domain was found to be increased vs. a previous preliminary analysis [1].

In agreement with earlier analysis, SCP-2 modulated the sterol exchange kinetics by decreasing both the halftimes; ${}^1t_{1/2}$ from 23.0 ± 2.1 to 9.1 ± 0.9 min, and ${}^2t_{1/2}$ from 140 ± 9 to 91.2 ± 8.0 min (Table I). The sterol fraction in f_1 increased from $4.7 \pm 0.4\%$ to $8.6 \pm 1.9\%$ at the expense of f_3 which showed a concomitant decrease from $64.8 \pm 1.8\%$ to $56.2 \pm 3.0\%$ (Table I). The relative size of f_2 remained unchanged. Previous preliminary analysis indicated larger changes in both f_1 and f_3 as well as a decrease in f_2 [1]. The effect of SCP-2 on sterol exchange was concentration dependent as indicated by the enhancement of the initial rate of sterol exchange (Fig. 4). This effect on initial rate was maximal near 3–4 μM SCP-2.

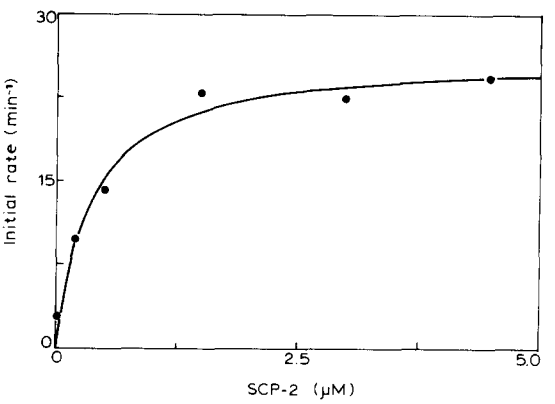

Fig. 4. Initial rate of dehydroergosterol exchange between donor and acceptor membranes as a function of SCP-2 concentration. The initial rates were determined for the first five minutes of four hour exchanges in the absence of SCP-2, and for the first minute of exchange in the presence of SCP-2. A representative plot is shown in Fig. 3. Conditions were as described in Fig. 3.

Effect of pH on spontaneous and SCP-2 mediated sterol exchange

Lowering the pH of the medium provides free hydrogen ions which can shield negatively charged surfaces from the bulk solution. In addition, the overall membrane will be less negatively charged through alteration of the ionization state. Thus, lowering the pH allows investigation of the relevance of ionic interaction between SCP-2 and the L-cell plasma membrane surface phospholipids. Decreasing the pH from 7.4 to 4.5 increased the rate of spontaneous exchange kinetics by decreasing ${}^1t_{1/2}$ and ${}^2t_{1/2}$ to 7.1 and 99.3 min,

TABLE I

Effect of pH, ionic strength, and sterol-lowering drugs on sterol exchange between plasma membranes isolated from L-cells

Exchange of dehydroergosterol from donor to acceptor membranes was measured by an increase of fluorescence polarization as described in Materials and Methods. Any effector present during the individual exchanges is given under conditions. Average values ±S.E. are for $n = 3$–19, the other values are for $n = 1$. An asterisk and double asterisk represent $P < 0.05$ and $P < 0.01$, respectively. Student's t-test was not possible for data with $n = 1$, in these cases a dagger indicates a change which appears to be significant.

