Sterol carrier protein-2 localization in endoplasmic reticulum and role in phospholipid formation

O. Starodub, C. A. Jolly, B. P. Atshaves, J. B. Roths, E. J. Murphy, A. B. Kier, and F. Schroeder. Sterol carrier protein-2 localization in endoplasmic reticulum and role in phospholipid formation. Am J Physiol Cell Physiol 279: C1259–C1269, 2000.—Although sterol carrier protein-2 (SCP-2; also called nonspecific lipid transfer protein) binds fatty acids and fatty acyl-CoAs, its role in fatty acid metabolism is not fully understood. L-cell fibroblasts stably expressing SCP-2 were used to resolve the relationship between SCP-2 intracellular location and fatty acid transacylation in the endoplasmic reticulum. Indirect immunofluorescence double labeling and laser scanning confocal microscopy detected SCP-2 in peroxisomes in phospholipid synthesis in the endoplasmic reticulum. SCP-2 enhances the rate-limiting step in microsomal phosphatidylethanolamine incorporation of oleic acid into phospholipids and triacylglycerols in overexpressing cells 1.6- and 2.5-fold, respectively, stimulated microsomal incorporation of oleic acid into phosphatidic acid in vitro 13-fold, and exhibited higher specificity for unsaturated versus saturated fatty acid-CoA. Overexpression of SCP-2 enhanced the rate-limiting step in microsomal phosphatidylcholine synthesis mediated by glycerol-3-phosphate acyltransferase. SCP-2 also enhanced microsomal acyl-chains of phosphatidylcholine twofold, depending on the specific fatty acid-CoA, but had no effect on other phospholipid classes. In summary, these results were consistent with a role for SCP-2 in phospholipid synthesis in the endoplasmic reticulum.

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indirectly via regulation of some other aspect of fatty acid metabolism.

In the present investigation, L-cell fibroblasts transfected with the cDNA encoding the 15-kDa pro-SCP-2 were used to 1) determine whether SCP-2 is localized in endoplasmic reticulum in addition to peroxisomes in intact cells, 2) show in intact cells whether SCP-2 expression enhanced incorporation of exogenous fatty acid into phospholipids as well as triacylglycerols, and 3) show whether SCP-2 directly enhanced microsomal transacylation of fatty acyl-CoAs into phosphatidic acid as well as phospholipid acyl-chain remodeling in vitro.

MATERIALS AND METHODS

Materials. Oleoyl-CoA, oleic acid, palmitoyl-CoA, arachidonyl-CoA, dioleoyl phosphatidic acid, oleoyl lysophosphatidic acid, dithiothreitol (DTT), NaF, and glycerol-3-phosphate were obtained from Sigma Chemical (St. Louis, MO). [1-14C]oleoyl-CoA, [1-14C]palmitoyl-CoA, and [1-14C]arachidonyl-CoA were purchased from NEN (Boston, MA). Sci-verse I scintillation cocktail was from Fisher Scientific (Pittsburgh, PA). Silica gel 60 thin-layer chromatography plates were from VWR Scientific (Houston, TX). Recombinant 13.2-kDa SCP-2 was purified as described previously (37). Primary antibody markers for organelles were obtained as follows: endoplasmic reticulum, monoclonal anti-76-kDa glycoprotein (Developmental Studies Hybridoma Bank, University of Iowa, IA) and anti-78-kDa glucose-regulated protein antibody (StressGen Biotechnologies, Victoria, BC, Canada); peroxisomes, anti-bovine PMP-70 (Zymed, San Francisco, CA) and sheep anti-bovine liver catalase (BioDesign International, Kennebunk, ME).

Secondary antibodies were purchased as follows: FITC-conjugated donkey anti-sheep IgG (Sigma), FITC-conjugated goat anti-mouse polyvalent Ig (Sigma), FITC-conjugated goat anti-rat IgG (Sigma), and Texas Red-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL).

Preparation and purification of anti-SCP-2 antibodies. For the preparation of polyclonal antisera to 13.2-kDa SCP-2, the protocols for the use of laboratory animals were approved by the appropriate institutional review committee and met American Association for Accreditation of Laboratory Animal Care guidelines. Freund’s adjuvant containing recombinant 13.2-kDa SCP-2 was injected into female New Zealand White rabbits (Hazleton Research Products, Denver, PA) as described previously (24). The resultant polyclonal antibodies were purified by incubations with mouse liver and L-cell homogenate blots from which the SCP-2 gene products had been cut out. This procedure eliminated cross-reactivity to other proteins such as the 30-kDa protein (48) and the 44-kDa creatine kinase (45).

