The Sterol Carrier Protein SCP-x/Pro-SCP-2 Gene Has Transcriptional Activity and Regulates the Alzheimer Disease γ-Secretase*

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The sterol carrier protein SCP-x/pro-SCP-2 gene is a fusion gene having two initiation sites that generate a long (SCP-x; 58.9-kDa) and a short (pro-SCP-2; 15.4-kDa) product, both containing the common SCP-2 module at the C terminus. Here, we show that SCP-x is processed on the peroxisomal surface to liberate a short C-terminal product of 12.9 kDa. This fragment has DNA binding activity in vivo and in vitro, as assessed by chromatin immunoprecipitation analysis, DNA-protein pull-down, electrophoretic mobility shift assay, and luciferase reporter activity. In addition, it is preferentially found in the nucleus where it regulates the transcription of CD147, the regulatory subunit of the Alzheimer disease γ-secretase. Overexpression of SCP-x increased, whereas antisense oligonucleotides against scp-x decreased, the generation of the above transcription factor. Both biochemical and genetic approaches indicate that pro-SCP-2 acts as a competitive inhibitor of SCP-x processing, thereby controlling the release of the 12.9-kDa transcriptionally active fragment. The transcription regulatory function of pro-SCP-2 requires a peroxisomal targeting sequence at the C terminus and a 20-amino-acid leader sequence at the N terminus. Finally, pro-SCP-2 has also cholesterol carrier activity, which is functionally separated from the transcription regulatory one. In conclusion, we have identified two novel functions (transcriptional and transcription regulatory) of the SCP-x/pro-SCP-2 gene that have impact on γ-secretase activity.

SCP-2 (sterol carrier protein 2) was initially identified both as a sterol carrier and a nonspecific lipid-binding protein. Subsequent studies showed that SCP-2 could bind and mediate the translocation of fatty acids and cholesterol between membrane systems both in vitro and in vivo (1). Further analysis revealed that SCP-2 is responsible for a rapid (10 min) protein-mediated translocation of cholesterol in cells and tissues (2–4) and that it can alter cholesterol metabolism in the liver and plasma (5). SCP-2 is part of a fusion gene with two initiation sites coding for a 15.4-kDa (pro-SCP-2) and a 58.9-kDa (SCP-x) protein containing the entire SCP-2 sequence at their C termini (6, 7).

The 58.9-kDa SCP-x is post-translationally cleaved to produce a 46-kDa N-terminal fragment that localizes in the peroxisomal matrix, where it acts as a 3-oxoacyl-CoA thiolase, and a 13.2-kDa C-terminal fragment containing SCP-2. The cleavage itself occurs on the peroxisomal surface, even though an extraperoxisomal cleavage has also been proposed (1). The peroxisomal targeting is provided by the last three amino acids (AKL) on the C-terminal tail of SCP-x acting as a peroxisomal targeting sequence (PTS1).2 The PTS1 is also removed, most likely following the initial N-terminal cleavage, thereby releasing the 12.9-kDa mature form of SCP-2 (1). A similar processing of the 15.4-kDa pro-SCP-2 has been suggested by immunocytochemistry; however, biochemical proof in vivo and in vitro is lacking.

Different aspects of cholesterol metabolism and distribution have been shown to regulate the production of the amyloid β-peptide (Aβ), a 38–43-amino acid-long peptide that is involved in the pathogenesis of Alzheimer disease, one of the most common forms of age-associated disorders (8). However, the possible role of intracellular cholesterol carriers (including SCP-2) in the regulation of Alzheimer disease neuropathology has not yet been evaluated.

