Interaction of the N-terminus of sterol carrier protein 2 with membranes: role of membrane curvature

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Although neither the physiological function nor the mechanism of action of sterol carrier protein 2 (SCP₂) is yet completely clear, it is thought that SCP₂ interacts with membranes to elicit its biological effects. The results presented here show that the SCP₂ N-terminus, composed of two amphipathic α -helices, interacted preferentially with highly curved but not lower-curvature membranes containing anionic phospholipid. CD spectra of SCP₂ showed up to 1.2-fold increased α -helical content, on the interaction of SCP₂ with small unilamellar vesicles (SUV) (median radius 10–14 nm) but less with large unilamellar vesicles (LUV) (median radius 52–60 nm). Although enhanced interaction with the SUV membranes was due in part to the radius of curvature and to the greater exposure of acidic phospholipid in the outer leaflet of the bilayer, simply increasing the molar percentage of acidic phospholipid in the LUV membranes had much less effect on SCP₂ binding. A similar preferential interaction was observed

with highly curved SUV as opposed to LUV for the SCP₂ N-terminal peptide ^{1–32}SCP₂ as well as structurally modified peptides in the order ^{1–32}SCP₂ = ^{10–32}SCP₂ > ^{1–24}SCP₂ > ^{1–E20–32}SCP₂. The CD results were confirmed with an independent filtration binding assay, which showed that SCP₂ bound 5-fold more to SUV than LUV, whereas its N-terminal peptides bound up to 4-fold better in the order ^{1–32}SCP₂ = ^{10–32}SCP₂ > ^{1–24}SCP₂ > ^{1–E20–32}SCP₂. Finally, cholesterol potentiated the binding of SCP₂ and N-terminal peptides to anionic-phospholipid-containing SUV but not LUV. These findings were consistent with the SCP₂ N-terminus being a membrane-binding domain that was highly dependent on membrane surface curvature as well as on lipid composition.

Key words: amphipathic peptide, cholesterol, circular dichroism, α -helix, lipid transfer protein.

INTRODUCTION

Although our understanding of lipid transport in the vasculature has advanced greatly over the past four decades, much less is known about intracellular lipid trafficking. Spontaneous movement of monomeric lipid does not account for the non-uniform distribution of lipids in cells (reviewed in [1–6]). Instead, intracellular lipid transport is thought to occur primarily via vesicular and/or protein-mediated pathways. One such protein, sterol carrier protein 2 (SCP₂) [also called non-specific lipid transfer protein ('nsLTP')] binds many types of lipid with high affinity: straight-chain [7,8] and branched-chain [9,10] fatty acids with *K_d* values of 200–400 nM [9,10], fatty-acyl-CoAs with *K_d* values near 5 nM [11], cholesterol (reviewed in [2,10,12–14]) and other isoprenoids [9] with *K_d* values as low as 5–10 nM, and phospholipids [15]. SCP₂ stimulates not only intermembrane cholesterol transfer (reviewed in [2,4]) but also fatty-acyl-CoA transesterification by several microsomal enzymes *in vitro* (reviewed in [2]).

SCP₂ is ideally localized intracellularly to mediate lipid transfer and metabolism. Immunogold electron microscopy as well as immunofluorescence double-labelling and laser scanning confocal microscopy indicate that SCP₂ is highly enriched in peroxisomes (reviewed in [16]). Peroxisomes are active sites of lipid metabolism including fatty acid oxidation, bile acid

formation, ether lipid synthesis and cholesterol. In addition, significant amounts of SCP₂ are extraperoxisomal, associated with endoplasmic reticulum and mitochondria (reviewed in [2,16]). The latter organelles are active in multiple aspects of lipid biosynthesis and oxidation respectively.

Studies with transfected cells demonstrate a role for SCP₂ in intracellular lipid transport and in the metabolism of sterols (reviewed in [2]) and fatty acids [17]. Furthermore, the insertion of anti-SCP₂ antibodies into adrenal cells inhibits corticosterone formation [18], whereas the treatment of normal human fibroblasts with SCP₂ antisense oligonucleotides inhibits the rapid (less than 10 min) transfer of cholesterol from the endoplasmic reticulum to the plasma membrane [19]. Animal studies also support a role for SCP₂ in the trafficking and metabolism of cholesterol (reviewed in [2]). Deletion of the gene for SCP₂ in mice decreases the cholesterol ester and triglyceride content of liver and inhibits peroxisomal α -oxidation of branched-chain fatty acids [10,20].

Despite these studies, little is known regarding the mechanism(s) by which SCP₂ enhances intracellular lipid transfer and metabolism. Some results suggest that the binding of lipid to SCP₂ as well as the interaction of SCP₂ with membranes might be essential for SCP₂-mediated intermembrane lipid transfer [21]. However, almost nothing is known about either the SCP₂ polypeptide domain involved in membrane binding or the

Abbreviations used: LUV, large unilamellar vesicles; PtdCho, phosphatidylcholine [1-palmitoyl-2-oleoyl *sn*-glycero-3-phosphocholine]; PtdSer, phosphatidylserine [1,2-dioleoyl *sn*-glycero-3-(phospho-L-serine)]; SCP₂, sterol carrier protein 2; ^{1–32}SCP₂, N-terminal 32 amino acid residues of SCP₂; ^{1–24}SCP₂, N-terminal 24 residues of SCP₂; ^{10–32}SCP₂, residues 10–32 of SCP₂; ^{1–E20–32}SCP₂, ^{1–32}SCP₂ containing a single Glu²⁰ substitution for Leu²⁰; SUV, small unilamellar vesicles; TFE, 1,1,1-trifluoroethanol.

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membrane structural requirements necessary for such interaction. Both amino acid sequence results [22] and NMR data [23] suggest that the N-terminus of SCP₂ is composed of two amphipathic α -helices. It is hypothesized that this N-terminal region interacts with acidic lipids in membranes, i.e. the membrane interaction site [22].

The present study addressed several important aspects of the hypothesis that the SCP₂ N-terminus represents a domain whose interaction with membrane is dependent on the structural properties of the membrane as well as those of the SCP₂ N-terminus.

MATERIALS AND METHODS

Materials

Human recombinant SCP₂ was prepared as described previously [24]. Phosphatidylcholine (PtdCho) and phosphatidylserine (PtdSer) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Cholesterol, dehydroergosterol, *o*-phthalaldehyde, trifluoroethanol and 2-mercaptoethanol were from Sigma (St. Louis, MO, U.S.A.). Microcon-100 filtration units were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Synthesis of peptides

A Millipore 9050 Plus (PerSeptive Biosystems) automated peptide synthesizer was used to synthesize peptides by fluorenylmethoxycarbonyl solid-phase chemistry (Dr. Judith Ball, Peptide Synthesis Core Facility, Department of Pathobiology, Texas A&M University, College Station, TX, U.S.A.). 1-Hydroxy-7-azabenzotriazole with di-isopropyl carbodi-imide were employed for the activation chemistry; the yield of pure peptide product was 0.3–0.5 g peptide/g of the poly(ethylene glycol) resin with Fmoc-aminomethyl-3,5-dimethoxyphenoxyvaleric acid (PAL) resin. The peptide was cleaved from the solid polymer support and side-chain-protecting groups were removed by treatment with trifluoroacetic acid/ethanedithiol/thioanisole/anisole (90:3:5:2, by vol.). After incubation for 2 h, the cleaved peptide was filtered into cold diethyl ether. Complete recovery was obtained by rinsing the resin repeatedly with trifluoroacetic acid followed by cold diethyl ether. The extracted peptide was then dried under N₂ and freeze-dried.

