Acidic Phospholipids Strikingly Potentiate Sterol Carrier Protein 2 Mediated Intermembrane Sterol Transfer†

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ABSTRACT: A liposomal membrane model system was developed to examine the mechanism of spontaneous and protein-mediated intermembrane cholesterol transfer. Rat liver sterol carrier protein 2 (SCP_2) and fatty acid binding protein (FABP, also called sterol carrier protein) both bind sterol. However, only SCP_2 mediates sterol transfer. The exchange of sterol between small unilamellar vesicles (SUV) containing 35 mol % sterol was monitored with a recently developed assay [Nemecz, G., Fontaine, R. N., & Schroeder, F. (1988) *Biochim. Biophys. Acta 943*, 511-541], modified to continuous polarization measurement and not requiring separation of donor and acceptor membrane vesicles. As compared to spontaneous sterol exchange, 1.5 μM rat liver SCP_2 enhanced the initial rate of sterol exchange between neutral zwwitterionic phosphatidylcholine SUV 2.3-fold. More important, the presence of acidic phospholipids (2.5-30 mol %) stimulated the SCP_2-mediated increase in sterol transfer approximately 35-42-fold. Thus, acidic phospholipids strikingly potentiate the effect of SCP_2 by 15-18 times as compared to SUV without negatively charged lipids. Rat liver FABP (up to 60 μM) was without effect on sterol transfer in either neutral zwitterionic or anionic phospholipid containing SUV. The potentiation of SCP_2 action by acidic phospholipids was suppressed by high ionic strength, neomycin, and low pH. The results suggest that electrostatic interaction between SCP_2 and negatively charged membranes may play an important role in the mechanism whereby SCP_2 enhances intermembrane cholesterol transfer.

Rat liver sterol carrier protein 2 and fatty acid binding protein (FABP,[1] also called sterol carrier protein) are intracellular hydrophobic ligand binding/transfer proteins. Although SCP_2 (Chanderbhan et al., 1982; Scallen et al., 1985; Schroeder et al., 1990) and FABP (Rustow et al., 1982; Schroeder et al., 1985; Fischer et al., 1985a) both bind sterol, it is unclear if sterol binding correlates with ability to transfer sterol between membranes by both proteins. In addition, little is known about the mechanism whereby these proteins may enhance sterol transfer.

SCP_2 facilitates the intermembrane transfer, in vitro, of many lipid classes including gangliosides (Bloj & Zilversmit, 1981), neutral glycosphinolipids (Bloj & Zilversmit, 1981), sphingomyelin (Crain & Zilversmit, 1980), phospholipids (Crain & Zilversmit, 1980), and cholesterol (Crain & Zilversmit, 1980; North & Fleischer, 1983; Muczynski & Stahl, 1983; Chanderbhan et al., 1982; Vahouny et al., 1984; Van Amerongen et al., 1989). SCP_2-mediated sterol transfer is important for microsomal conversion of lanosterol to cholesterol (Noland et al., 1980; Trzaskos & Gaylor, 1983), microsomal cholesterol esterification (Gavey et al., 1981), and adrenal mitochondrial steroidogenesis (Vahouny et al., 1987). Nevertheless, little is known about the mechanism and factors that modulate SCP_2-facilitated cholesterol transfer.

Liposomal model membrane systems are particularly attractive in studies designed to elucidate the mechanism whereby SCP_2 mediates cholesterol transfer. In the case of phospholipid transfer, it has been suggested that SCP_2 interacts with the donor membrane vesicle, thereby lowering the energy barrier for lipid monomers to dissociate from the vesicle membrane and thus establishing a faster phospholipid monomer-vesicle equilibrium (Nichols & Pagano, 1983). It is not known whether SCP_2 may enhance sterol transfer by a similar mechanism or by acting as an aqueous carrier (Vahouny et al., 1984; Schroeder et al., 1990). The work presented herein examines the effect of acidic phospholipids on the ability of SCP_2, a basic protein with p*I* near 8.6 (Noland et al., 1980), and FABP, with p*I* near 7.0 (Dempsey et al., 1981), to stimulate intermembrane cholesterol transfer.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (PC) and bovine heart cardiolipin (CL) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholesterol was from Applied Science Laboratories, Inc. (State College, PA). Bovine brain L-α-phosphatidyl-L-serine (PS) and bovine liver L-α-phosphatidylinositol (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Dehydroergosterol was synthesized as described previously (Fischer et al., 1985b). Prior to use, sterols were recrystallized in alcohol, and their purity was checked by HPLC (Fischer et al., 1985). SCP_2 was purified from rat liver according to Noland et al. (1980). FABP was generously provided by Dr. M. E. Dempsey and also isolated from *Escherichia coli* expressing the cDNA for

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[1] Abbreviations: SCP_2, sterol carrier protein 2; FABP, fatty acid binding protein; SUV, small unilamellar vesicles; PC, 1-palmitoyl-2-oleoylphosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin.

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rat liver FABP (Lowe et al., 1984). Both FABP preparations gave similar results.

SUV Preparation. Small unilamellar vesicles (SUV) were prepared as described earlier (Schroeder et al., 1987, 1988; Nemecz et al., 1988; Schroeder & Nemecz, 1989) except for two modifications: (i) All SUV preparations were sonicated until the suspensions were clear. For control SUV (PC/sterol, 65:35 mol %) and SUV containing acidic phospholipid (PC/acidic phospholipid/sterol, 55:10:35 mol %) this required a 30- and 2.5–4-min sonication time, respectively. Recovery of phospholipid in SUV, as measured according to Ames (1968), was similar for all SUV. (ii) The buffer (10 mM PIPES/0.02% NaN_3, pH 7.4) in which SUV were sonicated and redispersed was prefiltered with a 0.2-μm filter (Millipore, Bedford, MA).