The specificity of the purified anti-SCP-2 antibodies was determined by Western blotting performed as described previously (3). The antibodies reacted positively on dot or Western blots to the following standard proteins: 15.2-kDa pro-SCP-2, 13.2-kDa SCP-2, and 58-kDa SCP-x. This was expected because the 13.2-kDa SCP-2 comprises the entire COOH terminus of 15.2-kDa pro-SCP-2 as well as that of 58-kDa SCP-x. Western blots of L cells transfected with the cDNA encoding 15-kDa pro-SCP-2 detected only the mature 13-kDa SCP-2 (40). This was due to rapid posttranslational processing of 15-kDa pro-SCP-2 protein to 13-kDa SCP-2 in L cells, which is consistent with all other tissues examined (reviewed in Ref. 52). For immunocytochemistry, the working dilution of the purified polyclonal anti-SCP-2 antibody was 1:20 (1:40 for double labeling). FITC-conjugated (Texas Red-conjugated for double labeling) goat anti-rabbit IgG (1:100) was used as secondary antibody.

Cell culture. Transfected murine L cells (adenosine phosphoribosyl transferase deficient, thymidine kinase negative mutant) overexpressing 15-kDa pro-SCP-2 (posttranslationally completely cleaved to 13-kDa SCP-2) (40, 42) and mock transfected control L-cell fibroblasts were grown to preculture on glass coverslips in Higuchi medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) (3). In 15-kDa pro-SCP-2-expressing cells, the SCP-2 comprised 0.03% of total cytosolic protein as determined by quantitative Western blotting. In control cells, SCP-2 comprised <0.007% of total cytosolic protein (28, 40).

Cell preparation for indirect immunofluorescence. Cells were plated and grown on glass coverslips as well as chamber slides (Nunc; Fisher Scientific). The medium was then removed, and cells were washed three times with phosphate-buffered saline (PBS) followed by fixation and permeabilization by one of the two protocols: 1) 3.7% paraformaldehyde (Sigma) and 1.5% methanol (Fisher, Fair Lawn, NJ) in PBS (pH 7.2; Gibco BRL, Grand Island, NY) for 15 min and 1% Triton X-100 (Polysciences, Warrington, PA) in PBS for 5 min, followed by extensive washing (5 times in 30 min) with 0.1% Tween 20 (Fisher) in PBS, or 2) methanol for 5 min at −20°C and 57 mM borate buffer (pH 8.2) for rehydration and subsequent steps. Autofluorescence of residual aldehyde groups was quenched with 100 mM NH4Cl (Sigma) in PBS. Non specific reactivity was blocked by using either 2% ovalbumin or BSA (Sigma) in PBS or borate buffer, respectively. Incubations with primary antibodies diluted in 1% ovalbumin in buffer, as well as those with secondary antibodies (diluted in buffer only), were performed in a humid chamber for 30 min at 37°C, with subsequent extensive washing. For colocalization experiments, mixtures of primary and secondary antibodies were used. Cover glasses were mounted on slides with SlowFade medium (Molecular Probes, Eugene, OR) and sealed with fingernail polish.

Immunostaining specificity was established by omitting the primary antibody and using either one or both of the secondary antibodies, which allowed minimization of nonspecific adsorption of fluorescent antibodies, optimal separation of the fluorophore fluorescent signals, and optimization of fluorophore concentration to preclude self-quenching (43). The optimal concentrations of primary and secondary antibodies for immunolocalization and colocalization were determined at several dilutions to minimize nonspecific background and maximize specific signal and were 1:1 to 1:20 (anti-76-kDa glycoprotein), 1:10 to 1:100 (anti-SCP-2), 1:10 to 1:1,500 (anti-catalase), 1:200 (peroxisomal membrane protein-70), or 1:50 to 1:200 [anti-cytochrome oxidase, anti-heat shock protein 70 (HSP70), and anti-lysosome-associated membrane protein 2 (LAMP-2)]. Likewise, the optimal concentration of secondary antibody (FITC-conjugated donkey anti-sheep IgG, FITC-conjugated goat anti-mouse polyvalent IgG, or FITC-conjugated goat anti-rat IgG) was determined by titrations ranging from 1:50 to 1:500.

Epifluorescence microscopy. Immunostained cell preparations were examined by conventional epifluorescence microscopy with the use of an Olympus Vanox AHG53 microscope with a 100-W mercury lamp, a ×40 objective, and an Optronics Peltier-cooled three-chip charge-coupled device DEI-700 camera system, coupled with a 24-bit RGB Neotech frame grabber for image acquisition.
Laser scanning confocal microscopy. Laser scanning confocal microscopy was performed using an MRC-1024 point-scanning laser confocal microscopy system (Bio-Rad, Hercules, CA) equipped with a 15-mW krypton-argon laser. FITC was excited with the laser 488-nm band, and emission was detected through an OG515 long-pass filter; Texas Red was excited with the laser 568-nm band, and emission was detected through a 680/52 band-pass filter. Confocal images were obtained with a Zeiss Axiovert 135 inverted microscope fitted with a ×63, 1.4-NA oil-immersion lens. Image acquisition was made by using LaserSharp software (Bio-Rad). Serial horizontal sections (0.3 μm) were taken through the entire thickness of the cell. Figures 1–3 show single representative horizontal sections.