Here, we show that SCP-x undergoes post-translational processing on the peroxisomal surface liberating a short C-terminal product of 12.9 kDa that acts as a transcription factor. The generation of this fragment is tightly controlled by pro-SCP-2, which acts as a competitive inhibitor of SCP-x processing. Deletion mutant clones of pro-SCP-2 indicate that this novel regulatory function requires a peroxisomal targeting sequence at the C terminus and a 20-amino-acid leader sequence at the N terminus. The transcriptionally active 12.9-kDa SCP-x product is able to translocate to the nucleus where it activates transcription of CD147, the regulatory subunit of the γ-secretase complex. Expression of pro-SCP-2 abolished the activation of the transcriptionally active 12.9-kDa SCP-2 and shut down the transcription of CD147. This resulted in a 3–5-fold increase in γ-secretase activity in vitro and in vivo and

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‡ This abbreviation used is: PTS, peroxisomal targeting sequence; Aβ, amyloid β-peptide; ER, endoplasmic reticulum; PM, plasma membrane; LDI, lipoprotein-deficient serum; LDL, low density lipoprotein(s); ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; CHO, Chinese hamster ovary; SREBP, sterol regulatory element-binding protein; APP, amyloid precursor protein; mβ-CD, methyl β-cyclodextrin; NTD, N-terminal domain; CTD, C-terminal deleted; CND, N- and C-terminal deleted.

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increased production of Aβ. In addition to its transcription regulatory function, pro-SCP-2 translocates cholesterol to the cell surface and has a major impact on the intracellular cycling of cholesterol. However, in contrast to its transcription regulatory function, neither the peroxisomal targeting sequence at the C terminus nor the 20-amino acid leading sequence at the N terminus is required for the cholesterol carrier function of the protein.

**EXPERIMENTAL PROCEDURES**

Cells, cell culture techniques, Western blot analysis, Aβ determination by sandwich enzyme-linked immunosorbent assay, in vitro determination of β- and γ-secretase activities, and statistical analysis were described previously (9, 10). Analysis of cholesterol transport from the ER to the PM was described in (2), whereas cholesterol determinations in cell lysates and cholesterol-poor/cholesterol-rich domains (lipid rafts) were described in Refs. 11 and 10, respectively. Primer sequences are available upon request.

**Antibodies**—The following antibodies were used in this study: anti-SCP-x/SCP-2 (generous gift from Dr. F. Schroeder), anti-APP C-terminal (Chemicon), anti-P51 N-terminal (Santa Cruz Biotechnology), anti-CD147 (Chemicon), anti-Nicastrin (Santa Cruz), anti-Aph-1 (Chemicon), anti-Pen-2 (Zymed Laboratories), anti-Notch intracellular domain (Cell Signaling), anti-SREBP-2 (BD-Biosciences), anti-Myc (Sigma), and anti-actin (Cell Signaling).

**Incorporation of Acetate into Cholesterol and Oleate into Cholesterol Esters**—The rate of acetate incorporation into cholesterol in living cell cultures was determined as described previously (12). On day 1, cell monolayers (60–70% confluency) were washed with phosphate-buffered saline and switched to medium supplemented with 5% fetal bovine lipoprotein-deficient serum (LDS; produced as described before (2)). On day 2, the cells were washed twice with phosphate-buffered saline and switched to medium containing 5% LDS and 0.5 mM [14C]acetate. The specific activity of radiolabeled acetate (~400 cpm/pmol) was kept constant throughout all of the experiments. After incubation at 37 °C for 4 h, the cells were harvested for the analysis of cellular [14C]cholesterol, as described above.

For the incorporation of [14C]oleate into cholesterol [14C]oleate, the experiments were essentially performed as described above with the difference that radiolabeled oleate was preincubated with medium containing LDS and 5 mg/ml bovine serum albumin for 30 min before being added to the cell monolayers.