SUV Surface Charge. The average number of negative charges per lipid molecule in the pH range 4.5–10.5 was calculated from the acidic phospholipid content and the negative charge of the acidic lipids at the designated pH as calculated from the pK_a. Phosphatidylinositol has a pK_a at 2.5 (Abramson et al., 1968). The pK_a of cardiolipin (diphosphatidylglycerol) is derived from that of phosphatidylglycerol, 3.1 (Van Dijck et al., 1978). Phosphatidylserine has pK_a's at 3.5, 5.8, and 9.2; phosphatidylethanolamine has pK_a's at 3.6 and 6.8–7.5; phosphatidylcholine has a single pK_a near 3.5 (Papahadjopoulos, 1968).

The surface charge on SCP_2 was calculated from the pK_a values of the amino acids and the amino acid sequence (Pastuszyn et al., 1987). In this calculation the charged amino acid residues are all assumed to be exposed to the aqueous buffer. Since the three-dimensional structure of SCP_2 is not known, this calculation is an estimate that yields a pI slightly lower than that measured experimentally (Noland et al., 1980).

Sterol Exchange. Total lipid concentration was near 150 μM. There was a 10-fold excess of acceptor over donor SUV. The vesicle composition was 55 mol % PC, 35 mol % sterol (dehydroergosterol in the donor, cholesterol in the acceptor vesicles), and 10 mol % either PC, PE, PI, PS, or CL. The exchange of dehydroergosterol for cholesterol between donor and acceptor membranes was monitored at 24 °C in the absence and presence of SCP_2. Prior to use, lyophylized SCP_2 (Pastuszyn et al., 1987; Noland et al., 1980) was dissolved in distilled water to make a 100 μM stock solution. The method to measure sterol exchange was based on a fluorescence polarization technique described previously in this laboratory (Nemecz et al., 1988; Nemecz & Schroeder, 1988). Herein, this technique was modified by use of the T-format (rather than L-format previously used) and by continuous monitoring of steady-state polarization to obtain 540 data points in 0–3 h with an IBM-PC or Compaq-PC computer (rather than 15–20 data points taken manually as previously) (Nemecz et al., 1989). Initial rates of polarization change were obtained in 1-min (SCP_2 mediated) and 5-min (spontaneous) exchange curves. The excitation source was either a 450-W xenon arc or a He/Cd laser (Model 424 ONB, Liconix, Sunnyvale, CA). As shown earlier, photobleaching with this arc lamp did not occur under the conditions used (Schroeder et al., 1987). The output of the laser was attenuated to about 10% intensity in order to prevent photobleaching. The inner-filter effect and light scattering in the samples were made negligible by use of dilute vesicle suspensions (absorbance at the excitation wavelength, 325 nm, was less than 0.1) and by placing Janos GG-375 cutoff filters in the emission system.

Sterol Exchange Kinetics: Dependency of Dehydroergosterol Transfer on Polarization. It is clear from earlier work from our laboratory that dehydroergosterol polarization is not a linear function of dehydroergosterol content in the SUV (Schroeder et al., 1987; Nemecz et al., 1989; Butko et al., 1989). The relative amount of fluorescent sterol x_D present in the donor vesicles at any time is given as

$$x_D = c_D/c_T$$

where c_D and c_T are the dehydroergosterol concentration in the donor and the initial concentration in the donor (c_T = 35 mol %). The relative amount of dehydroergosterol present in the acceptor population x_A is

$$x_A = 1 - x_D = 1 - c_D/c_T$$

The dependency of polarization P on dehydroergosterol concentration can be described with the polynomial function

$$P = -ax_D^2 + bx_D + c$$

where the parameters have values of a = 0.185, b = 0.028, and c = 0.320. The regression coefficient r^2 = 0.999. The complete derivation of this equation is presented elsewhere (Nemecz et al., 1989; Butko et al., 1989). It is important to note that P changes almost linearly with x_D until about 20% of the dehydroergosterol leaves the donor vesicles. Thus, in the initial stages of exchange, the dehydroergosterol polarization increase reflects the quantity of sterol transferred.

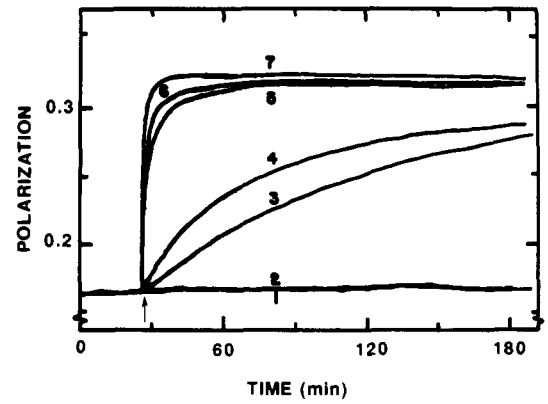

FIGURE 1: Changes in dehydroergosterol fluorescence polarization upon mixing the donor and acceptor vesicle populations of different lipid compositions: (curve 1) PC/dehydroergosterol (65:35) SUV, no acceptor SUV, no SCP_2; (curve 2) PC/dehydroergosterol (65:35) SUV, no acceptor SUV, 1.5 μM SCP_2 (this curve was the essentially the same as curve 1); (curve 3) spontaneous exchange of sterol between PC/dehydroergosterol (65:35) donor and PC/cholesterol (65:35) acceptor SUV, no SCP_2 [similar curves (not shown) were obtained when Pc/anionic phospholipid/dehydroergosterol (55:10:35) donor SUV were used]; (curve 4) 1.5 μM SCP_2-mediated exchange between PC/dehydroergosterol (65:35) donor and PC/cholesterol (65:35) acceptor SUV; (curve 5) 1.5 μM SCP_2-mediated exchange between PC/PS/dehydroergosterol (55:10:35) donor and PC/PS/cholesterol (55:10:35) acceptor SUV; (curve 6) 1.5 μM SCP_2-mediated exchange between PC/PI/dehydroergosterol (55:10:35) donor and PC/PI/cholesterol (55:10:35) acceptor SUV; (curve 7) 1.5 μM SCP_2-mediated exchange between PC/CL/dehydroergosterol (55:10:35) donor and PC/PS/cholesterol (55:10:35) acceptor SUV. The time of the addition of acceptor SUV (150 μM) and/or SCP_2 (1.5 μM) is indicated by an arrow. All initial rate determinations were at 24 °C.