Image processing. Image processing utilized the following software: LaserSharp (Bio-Rad), MetaMorph (Universal Imaging, Nikon, Melville, NY), Adobe Photoshop (Adobe Systems, Seattle, WA), and Claris Draw (Apple Computer, Cupertino, CA). To obtain a relative quantitative assessment for colocalization of SCP-2 with respective organelle markers, we subtracted the background fluorescence for each optical section and calculated correlation coefficients as described previously (36). The data were then fit to a pixel fluorogram constructed as described previously (36).

Interpretation of pixel fluorograms. Pixel fluorograms provide quantitative data on the overlap of anti-SCP-2 antibody reactivity and antibodies directed at specific organelles beyond the level of visual inspection of superimposed images (13, 36). Markers of near equivalent intensity that overlap completely are characterized by 1) the color/pixels falling almost entirely on the line of identity (13) and 2) correlation coefficients near 1. This ratio has been reported between 0.9 and 1.0 as complete overlap is approached (13, 17, 63). In contrast, markers that do not overlap at all are characterized by 1) no color/pixels falling along the line of identity (13, 36), 2) two widely divergent clouds of dots that appear along the x- and y-axes of the pixel fluorogram, and 3) correlation coefficients between 0.07 and 0.00 as overlap/colocalization approaches zero (13, 36, 53). Partial overlap/colocalization is characterized by 1) some color/pixels falling along the line of identity, 2) significant color/pixels not falling along the line of identity, and 3) intermediate ratios (13, 22, 36, 53).

Isolation of L-cell microsomes for Western blotting. Microsomes were isolated as described previously (18, 20). Protein was determined by the method of Lowry (35). Compared with the cell homogenate, the microsomal fraction was enriched four- to fivefold in NADPH-cytochrome-c reductase (18, 20). The SCP-2 content of isolated microsomal membranes was estimated by Western blotting (40). Quantitative densitometry of the Western blots was performed with NIH Image software as described earlier for other L-cell proteins (2, 23).

Incorporation of exogenous oleic acid into L-cell microbrosomes. The incorporation of exogenous [3H]oleic acid incorporated into L-cell microbrosomes was determined exactly as described previously (29), except that incorporation was measured 10 min after exogenous [3H]oleic acid was added.

Microsomal phosphatidic acid synthesis. Microsomes were isolated, washed, and purified by gel permeation chromatography (29). Microsomal glycerol-3-phosphate acyltransferase was measured using glycerol-3-phosphate and [1-14C]oleoyl-CoA, [1-14C]palmitoyl-CoA, or [1-14C]arachidonoyl-CoA as substrates as previously described (29). Briefly, unless otherwise specified the reaction mixture contained the appropriate concentration of [1-14C]fatty acyl-CoA, 735 μM glycerol-3-phosphate, 80 mM NaF, 10 mM DTT, 10 mM MgCl2, 70 mM Tris buffer, pH 7.4, and 10 μg of microsomal protein. The assay mixture was incubated for 15 min at 37°C in a shaking water bath. The microsomal lipids were extracted and resolved by thin-layer chromatography on silica gel 60 plates. The appropriate bands were scraped and quantitated by liquid scintillation counting.

Microsomal lysophosphatidic acid acyltransferase activity was measured as previously described (29). Briefly, lysophosphatidic acid, in chloroform plus 1% MeOH, 0.05% double-distilled H2O, and 0.025% triluoracetic acid, was placed in 1.5-ml Eppendorf tubes and dried under N2 to give a final concentration of 10 μM lysophosphatidic acid in the final assay volume. Phosphatidic acid formation, in the absence of glycerol-3-phosphate, was then carried out exactly as described for glycerol-3-phosphate acyltransferase.

Phospholipid acid chain remodeling. Preexisting phospholipids can be remodeled via acylation-deacylation cycles or transacylation to alter their fatty acid composition (60). Microsomal remodeling of phospholipid acyl chains was measured by the incorporation of [1-14C]oleoyl-CoA, [1-14C]palmitoyl-CoA, or [1-14C]arachidonoyl-CoA into phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin as described previously (30).

Statistical analysis. All data were analyzed by one-way ANOVA using Tukey’s multiple comparison test. P < 0.05 was considered significant. All analyses including linear regression were done using GraphPad Prism software (San Diego, CA).

RESULTS

Intracellular distribution of SCP-2 in transfected L-cell fibroblasts. To determine whether SCP-2 has a role in fatty acid metabolism outside the peroxisome, L-cell fibroblasts were chosen as a model system. Control and mock-transfected L cells express only very low amounts of SCP-2, as determined by Western blotting (40). In contrast, Western blotting shows that L cells transfected with a construct containing the 15-kDa pro-SCP-2 have SCP-2 amounts similar to physiological values (40). Unfortunately, these studies did not reveal the intracellular localization of SCP-2 in the transfected cells. Therefore, indirect anti-SCP-2 immunolabeling and epifluorescence microscopy were used to show the intracellular distribution of SCP-2 in cells grown to preconfluence (Fig. 1). Transfected L cells overexpressing SCP-2 exhibited a strong punctate pattern along with some diffuse cytoplasmic labeling (Fig. 1A). A similar but less intense immunostaining pattern was observed with control L-cell fibroblasts (Fig. 1B) as well as mock-transfected L cells (data not shown). The bright punctate pattern of SCP-2 immunolabeling was consistent with a large portion of SCP-2 being present in high concentration in organelle fractions rather than uniformly distributed through the cell cytoplasm.