**LDL Radiolabeling and Uptake**—LDL (density 1.025–1.063 g/ml) were isolated by sequential ultracentrifugation of fetal bovine serum as described (13). Before isolation, the serum was adjusted to 0.01% EDTA, 0.02% sodium azide, and 10 μM phenylmethylsulfonyl fluoride. Isolated LDL were then labeled with [3H]cholesteryl oleoyl ether following incubation with LDS and gentle rotation for 32 h at 37 °C (14). Thereafter, the labeled lipoproteins were reisolated by ultracentrifugation, filtered with 0.22-μm filters, and used immediately. The specific activity of lipoprotein labeled in cholesterol ester was ~5700 cpm/μg of protein. Radiolabeled LDL were added to serum-free cultures at 20 μg/ml concentration for 4 h. The cells were then washed twice with phosphate-buffered saline, scraped off the surface, and counted on a liquid scintillation counter.

**Chromatin Immunoprecipitation (ChIP) Analysis**—H4 cells were cultured in complete medium in 150-mm Petri dishes until ~70% confluent. The cells were then fixed by the addition of 280 μl of 37% formaldehyde (Sigma) to 10 ml of culture medium for 10 min at 37 °C, harvested, and processed for ChIP using a commercially available kit (Active Motif). SCP-2 DNA immune complexes were precipitated with a polyclonal antibody against SCP-x/SCP-2. PCR was carried out using primer sets centered on the 5′-untranslated region of the CD147 promoter (15, 16).

**Preparation of Cytosolic and Nuclear Fractions**—Cytosolic extracts were prepared as described by Shetty and Idell (17) with some modifications. The cells were homogenized in homogenization buffer containing 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, and a protease inhibitor mixture. The homogenates were centrifuged at 14,000 × g for 15 min, and supernatants were collected as cytosolic proteins.

 Nuclear extracts were prepared according to the protocol of Das et al. (18). Briefly, the cells were scraped into ice-cold phosphate-buffered saline and collected by centrifugation. The cell pellets were suspended in 2 volumes of lysis buffer (20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, pH 8.0, 0.2% Nonidet P-40, 10% glycerol, and a protease inhibitor mixture) followed by incubation on ice for 10 min. Cell suspensions were gently pipetted up and down; the lysates were then centrifuged at 14,000 × g for 5 min at 4 °C to obtain nuclear pellets. Nuclear pellets were washed twice with cell lysis buffer (lacking Nonidet P-40 and protease inhibitor mixture) and then resuspended in 2 volumes of nuclear extract buffer (20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, pH 8.0, 420 mM NaCl, 20% glycerol, and 10% protease inhibitor mixture). The nuclei were extracted by incubation at 4 °C for 30 min with gentle agitation followed by centrifugation at 14,000 × g at 4 °C for 5 min; the resultant supernatant fraction was used as a nuclear extract.

**Biotin-Streptavidin Pull-down Assay**—One microgram of biotin double-stranded DNA was used in pull-down assays performed as described previously (19). The probe used, corresponding to positions −492 to +10 of the human CD147 promoter, was incubated with 500 μg of nuclear and total cell extracts for 20 min at room temperature in a binding buffer containing 12% glycerol, 12 mM Hepes, pH 7.9, 4 mM Tris, pH 7.9, 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 μg of poly(dI-dC) competitor. Following the incubation, 30 μl of streptavidin-agarose beads (Pierce) were added to the reaction and incubated at 4 °C for 4 h. Prior to this step, 300 μl of the original streptavidin-agarose bead preparation were preadsorbed with 500 μl of bovine serum albumin (1 mg/ml) and 50 μg of poly(dI-dC) competitor for 30 min at 25 °C. The beads were washed three times and resuspended in 300 μl of the binding buffer. The protein-DNA-streptavidin-agarose complex was washed three times with binding buffer, and bound proteins were released by boiling in SDS loading buffer. The samples were then separated on a 4–12% Bis-Tris gel system (NuPAGE; Invitrogen) and analyzed by Western blot.