Results

Effect of Acidic Phospholipids on Spontaneous Sterol Exchange. Changes in polarization of DHE fluorescence upon mixing the donor and acceptor vesicle populations which have different compositions are shown in Figure 1. The initial rate of the polarization change is proportional to the initial rate of sterol exchange. The experimental and theoretical bases for this observation are presented in detail elsewhere (Nemecz et al., 1989; Butko et al., 1989). The polarization increases were not due to instability of the donor SUV. In the absence

Table I: Effect of Anionic Phospholipids on Protein-Mediated Sterol Exchange[a]

phospholipid (mol %)	sterol (mol %)	initial rate ($\times 10^3$ min^{-1})		
		no protein	+SCP_2	+FABP
PC (65)	35	1.8 ± 0.1	4.2 ± 0.1	1.9 ± 0.2
PC/PE (55:10)	35	1.6 ± 0.1	4.4 ± 0.1	1.8
PC/PS (55:10)	35	2.2 ± 0.0*	92.3 ± 4.3*	1.4
PC/PI (55:10)	35	2.4 ± 0.2*	93.8 ± 10.0*	1.4
PC/CL (55:10)	35	3.4 ± 0.2*	117.8 ± 8.9*	1.6

[a] SUV were prepared containing 55% PC, 35% sterol, and 10% PC, PE, PI, PS, or CL. Total lipid and protein concentrations were 150 and 1.5 μM, respectively. Numbers are the initial rate of polarization change determined at 24 °C as described in the legend to Figure 1. Values represent the mean ± SEM (n = 3–7). As asterisk refers to $p < 0.01$ by Student's t test as compared to PC/sterol SUV.

of acceptor SUV the fluorescence polarization of donor SUV was constant during the measurement (curve 1, Figure 1). Spontaneous dehydroergosterol exchange between PC/sterol SUV (curve 3, Figure 1) exhibited an initial rate of polarization change of 0.0018 min^{-1} (Table I). Inclusion of acidic phospholipid such as phosphatidylserine up to 10 mol % in the SUV increased the rate of spontaneous sterol exchange significantly (Table I). At 10 mol %, a variety of other acidic phospholipids stimulated spontaneous sterol exchange: phosphatidylethanolamine < phosphatidylserine, phosphatidylinositol < cardiolipin. The initial rates of the polarization change were between 0.0016 and 0.0034 min^{-1}, depending on the number of negative charges per molecule of acidic phospholipid present in the SUV (Table I). In summary, the effect of anionic phospholipids on spontaneous sterol exchange between SUV was modest (2-fold) as compared to the SCP_2-mediated sterol exchange documented in the following sections (35+-fold).

Protein-Enhanced Sterol Exchange in SUV Containing Neutral Zwitterionic and Acidic Phospholipid. SCP_2 and FABP both bind sterol with a 1:1 stoichiometry and K_d's of 1.2–2.7 and 0.2–0.7 μM (Schroeder et al., 1989a,b), respectively. The effect of SCP_2 on spontaneous sterol exchange in PC SUV (net neutral charge at pH 7.4) is illustrated in Figure 1 (curve 4 versus curve 3). FABP was without effect (curve identical with curve 3). SCP_2 increased the rate of polarization change in PC SUV (curve 4) more than 2-fold. More important, in PC SUV containing 10 mol % anionic phospholipid such as phosphatidylserine (curve 5), SCP_2 enhanced sterol transfer 42-fold. These polarization changes were not due to the binding of dehydroergosterol to SCP_2 with SCP_2–dehydroergosterol having a higher polarization. In the absence of acceptor PC SUV (Figure 1, curve 2) or of acceptors PS SUV (not shown), SCP_2 did not increase polarization of donor SUV over the time period examined.

Increasing SCP_2 over the range 0–10 μM increased the initial rate of sterol exchange from both PC/sterol (65:35) SUV and PC/PS/sterol (55:10:35) SUV (Figure 2). Double-reciprocal plots of initial rate versus SCP_2 concentration indicated that the V_{max} for these SUV was 23.9 and 133.3, respectively. FABP from 1.5 to 60 μM had no significant effect on initial rate of sterol transfer (data not shown). Increasing PS from 0 to 30 mol % resulted in increased initial rate of sterol transfer with a maximum near 20 mol % PS (Figure 3A). Thus, increasing mole percent PS potentiated SCP_2-mediated sterol transfer.

The dependence of SCP_2-stimulated sterol transfer on specific acidic phospholipids was examined. The presence of 10 mol % anionic phospholipids such as PI (curve 6, Figure 1) or CL (curve 7, Figure 1) greatly increased the rapidity of the polarization change. In the presence of acceptor SUV,

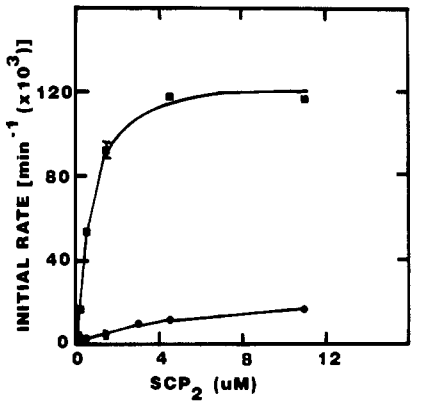

FIGURE 2: Effect of SCP_2 concentration on sterol exchange. The effect of increasing SCP_2 concentration on initial rate of polarization change was determined as described in Figure 1. Closed circles represent PC/dehydroergosterol (65:35) donor and PC/cholesterol (65:35) acceptor SUV. Closed squares represent PC/PS/dehydroergosterol (55:10:35) donor and PC/PS/cholesterol (55:10:35) acceptor SUV. All initial rate determinations were at 24 °C.