Conditions	Halftimes		Fractional domains		
	${}^1t_{1/2}$ (min)	${}^2t_{1/2}$ (min)	f_1 (%)	f_2 (%)	f_3 (%)
Control (pH 7.4)	23.0±2.1	140 ± 9	4.7±0.4	30.5±1.7	64.8±1.8
SCP-2 (1.5 μM)	9.1±0.9 **	91.2± 8.0 **	8.6±1.9 *	34.4±2.2	56.2±3.0 **
pH 4.5	7.1 †	99.3 †	5.2	22.2	71.1
pH 4.5 and SCP-2	5.3 †	41.8 †	14.6 †	17.2	65.7
KCl (0.6 M)	11.9±1.2 **	126 ± 8	1.8±0.2 **	36.5±1.5 *	62.6±0.8
KCl and SCP-2	19.0±1.2	101 ± 2 **	6.6±0.2 **	44.8±0.8 **	48.1±0.6 **
Neomycin (10 μM)	13.4±5.2	135±14	5.4±0.5	36.1±4.7	56.7±4.7
Neomycin and SCP-2	15.2±4.8	146 ± 6	8.2±0.3 **	32.6±1.6	57.8±1.5 **
Probucol (75 μM)	45.0±7.3 **	182 ± 1 **	7.4±1.7	37.4±4.1	55.3±5.5
Probucol and SCP-2	7.7 †	90.6 †	9.0	36.5	52.5

respectively (Table I). While not an additive effect, the SCP-2 and lowered pH in combination decreased the halftimes of exchange to a larger extent than either agent by itself. Lowering the pH of the medium to 4.5 lowered the halftimes to 5.3 and 41.8 min for the rapidly and slowly exchangeable domains, respectively. The relative content of f_1 increased to 14.6%, apparently at the expense of the slowly exchangeable domain (Table I). Thus, simple shielding of negatively charged groups on the membrane surface with protons was not effective in blocking SCP-2 effects on sterol domains. These data are in contrast to those obtained with model membrane systems in which lowering the pH both decreased the spontaneous exchange of sterol and blocked SCP-2 enhanced sterol exchange [28].

Ionic charge screening by KCl

Another approach to investigate the proposed mechanism is the direct screening of plasma membrane surface charge by a salt, KCl, added to the assay media. First, although KCl increased the rate of spontaneous exchange from the rapidly exchangeable domain (${}^1t_{1/2}$ from 23.0 ± 2.1 min to 11.9 ± 1.2 min), the size of this domain was decreased from $4.7 \pm 0.4\%$ to $1.8 \pm 0.2\%$ (Table I). The excess sterol was transferred to f_2 (30.5 ± 1.7 to $36.5 \pm 1.5\%$). Second, the combination of both KCl and SCP-2 served to lower the halftime of exchange ${}^2t_{1/2}$ from 140 ± 9 to 101 ± 2 min, while ${}^1t_{1/2}$ remained unchanged. The nonexchangeable domain, f_3, was decreased from 64.8 ± 1.8 to $48.1 \pm 0.6\%$. Both exchangeable domains increased, to 6.6 ± 0.2 and $44.8 \pm 0.8\%$ for f_1 and f_2, respectively. These changes are similar to the effect of SCP-2 alone with the exception that ${}^1t_{1/2}$ was not decreased in the additional presence of KCl. The results obtained with both SCP-2 and KCl present indicate either an incomplete blockage of the charged groups on the membrane or involvement of a more complex interaction.

Neomycin effects on SCP-2 mediated sterol exchange

Neomycin is a polyamine antibiotic that binds to negatively charged phospholipids [38,39]. Earlier work indicated that neomycin inhibited the SCP-2 enhancement of sterol exchange in model membranes by 98% [27]. Thus, neomycin should also screen charges on biomembrane lipids and may provide a better means to shield charges than KCl.

In contrast to a preliminary report from this laboratory [1] neomycin alone had no effect on spontaneous sterol exchange between L-cell plasma membranes (Table I). However, the presence of neomycin blocked the SCP-2 mediated decrease in $t_{1/2}$ values but did not effect the action of SCP-2 on relative sterol domain size in L-cell plasma membranes when both neomycin and SCP-2 were present. The size of f_1 increased to $8.2 \pm 0.3\%$ and f_3 decreased to $57.8 \pm 1.5\%$ (Table I). As only a portion of SCP-2's effects were inhibited by neomycin, there is a complex relationship between SCP-2, biological membranes, and sterol transfer.