**EMSA**—The EMSA for SCP-2 was performed using the Lightshift Chemiluminescent EMSA kit (Pierce) according to
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FIGURE 1. pro-SCP-2 affects cholesterol trafficking and metabolism in the cell. A, control and pro-SCP-2-expressing cells were pulsed with [3H]acetate for 7 min and then chased for the indicated times. The results are expressed as percentages of newly synthesized cholesterol translocated to the PM in each 10 min period and represent the averages of three different experiments. B, control and pro-SCP-2-expressing cells were labeled with [14C]acetate for 3 days (ad equilibrum); cholesterol-poor and cholesterol-rich (lipid rafts) domains were separated based on their solubilization with Triton X-100 and their migration properties on floating gradients. [14C]Cholesterol was analyzed by TLC and scintillation counting. The results were normalized per mg of protein and then expressed as percentages of control. Shown are the means \pm S.D. of at least four different experiments. The \textit{asterisk} indicates a significant difference from control. C, Western blot (W.b.) analysis showing the processing of SREBP-2 in control and pro-SCP-2 transfected cells. D, LDL uptake was determined by incubating cell monolayers with radiolabeled LDL (5700 cpm/µg protein) particles in the presence of serum-free medium. The results were normalized per mg of protein and then expressed as percentages of control. Shown are the means \pm S.D. of three different experiments. The \textit{asterisk} indicates a significant difference from control. E, the rate of acetate incorporation into the mevalonic pathway was analyzed by incubating cell monolayers with 0.5 mm [4C]acetate (–400 cpm/pmol) in the presence of lipoprotein-deficient serum. Cell [14C]cholesterol was analyzed by TLC and scintillation counting. The results were normalized per mg of protein and then expressed as percentages of control. Shown are the means \pm S.D. of three different experiments. The \textit{asterisk} indicates a significant difference from control. F and G, total cell cholesterol (F) and cholesterol-esters (G) were determined with the enzymatic assay. The \textit{asterisk} indicates a significant difference from control. H, schematic overview of the effect induced by pro-SCP-2 and described in the figure. I.P., immunoprecipitation.

the manufacturer’s protocol. PCR products corresponding to the 5’-untranslated region of the CD147 promoter were labeled using the biotin 3’ end labeling kit (Pierce) and incubated with an affinity-purified 12.9-kDa mature form of SCP-2 for 30 min. Additionally, increasing concentrations (50X, 100X, and 300X) of the unlabelled probe were added to confirm specificity of the interaction. The reaction mixture was then loaded onto a 6% retardation gel (Invitrogen) containing 0.5X Tris borate (TBE) and electrophoresed in 0.5X TBE prior to visualization according to the manufacturer.

Luciferase Reporter Assay—The ChIP-positive promoter fragments corresponding to region 3 and part c of Fig. 4A were cloned into a promoterless pGL3 plasmid (Promega). H4 cells were transfected with 1 µg of the promoter-reporter construct as well as the empty vector along with 0.1 µg of Renilla luciferase (Promega). Firefly and Renilla luciferase activities were measured 24 h after transfection with a dual luciferase kit (Promega) and expressed as relative luciferase activity. Co-transfected Renilla luciferase was used to normalize for transfection efficiency.

Antisense Treatment—Phosphodiester oligonucleotides, including scp-x antisense (3’-acgtgctacag-gagaag-5’) and sense (5’-tgcaacatg-tgccctc-3’), were synthesized at the University of Wisconsin Biotechnology Center and purified on reverse phase high performance liquid chromatography (10). Both oligonucleotides were designed to overlap the start codon of scp-x and used at 10 µm final concentration. Treatment was started 5 days before the experiment, and the oligonucleotides were added every day.

