Sterol carrier protein$_2$-like activity in rat intestine

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Abstract A sterol carrier protein$_2$ (SCP_2)-like activity has been demonstrated in rat intestinal mucosal homogenates and in isolated intestinal cells from both crypt and villus zones. The results indicate the presence of a protein with similar molecular weight and antigenicity to that of authentic SCP_2 purified from rat liver cytosol. Like liver SCP_2, mucosal cytosol stimulates pregnenolone production in rat adrenal mitochondria and acyl coenzyme A:cholesterol acyltransferase activity of liver and mucosal microsomes. The distribution of SCP_2-like activity as determined by radioimmunoassay indicates high levels in mitochondria and cytosol and relatively lower levels in microsomes and in brush-border membranes. The widespread distribution of SCP_2-like protein in the intestine is consistent with potential transfer functions in all phases of cholesterol processing.—**Kharroubi, A., J. A. Wadsworth, R. Chanderbhan, P. Wiesenfeld, B. Noland, T. Scallen, G. V. Vahouny, and L. L. Gallo.** Sterol carrier protein$_2$-like activity in rat intestine. *J. Lipid Res.* 1988. **29:** 287–292.

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The small intestine receives cholesterol via several routes. Like all tissues studied (1) the intestine synthesizes cholesterol de novo and receives cholesterol from serum lipoproteins (2, 3). Unlike other tissues, the intestine takes up luminal cholesterol from dietary and biliary sources (e.g., 4). Presumably, cholesterol derived from the three sources, to a greater or lesser extent, may be directed along several routes, including: retention to meet cellular requirements; exocytosis into the intestinal lumen; and exocytosis into the lymph as part of a lipoprotein package. Since cholesterol is relatively water-insoluble, transfer proteins may facilitate sterol movement along these pathways. Similar transfer functions of sterol carrier protein have been described in other tissues such as liver (5–7), adrenal (8), and ovary (9). Like intestine, cholesterol turnover in these tissues is high. In the present study, an SCP_2-like protein has been demonstrated in intestine. This protein shares functional and immunological properties with authentic liver SCP_2 and displays the same molecular size.

MATERIALS AND METHODS

Rat liver SCP_2 and anti-SCP_2 IgG

Rat liver SCP_2 was purified to homogeneity as previously reported (5). Antisera were raised in rabbits against SCP_2 electrostatically complexed to phosphorylated bovine serum albumin (BSA) (10). Control IgG and anti-SCP_2 IgG were purified by protein A-Sepharose affinity chromatography (11) and by albumin-Sepharose affinity chromatography. The characteristics and specificity of the anti-SCP_2 IgG have been described (12).

Animals

Male adult Wistar rats (Charles River Breeding Laboratories, Wilmington, MA) were acclimated under a normal light cycle for 2 weeks with free access to laboratory chow (Ralston-Purina Co., St. Louis, MO) and water prior to killing by decapitation.

Reagents

All chemicals and solvents were reagent grade (Fisher Scientific, Silver Spring, MD), unless otherwise indicated.

Preparation and characterization of subcellular organelles and isolated cells

Adrenal mitochondria were prepared as previously described (8). *Liver microsomes and cytosol* were prepared from a 5% homogenate as described by Teerlink et al. (12).

Intestinal subcellular organelles were prepared from a 1% homogenate of mucosa scraped from the proximal small intestine (≅32 cm). The mucosa was homogenized in iced 0.273 M mannitol containing 5 mM EDTA adjusted to pH 7.4. Nuclei and cell debris were sedimented by cen-

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; BSA, bovine serum albumin; IgG immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SCP_2, sterol carrier protein$_2$; DTT, dithiothreitol; RIA, radioimmunoassay; PBS, phosphate-buffered saline.

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trifugation (Model J21-B) at 1440 *g* for 10 min and washed once under the same conditions. The combined postnuclear supernatants were centrifuged at 6000 *g* for 15 min to collect the mitochondrial fraction, and the 6000 *g* supernatant was centrifuged at 55,000 *g* (Model LL3-50, Beckman Instruments, Fullerton, CA) for 2 hr to sediment the microsomal fraction. The 55,000 *g* supernatant was termed the mucosal cytosol. Organelles were resuspended either in the homogenization solution to give 10–20% suspensions based upon the original wet-weight mucosa or in RIA buffer, described below, as required by the individual methodologies. The concentration of the original homogenates and the centrifugation conditions selected, yielded the highest organelle purity based upon the distribution of standard organelle markers.