Immunocolocalization of SCP-2 with peroxisomes in transfected L-cell fibroblasts. SCP-2 appeared highly concentrated in punctate loci in transfected as well as control (mock transfected as well as untransfected) cells, suggesting significant localization of SCP-2 with peroxisomes. To determine whether SCP-2 was localized in peroxisomes, we labeled L cells overexpressing SCP-2 using primary antibodies to SCP-2 and catalase (a peroxisomal marker) and detected these cells using secondary antibodies conjugated with Texas Red and
FITC, respectively. Because epifluorescence shows the fluorescence signal from the entire thickness of the cell, laser scanning confocal microscopic images of successive single planes (0.3-μm thick) through the double-labeled cells were simultaneously obtained with separate photodetectors. Side-by-side comparison of representative single-slice, simultaneously acquired confocal images of SCP-2 (Fig. 2A, red) and catalase (Fig. 2B, green) revealed a striking similarity in staining patterns. This was confirmed by superposition of the two images (Fig. 2, A and B) to form Fig. 2C. Colocalization of SCP-2 and catalase appeared as yellow (red + green = yellow) fluorescence in the merged images (Fig. 2C). However, this superposition also indicated a considerable number of red or green bright punctate areas that did not overlap. This suggests that a large amount of 15-kDa pro-SCP-2 is not found in peroxisomes.

Fig. 1. The expression of sterol carrier protein-2 (SCP-2) in transfected L-cell fibroblasts. Indirect immunolabeling of the 15-kDa pro-SCP-2-expressing (A) and control (B) L cells with polyclonal rabbit anti-mouse 13-kDa SCP-2, followed by FITC-conjugated goat anti-rabbit IgG. Methanol was used for both fixation and permeabilization. A similar punctate staining pattern was obtained for both cell lines, although the pattern is stronger and more discrete on pro-SCP-2-overexpressing cells.

Fig. 2. Localization of SCP-2 in transfected L cells expressing peroxisomal and extraperoxisomal SCP-2: double-label confocal image analysis with pixel fluorogram. The 15-kDa pro-SCP-2-expressing cells were simultaneously labeled for SCP-2 with Texas Red-conjugated secondary antibody (A) and for catalase with FITC-conjugated secondary antibody (B). The punctate pattern obtained for SCP-2 was significantly superimposable to that with the peroxisomal marker over the same cells (C). The superposition was delineated graphically as a pixel fluorogram (D), where colocalization of SCP-2 (red) with catalase (green) resulted in yellow to orange points that predominantly fall along the diagonal line. Points falling outside the diagonal represent non-superposition. Correlation coefficients representing the extent of colocalization were expressed as red-to-green (0.4) and green-to-red (0.6) ratios.
This point is further illustrated by using the diagonal of the fluorogram (Fig. 2D). The areas with complete or perfect superposition corresponded to the pattern of yellow pixels on the diagonal of the fluorogram (Fig. 2D). The values in Fig. 2D represent correlation coefficients. These ratios indicate that ~42% of the red intensity (SCP-2) colocalized with the green intensity (peroxisomal catalase marker), whereas 61% of the green intensity (peroxisomal catalase marker) overlapped with the red intensity (SCP-2).

As expected from the presence of the COOH-terminal peroxisomal targeting sequence in pro-SCP-2, SCP-2 was localized in the highest concentration (brightest staining punctate structures) in peroxisomes. However, the fluorogram demonstrates that an appreciable pool (>50%) of SCP-2 was extraperoxisomal (not colocalized with catalase). Therefore, immunocolocalization and cell fractionation studies were performed to determine the localization of the extra-peroxisomal SCP-2.

**Immunocolocalization of SCP-2 with endoplasmic reticulum in transfected L-cell fibroblasts.** Transfected L cells overexpressing SCP-2 were examined by indirect double immunolabeling with primary antibodies to SCP-2 and 76-kDa glycoprotein (rough endoplasmic reticulum marker), followed by detection with Texas Red- and FITC-conjugated secondary antibodies, respectively. Epifluorescence (data not shown) and confocal (Fig. 3A) images show that SCP-2 was both brightly punctate and diffuse. Epifluorescence (data not shown) and confocal (Fig. 3B) images of the rough endoplasmic reticulum marker, anti-76-kDa glycoprotein, show an intense staining pattern, especially near the nucleus, that is typical of that expected for rough endoplasmic reticulum (14, 62). Side-by-side comparison of single-slice confocal images reveals that the punctate pattern of SCP-2 immunolabeling (Fig. 3A, red) partially coincides with the pattern of rough endoplasmic reticulum (Fig. 3B, green) immunolabeling. Superposition of these images (Fig. 3, A and B) shows significant overlap of the SCP-2 (red) and 76-kDa glycoprotein (green) as yellow structures (Fig. 3C). In the fluorogram these regions are represented as yellow intensity aligned along the diagonal (Fig. 3D). The correlation coefficients in the fluorogram (Fig. 3D) indicate that 31% of the red intensity (SCP-2) colocalized with the green intensity, whereas 47% of the green intensity (rough endoplasmic reticulum marker) overlapped with the red intensity. Examination of colocalization in the large, round structures near the nucleus (Fig. 3C, arrows) reveals that some, but not all, of these rough endoplasmic reticulum structures contained SCP-2. With enlargement, these structures are more clearly shown to be knoblike (see Fig. 3, insets a–c). Furthermore, fluorograms of these enlarged, knoblike structures reveal a pattern of yellow intensity considerably grouped on the diagonal (Fig. 3a) as well as patterns of knoblike structures showing separate populations of mostly red (SCP-2) (Fig. 3b) or mostly green (76-kDa glycoprotein) (Fig. 3c) pixels representing other structures concentrated near the nucleus.