RESULTS

Pro-SCP-2 Regulates the Enzymatic Activity of γ-Secretase—We initially wanted to assess whether the cholesterol binding/carryer function of pro-SCP-2 was able to affect Aβ metabolism. Accordingly, we decided to characterize the effect that the stable transfection of pro-SCP-2 had on cellular cholesterol metabolism. Fig. 1A shows that, when transfected into Chinese hamster ovary (CHO) cells, pro-SCP-2 enhanced the translocation of cholesterol from the ER to the PM, resulting in a parallel increase of sterols in cholesterol-rich domains (also called lipid rafts) (Fig. 1B). The continual removal of cholesterol from the ER membrane caused the proteolytic activation of the sterol regulatory element-binding protein-2 (SREBP-2), an ER membrane-based transcription factor that controls cholesterol levels in the ER membrane and regulates both biosynthesis and uptake of cholesterol (20) (Fig. 1C). As a consequence, we also observed an increase in both receptor-mediated internalization of extracellular lipoproteins (Fig. 1D) and acetate incorporation into the mevalonic pathway (Fig. 1E). These effects led to a ∼3-fold increase in the levels of total cell cholesterol (Fig. 1F); however, no overall effect was observed on cholesterol distribution between the pool of free and ester derivatives (Fig. 1G). Therefore, the above results
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confirm the ability of pro-SCP-2 to stimulate the cycling of intracellular cholesterol, producing major changes in cholesterol metabolism and distribution (schematically shown in Fig. 1H; see also Refs. 1–5).

Transfection of pro-SCP-2 into CHO cells harboring the human amyloid precursor protein (APP751) revealed a marked reduction in the steady-state levels of both C99 and C83 (Fig. 2A) without any apparent change in the expression levels of either the immature or mature forms of APP (Fig. 2A). C99 and C83 are proteolytic intermediates resulting from β and α cleavage of APP, respectively. They also act as the immediate substrates for γ cleavage, which is the final proteolytic step for the generation of Aβ from the C99 fragment of APP (8, 21). The decrease in C99 was paralleled by an increase in Aβ generation/secretion (Fig. 2B), suggesting a direct effect on γ, rather than β, cleavage of APP. This last conclusion is also supported by the fact that pro-SCP-2 affected the steady-state levels of both C99 and C83. No overall effect was observed on the Aβ42/Aβtotal ratio (Fig. 2B), excluding any apparent interference with the affinity of γ secretase for its immediate substrate, C99.

To confirm the above conclusions, we decided to use human neuroglioma (H4) cells expressing either full-length (H4-APP751) or the last 105 amino acids of the C-terminal end (H4-APP105) of human APP. It is worth remembering that APPC105 mimics C99 and acts as γ (but not β) substrate (11, 22). When expressed in H4-APP751 cells, pro-SCP-2 reduced the steady-state levels of both C99 and C83 (Fig. 2C), confirming the results obtained with CHO cells (Fig. 2A). The dramatic decrease in APPC105 levels following expression in H4-APP105 cells (Fig. 2D) further demonstrates the ability of pro-SCP-2 to activate γ cleavage of APP. This conclusion was also confirmed by the increased levels of the Notch intracellular C-terminal domain, another product of γ cleavage, observed in the cytosol of H4 cells following pro-SCP-2 expression (Fig. 2E), and by the ability of the γ-secretase inhibitor N-[N-3,5-difluorophenacyl]-l-alanyl-S-phenylglycine methyl ester (DAPM) to restore the levels of APPC105 (Fig. 2F). Finally, in vitro analysis of γ-secretase activity from CHAPS-solubilized membranes showed a ~3-fold increase in pro-SCP-2-expressing cells, compared with control (Fig. 2G). No effect was observed on the in vitro activity of the β-secretase BACE1 (data not shown).

Analysis of the steady-state levels of the different members of the γ-secretase complex (PS1, Nicastrin, Aph-1, Pen-2, and CD147) revealed a marked decrease in the levels of both the low and high glycosylated forms of CD147 (Fig. 2H), the regulatory subunit of the enzymatic complex (23). No apparent effect was observed on the levels of PS1, Nicastrin, Aph-1, or Pen-2. As expected, N-[N-3,5-difluorophenacyl]-l-alanyl-S-phenylglycine methyl ester (DAPM) inhibited the enzymatic activity of the γ-secretase complex but did not affect the expression levels of CD147 (Fig. 2F).