Intestinal cells were isolated from the crypt and villus zones of proximal small intestine (≅32 cm) as described by Weiser (13). The segment of intestine was washed initially with 150 ml of 0.154 M NaCl containing 1 mM DTT. Then, with buffers A and B as defined (13), the intestinal segment was filled with buffer A, each end was tied, and the segment was placed into a polystyrene flask containing buffer A and incubated for 15 min at 10°C in an oscillating (2 cycles/sec) Dubnoff metabolic shaker. Dislodged cells, designated Fraction 1, were collected by rinsing the segment with 50 ml of buffer A. The segment, with minimal handling, was filled with buffer B incubated for 15 min at 10°C with shaking, drained, and flushed with 50 ml of buffer B. The dislodged cells were designated Fraction 2. The buffer B cycle was repeated six times for periods of 15, 10, 10, 5, 5, 5 min and six additional populations of cells were collected and designated Fractions 3–8. Cells were pelleted by centrifugation at 900 *g* for 5 min. An estimate of cell type in each fraction was assessed with the crypt-zone marker enzyme, thymidine kinase (14), and villus zone marker enzyme, alkaline phosphatase (13). The marker enzymes were assayed in a 10% homogenate (phosphate-buffered saline) of each packed cell pellet. In the individual experiments, the cell pellets were homogenized in the buffer and at the concentration indicated.

Brush border membranes were prepared from rat jejunal mucosa by the method of Kessler et al. (15). The purity of the brush border membranes was assessed by measuring the marker enzymes sucrase and alkaline phosphatase. The specific activity of these enzymes was 20- and 15-fold higher as compared to the whole homogenate. In contrast, recovery of ACAT, succinate dehydrogenase, and DNA in these membranes was less than 0.5% of the total activity. These results were comparable to those of Kessler et al. (15).

Assays

Acyl coenzyme A:cholesterol acyltransferase activity (ACAT) in mucosal and liver microsomes was determined by the rate of incorporation of [1-^{14}C]oleic acid into cholesteryl ester (16). Siliconized 50-ml glass conical tubes containing 5.1 mM ATP, 512 μM coenzyme A, 5.0 mg of BSA (each from Sigma Chemical Co., St. Louis, MO), 10.5 mM $MgCl_2 \cdot 6H_2O$, 0.2 M potassium phosphate, pH 7.4, 227 mM [1-^{14}C]oleic acid, 0.4 μCi (57.4 mCi/mmol, Amersham Corp., Arlington Heights, IL) added in 10 μl acetone, and 334 μM cholesterol (Serdary Research Laboratories, London, Ontario) added in 5 μl of dioxane (freshly distilled)–propylene glycol 2:1 (v/v) were preincubated at 37°C for 15 min. The ACAT-catalyzed reaction was initiated by the addition of 80 μl of a 10% microsomal suspension which gave a final incubation volume of 0.55 ml. Digests were incubated at 37°C in an oscillating Dubnoff metabolic shaker, and reactions were terminated by the addition of 20 volumes of chloroform–methanol 2:1 (v/v) (17) either after 3 min or with time over a 15-min period. The 3-min reaction time was determined previously to fall on the linear region of the rate curve. [1,2-^{3}H]Cholesteryl oleate (20,000 dpm), prepared and purified by thin-layer chromatography just prior to use as described by others (15, 16, 18, 19), was added to the extraction mixture as the internal standard to correct for procedural losses (not > 10%). Zero-time and buffer controls were included and each assay was run in triplicate. Lipids were extracted according to Folch, Lees, and Sloane Stanley (17). The solvent was evaporated to dryness under nitrogen and the lipid residue was dissolved in hexane. To determine the quantity of [1-^{14}C]oleic acid incorporated into cholesteryl ester, the lipids were separated on Silica Gel G precoated thin-layer chromatography plates (Uniplates, Analtech, Newark, DE). The silicic acid areas corresponding to cholesteryl esters, as identified with authentic standards (Nu-Chek Prep Inc., Elysian, MN) were scraped into counting vials. Radioactivity was measured in a liquid scintillation counter (Model LS-250, Beckman Instruments, Fullerton, CA). The external standard-channels ratio method of quench calibration was used to monitor the dual-labeled samples.