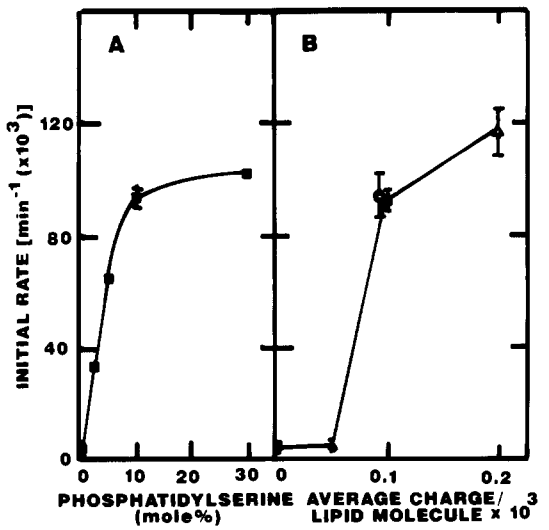

FIGURE 3: Role of concentration and molecular species of anionic phospholipid on SCP_2-mediated sterol exchange. All procedures were as described in the legend to Figure 1. (Panel A) Initial rate of exchange was measured for SUV containing increasing mole percent PS. (Panel B) Initial rate was measured for SUV containing 10 mole percent PC (closed circle), PE (closed triangle), PS (closed square), PI (open circle), or CL (open triangle). Average membrane charge/lipid molecule was calculated at pH 7.4 from the known pK_a's as described under Materials and Methods. The ratio of donor to acceptor SUV was 1:10. SCP_2 was at 1.5 μM. All initial rate determinations were at 24 °C. Values represent the mean ± SEM (n = 3–7).

SCP_2 increased the initial rate of the polarization change in PC SUV 2.3-fold (Table I). In PC/CL/sterol (55:10:35) SUV the initial rate of SCP_2-mediated sterol transfer was 117.8, 35-fold faster than spontaneous transfer in PC/CL/sterol SUV and 65-fold faster than spontaneous transfer in PC/sterol SUV (Table I). FABP was without effect on sterol transfer in neutral zwitterionic PC or in anionic phospholipid containing SUV. Comparison of the rates of the SCP_2-mediated exchanges in the presence and absence of PS, PI, or CL leads to the conclusion that the presence of 10 mol % acidic phospholipid in the donor and acceptor membranes enhances the effect of SCP_2 about 40 times. Of the three acidic phospholipids, CL with two net negative charges at neutral pH appeared to be the most effective, resulting in the highest SCP_2-mediated sterol transfer rates (Figure 3B and Table I). Thus, it appears that SCP_2-mediated sterol transfer may be

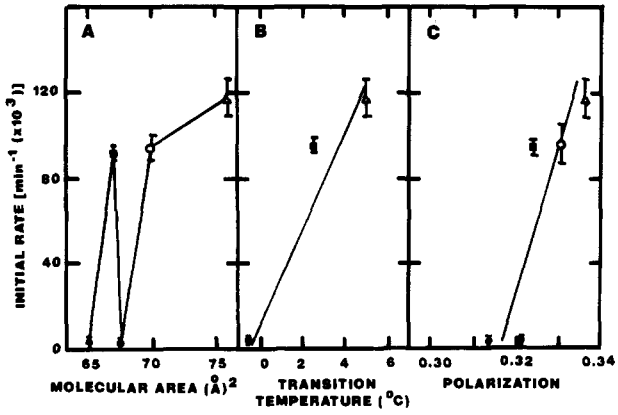

FIGURE 4: Effect of anionic phospholipid molecular area and fluidity on SCP_2-mediated sterol exchange. All conditions and symbols were as described in the legend to Figure 3B. (Panel A) Initial rates of polarization change were plotted versus average phospholipid molecular area. The average phospholipid molecular area was calculated from the composition of the SUV and from the known molecular areas of individual phospholipids. The molecular areas of PC (Macdonald & Seelig, 1987), PE (Hauser & Dawson, 1967), PS (Demel et al., 1987), PI (Fagan & Keough, 1988), and CL (Shah & Schulman, 1965) are 68, 50, 62, 80, and 120, respectively. The contribution of sterol to the SUV lipid molecular area is not included because (a) the mole percent sterol was the same in all SUV, (b) dehydroergosterol and cholesterol have very similar condensing effects on PC monolayers (F. Schroeder and P. Slotte, unpublished observation), and (3) the relative condensing effect of sterol in the various types of SUV is not known. (Panel B) Initial rates of polarizationchange were plotted versus average phospholipid phase transition temperature (contribution of sterol was not included in this calculation). The average transition temperature was calculated from the mole percent phospholipid composition and the known phase transition temperatures. Transition temperatures of pure PC (Davis et al., 1981), PS (Demel et al., 1987), and CL (Ioannou & Golding, 1979) are −2.6, 14.5, and 50 °C, respectively. (Panel C) Initial rates of polarization change were plotted versus dehydroergosterol polarization in PC/anionic phospholipid/cholesterol/dehydroergosterol (55:10:31.5:3.5) SUV. Dehydroergosterol polarization and sterol exchange were determined at 24 °C. SCP_2 was at 1.5 μM.

dependent on membrane surface charge per lipid molecule. However, the relationship between calculated average surface charge per lipid molecule and the SCP_2-mediated initial rate of sterol exchange was nonlinear, independent of the type of acidic phospholipid used (Figure 3B). The effect of acidic phospholipids on SCP_2 action seems to be nonspecific for the type of negatively charged polar head group on the phospholipid.