The localization of SCP-2 to endoplasmic reticulum in transfected L-cell fibroblasts overexpressing SCP-2 was also examined by cellular subfractionation followed by Western blotting. In 15-kDa pro-SCP-2-expressing cells, Western blots of purified microsomes detected the 13-kDa SCP but not the 15-kDa pro-SCP-2 (Fig. 4, lane 2).

The merged images and pixel fluorograms of intact cells as well as Western blots of isolated microsomal subfractions indicate that significant amounts of SCP-2 were colocalized with endoplasmic reticulum.

**Immunolocalization of SCP-2 with other intracellular organelles in transfected L-cell fibroblasts.** To determine whether some of the punctate anti-SCP-2 shown in Figs. 1 and 2 was associated with additional membrane structures, we performed immunocytochemical labeling for colocalization with mitochondria and lysosomes. Mitochondria were chosen because some data in the literature suggest that SCP-2 may play a role in mitochondrial cholesterol oxidation (reviewed in Refs. 11 and 46). Both epifluorescence and laser scanning confocal microscopy of L cells expressing SCP-2 and immunostained with monoclonal antibodies to the mitochondrial markers cytochrome oxidase or HSP70 revealed a typical mitochondrial reticular pattern with interconnected rodlike structures. Colocalization studies with anti-SCP-2 indicated that SCP-2 was found in mitochondria, but in amounts less than those observed with peroxisomes or rough endoplasmic reticulum. Western blots of mitochondria purified from the SCP-2-expressing cells confirmed the presence of 13-kDa SCP-2 in mitochondria (data not shown).

Finally, lysosomes were chosen as a negative control organelle because immunogold electron microscopy did not detect SCP-2 association with lysosomes (32). Neither epifluorescence nor laser scanning confocal microscopic fluorescence images of L cells overexpressing SCP-2 showed significant colocalization of SCP-2 with LAMP-2, a lysosomal membrane protein (data not shown). Consistent with this, Western blotting of lysosomes isolated from transfected L-cell fibroblasts expressing SCP-2 also showed very limited SCP-2 immunoreactivity (data not shown).

Results from immunofluorescence imaging microscopy of intact cells and Western blotting of isolated subcellular fractions from transfected L-cell fibroblasts overexpressing SCP-2 indicate that SCP-2 was membrane associated. Membrane-associated SCP-2 was most highly concentrated in organelles such as peroxisomes and endoplasmic reticulum. In contrast, SCP-2 was only weakly associated with mitochondria and was virtually absent from lysosomes. The ability of SCP-2 to bind fatty acyl-CoA (15), taken together with its presence in endoplasmic reticulum, was consistent with the possibility that this protein may play a role in fatty acyl-CoA transacylation reactions in these organelles.

**Incorporation of exogenous fatty acid into glycerides in L cells expressing SCP-2.** To determine the effect of SCP-2 on endoplasmic reticulum incorporation of fatty

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acid into glycerides in intact cells, we exposed control (mock transfected) L cells and SCP-2-overexpressing L cells to exogenous [3H]oleic acid for a short time, 10 min (Fig. 5). Control cells incorporated 162.0 ± 8.0 and 6.7 ± 1.1 pmol of exogenous [3H]oleic acid/mg cell protein into phospholipid and triacylglycerol, respectively (Fig. 5). Thus exogenous oleic acid was incorporated 24-fold more effectively into phospholipids than triacylglycerols. In SCP-2-overexpressing L cells, the incorporation of exogenous [3H]oleic acid into phospholipid and triacylglycerol was stimulated 1.6- and 2.5-fold compared with control cells. Furthermore, overexpression of SCP-2 reduced the preferential incorporation of [3H]oleic acid into phospholipids compared with tri-
acylglycerol by a factor of two. Thus these data with intact SCP-2-expressing L-cell fibroblasts were consistent with SCP-2 enhancing the incorporation of exogenous fatty acid into glycerides, lipids synthesized primarily in the endoplasmic reticulum. However, as was pointed out in the Introduction, it was not known whether this stimulation was a direct effect of SCP-2 or a secondary effect due to upregulation of other protein(s) involved in fatty acid metabolism. To resolve this issue, we undertook studies undertaken with isolated microsomes.