SCP_2-like protein levels and distribution were estimated in subcellular organelles from intestinal mucosa and in isolated intestinal cells by radioimmunoassay (20). Liver SCP_2 was radioiodinated (21) with Bolton-Hunter reagent (Amersham Corp., Arlington Heights, IL) to a specific activity of 20–50 μCi/μg and the ^{125}I-labeled SCP_2 was assessed as pure based upon migration as a single radioactive band upon SDS-PAGE and autoradiography.

Subcellular organelles from mucosa or isolated intestinal cells were resuspended in RIA buffer (10 mM sodium phosphate, pH 7.2, containing 0.15 M NaCl, 1 μg/ml leupeptin, 0.1% BSA, and 0.01% Triton X-100) lacking only BSA, sonicated (Branson, Danbury, CT) for 20 sec at cycle 50, and diluted in RIA buffer to give protein concentrations of 0.2–1.0 mg/ml. Anti-SCP_2 IgG was diluted in the same buffer to a concentration (8 μg/ml) that gave 40–50% binding of ^{125}I-labeled SCP_2 in the absence of competing ligand. In polypropylene test tubes, 0.1 ml of

tissue fractions was mixed with 0.1 ml of the diluted anti-SCP_2 IgG and 50 μl of ^{125}I-labeled SCP_2 (3–4 $\times$ 10^4 cpm) was then added. The tubes were incubated at 4°C for 24 hr. Antibody-bound ^{125}I-labeled SCP_2 was separated from free ^{125}I-labeled SCP_2 by overnight precipitation at 4°C with 0.1 ml goat anti-rabbit IgG (1.2 mg/ml) (Cooper Biomedical Inc., Malvern, PA). After dilution with 2 ml of ice-cold RIA buffer, the immunoprecipitate was collected by centrifugation at 2,800 rpm for 40 min (IEC-centrifuge) and the supernatant was decanted. Radioactivity was counted in a Beckman Gamma Counter (Model 5500). The RIA data were analyzed using a logit-log computer routine (22). All assays were carried out in triplicate.

SCP_2-identity in subcellular organelles from intestinal mucosa was substantiated by electrophoretic blotting (20, 23). Organelle suspensions sonicated for 20 sec at cycle 50 and treated with a mixture of 0.4% SDS and 1% 2-mercaptoethanol for 5 min at 90°C were applied to an SDS (0.15%)–polyacrylamide gradient (10–15%) slab gel. The organelle proteins were resolved by electrophoresis (24) and were transferred (23) from the gel to nitrocellulose sheets, 0.45 μm pore size (Bio-Rad Laboratories, Richmond, CA). Nonspecific protein binding sites on the sheet were blocked with 1% BSA contained in 10 mM Tris-HCl and 0.15 M NaCl, pH 7.4, (buffer C) by incubation at room temperature for 30 min.

The nitrocellulose sheets were subjected to a series of treatment and wash cycles performed at room temperature as follows: treated for 90 min with 50 ml (44.5 ng/ml) of either rabbit anti-SCP_2 IgG or rabbit control IgG in buffer C containing 10% horse serum, and washed five times for 5 min each with PBS containing 1% horse serum; treated for 60 min with 50 ml (30 μg/ml) of goat anti-rabbit IgG in buffer C containing 10% horse serum and washed as above; treated for 60 min with 50 ml peroxidase-anti-peroxidase IgG (Cooper Biomedicals, Westchester, PA; 1:2000) in buffer C containing 10% horse serum and washed as above; and treated for 30–60 sec with the peroxidase substrate mixture, 0.02% diaminobenzidine tetrahydrochloride, 0.02% imidazole (Sigma Chemical Co., St. Louis, MO) and 0.01% H_2O_2 in buffer C without BSA, and then washed with distilled water and dried.

Protein was determined by the method of Lowry et al. (25) with BSA as the standard.

TABLE 1. Effect of mucosal cytosol on pregnenolone production in adrenal mitochondria

Additions	Pregnenolone Production
	ng/mg protein per 25 min
None	50.4 ± 2.8[a]
Mucosal cytosol	200.0 ± 10.6
Liver cytosol	153.0 ± 4.2
Liver SCP_2	190.0 ± 3.5
Mucosal cytosol + anti-SCP_2 IgG	50.4 ± 2.8
Mucosal cytosol + control IgG	176.0 ± 15.6

Adrenal mitochondria (2.48 mg protein) were incubated in a 2.0-ml reaction volume for 25 min at 37°C with mucosal cytosol (5.6 mg of protein), liver cytosol (5.6 mg of protein), liver SCP_2 (20 μg), mucosal cytosol (5.6 mg of protein) treated with anti-SCP_2 IgG (28 μg) or control IgG (28 μg). Pregnenolone was measured by RIA (36).