Therefore, the above results indicate that pro-SCP-2 can impact on γ-secretase activity by affecting the steady-state levels of CD147. Interestingly, normalization of cholesterol levels...
The 12.9-kDa C-terminal Product of SCP-x Has Transcriptional Activity—The fact that the steady-state levels of CD147 were not restored in conditions that successfully normalized cholesterol levels led us to speculate that pro-SCP-2 itself (or one of its products) can act as transcriptional regulator of CD147. To explore such a possibility, we performed a ChIP assay, which allows immunoprecipitation of protein-DNA complexes following in vivo cross-linking with formaldehyde, cell lysis, and DNA shearing. The cross-linked DNA (Fig. 4B) was immunoprecipitated with a polyclonal antibody against SCP-2, reversed, purified, and amplified with specific primers designed to overlap different 5′ areas of the promoter region of CD147 (Fig. 4A). Only those that included the region immediately upstream of the start codon were able to generate a PCR product of the expected size (Fig. 4C), further indicating that such interaction requires a specific segment of the 5′-untranslated region of CD147.

Western blot analysis of nuclear extracts detected both the 15.4- and the 12.9-kDa forms of SCP-2 (Fig. 4D); interestingly, the 15.4-kDa form (pro-SCP-2) appeared mostly associated with the cytosol and cell membranes, whereas the 12.9-kDa form (mature SCP-2) appeared highly enriched in the nuclear fraction. The fact that full-length pro-SCP-2 could also be observed in the nuclear extract might be due to its ability to bind to cholesterol and fatty acids in the lipid bilayer of the nucleus. Indeed, a similar approach failed to detect the precursor form of SREBP-2 (Fig. 4E, lower panel), which does not posses lipid binding properties and is known to localize only to the ER (20).

Next, we performed a pull-down experiment using the PCR product corresponding to region 3 of Fig. 4A, which was biotinylated prior to incubation with either a nuclear extract or a total cell lysate. The biotinylated DNA-protein complex was purified with streptavidin, digested with DNAses, and then analyzed by SDS-PAGE and immunoblotting with a specific antibody against SCP-2. Interestingly, only the 12.9-kDa isoform of SCP-2 was consistently pulled down following incubation with the PCR probe (Fig. 4E, upper panel). As a control, we used the same strategy to pull down SREBP-2. As expected, nuclear extracts contained the cleaved and transcriptionally active form of SREBP-2, whereas the precursor was only observed in total cell lysates (Fig. 4E, lower panel). However, SREBP-2 was never observed following incubation with the 5′ promoter region of CD147 and the consequent pull-down (Fig. 4E, lower panel), supporting the validity and specificity of the results obtained with 12.9-kDa SCP-2 and confirming that SREBP-2 does not act as a transcriptional regulator of CD147. These results are also supported by the fact that CD147 does not contain a SRE element on its 5′-untranslated region.

Finally, we analyzed the DNA binding properties of mature SCP-2 on an EMSA following incubation of affinity-purified 12.9-kDa SCP-2 with PCR products corresponding to different areas of region 3 (Fig. 4A), which was shown to interact with SCP-2 by ChIP (Fig. 4C). The probe corresponding to nucleotides −245 to −95 (labeled with c) was the only one to display a strong mobility shift when incubated with the transcriptionally active fragment of SCP-2 (Fig. 4F, arrowhead); no effect was observed with PCR products corresponding to nucleotides −492 to −392 (labeled with a) and −94 to +10 (labeled with d). Interestingly, a weak shift could also be observed when we used a probe corresponding to nucleotides −392 to −242 (labeled with b), suggesting the existence of low affinity binding sites for 12.9-kDa SCP-2 on that area of the promoter (Fig. 4F). The specificity of the mobility shift was further proved by displacing the DNA-protein interaction with increasing concentrations of unlabeled oligonucleotides (Fig. 4G, lanes 3–5).