Peptide purification and characterization

The above peptides were further purified by Sephadex G25 gel-filtration chromatography (Sigma) to remove organic contaminants and to partly separate incomplete peptides on the basis of molecular mass. Fractions corresponding to absorbance peaks at 215 nm were collected, freeze-dried and further purified by reverse-phase HPLC with a C₄ column (Waters, Milford, MA, U.S.A.).

Plasma desorption MS was used to differentiate the full-length peptide product and any by-product that differed from the target peptide's theoretical molecular mass. Plasma desorption mass spectra were obtained for each peptide at the Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University, College Station, TX, U.S.A.

The concentration of peptide stock solution was determined by UV absorption at 280 nm. The concentration of those peptides that did not contain Tyr or Trp was determined by amino acid analysis performed at the Protein Chemistry Laboratory, Department of Chemistry, Texas A&M University, College Station, TX, U.S.A.

Preparation of small unilamellar vesicles (SUV) by sonication

Sonicated SUV composed of PtdCho/cholesterol (molar ratio 65:35) or PtdCho/cholesterol/PtdSer (specific molar ratios as indicated in the Figures and Tables) were prepared essentially as described previously [25,26], with some modifications. Lipids dissolved in chloroform stocks were mixed in an amber vial, dried under N₂ to form a thin film on the wall of the vial, and rehydrated in 10 mM Mops buffer, pH 7.4, prefiltered through a 0.2 μ m filter (Millipore, Bedford, MA, U.S.A.). The rehydrated sample was then vortex-mixed and then sonicated under N₂ at 4 °C with a Sonic Dismembrator Model 550 (Fisher Scientific, Pittsburgh, PA, U.S.A.). The microprobe energy was maintained at level 4. Pauses of 1 min between 2 min sonication periods prevented overheating. Multilamellar vesicles and titanium debris from the sonicator probe were removed by centrifugation at 110000 g (35000 rev./min) for 4 h with a 40Ti rotor (Beckman Instruments, Fullerton, CA, U.S.A.). A lipid phosphate assay was used to determine the lipid concentration of the final SUV solution.

Preparation of large unilamellar vesicles (LUV) by extrusion

LUV composed of PtdCho/cholesterol or PtdCho/cholesterol/PtdSer (all ratios given are molar percentages) were prepared by extrusion through polycarbonate membranes. The appropriate amount of lipids were mixed, dried and hydrated in Mops buffer as described for SUV. The lipid suspension was then extruded with a handheld mini-extruder (Avanti). The extrusion was repeated 15 times back and forth through double layers of 0.1 μ m polycarbonate membranes (Avanti).

Photon correlation spectroscopy to determine the size of SUV and LUV

Photon correlation spectroscopy with a Coulter N4 Plus Photon Correlation Spectrometer (Beckman-Coulter, Miami, FL, U.S.A.) was used to determine the particle size distribution of SUV and LUV. Photon correlation spectroscopy optimally measures particle size in the range 3–3000 nm in diameter, well within the size range of the SUV and LUV.

CD

CD spectra were obtained as described [8], with the following modifications. The samples contained peptide (15 μ M) or protein (4 μ M) with or without lipid vesicles (1 mM). All spectra were corrected for background with samples consisting of buffer or vesicles without peptide or protein. A J-710 Spectropolarimeter (JASCO, Easton, MD, U.S.A.) was used to obtain CD measurements at room temperature for samples in a 1 mm circular quartz cell. The spectra were obtained from 185 to 260 nm under the following conditions: 1 nm step resolution; 50 nm/min; response 1 s; bandwidth 2.0 nm; sensitivity 0.01°. Five to ten scans were averaged, smoothed, background-subtracted and converted to mean residue molar ellipticity [θ] (degrees $\cdot$ cm² $\cdot$ dmol⁻¹) for each measurement. The percentage α -helix for the N-terminal peptides was estimated from $\theta_{222} = (f_h - i\kappa/N)[\theta_{h222\infty}]$, where θ_{222} is the mean residue molar ellipticity at 222 nm, f_h is the fraction in α -helical form, i is the number of helices, κ is a wavelength-specific constant with a value of 2.6 at 222 nm, N is the number of residues in the peptide, and $\theta_{h222\infty}$ is the molar ellipticity for a helix of infinite length at 222 nm and has a value of -39500 degrees $\cdot$ cm² $\cdot$ dmol⁻¹. The percentage α -helix for the SCP₂ protein

was estimated by using the SELCON program provided by JASCO.

Transbilayer distribution PtdSer

In both SUV and LUV the PtdSer transbilayer distribution was determined by reaction of the PtdSer amino group with a non-penetrating reagent (2,4,6-trinitrobenzenesulphonic acid), as described previously [27].

Peptide and SCP₂ binding to SUV or LUV membranes

Peptide and SCP₂ binding to SUV and LUV membranes was performed essentially as described previously [28], with the following modifications. Samples (200 μ l) containing 12 nmol of peptide and 800 nmol of SUV or LUV lipid in 10 mM Mops buffer, pH 7.4, were incubated at room temperature for 10 min, transferred to a Microcon-100 filtration unit (molecular mass cut-off 100 kDa) and centrifuged at 3000 *g* for 4–7 min until all except 5–10 μ l had passed through the filter. Then the filter was rinsed with buffer (100 μ l) and the filtration unit was recentrifuged for a further 2–3 min. Unbound peptide in the flow-through fraction was quantified with an *o*-phthalaldehyde assay. SCP₂ binding was determined similarly except that the amount of SCP₂ was 2 nmol and the amount of SUV or LUV was 250 nmol in the sample.

Standard curves composed of 1–6 nmol of the peptide or SCP₂ to be assayed were analysed with each experiment. For each 100 μ l sample aliquot, 0.5 ml of 0.05 M sodium borate, pH 10, and 0.5 ml of 0.5% (v/v) 2-mercaptoethanol in ethanol were added to prepare the sample for peptide quantification. The sample was vortex-mixed after each addition. A stock solution of *o*-phthalaldehyde (10 mg/ml in methanol) was diluted 1:10 in 0.05 M borate, pH 10. While the above sample was again vortex-mixed, a 0.5 ml of the diluted *o*-phthalaldehyde solution was added followed by incubation at room temperature for 20 min. The fluorescence intensity (excitation at 340 nm, emission at 440 nm), measured with a PC1 Photon Counting fluorescence spectrophotometer (ISS, Champaign, IL, U.S.A.), was proportional to the peptide concentration over the range used.