Modulation of L-cell plasma membrane sterol domains by probucol

Probucol is also a cholesterol lowering drug. However, probucol is hydrophobic and will not provide any charge shielding. In contrast to other agents investigated, probucol inhibited the rate of sterol exchange (Table I). The exchange halftimes, ${}^1t_{1/2}$ and ${}^2t_{1/2}$, increased from 23.0 ± 2.1 min to 45.0 ± 7.3 min and from 140 ± 9 min to 182 ± 1 min, respectively. Probucol was ineffective as an antagonist of the action of SCP-2 on the exchange halftimes as these decreased to 7.7 and 90.6 min for ${}^1t_{1/2}$ and ${}^2t_{1/2}$, respectively (Table I). These decreases are on the same order of magnitude as in the presence of SCP-2 alone. Concomitant treatment of L-cell plasma membranes with probucol and SCP-2 also had no effect on the SCP-2 mediated alterations in the relative sizes of the domains. Thus, although probucol altered spontaneous sterol transfer, it was incapable of inhibiting the SCP-2-mediated effects.

Effect of amphipathic peptides on L-cell plasma membrane sterol domains

The above studies employed charge blocking agents to investigate the importance of charge-charge interactions in SCP-2-mediated enhancement of sterol transfer. Assuming this interaction is necessary, an alternative approach is to investigate sterol transfer in the presence of amphipathic peptides which are likely to interact with charged regions of the membrane. Amphipathic peptides may form an amphipathic helix. The helical structure of the synthetic peptide MDP is shown as a wheel diagram in Fig. 5. This structure shows that the charged groups lie largely on one face of the helix. Similar helices are designated as Class A or apolipoprotein-like helices and have been shown to interact with membranes [40]. The charged peptide residues are expected to interact with charged components on the membrane surface. Therefore, this peptide was synthesized and its effects on L-cell plasma membrane sterol kinetics was examined.

Consistent with a previous analysis [1], MDP appeared to lower f_3 from 64.8 ± 1.8 to 50.3% (Table II). The contents of f_1 and f_2 showed very modest increases to 7.8 and 41.3%, respectively. However, in contrast to the effects of SCP-2, ${}^2t_{1/2}$ increased from 140 ± 9 min to 223 min. The halftime of the rapidly exchangeable domain appeared to remain unchanged.

Although the SCP-2 tertiary structure is unknown, the amino acid sequence [41] suggests a region of amphipathic character, residues 13–24, which may form an amphipathic helix and could represent a membrane

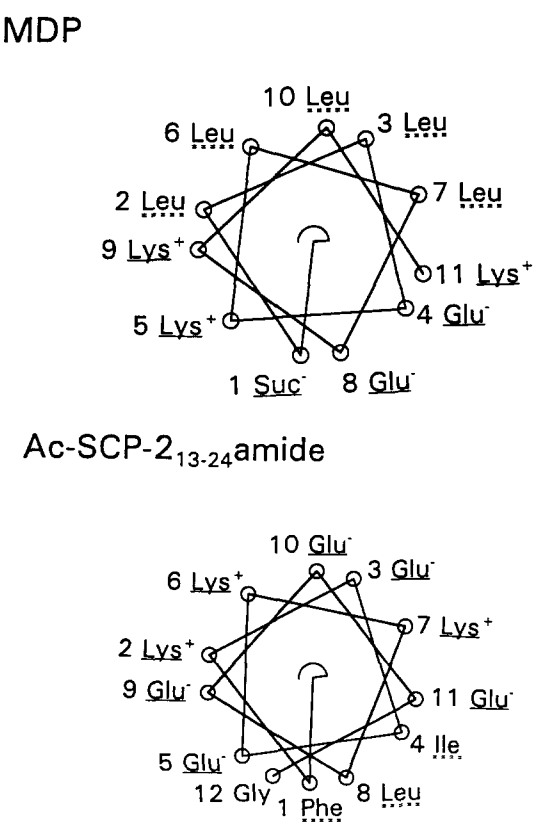

Fig. 5. Helix wheel diagrams of the peptides MDP and Ac-SCP-2_{13-24}amide. The diagrams were generated using the MacProt program (Dr. A. Luttke, Koln, Germany). The underlined residues are charged; dotted underlined residues are hydrophobic in nature.