[a] Mean ± SD; n = 2.

TABLE 2. Effect of mucosal cytosol on mucosal and liver ACAT activity

Additions	ACAT Activity[a]
	pmol cholesteryl ester/min per mg
Mucosal microsomes	538 ± 38
Mucosal microsomes + mucosal cytosol	550 ± 37[b]
Liver microsomes	130 ± 10
Liver microsomes + mucosal cytosol	300 ± 21
Mucosal cytosol	1.4 ± 1

Liver and mucosal microsomes and mucosal cytosol were prepared as described under Methods. Each assay contained in a final volume of 0.55 ml:5.1 mM ATP, 512 μM coenzyme A, 5.0 mg of BSA, 10.5 mM $MgCl_2 \cdot 6H_2O$, 227 mM [1^{14}C]oleic acid (0.4 μCi), 334 μM cholesterol, 0.2 M potassium phosphate, pH 7.4, and either microsomal suspension (200 μg of protein) ± mucosal cytosol (6 mg of protein) or mucosal cytosol alone (6.0 mg of protein). Reactions were terminated with time over a 15-min period by addition of 20 volumes of chloroform–methanol 2:1 (v/v). Cholesteryl ester was extracted and analyzed as described under Methods.

[a] Expressed as pmol of cholesteryl ester formed/min per mg protein, mean ± SD, n = 2.

[b] Represents a doubling (4 min to 8 min) in time over which initial reaction rate was linear.

RESULTS

Effect of mucosal cytosol on pregnenolone production

Addition of mucosal cytosol (5.6 mg of protein) to adrenal mitochondria resulted in a fourfold stimulation in pregnenolone production from endogenous cholesterol compared with mitochondria incubated under the same conditions but without added cytosol. The level of the stimulatory effect produced by mucosal cytosol was equivalent to that produced by purified liver SCP_2 (20 μg) and greater than the threefold stimulation produced by the same amount of liver cytosol protein as shown in **Table 1.**

When mucosal cytosol was pretreated with anti-SCP_2 IgG, its stimulatory effect on mitochondrial pregnenolone production was abolished completely, shown in Table 1. However, cytosol treated with control IgG was fully stimulatory.

Effect of mucosal cytosol on ACAT activity

The addition of mucosal cytosol to assays for ACAT activity in mucosal and liver microsomes mimicked the stimulatory effect reported earlier for authentic liver SCP_2 (26). As shown in **Table 2,** the addition of mucosal cytosol

to liver microsomes more than doubled the initial rate of ester formation (130 vs. 300 pmol/min per mg of protein). In contrast, the addition of mucosal cytosol to mucosal microsomes, while not altering the initial rate of the ACAT-catalyzed esterification, doubled the time (4 to 8 min, rate curve not shown) that the initial reaction rate was linear. In four other experiments linearity was extended from as little as 2 min to as much as an additional 7 min.

Distribution of SCP_2-like protein in intestinal cells

Consistent with a general role for SCP_2 in cholesterol transport is its presence in villus tip and crypt populations of isolated intestinal cells as shown in **Table 3.** Cell fractions 1 and 2, which were predominantly from the villus zone, and cell fractions 7 and 8, which were predominantly from the crypt zone, contained an average of 650 ng of SCP_2 and 1300 ng of SCP_2 per mg of cell protein, respectively. Such a dual occurrence of SCP_2 permits participation in cholesterol biosynthesis, reported to take place primarily in crypts (27), cholesterol esterification, which occurs in both crypts and villus cells (26–28), and any additional cholesterol transfer function that may occur in both cell types.

Distribution of SCP_2-like protein in intestinal subcellular organelles

SCP_2-like protein was present in the four major subcellular organelles prepared from intestinal mucosa. As shown in **Table 4,** the highest SCP_2-like protein specific activity resided in the mitochondria which was fivefold greater than in nuclei, and seven- to ninefold greater than in microsomes and cytosol. Of the total homogenate SCP_2-like protein, over 70% was equally divided between mitochondria and cytosol, 25% was localized in the nuclei and cell debris, and just 3% was found in the microsomal fraction. The recovery of SCP_2-like protein in the organelles averaged no less than 75% of that measured in the homogenate. The brush border membranes have very low levels of SCP_2-like protein.