The SCP_2-mediated stimulation of sterol transfer in acidic phospholipid containing SUV was not related to molecular area (Figure 4A). Although PS has a smaller molecular area than PC, PS stimulated sterol transfer 42-fold while in the absence of PS sterol transfer was stimulated only 2-fold. Cardiolipin contains four acyl chains as compared to only two acyl chains in PC, PE, PI, and PS. The four acyl chains of CL occupy nearly twice the molecular area (120 Å) as phospholipids with two acyl chains (50–68 Å) (see legend to Figure 4). However, even if the initial rate for SCP_2-mediated sterol exchange in CL containing SUV was divided by 2, the initial rate is still 1 order of magnitude higher than that for PC SUV.

Last, the effect of membrane fluidity on initial rate of SCP_2-mediated sterol transfer was examined (Figure 4). When initial rate was plotted versus the phase transition temperature of the pure phospholipid (obtained from published data), initial rate increased with increasing phase transition temperature (Figure 4A). However, in the presence of 35 mol % sterol such phase transitions are not present. Therefore, the effect of fluidity was directly verified in the SUV with dehydroergosterol as a probe molecule. At low mole percent

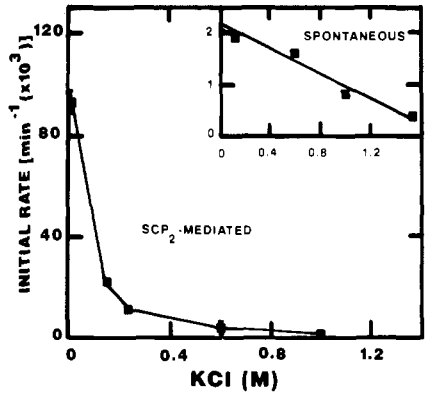

FIGURE 5: High ionic strength decreases both spontaneous and SCP_2-mediated sterol exchange between phosphatidylserine-containing SUV. All conditions are as described in Figure 1 for PC/PS/sterol (55:10:35) SUV, except that an increasing concentration of KCl was included. SCP_2 was at 1.5 μM. All initial rate determinations were at 24 °C. (Insert) Spontaneous sterol exchange.

dehydroergosterol, polarization is sensitive to the order of the surrounding lipids in SUV (Schroeder et al., 1987; Kier et al., 1986). The initial rate of sterol exchange increased with increasing polarization of dehydroergosterol in SUV containing the various phospholipids, 31.5 mol % cholesterol, and 3.5 mol % dehydroergosterol (Figure 4C). From the data shown in Figure 4B,C, one might erroneously conclude that decreased fluidity leads to an increased sterol transfer rate. However, it is well established that decreased fluidity (McLean & Phillips, 1982, 1984; Bar et al., 1986) decreases spontaneous sterol transfer. Thus, the membrane charge per lipid molecule and not phospholipid molecular area or fluidity appears to be the predominant factor potentiating SCP_2-mediated sterol exchange between SUV. This charge effect was further examined in the following sections.

Ionic Effects. SCP_2 is positively charged at neutral pH, since its isoelectric point is 8.6 (Noland et al., 1980). Acidic phospholipids bear a net negative charge at neutral pH. Electrostatic attraction between the two species with opposite charges should be expected. If this accounts for the stimulatory effect of acidic phospholipids on the SCP_2-mediated sterol exchange, then the effect should be suppressed by screening of the charges with high salt. The surface charge on SUV and/or SCP_2 was screened by three different methods.

First, the effect of charge screening by KCl was determined. The spontaneous sterol exchange rate was decreased about 2.8-fold at 1 M KCl (Figure 5, insert). More important, increasing concentration of KCl inhibited the SCP_2-mediated sterol transfer between PS containing SUV by more than 50-fold (Figure 5) such that, in the presence of 1 M KCl, the SCP_2-mediated sterol transfer was only 2.3-fold greater than that of the spontaneous transfer. The presence of 1 M KCl also reduced the initial rate of spontaneous and SCP_2-mediated sterol transfer in PC/sterol SUV, but the protein-mediated transfer was still nearly 3-fold faster than the spontaneous transfer in the presence of high salt. The same qualitative effect observed with PS containing SUV was obtained for PI containing SUV as well (data not shown).

Second, the divalent metal ion effect of Ca^{2+} on SCP_2-mediated sterol exchange was examined. Not only may Ca^{2+} also screen negatively charged lipids in SUV, under some conditions Ca^{2+} can induce formation of nonbilayer forms or of phase-separated PS in model membranes. In addition, Ca^{2+} might possibly form divalent metal ion bridges between SCP_2 and the negatively charged SUV. Some of these effects could inhibit SCP_2-mediated sterol transfer. In the presence of 1

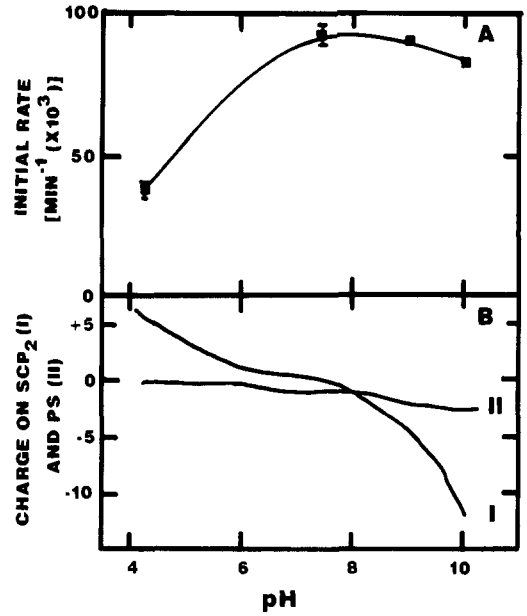

FIGURE 6: pH dependence of SCP_2-mediated sterol exchange between phosphatidylserine-containing SUV. All conditions were as described in the legend to Figure 5, except that the pH was varied between 4.5 and 10 (panel A). (Panel B) The average surface charge on the SUV (curve II) and the average surface charge on the protein (curve I) were calculated as a function of pH as described under Materials and Methods. SCP_2 was at 1.5 μM. All initial rate determinations were at 24 °C.

mM $CaCl_2$, the initial rate of SCP_2-mediated sterol transfer was decreased 24% and 64% for PC/sterol (65:35) and PC/PS/sterol (55:10:35) SUV, respectively.