interaction site. The structure of this peptide, designated as Ac-SCP-2_{13-24}amide, is shown as a wheel diagram in Fig. 5. This helix is similar to that of MDP. The effect of Ac-SCP-2_{13-24}amide on sterol exchange was investigated to determine if an SCP-2 derived peptide could better mimic SCP-2 action than the general amphipathic helix MDP. As in the case of neomycin and in agreement with preliminary analysis [1], it was determined that Ac-SCP-2_{13-24}amide had no effect on spontaneous sterol exchange. In summary, the actions of SCP-2 in enhancing sterol exchange were not mimicked by simple amphipathic peptides capable of interacting with membrane surfaces.

Discussion

The primary purpose of this investigation was the examination of ionic interactions in SCP-2 effects on plasma membranes which were previously observed in model membrane studies. In the present study a more sophisticated data treatment was used which provided more accurate results. As discussed in Materials and Methods, the computer analysis indicates the presence of three kinetic sterol pools. Multiple fluorescence lifetimes of dehydroergosterol [1] are consistent with the multiple sterol pools in L-cell plasma membranes detected in this study. The nonexchangeable pool in L-cell plasma membranes was larger (64.8%) than reported with a preliminary analysis [1].

Although these kinetic studies are incapable of identifying the exact physical nature of the sterol domains, several possibilities may be drawn from the published literature. (i) Electron micrographs indicate cholesterol increases the ripple repeat distance in DMPC vesicles [42,43]. The smooth portion of the ripple was suggested to be cholesterol by Mortensen et al. [44]. However, the resolution is not at the molecular level. (ii) Phase diagrams (NMR, ESR, DSC) also indicate the co-existence of up to three lipid phases in similar systems [43,45]. (iii) Houslay and Stanley [43] proposed that cholesterol existed as monomers when the total cholesterol was lower than 22%, as dimers between 22% and 31%, and as larger aggregates, possibly even pure cholesterol patches, above 31%. (iv) DPPC/cholesterol membrane models determined by Monte Carlo simulation [46] indicated that cholesterol accumulates at lipid domain interfaces. (v) In the complex erythrocyte plasma membrane [43], interaction of cholesterol with phospholipids was cited as a potential explanation for the presence of multiple kinetic pools of sterol. (vi) In contrast, studies of intestinal brush

TABLE II

Effect of sterol carrier protein and helical peptides on sterol exchange between plasma membranes isolated from L-cells

Exchange of dehydroergosterol from donor to acceptor membranes was measured by an increase of fluorescence polarization as described in Materials and Methods. Any effector present during the individual exchanges is given under conditions. Average values ±S.E. are for $n = 5$–19, the other values are for $n = 1$. An asterisk and double asterisk represent $P < 0.05$ and $P < 0.01$, respectively. Student's t-test was not possible for data with $n = 1$, in these cases a dagger indicates a change which appears to be significant.

Conditions	Halftimes		Fractional domains		
	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)	f_1 (%)	f_2 (%)	f_3 (%)
Control	23.0±2.1	140 ±9	4.7±0.4	30.5±1.7	64.8±1.8
+SCP-2 (1.5 μM)	9.1±0.9 **	91.2±8.0 **	8.6±1.9 *	34.4±2.2	56.2±3.0 **
+SCP-2_{13-24} [a] (50 μM)	28.4	110	2.0	27.9	69.7
+MDP (15 μM)	30.7	223	7.8	41.3	50.3

[a] SCP-2_{13-24} is an abbreviation for Ac-SCP-2_{13-24}amide.

border membranes indicated that membrane components other than phospholipids, such as proteins, may be responsible [8]. (vii) Nemecz et al. [11] have proposed a simplified model in which the rapidly exchangeable pool is composed of sterol monomers, the slowly exchangeable pool comprises sterol enriched portions of the membrane, and the nonexchangeable pool is made up of nearly pure cholesterol patches. The factors responsible for this segregation may be among those described above or others hitherto unknown.