In conclusion, when taken together, the above results indicate that the 12.9-kDa mature form of SCP-2 has DNA binding activity both in vivo and in vitro and is found in nuclear extracts, supporting the notion that it can function as a transcription factor. Finally, they also indicate that the interaction with the
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promoter of CD147 is restricted to a specific area of the 5'-untranslated region.

Pro-SCP-2 Has Transcription Regulatory Activity—To further clarify the mechanisms that regulate the transcriptional activity of the SCP-x/pro-SCP-2 gene, we generated three different deletion mutant versions of pro-SCP-2 (Fig. 5A) lacking the 20-amino acid N-terminal leading sequence (NTD), the C-terminal PTS1 sequence (CTD), and a combination of both mutations (CND). Analysis of stable-transfected H4 clones indicated that only full-length pro-SCP-2 was able to reduce the steady-state levels of the mature 12.9-kDa SCP-2 (Fig. 5B, bottom panel). This effect was accompanied by an increase in the steady-state levels of the 58-kDa SCP-x (Fig. 5B) and by the almost complete disappearance of the 46-kDa SCP-x N-terminal product of SCP-x (Fig. 5B, Thiolase), suggesting that pro-SCP-2 interferes with the proteolytic generation of mature SCP-2 and the 46-kDa thiolase from the common precursor, SCP-x. Neither NTD nor CTD could elicit the same effect. It is worth noting that only full-length pro-SCP-2 was able to affect the expression levels of CD147 (Fig. 5C).

Analysis of nuclear extracts revealed that the mature 12.9-kDa SCP-2 was completely lacking in cells expressing full-length pro-SCP-2, whereas no apparent effect was observed with NTD, CTD, or CND deletion mutants (Fig. 5D). The steady-state levels of mature 12.9-kDa SCP-2 did not appear to be increased by either of the above deletion mutant versions, suggesting that they do not participate in the generation of the 12.9-kDa form (Fig. 5, B and D). However, neither NTD nor CND resulted in very high expression levels following stable transfection, suggesting a rapid turnover of these mutant forms. Interestingly, an additional and previously unidentified band was observed immediately below mature 12.9-kDa SCP-2 in both CTD and CND clones that could be a proteolytic degradation product (Fig. 5D, question mark).

The N-terminal leading sequence of pro-SCP-2 acts as an amphipathic α-helix that penetrates membrane bilayers (24, 25); in contrast, the C-terminal AKL sequence provides peroxisomal targeting. Therefore, based on the above results, it is conceivable that most of the mature 12.9-kDa SCP-2 derives from SCP-x following post-translational processing on the peroxisomal surface. The 15.4-kDa pro-SCP-2 containing both the N-terminal amphipathic α-helix and PTS1 can reach the peroxisomes and act as a competitive inhibitor/regulator of SCP-x processing. In contrast, neither the CTD, which lacks peroxisomal targeting, nor the NTD, which lacks the leading α-helix, mutants can elicit the same function. It is worth noting that the CTD mutant was found mostly associated with the cytosol (Fig. 5D) and was not able to reach the peroxisomes (data not shown). Interestingly, CTD and NTD were still able to translocate cholesterol from the ER to the PM (Fig. 6A) and affect cholesterol metabolism (Fig. 6B), indicating that neither the 20-amino acid N-terminal leading sequence nor the C-terminal PTS1 sequence is required for the cholesterol carrier function of pro-SCP-2. These findings are consistent with the fact that the lipid-binding pocket of pro-SCP-2 is imbedded in the center of the protein and is affected by neither the N-terminal leading sequence nor the C-terminal PTS1 sequence (26–28). Finally, the fact that both CTD- and NTD-SCP-2 were able to affect cholesterol metabolism (Fig. 6, A and B), but not the steady-state levels of CD147 (Fig. 5C) or the γ cleavage of APPC105 (Fig. 6C), further confirms that the ability of pro-SCP-2 to influence the processing of APP is independent of its cholesterol binding and carrier properties.