Third, screening of SUV negative charge by neomycin was determined. The polyamine antibiotic neomycin binds to negatively charged lipids (Schacht, 1978; Palmer, 1981). Increasing concentrations of neomycin inhibit the SCP_2-mediated sterol transfer in PS-containing vesicles up to 98% at 1 mM neomycin. Thus, by interacting with PS, the neomycin shields PS negative charges in the SUV, thereby preventing the interaction of SUV with SCP_2.

Effect of pH. Increasing the pH from 4.5 to 7.4 increases the initial rate of polarization change of SCP_2-mediated sterol transfer in PS-containing SUV more than 2-fold from 37.8 ± 1.7 to 92.3 ± 4.3 (Figure 6A). PS is nearly neutral at pH 4.5, has one negatively charge per molecule at pH 7.4, and has two negative charges at pH 10 (Figure 6B). In contrast, an increase of pH from 4.5 to 10 decreased the overall charge on the SCP_2 protein from +5 to −20. Thus, at low pH the SCP_2 and the PS-containing SUV do not have opposite charge, and electrostatic attraction between the transfer protein and the vesicle surface is expected to be minimal, resulting in a decreased initial rate (Figure 6B). At pH 7.4, the SCP_2 and the SUV appear to have opposite charge, and electrostatic attraction is expected to be maximal, resulting in maximal initial rate of sterol transfer (Figure 6B). In contrast, at high pH the SCP_2 and the SUV both have their greatest negative charge, and electrostatic repulsion is expected to be maximal and initial rate of sterol transfer should be minimal. However, only a small decrease in initial rate was observed at pH 10. Thus, two effects appear to be operating: First, increasing the SUV negative charge at higher pH is expected to stimulate the initial rate of SCP_2-mediated sterol transfer. This is based on the data in Figure 1 and Table I, which indicate that increasing PS or anionic phospholipids with greater charge (e.g., CL) stimulate SCP_2-mediated sterol transfer as much as 35–42-fold. Second, even at pH 10 the SCP_2 must have a positively charged region that still allows interaction with the SUV. The attraction of this region of the protein for the SUV is expected to be greatest when the PS has two negative charges per molecule (e.g., pH 10). The doubling in SUV negative charge at pH 10 (stimulatory effect) and a postulated positively charged SUV binding domain on the SCP_2 therefore appear to compensate for the expected overall electrostatic repulsion between the protein and the SUV surface at high pH. Thus, an increase in the overall negative charge of the protein has an effect on the SCP_2 interaction with the vesicle surface which is not the same as that observed when the negative charge on the surface itself increases. Apparently, the overall change on the SCP_2 is not as important as the charge on a putative positively charged membrane binding domain (see Discussion).

Discussion

The present investigation was undertaken to examine the effect of acidic lipids on intracellular sterol carrier protein mediated sterol transfer between membranes. A liposomal membrane system using a fluorescent sterol, dehydroergosterol, and not requiring separation of donor and acceptor membranes (Nemecz & Schroeder, 1988; Nemecz et al., 1988) was used for this purpose. The method was modified to continuous monitoring of fluorescence polarization changes (Schroeder et al., 1990; Nemecz et al., 1989) in order to obtain initial rates of polarization change. Polarization of the dehydroergosterol fluorescence is dependent on the dehydroergosterol concentration in the SUV (Schroeder et al., 1987, 1989, 1990; Nemecz & Schroeder, 1988; Nemecz et al., 1988, 1989; Muczynski & Stahl, 1983; Butko et al., 1989). The relationship of polarization to mole percent dehydroergosterol in SUV containing increasing molar ratio of sterol/phospholipid is sigmoidal (Schroeder et al., 1987, 1989; Nemecz & Schroeder, 1988; Nemecz et al., 1988; Muczynski & Stahl, 1983) and also deviates from linearity in SUV containing a constant molar ratio of sterol/phospholipid at 0.35 (Nemecz et al., 1989; Butko et al., 1989). However, as described under Materials and Methods, a linear relationship of polarization to mole percent dehydroergosterol is obtained under conditions where less than 20% of the dehydroergosterol is transferred between donor and acceptor SUV, both containing 35 mol % total sterol. Thus, the initial rate of the polarization changes during exchange is proportional to the mass of sterol transferred (Nemecz et al., 1989; Butko et al., 1989). This observation is consistent with earlier studies in which polarization changes (in the assay not requiring separation of donor and acceptor), dehydroergosterol fluorescence intensity, and radiolabeled cholesterol (in assays requiring separation of donor and acceptor SUV) showed similar sterol exchange kinetics (Nemecz et al., 1988; Nemecz & Schroeder, 1988; Bar et al., 1989). The SUV model system allowed examination of the effect of individual variables on spontaneous and protein-mediated sterol transfer.

In the past, most investigations of sterol exchange between membranes used methods requiring separation of donor and acceptor SUV, usually based on differences in charge of donor and acceptor. Consequently, there are no previous reports, to our knowledge, of the effect of negatively charged lipids on sterol exchange. However, one report, utilizing donor and acceptor SUV differing in charge and possibly fluidity, concluded no effect of negatively charged lipid on spontaneous cholesterol transfer (Bar et al., 1986). In the assay used herein, donor and acceptor did not differ in charge or fluidity and did not require separation of donor and acceptor SUV. The spontaneous transfer of sterol between PC/sterol SUV is en-

hanced by the presence of negatively charged lipids approximately up to 1.9-fold (CL containing SUV) over that occurring in PC/sterol SUV without negatively charged vesicles. Although it is a negatively charged phospholipid, PE did not enhance spontaneous sterol transfer, possibly due to insufficient negative charge contributed to the SUV at 10 mol %. Consistent with this possibility, PE at 30 mol % stimulated spontaneous sterol transfer 6-fold.