RESULTS

The results presented here address the hypothesis that the N-terminus of SCP₂ is a domain whose interaction with membranes is dependent on the structural properties of the membrane as well as the structure of the N-terminus. This was achieved by (1) the preparation of membranes differing in composition and surface curvature, (2) the synthesis of an SCP₂ N-terminal peptide representing the membrane interaction domain, and (3) the synthesis and characterization of several variants of the SCP₂ N-terminal peptide to determine the structural specificity of this peptide for membranes differing in surface curvature.

Size distribution of SUV and LUV determined by photon correlation spectroscopy

To elucidate the effect of membrane curvature on the interaction of SCP₂ and N-terminal peptides from SCP₂ with membranes, two types of model membrane lipid vesicle were prepared as described in the Materials and methods section: SUV prepared by sonication and LUV prepared by extrusion through two layers of polycarbonate membranes. The size distributions of the SUV and LUV were determined by photon correlation spectroscopy, also as described in the Materials and methods section.

Figure 1 Size distribution of SUV and LUV lipid vesicles determined by photon correlation spectroscopy

(A) Sonicated SUV (PtdCho/cholesterol/PtdSer molar ratio 55:35:10); (B) LUV (PtdCho/cholesterol/PtdSer 55:35:10) extruded through a double layer of polycarbonate membrane with a 0.1 μ m pore size.

Table 1 Size distribution of lipid vesicles

SUVs were prepared by sonication as described in the Materials and methods section; LUVs were prepared by extrusion through a stack of two polycarbonate membranes with a pore size of 0.1 μ m, as described in the Materials and methods section. Vesicle lipid compositions are expressed as molar ratios of PtdCho/cholesterol/PtdSer. Vesicle size distributions were determined by photon correlation spectroscopy as described in the Materials and methods section. Values are means $\pm$ S.D.

Vesicle lipid composition	Median vesicle diameter (nm)	
	SUV	LUV
65:35:0	29 $\pm$ 5	105 $\pm$ 20
55:35:10	25 $\pm$ 5	120 $\pm$ 20
40:50:10	27 $\pm$ 6	120 $\pm$ 10
35:35:30	19 $\pm$ 5	110 $\pm$ 15

SUV, prepared by the sonication of PtdCho/cholesterol/PtdSer (55:35:10), had a very narrow size distribution with a median diameter of 25 nm (mean radius of curvature 13 nm) and peak width at half height of 14 nm (Figure 1A). This size distribution varied with the lipid composition. Increasing the amount of anionic phospholipid from 0 to 10 to 30 mol% decreased the SUV size, as shown by median diameters of 29, 25 and 19 nm respectively (Table 1). In contrast, increasing the cholesterol content from 35 to 50 mol% did not significantly affect the SUV size.

In contrast with SUV, the preparation of LUV by extrusion resulted in much larger vesicles with nearly 4-fold greater median diameters of 105 nm (mean radius of curvature 53 nm) and a broader distribution (peak width at half height of 31 nm) (Figure 1B). However, this size distribution was not significantly

Table 2 Transbilayer distribution of PtdSer in lipid vesicles

SUVs were prepared by probe sonication as described in the Materials and methods section; LUVs were prepared by extrusion through a stack of two polycarbonate membranes with a pore size of 0.1 μm , as described in the Materials and methods section. Vesicle lipid composition is expressed as molar ratios of PtdCho:cholesterol:PtdSer. Negatively charged lipid PtdSer distributions were determined by 2,4,6-trinitrobenzenesulphonic acid assay as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 3$).

Vesicle lipid composition	Phosphatidylserine transbilayer distribution					
	SUV			LUV		
	Outer leaflet (nmol)	Total (nmol)	Outer leaflet/total	Outer leaflet (nmol)	Total (nmol)	Outer leaflet/total
55:35:10	113.0 $\pm$ 0.7	147.5 $\pm$ 4.7	0.77 $\pm$ 0.02	53.6 $\pm$ 0.5	146.4 $\pm$ 3.0	0.37 $\pm$ 0.01
40:50:10	112.3 $\pm$ 1.4	142.5 $\pm$ 4.6	0.79 $\pm$ 0.03	53.9 $\pm$ 3.0	159.8 $\pm$ 2.2	0.34 $\pm$ 0.02
35:35:30	167.6 $\pm$ 3.0	438.9 $\pm$ 2.7	0.38 $\pm$ 0.01	85.1 $\pm$ 3.9	445.1 $\pm$ 2.5	0.19 $\pm$ 0.01

dependent on the lipid composition. Increasing the amount of anionic phospholipid from 0 to 10 to 30 mol % did not alter the mean size distribution of the LUV (Table 1); neither did increasing the cholesterol content from 35 to 50 mol % (Table 1). Most importantly for the studies presented here, the size distribution of the LUV (Figure 1B) essentially showed no overlap with that of the SUV (Figure 1A).

Effect of membrane vesicle curvature on transbilayer distribution of anionic phospholipid

Because the amphipathic α -helical N-terminus of SCP₂ is hypothesized to be a membrane-binding domain, it was important to determine the effect of vesicle radius of curvature and anionic phospholipid content on the exposure of anionic phospholipid at the membrane surface. As shown in Table 2, the ratio of PtdSer in the outer leaflet to total PtdSer in the SUV was approx. 0.77:1. This ratio was consistent with the total lipid distribution across the SUV bilayer, i.e. 70 % and 30 % of the total lipid are in the outer and inner leaflets respectively [27,29]. The ratio of PtdSer in the SUV outer leaflet to total PtdSer decreased by nearly 50 % at 30 mol % PtdSer (Table 2). However, the quantitative amount of PtdSer in the outer leaflet increased concomitantly from 113 nmol at 10 % PtdSer to 167 nmol at 30 mol % PtdSer in the SUV. Because increasing the molar percentage of PtdSer 3-fold increased the amount of PtdSer in the SUV outer leaflet only 1.5-fold, this was consistent with PtdSer's exhibiting a preference (at high molar percentages of PtdSer) for the cytofacial leaflet in the SUV. In contrast, neither this ratio nor the amount of PtdSer in the outer leaflet were significantly affected by increasing the molar percentage of cholesterol (Table 2).

A similar pattern of PtdSer transbilayer distribution was observed in LUV. The exposure of PtdSer in the outer leaflet increased from 53.6 nmol at 10 mol % PtdSer in the LUV to 85 nmol at 30 mol % PtdSer in the LUV. However, this amount as well as the ratio of PtdSer in the LUV outer leaflet to total PtdSer was approximately half that observed in SUV with the same lipid composition (Table 2). This was true regardless of the molar percentage of cholesterol or PtdSer present. Thus SUV had a greater exposure of PtdSer in the outer leaflet, both as a fraction of the total PtdSer and as a total amount, than did the LUV. Even a 3-fold increase in PtdSer from 10 to 30 mol % in the LUV did not completely increase the amount of PtdSer in the LUV outer leaflet to equal that of 10 mol % PtdSer containing SUV.