SCP-2 stimulation of microsomal fatty acyl-CoA transacylation: phosphatidic acid. The possibility that SCP-2 may directly affect the utilization of fatty acyl-CoA by microsomal acyltransferases to form phosphatidic acid was examined by measuring the effect of SCP-2 on microsomal incorporation of [14C]oleoyl-CoA into glycerol-3-phosphate and lysophosphatidic acid (Fig. 6). In the absence of SCP-2, basal activities of microsomal glycerol-3-phosphate acyltransferase (GPAT) (Fig. 6A) and lysophosphatidic acid acyltransferase (LAT) (Fig. 6B) were near 100 and 1,200 pmol min⁻¹ mg microsomal protein⁻¹, respectively. SCP-2 enhanced microsomal phosphatidic acid biosynthesis from glycerol-3-phosphate and [14C]oleoyl-CoA up to 13-fold in a dose-dependent, hyperbolic manner (Fig. 6A). Maximal activity (1,403 ± 32 pmol min⁻¹ mg microsomal protein⁻¹) was obtained with 20 µM SCP-2. To resolve whether SCP-2 stimulated microsomal GPAT, LAT, or both to form phosphatidic acid, we measured separately the effect of SCP-2 on the second acylation step of glycerol-3-phosphate, i.e., LAT (Fig. 6B). SCP-2 only stimulated lysophosphatidic acid acyltransferase activity twofold (Fig. 6B). Thus SCP-2 differentially modulated the two fatty acyl-CoA acyltransferases, which shows that SCP-2 primarily stimulated the rate-limiting step in microsomal phosphatidic acid formation mediated by glycerol-3-phosphate acyltransferase.

Fatty acyl-CoA specificity of SCP-2 mediated microsomal fatty acyl-CoA transacylation: phosphatidic acid. The acyl-CoA specificity of SCP-2 in stimulating microsomal phosphatidic acid biosynthesis was determined using a saturated (palmitoyl-CoA), monounsaturated (oleoyl-CoA), and polyunsaturated (arachidonoyl-CoA)
fatty acyl-CoA. Basal microsomal activity of GPAT showed very little fatty acyl-CoA specificity as indicated by less than twofold differences in fatty acyl-CoA utilization, with oleoyl-CoA having the lowest activity (Table 1). In contrast, basal LAT showed a preferential utilization, with oleoyl-CoA having the lowest activity cated by less than twofold differences in fatty acyl-CoA showing very little fatty acyl-CoA specificity as indi-fatty acyl-CoA. Basal microsomal activity of GPAT, suggesting that depending on the concentration of arachidonoyl-CoA, basal GPAT activity may not nec- so low that it was only twofold faster than that of GPAT, suggesting that depending on the concentration of arachidonoyl-CoA, basal GPAT activity may not neces-sarily be rate limiting in microsomal incorporation of arachidonoyl-CoA into phosphatidic acid.

SCP-2 dramatically altered the microsomal fatty acyl-CoA incorporation pattern into phosphatidic acid (Table 1). SCP-2 stimulated more than 10-fold microsomal GPAT utilization of fatty acyl-CoAs in phospha-tidic acid formation in the following order: oleoyl-CoA > arachidonoyl-CoA > palmitoyl-CoA. In contrast, SCP-2 stimulated LAT utilization of fatty acyl-CoAs only up to twofold in the following order: oleoyl-CoA > arachidonoyl-CoA, palmitoyl-CoA. SCP-2 dramatically shifted the pattern of fatty acyl-CoA specificity of microsomal GPAT but not that of LAT.

Fatty acyl-CoA specificity of SCP-2 on microsomal phospholipid remodeling. Microsomes also contain the enzymes required for acyl-chain remodeling of previously synthesized phospholipids (60). Therefore, the ability of SCP-2 to induce microsomal phospholipid remodeling was also determined (Table 2). Basal phospholipid acyl-chain remodeling was highest with phos-phatidyethanolamine, as much as 35-fold higher than that for other phospholipids (Table 2). Furthermore, the fatty acyl-CoA specificity of phosphatidylethanolamine was up to 10-fold higher with palmitoyl-CoA than with oleoyl-CoA and arachidonoyl-CoA (Table 2).

SCP-2 selectively stimulated phospholipid acyl-chain remodeling, depending on the specific phospholipid and fatty acyl-CoA. SCP-2 stimulated phosphatidyethanolamine remodeling 1.9- and 4.7-fold with palmitoyl-CoA and oleoyl-CoA, respectively, but not with arachidonoyl-CoA (Table 2). SCP-2 also stimulated the incorporation of oleoyl-CoA into phos-phatidylserine 1.9-fold. In contrast, SCP-2 inhibited incorporation of arachidonoyl-CoA into sphingomyelin by 63%.

**DISCUSSION**

The discovery that the SCP-2 gene has two initiation sites, encoding for a 58-kDa SCP-x and a 15-kDa pro-SCP-2, has fueled interest in a new potential role(s) for this protein family discovered two decades ago by Chanderbhan and co-workers (reviewed in Ref. 11). Whereas the SCP-2 gene product(s) was originally thought to be primarily involved in cholesterol metabolism, recent major advances have suggested new functions in fatty acid metabolism.