The results presented herein provide the first direct evidence on a potentiating effect of negatively charged phospholipids on the SCP_2-mediated exchange of sterol between membranes. Five points can be made regarding this effect: (1) It does not appear to be specific for the type of negatively charged phospholipids in the SUV. (2) It appears to be dependent on the quantity of the acidic phospholipid and/or the SUV surface charge per lipid molecule. (3) It can be abolished by high ionic strength, divalent metal ion, or neomycin (a polycationic antibiotic that binds to negatively charged lipids). (4) The effect of anionic lipids cannot be accounted for by the membrane rigidifying effect of anionic lipids on SUV membrane fluidity. This result is consistent with that observed for another lipid transfer protein, phosphatidylinositol transfer protein, whose transfer rate is increased with increasing membrane fluidity (Helmkamp, 1980). (5) It does not appear to be dependent on phospholipid molecular area. This strongly suggests that the enhanced performance of SCP_2 in the presence of acidic phospholipids might primarily be due to electrostatic attraction between negatively charged membrane surfaces and SCP_2.

The pH dependence of the SCP_2-mediated sterol transfer in PS-containing SUV is interesting in regard to the mechanism of SCP_2-mediated sterol transfer. Apparently, the charge on both the SUV and the protein are important. Three possibilities were tested: First, at pH 4.5 (below the isoelectric point of the SCP_2) the transfer activity was markedly diminished. This decrease may be due either to (1) lack of strong binding of the overall positively charged SCP_2 to the nearly neutral charged SUV or (2) to a change in protein conformation. Second, at pH 7.4 the SCP_2-mediated transfer activity was maximal. At this pH the protein has less positive charge while the SUV has a single negative charge per PS molecule in the SUV, thereby maximizing potential electrostatic attraction. Third, at pH 10 (above the isoelectric point of the protein) the initial rate of sterol transfer was decreased less than 10% from the maximum near pH 7.4. This result is surprising since at pH 10 the net charge on the SCP_2 is negative and in the SUV the PS has two negative charges per molecule at pH 10. Electrostatic repulsion and greatly diminished transfer rate were expected at pH 10. Thus, it appears that negative charge on the SUV is much more important in determining SCP_2-mediated sterol transfer than the overall protein charge. One may speculate that the SCP_2 therefore has a surface region that remains positively charged even at high pH. Such a region could provide a membrane binding site. Indeed, Pastuszyn et al. (1987) have identified an amphipathic α-helical region (Residues 21–34) in SCP_2 that may provide such a membrane binding site. Very similar observations for pH effects on lipid transfer have been made with phosphatidylinositol transfer protein stimulated transfer of PI (Van Paridon et al., 1988).

Although SCP_2 is a water-soluble protein, its electrostatic association with membranes is apparently very important for its function [as also paralleled, e.g., by cytochrome *c* and mitochondrial membranes (Mustonen et al., 1987; Gupte & Hackenbrock, 1988a,b; Demel et al., 1989)]. There have been reports regarding SCP_2 binding to isolated mitochondria (Megli et al., 1986) or increased concentration of SCP_2 around mitochondria in vivo (Fournier & Rahim, 1983). In view of the high content of negatively charged lipids in the outer mitochondrial membrane, 14.3 mol % PS + PI and 4.8 mol % (Demel et al., 1989) to 17 mol % CL (Vahouny et al., 1984), SCP_2 has been proposed to enhance sterol transfer between outer and inner mitochondrial membranes (Vahouny et al., 1984).

Direct interaction of SCP_2 with membranes also has important consequences to the mechanism whereby SCP_2 enhances sterol transfer. Recently, it was demonstrated that SCP_2 binds to phospholipid monolayers containing 10 mol % negatively charged phospholipid and increases the monolayer surface pressure (Van Amerongen et al., 1989). Thus, SCP_2 is high surface active. If SCP_2 similarly increases the surface pressure in the outer monolayer of SUV, the surface area may increase in order to maintain membrane stability. Expansion of the area occupied by each lipid molecule is similar to the effect of elevating temperature. The rate of sterol desorption from membranes increases with increasing temperature (McLean & Phillips, 1982, 1984; Bar et al., 1986). Alternately, cholesterol could preferentially be forced to desorb more rapidly from the membrane when SCP_2 increases the surface pressure in the outer leaflet. This is because cholesterol has an order of magnitude faster spontaneous desorption rate from membranes than phospholipids.

It should be noted that SCP_2 but not FABP stimulates sterol transfer in PC SUV. More important, anionic phospholipids potentiate the SCP_2-mediated transfer of sterol without effect on FABP. The latter observation is significant for two reasons. First, both SCP_2 and FABP bind sterol (Schroeder et al., 1985, 1989a,b; Fischer et al., 1985a) but only SCP_2 enhances sterol transfer. Thus, sterol binding and enhancement of sterol transfer seem to be distinct properties in these proteins. The reasons for this difference between the two proteins are not known but may be due to the 6–10-fold higher affinity of FABP for sterol (making it less able to release bound sterol) or possibly the lack of an anionic phospholipid binding site. It should be noted that FABP also binds fatty acids, but the presence of PS did not increase the FABP-mediated off-rate of radiolabeled oleic acid from PC monolayers (Peeters & Veerkamp, 1989). These important observations are consistent with the conclusion that ligand binding does not necessarily correlate with ligand transfer ability of a binding protein. A similar conclusion was recently reported with interphotoreceptor retinol binding protein and retinol binding/transfer (Ho et al., 1989).