Figure 2 CD spectra of SCP₂ in the presence of neutral lipid vesicles (PtdCho/cholesterol molar ratio 65:35)

CD spectra were obtained as described in the Materials and methods section with 4 μM SCP₂ in buffer only (●); SUV (○); LUV (▼).

Interaction of SCP₂ with SUV and LUV

The effect of membrane curvature and PtdSer transbilayer distribution on the interaction of the complete SCP₂ protein with membranes was examined by CD. The CD spectra of SCP₂ in aqueous buffer displayed significant negative molar ellipticity ($-7.9 \pm 0.1 \times 10^3$ ($n = 3$) degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ at 222 nm (values given as means $\pm$ S.D.), and a large positive molar ellipticity at 190 nm (Figure 2, ●). Curve-fitting analysis as described in the Materials and methods section showed that SCP₂ in aqueous buffer had 29.4 ± 1.0 % ($n = 3$) α -helix.

The effect of the radius of curvature of the membrane on the interaction of SCP₂ with neutrally charged membranes (PtdCho/cholesterol molar ratio 65:35) was determined (Figure 2). In the presence of neutrally charged SUV membranes the CD spectra of SCP₂ were not significantly different from those of SCP₂ in buffer alone (Figure 2, ○ and ●). Likewise, in the presence of neutrally charged LUV membranes the CD spectra of SCP₂ were not

Figure 3 CD spectra of SCP₂ in the presence of negative lipid vesicles (PtdCho/cholesterol/PtdSer molar ratio 55:35:10)

CD spectra were obtained as described in the Materials and methods section with 4 μ M SCP₂ in buffer only (●); SUV (○); LUV (▼).

Table 3 α -Helical content of SCP₂ on interaction with lipid vesicles: -effect of lipid composition and membrane curvature

The experiments were performed with 4 μ M SCP₂ and 1 mM liposomes, as described in the Materials and methods section. SUV were prepared by sonication and LUV by extrusion, as described in the Materials and methods section. Lipid compositions are expressed as molar ratios of PtdCho/cholesterol/PtdSer. The α -helix content was estimated from CD spectra by using the SELCON program as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 3$ or 4).

Type of lipid vesicles		α -Helix content (%)
None (buffer)		29.4 $\pm$ 1.0
Neutral vesicles	SUV 65:35:0	31.2 $\pm$ 0.5
	LUV 65:35:0	31.9 $\pm$ 2.0
Negative vesicles	SUV 55:35:10	35.2 $\pm$ 0.1
	LUV 55:35:10	33.2 $\pm$ 0.3
	LUV 40:50:10	31.6 $\pm$ 2.2
	LUV 35:35:30	32.9 $\pm$ 0.9

significantly different from those of SCP₂ in buffer alone (Figure 2, ▼ and ●). Thus SCP₂ did not display alterations in secondary structure in the presence of neutral membrane vesicles, regardless of the membrane's radius of curvature.

The effect of membrane curvature on the interaction of SCP₂ with acidic membrane vesicles (PtdCho/cholesterol/PtdSer molar ratio 55:35:10) of different radii of curvature differed markedly from that for neutral membrane vesicles (Figure 3). In the presence of acidic-phospholipid-containing SUV membranes, the CD spectra of SCP₂ were altered markedly from those of SCP₂ in buffer alone (Figure 3, ○ and ●). The molar ellipticity at 222 nm became significantly more negative [$(-1.03 \pm 0.05) \times 10^4$ degrees $\cdot$ cm² $\cdot$ dmol⁻¹ ($n = 4$) compared with $(-7.9 \pm 0.1) \times 10^3$ degrees $\cdot$ cm² $\cdot$ dmol⁻¹ ($n = 3$)], whereas that of molar ellipticity at 190 nm increased. Curve-fitting analysis to determine the proportion of α -helix as described in the Materials and

Figure 4 CD spectra of SCP₂ in the presence of LUV with increasing amounts of cholesterol and negatively charged lipid (PtdSer)

CD spectra were obtained as described in the Materials and methods section with 4 μ M SCP₂ in LUV (PtdCho/cholesterol/PtdSer molar ratio 55:35:10) (●); LUV (PtdCho/cholesterol/PtdSer 40:50:10) (○); LUV (PtdCho/cholesterol/PtdSer 35:35:30) (▼).

methods section revealed that, on interaction with the acidic-phospholipid-containing SUV membranes, SCP₂ underwent a significant change in secondary structure. The content of α -helix increased from 29.4 $\pm$ 1.0% ($n = 3$) to 35.2 $\pm$ 0.1% ($n = 4$) (Table 3). In contrast, in the presence of acidic-phospholipid-containing LUV membranes, the CD spectra of SCP₂ differed only slightly from those of SCP₂ in buffer alone (Figure 3, ▼ and ●). To determine whether this lack of effect might be due to the lower exposure of PtdSer in the LUV (see above), the acidic phospholipid content of the LUV was increased from 10 to 30 mol%. This increased the exposure of PtdSer in the outer leaflet of the LUV by 1.6-fold (Table 2) but resulted in only a small alteration in the SCP₂ CD spectrum that was suggestive of slightly increased α -helical content (Figure 4, ▼). Calculation of the α -helical content showed that this change versus buffer was statistically significant (Table 3). Increasing the cholesterol content of LUV to 50 mol% did not elicit significant alterations in SCP₂ CD spectra compared with LUV (PtdCho/cholesterol/PtdSer 55:35:10) (Figure 4).

In summary, the results showed for the first time that the complete SCP₂ protein underwent large changes in secondary structure on interaction with highly curved, anionic-phospholipid-containing SUV, but much less so with the low-curvature LUV.

Synthesis, purification and identity of the SCP₂ N-terminal peptides

As pointed out in the Introduction section, the SCP₂ N-terminus contains an amphipathic α -helical domain, comprising the first 32 amino acid residues of the protein. This region has been postulated to be a membrane interaction domain in SCP₂ [22]. To test this possibility, several SCP₂ N-terminal peptides were synthesized. Peptide 1-32SCP₂ was identical with the first 32 residues in SCP₂ and contained both putative amphipathic N-terminal α -helices (residues 9-22 and 25-30). Several of the

Figure 5 CD spectra of SCP₂ N-terminal peptides in aqueous buffer (A) and water/TFE (1:1, v/v) (B)

CD spectra were obtained as described in the Materials and methods section for the following peptides: 1-32SCP₂ (●); 1-24SCP₂ (○); 1-E20-32SCP₂ (▼); 10-32SCP₂ (▽).

structural requirements of this peptide for membrane interaction and/or intermembrane sterol transfer were tested by synthesis of modified N-terminal peptides. Peptide 1-24SCP₂ contained only the first α -helix, residues 9–22. Peptide 1-E20-32SCP₂ contained a single Glu²⁰ substitution for Leu²⁰. This substitution in intact SCP₂ inactivated the protein [30]. Peptide 10-32SCP₂ had the non-helical N-terminal nine amino acids deleted but contained both α -helices. Site-directed mutagenesis to delete this segment from the intact SCP₂ protein inactivated the protein [30]. The purity of all peptides was confirmed by HPLC as described in the Materials and methods section. The amino acid composition of each peptide, also determined as described in the Materials and methods section, was the same as that expected on the basis of the amino acid sequence. The expected masses of the peptides were confirmed by matrix-assisted laser desorption/ionization-time-of-flight MS to be 3585, 2655, 2717 and 3601 Da for 1-32SCP₂, 1-24SCP₂, 10-32SCP₂ and 1-E20-32SCP₂ respectively.