First, SCP-2 gene product(s) may be involved in peroxisomal α-oxidation of branched-chain fatty acids. Both the 58-kDa SCP-x and 15-kDa pro-SCP-2 contain the COOH-terminal peroxisomal targeting sequence (PTS-1) (reviewed in Ref. 58). Receptors for the PTS-1 targeting sequence are associated with the cytosolic face of the peroxisomal membrane (reviewed in Ref. 58). These observations predict that the 58-kDa SCP-x, via its PTS-1 targeting sequence, would be highly lo-calized in peroxisomes. Indeed, immunofluorescence, immunogold, and cellular subfractionation studies con-firm that 58-kDa SCP-x is highly localized to peroxi-somes and copurifies with catalase, a peroxisomal marker, on subcellular fractionation (reviewed in Refs. 3 and 52). Studies in vitro with purified 58-kDa SCP-x indicate that this protein has much higher 3-ketoacyl-CoA thiolase activity with branched-chain fatty acyl-CoAs than other peroxisomal thiolases (1, 61). SCP-2 gene-ablated mice, expressing neither 58-kDa SCP-x nor 15-kDa pro-SCP-2, exhibit impaired peroxisomal α-oxidation of branched-chain fatty acyl-CoAs (56). These data are consistent with the conclusion that the 58-kDa SCP-x is a 3-ketoacyl-CoA thiolase specifically involved in peroxisomal α-oxidation of branched-chain

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**Table 1. Sterol carrier protein-2 alters acyl-CoA selectivity of microsomal acyltransferases involved in phosphatidic acid formation**

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<tr>
<th>Acyl-CoA</th>
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<th>SCP-2</th>
<th>No protein</th>
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<td>18:1</td>
<td>148 ± 16</td>
<td>1,417 ± 61†</td>
<td>1,166 ± 129</td>
<td>2,420 ± 203†</td>
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<td>16:0</td>
<td>239 ± 12</td>
<td>290 ± 8†</td>
<td>4,999 ± 210</td>
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<td>20:4</td>
<td>269 ± 10</td>
<td>455 ± 46†</td>
<td>528 ± 73</td>
<td>748 ± 18</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 experiments. GPAT, glycerol-3-phosphate acyltransferase; LAT, lysophosphatidic acid acyltransferase; SCP-2, sterol carrier protein-2. Specific activity was measured as picomoles of phosphatidic acid formed per minute per milligram of protein. *P < 0.05, †P < 0.01 vs. no protein.

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**Table 2. Effect of SCP-2 on phospholipid transacylation**

<table>
<thead>
<tr>
<th>Phospholipid</th>
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<th>Oleoyl-CoA</th>
<th>Arachidonoyl-CoA</th>
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<td></td>
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<td>SCP-2</td>
<td>Control</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1,992 ± 151</td>
<td>3,866 ± 1207*</td>
<td>121 ± 31</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>84 ± 7</td>
<td>95 ± 13</td>
<td>141 ± 49</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>88 ± 5</td>
<td>72 ± 11</td>
<td>138 ± 40</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>68 ± 3</td>
<td>69 ± 3</td>
<td>162 ± 90</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>57 ± 9</td>
<td>68 ± 1</td>
<td>62 ± 34</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 experiments. Acelyltransferase activity was determined as described in MATERIALS AND METHODS. *P < 0.05, †P < 0.01 vs. control.
fatty acyl-CoAs. However, it is not known whether the other gene product, 15-kDa pro-SCP-2, or its cleavage product, 13-kDa SCP-2, has a role in peroxisomal α-oxidation of branched-chain fatty acyl-CoAs.

Second, SCP-2 is not exclusively localized in peroxisomes, and the observation that it is present in significant concentration in endoplasmic reticulum suggests potential role(s) therein. In almost all tissues and cells examined, the 15-kDa pro-SCP-2 is not detected because it is rapidly posttranslationally cleaved to yield the mature 13-kDa SCP-2 (reviewed in Refs. 40 and 52). Although the COOH-terminal PTS-1 site is present in 15-kDa pro-SCP-2, targeting to peroxisomes was not exclusively to peroxisomes. The data presented herein suggest that more than one-half of SCP-2 is localized outside the peroxisome (Figs. 1 and 2), with as much as 31% of SCP-2 immunocolocalized with an endoplasmic reticulum marker (Fig. 3). Furthermore, SCP-2 was detected in Western blots of microsomes isolated from these cells. The observation that some SCP-2 was diffusely distributed (Figs. 1 and 2) was also consistent with some cytoplasmic localization. This pattern of SCP-2 intracellular localization was not due to nonphysiological overexpression of SCP-2. Western blots of control and mock-transfected L-cell fibroblasts detected SCP-2 at <0.007% of soluble protein (28), a level ~10-fold lower than that expressed in liver but similar to that expressed in peripheral tissues (reviewed in Ref. 44). Not only was the level of SCP-2 in transfected L cells (0.030% of soluble protein) in the physiological range (40), but the relative distribution of SCP-2 immunofluorescence in transfected L cells yielded a rank order of highest to lowest intensities that was also very similar to that shown by immunogold electron microscopy and cellular subfractionation of liver, intestine, and steroidogenic tissues: peroxisomes > endoplasmic reticulum ≥ mitochondria > cytoplasm > lysosomes (reviewed in Refs. 33 and 52). The localization of significant amounts of SCP-2 to endoplasmic reticulum places this fatty acyl-CoA binding protein (16, 55–57) in the same subcellular organelle as a variety of fatty acyl-CoA acyltransferases involved in synthesis of phospholipids, triacylglycerols, and cholesterol esters.