Four points concerning our assay and the way of data evaluation merit further discussion. First, SCP_2 is a nonspecific lipid transfer protein. It binds and/or transfers many lipids, including PC and PI. Our assay, however, is specific for intermembrane movement of sterol. Due to the symmetrical composition of donor and acceptor SUV, exchange of any phospholipid (be it PC, PI, PS, or CL) would not change the fluorescence polarization of dehydroergosterol. Second, SCP_2 binding to negatively charged vesicles could increase polarization. However, the data (Figure 1, curve 2) indicate that this did not occur. Third, in the assay mixture the amount of SCP_2 is 1.5 μM relative to 5.25 μM dehydroergosterol (donor SUV) and 52.5 μM cholesterol (acceptor SUV). Elsewhere, we have shown that SCP_2 binds dehydroergosterol with 1:1 stoichiometry and K_d = 1.2–2.7 μM (Schroeder et al., 1989a). The polarization of SCP_2-bound dehydroergosterol is 0.154 (Schroeder et al., 1989a). Depending on the SUV preparation and experimental conditions, the initial polariza-

tion of SUV containing 35 mol % dehydroergosterol is between 0.15 and 0.16 (e.g., see Figure 1). Consequently, addition of SCP_2 to donor SUV in the absence of acceptor SUV (Figure 1, curve 2) does not change polarization. When acceptor SUV are present, the donor dehydroergosterol and acceptor cholesterol (10-fold excess) can both bind to the SCP_2, but only the former fluoresces when bound to SCP_2. The ratio of bound dehydroergosterol/SCP_2 under the latter conditions (calculated from the K_d and the above concentrations) is approximately 0.05. Thus, fluorescence of dehydroergosterol bound to SCP_2 would not account for the large increase in polarization observed with negatively charged SUV. Consequently, it is solely the exit of dehydroergosterol from donor SUV and its incorporation into the acceptor SUV that can bring about the polarization changes monitored in the assay. Fourth, the polarization data were expressed in terms of initial rates. Therefore, possible differential effects of SCP_2 on multiple sterol pools [recently found by Nemecz et al. (1988)] are not resolved. This possibility is presently being investigated.

In summary, the presented data suggest that electrostatic interaction between SCP_2 and negatively charged membranes may play an important role in efficient functioning of SCP_2. In contrast, FABP (another sterol binding protein) did not enhance sterol transfer under any of the conditions tested. The presence of negatively charged lipids enhanced SCP_2-mediated sterol transfer approximately 40-fold. In comparison, negatively charged lipids enhanced the SCP_2-mediated transfer of phosphatidylcholine only 1.3-fold (Altamura & Landriscina, 1986) while negatively charged lipids were themselves not transferred by SCP_2 (Altamura & Landriscina, 1986; Nichols & Pagano, 1983). This may indicate a much greater specificity of SCP_2 in mediating sterol transfer as compared to phospholipid transfer than previously suspected.

Registry No. PC, 26853-31-6; cholesterol, 57-88-5; dehydroergosterol, 516-85-8.

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A Possible Receptor-Binding Function for the N-Terminus of Connective Tissue Activating Peptide III†

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ABSTRACT: Connective tissue activating peptide III (CTAP-III) is an 85-residue peptide which has been purified from platelets and shown to possess mitogenic activity toward a variety of fibroblastic cell lines. β-Thromboglobulin (βTG) is an 81-residue peptide which is derived from CTAP-III by cleavage of the N-terminal tetrapeptide Asn-Leu-Ala-Lys which results in the loss of mitogenic activity. The near-UV CD spectra for the two proteins indicated that the conformations as well as the electronic environments of the two disulfide bonds, and also of the single aromatic tyrosine residue, were similar in CTAP-III and βTG. However, differences in the far-UV CD spectra of these proteins indicated a substantial decrease in α-helical content for βTG (29%) as compared to CTAP-III (38%). Structure prediction analysis also suggested that the longer N-terminal segment of CTAP-III may form an α-helix. The N-terminal region of βTG, which lacks this tetrapeptide, was predicted to be in an unordered, or possibly a turn, conformation. This predicted structural difference appears to be due to the high helix-forming potential of the N-terminal tetrapeptide Asn-Leu-Ala-Lys in CTAP-III. These results suggest a possible structural role for the N-terminal region of CTAP-III in the expression of the biologic activities of this protein. On the basis of these studies, a reasonable hypothesis to account for the difference in mitogenic activity between βTG and CTAP-III is that the N-terminal region must be helical for receptor binding to occur.

Connective tissue activating peptide III (CTAP-III)[1] (also known as low-affinity platelet activating factor), β-thromboglobulin (βTG), platelet basic protein (PBP), and platelet factor 4 (PF4) are structurally related proteins located in the α-granules of human platelets and released by a number of factors that stimulate platelet activation (Paul et al., 1980). The amino acid sequence of CTAP-III is known (Castor et al., 1983), and its molecular weight determined from the primary sequence (9278) agrees with that estimated by SDS–PAGE (Castor et al., 1977). PBP is a probable precursor to CTAP-III and contains nine additional amino-terminal residues (Holt et al., 1986), while βTG is probably a product of CTAP-III since it lacks the amino-terminal sequence Asn-Leu-Ala-Lys found in CTAP-III but is otherwise identical in sequence with this peptide (Castor et al., 1983; Niewiarowski et al., 1980). The conversion of PBP to CTAP-III to βTG may be due to a platelet-derived heat-labile protease(s) (Niewiarowski et al., 1979). CTAP-III exhibits about 50% amino acid sequence homology with PF4 (Lawler, 1981; Niewiarowski & Levine, 1979), which is a high-affinity heparin-binding protein whose three-dimensional structure has recently been determined (St. Charles et al., 1989).

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[1] Abbreviations: CTAP-III, connective tissue activating peptide III; βTG, β-thromboglobulin; PF4, platelet factor 4; CD, circular dichroism.

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