CD spectra of SCP₂ N-terminal peptides in aqueous buffer and aqueous trifluoroethanol

The ability of the SCP₂ N-terminal peptides to form α -helical structure spontaneously was examined in aqueous buffer. CD spectra of the SCP₂ N-terminal peptides 1-32SCP₂, 1-24SCP₂, 1-E20-32SCP₂ and 10-32SCP₂ in aqueous buffer showed a large negative peak at approx. 200 nm and a small negative peak at 222 nm, which indicated that these peptides adopted primarily random coil structures (Figure 5A). The content of α -helix for each peptide in aqueous buffer was estimated to be 15% for 1-32SCP₂ and 1-24SCP₂, 12% for 1-E20-32SCP₂ and 18% for

Table 4 SCP₂ N-terminal peptide CD and secondary structure in water/TFE

CD spectra were taken with 15 μ M peptides in water/TFE (1:1, v/v). The molar ellipticities at 222 nm are expressed as means $\pm$ S.D. ($n = 3$). The α -helical contents were estimated on the basis of molar ellipticity at 222 nm, as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 3$).

Peptide	$10^{-4} \times (-[\theta]_{222})$	Estimated α -helical content (%)	Estimated numbers of helical/non-helical residues
1-32SCP ₂	2.68 ± 0.07	75.9 ± 1.8	24/8
1-24SCP ₂	2.18 ± 0.02	66.6 ± 0.5	16/8
1-E20-32SCP ₂	2.19 ± 0.01	40.7 ± 0.2	13/19
10-32SCP ₂	2.95 ± 0.02	86.0 ± 0.5	20/3

10-32SCP₂ on the basis of molar ellipticity at 222 nm (see Figure 7, open columns).

To determine the propensity for SCP₂ N-terminal peptides to form helical structures in an environment more closely resembling the interior core of proteins, the above experiments were repeated in water/1,1,1-trifluoroethanol (TFE) (1:1, v/v) (Figure 5B). Water/TFE (1:1) favours secondary structure in peptides and proteins by promoting intramolecular hydrogen-bonding [31]. In water/TFE (1:1), the CD spectra of all peptides showed the characteristics of α -helical structure: double negative minima at 208 and 222 nm and a positive maximum at 190 nm. The molar ellipticity at 222 nm, the estimated α -helical content and the estimated number of helical and non-helical amino acids for each peptide are summarized in Table 4. The α -helical content of each peptide in water/TFE (1:1) was estimated on the basis of the molar ellipticity at 222 nm as described in the Materials and methods section. In water/TFE (1:1), the α -helical content of peptide 1-32SCP₂ was 76%, with eight non-helical amino acids and 24 helical amino acids. This was consistent with the NMR data for the SCP₂ protein [23] which suggested that the first nine residues of the SCP₂ N-terminus are non-helical. Thus, in a more hydrophobic environment, the SCP₂ N-terminal peptide comprising the entire putative membrane interaction domain had an α -helix-forming potential very similar to that predicted from the intact protein. The α -helical content of peptide 1-24SCP₂ in water/TFE (1:1) was 67%, which is consistent with 16 helical amino acids and 8 non-helical amino acids. Both 1-32SCP₂ and 1-24SCP₂ had 8 non-helical amino acids; the remaining amino acids were α -helical. Thus the deletion of the C-terminal putative α -helical region of peptide 1-32SCP₂ to form peptide 1-24SCP₂ had no effect on the predicted ability of the N-terminal putative α -helical region to form an α -helix. In contrast, peptide 1-E20-32SCP₂ (replacement of Leu²⁰ with Glu²⁰) showed only 41% α -helix, which is consistent with 13 helical amino acids and 19 non-helical amino acids. Thus the replacement of Leu²⁰ with Glu²⁰ substantially decreased the ability of the peptide to form α -helical structure. Finally, peptide 10-32SCP₂ showed 86% α -helix (Table 2), suggesting 20 helical amino acids and 3 non-helical amino acids. This indicated that the first 9 non-helical amino acids were not necessary for the rest of the peptide to form an α -helical structure.

Interaction of SCP₂ N-terminal peptide 1-32SCP₂ with SUV and LUV

The N-terminal 32 residues of the intact SCP₂ contains two amphipathic α -helical regions, representing 75% of the protein's total α -helices as determined by NMR [23]. To test whether this region in the protein interacts with membranes, peptide 1-32SCP₂ (comprising both N-terminal α -helical regions residues 9–22 and

Figure 6 CD spectra of SCP₂ N-terminal peptides in the presence of SUV and LUV

(A) 1-32SCP₂; (B) 1-24SCP₂; (C) 1-E20-32SCP₂; (D) 10-32SCP₂. Symbols: ●, buffer only; ○, SUV (PtdCho/cholesterol/PtdSer molar ratio 55:35:10); ▼, LUV (PtdCho/cholesterol/PtdSer 55:35:10); ▽, LUV (PtdCho/cholesterol/PtdSer 35:35:30).

25–30) was synthesized. The specificity of peptide 1-32SCP₂ to interact with SUV and/or LUV was examined by CD for comparison with that of the intact SCP₂ protein. In the presence of anionic-phospholipid-containing SUV membranes (PtdCho/cholesterol/PtdSer 55:35:10), CD spectra of peptide 1-32SCP₂ (Figure 6A) showed increased negative molar ellipticity near 222 nm and increased positive molar ellipticity near 190 nm. Both spectral alterations were consistent with increased α -helical structure. On the basis of the molar ellipticity at 222 nm, the α -helical content increased from 15% to 38% (Figure 7).

In contrast, in the presence of anionic-phospholipid-containing LUV membranes (PtdCho/cholesterol/PtdSer 55:35:10), the molar ellipticity at 222 nm and estimated α -helical content of the peptide were not significantly different from those in aqueous buffer (Figures 6A and 7). When the PtdSer content of LUV was increased to 30%, a very small increase in molar ellipticity at 222 nm was observed (Figure 6A, ▽). The α -helical content was estimated to be 17%, slightly higher than that in aqueous buffer and much smaller than that in the presence of negative SUV (Figure 7). Therefore the interaction of peptide 1-32SCP₂ with membranes depended on membrane curvature and the presence of negatively charged lipids.

This pattern of preferential interaction of peptide 1-32SCP₂ with anionic-phospholipid-containing membranes of high surface curvature (SUV) but not those of lower curvature (LUV) was basically similar to that exhibited by the complete SCP₂ protein described above.