TABLE 3. SCP_2-like protein in villus and crypt cells

Fraction	SCP_2-like Protein	AP[a]	TK[a]	Protein
	ng/mg	*% of total*		*mg*
1	200	22	0	16
2	1100	22	7	27
7	1700	7	38	31
8	900	0.1	12	5

Fractions 1 and 2 are enriched in villus zone cells, 7 and 8 are enriched in crypt zone cells, and 3–6 are a mixed cell population (not shown). SCP_2-like protein was determined by RIA. The cell isolation procedure, marker enzyme activity assays, and protein determination are described under Methods.

[a]AP, alkaline phosphatase; TK, thymidine kinase.

TABLE 4. Distribution of SCP_2-like protein in subcellular organelles from intestinal mucosa

Organelle	SCP_2-like Protein	Distribution
	ng/mg protein	*%*
Experiment 1		
Homogenate	415 ± 81[a]	
Nuclei and cell debris	279 ± 29	25.3 ± 3.5
Mitochondria	1470 ± 368	34.0 ± 3.1
Microsomes	169 ± 13	3.3 ± 0.02
Cytosol	196 ± 3.0	37.4 ± 0.4
Experiment 2		
Brush border membranes	16.6 ± 3.0[b]	

Organelles were prepared by ultracentrifugation and their SCP_2-like protein was determined in triplicate by RIA. Organelles (0.1 ml containing 20 to 100 μg of protein) were mixed with anti-SCP_2 IgG (0.1 ml containing 0.8 μg of protein) and 50 μl of ^{125}I-labeled SCP_2 (3–4 × 10^4 cpm). After incubation at 4°C for 24 hr, goat anti-rabbit IgG (0.1 ml containing 120 μg of protein) was added and incubation at 4°C was continued for an additional 24 hr. The immunoprecipitate was collected and radioactivity was monitored. The RIA data were analyzed using a logit-log computer routine.

[a]Mean ± SD, n = 2.

[b]Mean ± SD, n = 3.

Electrophoretic blotting analysis for SCP_2-like protein in intestinal subcellular organelles

Of the solubilized proteins in the four major subcellular organelles that were resolved by SDS-PAGE and transferred to nitrocellulose strips, the only protein recognized by anti-SCP_2 IgG displayed the same molecular size as authentic liver SCP_2. This result is shown in **Fig. 1.** The intensity of the stain for SCP_2-like protein in the organelles is consistent with the quantitative distribution for SCP_2-like protein determined by RIA.

DISCUSSION

Numerous low molecular weight proteins function in intracellular lipid transfers (e.g., 29). By performing this role, they facilitate cellular processes dependent upon lipid availability. In particular, SCP_2 and/or SCP_2-like proteins are present in tissues active in cholesterol metabolism (5, 8, 9) and have been shown in the adrenal to transfer cholesterol from cytoplasmic lipid droplets to mitochondria and from the outer to the inner mitochondrial membrane (29); in liver (30, 31) and intestine (26) SCP_2 facilitates ACAT-catalyzed cholesterol esterification; and in liver, SCP_2 participates in cholesterol (5) and bile acid (32) synthesis.

In the current study, an SCP_2-like protein(s) has been detected in rat intestinal mucosa. The original detection of SCP_2-like protein in intestine was based upon the ability of mucosal cytosol to mimic authentic liver SCP_2 in stimulating pregnenolone production in adrenal mitochondria and the subsequent complete loss of this stimu-