Further studies were undertaken to investigate the mechanism of SCP-2 action in mediating sterol exchange. The results of these studies clearly show that the lipid binding protein, SCP-2, enhances transfer of sterol between isolated L-cell plasma membranes. This enhancement was found to be concentration dependent with maximal results in a concentration range which is physiologically reasonable, as calculated from Refs. [33,47,48].

SCP-2-mediated sterol transfer between model vesicle membranes has been shown in the past to be enhanced when anionic phospholipids are present in the membranes [28]. To determine if ionic effects are also operative in the plasma membrane system, further data were collected in experiments with charge shielding agents added to the assay mixture. The three agents; protons (lowered pH), KCl, or neomycin, were all incapable of completely inhibiting SCP-2's effects. Therefore, simple charge shielding is insufficient to block SCP-2 action.

These results are in contrast to those observed with the model vesicle membranes where the charge shielding agent was able to inhibit SCP-2 50-fold [28]. Indeed, the combination of lowered pH and SCP-2 resulted in the halftimes decreased beyond that in the presence of only one agent. As the pH is lowered, SCP-2 ($pI = 8.6$ [27]) becomes increasingly positively charged. This increase in charge may increase charge-charge interactions between SCP-2 and the membrane.

The results obtained using KCl to shield the charges were even more complex. The presence of KCl alone lowered the value of $^{1}t_{1/2}$ similar to the results of SCP-2 alone. However, when in combination this decrease did not occur. Apparently, the KCl and SCP-2 interact in some unknown manner which results in a slowing of sterol transfer. Although confirming experiments are beyond the scope of this paper, it may be possible that the effect could stem from either the change in the salt gradient or a reordering of the membrane lipids which could effect the sterol domains.

A second method to investigate ionic interactions between SCP-2 and the plasma membrane involves study of a peptide derived from the SCP-2 primary sequence. The twelve residue peptide, Ac-SCP-2_{13-24}amide, contains 22% of all the charged residues in the SCP-2 sequence [49]. Ac-SCP-2_{13-24}amide has eight charged residues and is likely to form an amphipathic helix [50]. Thus, if ionic interactions of SCP-2 with the membrane are important, this peptide may be actively involved. However, Ac-SCP-2_{13-24}amide was unable to alter sterol exchange kinetics. A second unrelated amphipathic helix, MDP, was capable of affecting sterol exchange. Three possible explanations are (1) the SCP-2 region 13–24 is not involved in membrane interactions, (2) this region adopts a different conformation in the whole protein as opposed to the peptide which allows for interaction with the membrane, or (3) ionic interaction with the plasma membrane is not sufficient for SCP-2 to effect sterol exchange.

The polyamine cholesterol lowering agent neomycin, which was chosen to shield negative charges on the membrane, had no effect on sterol exchange kinetics or sterol domain size. In contrast, a second cholesterol lowering agent, probucol, effectively decreased the rate of sterol exchange from both the exchangeable domains. Reverse cholesterol movement from non-hepatic cells through the bloodstream to the liver, requires sterol movement through the plasma membrane. It is possible that cholesterol lowering agents could act at any of the points in this sequence of transport. Thus, probucol could conceivably regulate blood plasma sterol levels by interaction with the plasma membrane. Such an interaction could affect the rate of sterol movement out of the cell. Although probucol is believed to act by lowering blood plasma LDL concentrations [51], many drugs act at more than one site in the body. A common example is aspirin which has among other functions, direct action on platelets, smooth muscle, and the adrenal cortex [52–54]. It is therefore possible that probucol may have actions other than those which specifically lower LDL concentrations.

In summary, the lipid binding protein SCP-2 enhanced sterol transfer from L-cell plasma membranes. The action of SCP-2 was not fully inhibited through charge shielding of the membrane. This indicates ionic interaction of SCP-2 with the membrane does not account for the complete mechanism of enhancement. In addition, a cholesterol lowering agent probucol was effective in altering the kinetics of sterol exchange.

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