Effect of N-terminal peptide modifications on interaction with SUV and LUV

The effect of membrane curvature on the ability of modified N-terminal peptides 1-24SCP₂, 1-E20-32SCP₂ and 10-32SCP₂ to interact

Figure 7 α -Helical content of SCP₂ N-terminal peptides in the presence of SUV and LUV

α -Helical content was estimated on the basis of the CD molar ellipticity of the peptides at 222 nm as described in the Materials and methods section. From left to right for each peptide the following symbols were used: open columns, buffer only; hatched columns, SUV (PtdCho/cholesterol/PtdSer molar ratio 55:35:10); filled columns, LUV (PtdCho/cholesterol/PtdSer 55:35:10); squared columns, LUV (PtdCho/cholesterol/PtdSer 35:35:30).

with membranes was examined (Figure 6). In the presence of anionic-phospholipid-containing SUV membranes (PtdCho/cholesterol/PtdSer 55:35:10), the CD spectra of all the peptides changed to patterns indicative of a higher α -helical content: increased negative molar ellipticity at 222 nm and increased positive molar ellipticity at 190 nm. However, the SCP₂ N-terminal peptide containing only the first α -helix (1-24SCP₂, α -helix residues at amino acids 9–22; Figure 6B) as well as the peptide with replacement of Leu²⁰ by Glu (1-E20-32SCP₂; Figure 6C) interacted less well with acidic-phospholipid-containing SUV membranes than peptide 1-32SCP₂, which contained both N-terminal α -helices (residues 9–22 and 25–30) (Figure 6A). Deletion of the N-terminal nine non-helical amino acids (10-32SCP₂; Figure 6D) resulted in no significant changes in membrane interaction from that of peptide 1-32SCP₂.

In contrast, no significant changes in CD spectra were observed with these peptides in the presence of anionic-phospholipid-containing LUV membranes (PtdCho/cholesterol/PtdSer 55:35:10) (Figure 6, ▼). Even a 3-fold increase in content of anionic phospholipid in the LUV membranes (PtdCho/cholesterol/PtdSer 35:35:30) only slightly altered the CD spectra of these peptides in comparison with those in aqueous buffer (Figure 6, ▽). Calculations based on molar ellipticity at 222 nm also showed no significant changes in the α -helical content of the peptides in the presence of these 30% anionic-phospholipid-containing LUV membranes in comparison with peptides in buffer only (Figure 6).

In summary, the CD spectra and calculated α -helical content indicated the following relative order of SCP₂ N-terminal peptide interaction with anionic-phospholipid-containing SUV membranes: 1-32SCP₂ = 10-32SCP₂ > 1-24SCP₂ > 1-E20-32SCP₂. However, none of the peptides interacted with acidic-phospholipid-containing LUV membranes.

Figure 8 Binding of SCP₂ to model membranes

The binding assay was done as described in the Materials and methods section. For each type of liposome from left to right the following symbols were used: solid columns, SUV; open columns, LUV. The ratios are molar ratios of PtdCho (POPC)/cholesterol/PtdSer (DOPS).

Binding of SCP₂ to SUV and LUV membranes

The lack of large changes in CD observed with SCP₂ on the addition of LUV could be due either to a lack of binding of the protein to the vesicle membranes or to binding without alteration to the protein secondary structure. This issue was resolved by using an independent protein binding assay as described in the Materials and methods section. SCP₂ was incubated with SUV and LUV separately. Bound and unbound SCP₂ were then separated by filtration through a 100 kDa cutoff filter, followed by quantification of protein in the flow-through with an *o*-phthalaldehyde assay. To ensure that the 100 kDa filter excluded lipids, a control filtration-binding experiment was performed. A lipid phosphate assay [32] was used to measure the phospholipid in both the flow-through and the retained fractions $\pm$ SCP₂. SUV (PtdCho/cholesterol/PtdSer 35:35:30) (800 nmol) were treated in exactly the same manner as in the filtration binding assay described in the Materials and methods section. All the phospholipid was retained on the filter; no phospholipid was detected in the flow-through fraction.

SCP₂ did not bind to neutrally charged SUV or LUV (PtdCho/cholesterol 65:35) membranes (Figure 8). However, increasing the anionic phospholipid content of the SUV from 0 to 10 to 30 mol % increased the binding of SCP₂ from 0 % to 62 % and 92 % respectively (Figure 8). The effect of anionic phospholipid was potentiated by increasing the cholesterol content (Figure 8). Increasing the anionic phospholipid content and the cholesterol content of LUV did not significantly affect SCP₂ binding until the content of PtdSer was increased to 30 mol % or the cholesterol content of the anionic-phospholipid-containing LUV was increased to 50 mol % (Figure 8). However, even under these conditions the binding of SCP₂ to LUV was only 20–33 % of that to SUV. Thus SCP₂ bound significantly less to LUV than to SUV with a similar lipid composition.

Binding of peptide to SUV and LUV membranes

The lack of CD changes observed with N-terminal peptides on the addition of LUV could also be due either to a lack of binding of the peptides to the vesicle membranes or to binding without alteration to the peptide secondary structure. We therefore incubated the peptides with sonicated SUV and extruded LUV

Figure 9 Percentage of free peptides in the binding assay

The binding assay was performed as described in the Materials and methods section with N-terminal peptides of SCP₂ (12 nmol) and lipid vesicles (800 nmol). The free peptides were separated from the bound peptides by passage through a 100 kDa cut-off filter and quantified by the *o*-phthalaldehyde assay. From left to right for each peptide the following symbols were used: solid columns, LUV (PtdCho/cholesterol molar ratio 65:35); open columns, LUV (PtdCho/cholesterol/PtdSer 35:35:30); hatched columns, SUV (PtdCho/cholesterol/PtdSer 35:35:30).

separately, unbound peptide was separated from the bound peptide by filtration through a 100 kDa molecular cut-off filter, and free peptides in the flow-through portion were then quantified with *o*-phthalaldehyde assay as described in the Materials and methods section. To determine whether the presence of amphipathic peptides might disrupt membrane vesicles and allow vesicle fragments to pass through the membrane, the phospholipid in both the flow-through and the retained fractions was determined in the presence or absence of peptides. SUV (PtdCho/cholesterol/PtdSer 35:35:30) (800 nmol) were incubated with 12 nmol of 1-32SCP₂, 1-24SCP₂, 1-E20-32SCP₂ or 10-32SCP₂ in a total volume of 200 μ l, exactly as described for the filtration binding assay (see the Materials and methods section). All the phospholipids were retained on the filter; no phospholipid was detected in the flow-through fraction. This control experiment with phosphate assay demonstrated (1) that the 100 kDa filter retained all the phospholipid vesicles and (2) that, in the presence of amphipathic SCP₂ N-terminal peptide 1-32SCP₂ and other modified N-terminal peptides, there was no vesicle breakdown that allowed phospholipids to pass through the membrane filter.