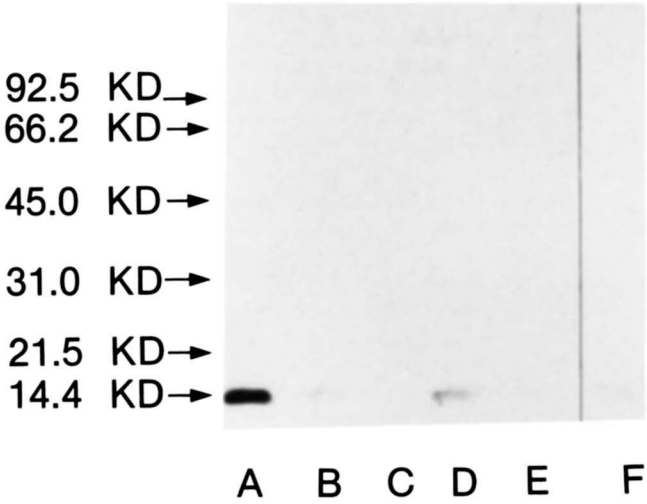

Fig. 1. Western blot analysis of SCP_2-like protein in subcellular organelles from intestinal mucosa; A, liver SCP_2 (0.8 μg); B, homogenate; C, nuclei; D, mitochondria; E, microsomes; F, cytosol. Each organelle represents 150 μg of protein.

latory effect when mucosal cytosol was pretreated with anti-SCP_2 IgG. Moreover, mucosal cytosol was stimulatory to ACAT-catalyzed cholesterol esterification in both liver and intestinal microsomes, a reported function of authentic SCP_2 (5, 26). Intestinal SCP_2 on Western blots displayed a molecular size (14,000 daltons) which was indistinguishable from pure liver SCP_2.

When the distribution of mucosal SCP_2-like protein was determined for subcellular organelles, cytosol was shown to contain about 40% (Table 4) of the total. Therefore, one might speculate that SCP_2-like protein functions in the transfer of absorbed (dietary and biliary) cholesterol between its sites of cellular entry and utilization. By comparison, liver nonspecific lipid transfer protein (which is identical to SCP_2) has been shown to facilitate the exchange of cholesterol between brush border membranes and acceptor vesicles (33). Specifically in intestine, since a large amount of absorbed sterol is destined for lipoprotein packaging, a necessity for sterol transfer from the brush border to the site of its obligatory esterification is anticipated. Cholesterol esterase, an intestinal enzyme thought to esterify luminal sources of cholesterol, is localized in the cytosol. However, we were unable to assess the effect of SCP_2-like protein on this activity, since the enzyme assay utilizes bile salts which are known to inactivate SCP_2. In contrast, mucosal ACAT, which esterifies microsomal cholesterol, was stimulated by mucosal cytosol SCP_2-like protein, which presumably delivers exogenous cholesterol to the microsomes. Moreover, in an earlier study (26), authentic liver SCP_2, not only in the presence but also in the absence of added exogenous cholesterol, produced a similar but less dramatic effect on mucosal ACAT which suggests an SCP_2-facilitated movement of an intramicrosomal pool of sterol to the enzyme. A comparison of the effects of mucosal cytosol SCP_2-like protein on liver and intestinal microsomal ACAT activity in the presence of exogenous cholesterol reveals an increase in the initial reaction rate in liver and an extension of the initial reaction rate in mucosal microsomes. This suggests that the available endogenous cholesterol is not saturating in the liver system and that mucosal cytosol SCP_2-like protein delivers exogenous cholesterol so that saturation is approached, whereas in the intestine, mucosal cytosol SCP_2-like protein prolongs the supply of cholesterol to the initially substrate-saturated enzyme. This interpretation of the SCP_2-like protein effect is based on the earlier observation (27) that exogenous cholesterol (added in solvent) extends the initial reaction rate of ACAT in mucosal microsomes beyond that seen in its absence but increases the initial reaction rate in liver microsomes (34).

Another microsomal function for SCP_2-like protein may be the stimulation of cholesterol biosynthesis, a reported function of SCP_2 in liver (5). However, the apparent low concentration of SCP_2-like protein in intestinal microsomes (169 ng/mg of protein) relative to liver (2470 ng/mg of protein, unpublished results from this laboratory) speaks against this function unless the protein has the ability to facilitate the synthetic processing without microsomal residence or unless the level of SCP_2 in or associated with microsomes may be dependent upon the cellular demand for cholesterol biosynthesis, i.e., a dynamic process.

The high concentration of SCP_2-like protein in the mitochondria is similar to what is found in the adrenal (20, 35). However, a mucosal function for mitochondrial SCP_2 is not immediately apparent but might be related to its reported phospholipid exchange activity (29).

Not only was SCP_2-like protein associated with several intracellular organelles, but it was also present in an enriched population of either villus or the crypt zone intestinal cells. These varied intracellular and multicellular locations are consistent with a role for SCP_2-like protein in many phases of cholesterol processing in the intestine.

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