Third, the present findings show that the 15-kDa pro-SCP-2 has a role in glyceride synthesis in the endoplasmic reticulum. This conclusion was supported by two types of evidence. 1) Exogenous [3H]oleic uptake and incorporation into phospholipids and triacylglycerols was enhanced in L cells transfected with the cDNA encoding the 15-kDa pro-SCP-2. These lipids are synthesized by enzymes located in the endoplasmic reticulum. When the 15-kDa pro-SCP-2 is overexpressed in Escherichia coli, fatty acids and phosphatidylglycerol are increased two- to threefold (37). In contrast, in mice that have the SCP-2 gene ablated, liver triacylglycerols are reduced twofold (56). 2) SCP-2 stimulated microsomal phosphatidic acid biosynthesis in vitro. This observation, together with effects in transfected cells and gene-ablated mice, indicates that the action of SCP-2 on glyceride synthesis was not indirect but, rather, that SCP-2 directly enhanced fatty acyl-CoA acyltransferase reactions in the endoplasmic reticulum. 3) SCP-2 stimulated microsomal phospholipid acyltransferases in vitro. 4) The SCP-2 altered the type of fatty acid esterified to the phospholipids synthesized by GPAT and phospholipid acyltransferase. For example, basal GPAT specificity for acyl-CoAs was 20:4 > 16:0 > 18:1. In contrast, for SCP-2-stimulated GPAT, the order of acyl-CoA fatty acid chain preference was 18:1 > 20:4 > 16:0 (Table 1). Likewise, SCP-2 stimulated phospholipid transacylation to phosphatidylethanolamine and other phospholipids with the highest preference for 18:1 acyl-CoA (Table 2). However, this altered pattern of the type of fatty acyl-CoAs incorporated into phospholipids was not simply related to the order of SCP-2 affinities for these fatty acyl-CoAs. SCP-2 exhibited <1.5-fold differences in affinities for the 18:1, 20:4, and 16:0 fatty acyl-CoAs, with the 20:4 fatty acyl-CoA being bound with the slightly higher affinity (16). This suggests that perhaps the fatty acyl-CoA-SCP-2 complex itself interacts in a more specific way with the respective acyltransferase enzymes. Because 18:1-CoA is known to alter the tertiary structure of SCP-2 (15), the degree of structural change in SCP-2 may be dependent on the specific acyl-CoA species. It may be speculated that this, in turn, might alter the interaction and/or ability of the holo-SCP-2 containing bound acyl-CoA to stimulate the transacylase enzymes in the endoplasmic reticulum. Consistent with this suggestion, the holo- rather than the aporetiol binding protein interacts with retinol acyltransferase to stimulate retinol ester formation in the endoplasmic reticulum (25).

In summary, the immunofluorescence data presented herein show, for the first time in cultured cells, that the 13-kDa SCP-2, derived from the 15-kDa SCP-2 gene product, was localized not only in the peroxisome but also in endoplasmic reticulum. Supporting the physiological significance of this observation, immunogold electron microscopy of liver, intestine, and steroidogenic tissues revealed substantial extraperoxisomal SCP-2 (reviewed in Refs. 33, 50, 52, and 59). SCP-2 expression not only stimulated glyceride synthesis in the endoplasmic reticulum of intact cells but also enhanced microsomal glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase 13- and 2-fold, respectively, in vitro. Thus this effect of SCP-2 was direct. Furthermore, SCP-2 demonstrated marked specificity for fatty acyl-CoA. Basal microsomal activity of GPAT showed very little fatty acyl-CoA specificity. In contrast, SCP-2 exhibited higher specificity for unsaturated versus saturated fatty acyl-CoAs. This enhancement was primarily due to SCP-2 stimulating GPAT. These data suggest that SCP-2, by stimulating microsomal fatty acyl-CoA acyltransferases and altering its fatty acyl-CoA specificity in phosphatidic acid formation, may in part influence not only the total amount of phospholipid and other glycerides (triacylglycerols) formed in the endoplasmic reticulum but also their fatty acid composition. Thus SCP-2 is not only localized in the endoplasmic reticu-
lum but appears to directly enhance phosphatidic acid biosynthesis and may alter the fatty acid composition of phospholipids by modulating the acyl-chain selectivity of microsomal acyltransferases.

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REFERENCES