When the peptides were incubated with anionic-phospholipid-containing SUV membranes (PtdCho/cholesterol/PtdSer 35:35:30), the proportion of bound peptides greatly increased to 73 % for 1-32SCP₂, 47 % for 1-24SCP₂, 22 % for 1-E20-32SCP₂ and 70 % for 10-32SCP₂ (Figure 9). These peptide binding results with anionic-phospholipid-containing SUV were consistent with the observed changes in CD spectra and secondary structure of the peptides on interaction with anionic-phospholipid-containing SUV membrane. The results indicated that all peptides bound preferentially to membrane vesicles with a high radius of curvature and containing anionic phospholipids. The binding of peptides with SUV containing anionic lipids was in the following order: 1-32SCP₂ = 10-32SCP₂ > 1-24SCP₂ > 1-E20-32SCP₂.

In contrast, only weak binding was observed after incubation of the peptides with LUV of the same lipid composition. After incubation with neutrally charged LUV (PtdCho/cholesterol 65:35) membranes, 0 % of 1-32SCP₂, 0 % of 1-24SCP₂, 3 % of

Figure 10 Effect of SCP₂ and peptide ¹⁻³²SCP₂ on the size distribution of SUV lipid vesicles determined by photon correlation spectroscopy

SUV were composed of PtdCho/cholesterol/PtdSer (molar ratio 35:35:30). (A) SUV without protein or peptide; (B) SUV plus 4 μM SCP₂ added; (C) SUV plus 15 μM peptide ¹⁻³²SCP₂.

^{1-E20-32}SCP₂ and 10 % of ¹⁰⁻³²SCP₂ were bound (Figure 9). Therefore none of the peptides bound significantly to the neutral LUVs. This agrees with the observed lack of significant changes in CD spectra and secondary structure of the peptides on interaction with neutral LUV. In the presence of anionic-phospholipid-containing LUV (PtdCho/cholesterol/PtdSer 35:35:30) membranes, the percentage of bound peptides increased to 3 % for ¹⁻³²SCP₂, 3 % for ¹⁻²⁴SCP₂, 8 % for ^{1-E20-32}SCP₂ and 12 % for ¹⁰⁻³²SCP₂ (Figure 9). However, these slight increases in peptide binding were not significantly different from those for each peptide's binding to the corresponding LUV containing no anionic phospholipid (Figure 9, compare open bars with solid bars). This much weaker binding of the peptides to LUV than to SUV was consistent with the observed CD alterations described above.

Effect of peptide and SCP₂ binding on SUV membrane structure

To ascertain whether the interaction of the SCP₂ or its active amphipathic peptide ¹⁻³²SCP₂ with SUV might disrupt vesicle structure, light-scattering experiments were performed. Measurements of light scattering permitted the determination of whether SUV size or distribution was altered by exposure to peptides or SCP₂ under the same conditions as the CD measurements described above. SUV (PtdCho/cholesterol/PtdSer 35:35:30) (1 mM) were incubated with SCP₂ (4 μM) or ¹⁻³²SCP₂ (15 μM); vesicle size distribution was measured by light scattering.

The mean size of the SUV in the absence of added SCP₂ or peptide was 21.8 nm, with a narrow distributional width as indicated by an S.D. of ±5.1 nm (Figure 10A). In the presence of SCP₂, the mean size of the SUV was increased to 30.3 nm, with a broader distributional width as shown by an S.D. of ±9.9 nm

(Figure 10B). Previous studies showed that SCP₂ is slightly ellipsoidal, with a diameter of 41 Å (4.1 nm) [33]. Thus the increase in size of the SUV was consistent with the binding of multiple SCP₂ molecules, which would increase the average size from 21.8 nm to approx. 30 nm. More importantly, SCP₂ binding to the SUV did not decrease the SUV size, which would have indicated disruption of the SUV membrane structure.

In the presence of amphipathic SCP₂ N-terminal peptide (¹⁻³²SCP₂), the mean size of the SUV was 23.2 nm, with an intermediate distributional width as shown by an S.D. of ±7.2 nm (Figure 10C). The three-dimensional structure and the exact size of the peptide are not known. Because ¹⁻³²SCP₂ represents about 26 % of the SCP₂ sequence of the protein, this would explain the slight increase in size of the SUV on peptide binding. Most importantly, peptide ¹⁻³²SCP₂ did not disrupt the SUV membrane structure.

In summary, the light-scattering experiment demonstrated that in the presence of SCP₂ or its N-terminal peptide ¹⁻³²SCP₂ there was no vesicle breakdown or vesicle aggregation into large particles under the experimental conditions used. The small size increases consequent on the binding of either protein or peptide were consistent with the addition of protein or peptide to the vesicle surface to increase the mean size of the vesicles slightly.

DISCUSSION

Despite several decades of investigation into the role of SCP₂ in lipid metabolism, almost nothing is known about the molecular details of SCP₂ action (reviewed in [2]). Current thought indicates that SCP₂ might have at least two important structural domains: (1) a ligand-binding domain whose properties have been the focus of intense investigation action (reviewed in [8–11,15]) and (2) a membrane-binding domain whose postulated properties [22,23] are only beginning to be elucidated [9]. The putative membrane-binding domain of SCP₂ has been proposed to be composed of the N-terminal two amphipathic α-helices, which are predicted to interact with acidic lipids in membranes. However, almost nothing is known about the membrane's structural properties necessary for the interaction of the putative SCP₂ N-terminal binding domain. The results presented here contribute significantly to our understanding of these issues, as follows.

First, two independent methods directly confirmed the prediction that SCP₂ bound preferentially to SUV membranes enriched in acidic phospholipid. The secondary structure of SCP₂ was significantly altered by this interaction, as indicated by the increase in the content of α-helix in the SCP₂ protein. This interaction was further supported by an independent filtration binding assay. The significance of these observations to biological membranes was demonstrated previously with isolated plasma membrane vesicles to which SCP₂ bound *in vitro* [21].

The above observation of the binding of SCP₂ to anionic membranes is also important in the context of lipid transfer activity. SCP₂ is a basic protein (pI 8.6); previous studies have shown that the incorporation of acidic lipids into the membrane vesicles greatly enhanced SCP₂-mediated lipid transfer activity between sonicated SUV [26,34–36] and SUV produced by ethanol injection [37]. In contrast, SCP₂ was unable to transfer cholesterol between anionic membranes of low surface curvature, i.e. monolayer membranes [38]. Furthermore, at equilibrium very little lipid ligand–SCP₂ complex was associated with the model membrane surface [39]. On the basis of this and other evidence, a three-step kinetic model of SCP₂-mediated lipid transfer was proposed [35]: (1) interaction of the basic SCP₂ with the acidic membrane surface, (2) equilibration of lipid monomers between

the membrane and membrane-bound SCP₂, and (3) rapid dissociation of the lipid-monomer-SCP₂ complex from the membrane surface. That the interaction of SCP₂ with the donor membrane is important for the lipid transfer activity was demonstrated further by the inhibition of SCP₂-mediated lipid transfer on increasing the anionic lipid content in the acceptor membrane or by the use of acceptors consisting entirely of anionic phospholipids [35]. It was proposed that in the presence of more acceptors with greater negative charge than the donor membranes, SCP₂ bound more effectively to the more negatively charged acceptor membranes, thereby making SCP₂ unavailable for interaction with donor membranes and the stimulation of lipid transfer [35].

Secondly, SCP₂ binding to anionic-phospholipid-containing SUV (but not LUV) was potentiated by increased cholesterol content. Consistent with this binding assay was the observation that increasing the cholesterol content of anionic SUV (but not LUV) increased the α -helical content of SCP₂ (results not shown).

Thirdly, SCP₂ N-terminal peptides basically mimicked the interaction of the complete SCP₂ with highly curved SUV enriched with acidic phospholipid and cholesterol. The structural properties of the SCP₂ N-terminal peptides necessary for optimal interaction with acidic-phospholipid-containing SUV paralleled those in the intact protein. SCP₂ N-terminal peptides adopted a more α -helical structure and bound to anionic-phospholipid-containing SUV membranes in the order $^{1-32}\text{SCP}_2 = ^{10-32}\text{SCP}_2 > ^{1-24}\text{SCP}_2 > ^{1-20-32}\text{SCP}_2$. Increased cholesterol content potentiated N-terminal peptide binding to anionic-phospholipid-containing SUV (results not shown). The effects of these structural modifications of SCP₂ N-terminal peptides paralleled those of analogous structural modifications by the site-directed mutagenesis of SCP₂ [30], with one exception. Site-directed mutagenesis to delete the N-terminal non-helical nine residues (analogous to $^{10-32}\text{SCP}_2$) abolished SCP₂ protein lipid transfer activity [30] but had no effect on peptide binding (see the Results section). These results indicate that the N-terminal amphipathic α -helical region of SCP₂ is a membrane interaction site. However, membrane binding alone seemed to be at least partly independent of lipid transfer activity. Future studies beyond the scope of the present investigation will examine whether the N-terminal peptides themselves are competent to promote lipid transfer. This is especially important if SCP₂ can, in part, promote lipid transfer across the aqueous phase by increasing desorption rates.

Fourthly, the degree of membrane surface curvature profoundly affected the interaction of SCP₂ and its N-terminal peptides with the membranes. LUV membranes had nearly 4-fold larger radii of curvature than the SUV membranes. Overall, the complete SCP₂ protein and the N-terminal peptides showed small or no significant changes in CD spectra with LUV membranes, regardless of the membrane lipid composition. However, the lack of change in CD spectra or diminished binding with SCP₂ or its N-terminal peptides in the presence of LUV was not due to the absence of acidic phospholipid from the outer leaflet.

In comparison with LUV, SUV have unique physical properties resulting from high curvature, such as: (1) SUV have a more mobile packing of the phospholipid head group in the outer monolayer and a relative predominance of immobilized fraction in the inner monolayer of the vesicles (in contrast, the molecular packing in the outer and inner layer of LUV is the same and is tighter than that in the outer monolayer of SUV [40,41]); (2) the packing constraints of SUV result in an increased exposure of the hydrophobic core of the bilayer, probably facilitating the interaction of the vesicles with hydrophobic regions of proteins [42]; (3) owing to their high curvature, SUV bilayers experience

molecular packing defects, which also facilitate the insertion of proteins; (4) SUV have decreased lateral pressure in the bilayer, again favouring interaction with proteins [43]; (5) SUV exhibit different phase transition behaviour from that of LUV [43].

The possibility that SCP₂ and its N-terminal peptides exhibit specific structural requirements for interaction arising from membrane curvature has a precedent in other lipid transfer proteins, which are also membrane-binding proteins, a plant homologue of SCP₂ and a PtdCho exchange protein. The interaction of the wheat non-specific lipid transfer protein, a plant counterpart of SCP₂, with monolayers was shown to be highly dependent on the surface pressure, i.e. the packing density [44]. The inhibitory effect of PtdSer on the activity of PtdCho exchange protein was greatly dependent on the membrane curvature [40]: SUV with a diameter of 17 nm were more than 20-fold more inhibitory than those with a diameter of 22 nm. In contrast, the inhibitory effect of LUV was very small. The size dependence was ascribed to a stronger binding of the PtdCho exchange protein to membranes with higher curvature, i.e. SUV. Several other membrane-binding proteins and peptides also exhibit specific structural requirements for membrane curvature [40,42]. For example, blood clotting factor Va light chain [45], cytochrome *b₅* [46,47] and phospholipase A2 [48] exhibit a substantial preference for binding to SUV over LUV. Glucosylceramidase bound anionic-phospholipid-containing SUV, but not LUV; the smallest SUV were the most effective [42].

The preferential interaction of SCP₂ and its N-terminal peptides with highly curved anionic-phospholipid-containing membranes has physiological relevance. It has been speculated that regions of high curvature in cell membranes provide a special environment for protein binding or function. It is now well known that most cell membranes are heterogeneous in membrane curvature and lipid distribution. Several regions of high membrane curvature, specific lipid composition and selective functions have been identified in the plasma membrane: clathrin-coated pits (reviewed in [49–51]), caveolae (reviewed in [52]) and shed vesicles from spiculated erythrocytes [53]. The protein-mediated transfer of phospholipid from SUV to spiculated erythrocyte ghosts was about 4-fold faster than that to cup-shaped ghosts [40]. Furthermore, the isolation of membrane projections called spicules from deoxygenated erythrocytes revealed that SCP₂ rapidly enhanced intermembrane sterol transfer from the highly curved spicule vesicles but not from the erythrocyte ghost membrane [53]. It has been suggested that a protein believed to be identical with SCP₂ [54,55] mediates the absorption of cholesterol in the highly curved intestinal brush border (microvillus) membrane [55,56]. Finally, intracellular membranes such as the endoplasmic reticulum and the Golgi also exhibit highly curved regions.

In summary, the results presented here contribute significantly to our understanding of the mechanism of action of SCP₂. First, two independent methodologies clearly demonstrated that SCP₂ binds to lipid membranes. A protein receptor was not required. Secondly, a primary structural feature of membranes necessary for the binding of SCP₂ was a high curvature in the membrane. Thirdly, an equally important membrane feature essential for this interaction was the presence of anionic phospholipid in the membrane. However, SCP₂ did not bind to low-curvature membranes even in the presence of anionic phospholipid in the membrane. Fourthly, studies with a peptide composed of the SCP₂ N-terminal 32 residues were consistent with this amphipathic helical region's being a structural component of this protein that interacts preferentially with highly curved acidic-phospholipid-containing membranes. Structural modifications of this peptide that disrupted the amphipathic helix (i.e.

1–E20–32SCP₂) of the peptide inhibited membrane binding markedly, which is consistent with site-directed mutagenesis studies showing that the analogous substitution in SCP₂ abolished lipid transfer activity [30]. However, deletion of the non-helical N-terminal nine residues from the peptide did not abolish membrane binding, whereas the analogous mutation in SCP₂ abolished lipid transfer activity [30]. This suggests that the latter mutation altered another part of the SCP₂ protein structure, e.g. a lipid-binding site, to inhibit lipid transfer activity [30]. Future studies will delineate the significance of these findings to the increasing acceptance that membranes are composed of lipid and protein domains differing in structural and functional properties (reviewed in [2]).

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