To further demonstrate that pro-SCP-2 interferes with the post-translational cleavage of SCP-x, we transiently transfected H4 cells with different concentrations of pro-SCP-2, CTD-
SCP-2, and NTD-SCP-2 cDNA (Fig. 5, E and F). Consistently with the experiments performed with stable transfected cells, pro-SCP-2 successfully reduced the steady-state levels of 12.9-kDa SCP-2 (Fig. 5E). This effect was gene dose-dependent, and paralleled by an increase in the steady-state levels of SCP-x and a decrease in the 46-kDa N-terminal product (Fig. 5E, Thiolase). Finally, the decrease in CD147 and APPC105 (Fig. 5E) was concomitant with the progressive decrease in 12.9-kDa SCP-2. The above effects were not observed when we used the same strategy with CTD-SCP-2 or NTD-SCP-2 cDNA (Fig. 5F).

When taken together, the above results indicate that pro-SCP-2 inhibits the generation of the transcriptionally active 12.9-kDa SCP-2 fragment, most likely competing with the processing of SCP-x. Therefore, pro-SCP-2 seems to provide a regulatory function to the transcriptional activity of the SCP-x/mature 12.9-kDa SCP-2 system. This conclusion is also supported by the fact that when the ChIP analysis was per-
formed in pro-SCP-2-expressing cells, we detected a marked reduction in the PCR product recovered following immuno-precipitation of protein-DNA complexes with an anti-SCP-2 antibody (Fig. 5G).

If the above arguments are correct, we would expect to increase the steady-state levels of 12.9-kDa SCP-2 by overexpressing SCP-x. Indeed, Fig. 7A shows that stable transfection of SCP-x led to a parallel increase in the levels of both the 46-kDa thiolase and the mature 12.9-kDa SCP-2 products (Fig. 7A). As a result, we observed increased levels of CD147 and reduced γ cleavage of APPC105 (Fig. 7A). This effect was not observed when the peroxisomal targeting function of PTS1 was blocked by the addition of a Myc tag at the C terminus of SCP-x (data not shown), confirming the requirement of the peroxisomes for the processing of SCP-x. Finally antisense oligonucleotides designed to overlap the start codon of SCP-x decreased the levels of the mature 12.9-kDa SCP-2 and resulted in reduced transcription of CD147 and increased γ cleavage of APPC105 (Fig. 7B). Gene-silencing approaches against pro-SCP-2 were not attempted because they are known to interfere with the synthesis of both SCP-x and pro-SCP-2 (2–4).

Because the processing of SCP-x is known to occur on the peroxisomes (1), it is conceivable that the peroxisomal membrane has binding sites for docking, together with a proteolytic machinery for the processing of SCP-x. It is also conceivable that pro-SCP-2 competes with the “docking” rather than the “processing” machinery because the expression of pro-SCP-2 does not seem to generate any proteolytic product. To test this assumption, we decided to analyze the levels of SCP-x in highly purified peroxisomal fractions (Fig. 7, C and D). As shown in Fig. 7D, under normal conditions peroxisomes contained SCP-x and its immediate products, the 46-kDa thiolase and the 12.9-kDa transcriptionally active SCP-2. The presence of the 12.9-kDa form is consistent with the fact that it is generated on the peroxisomal surface prior to its translocation to the nucleus. However, expression of full-length pro-SCP-2 dramatically reduced the levels of both SCP-x and its products (Fig. 7D), indicating that it interferes with the ability of SCP-x to dock on the peroxisomal surface. Once again, we did not observe any proteolytic product of pro-SCP-2, suggesting, but not proving, that pro-SCP-2 does not compete with the proteolytic machinery that processes SCP-x.

Even though the transfection of pro-SCP-2 into CHO and H4 (human neuroglioma) cells resulted in similar phenotypes, we always observed a more dramatic response with H4. We also noticed that CHO and H4 cells express different levels of endogenous pro-SCP-2 and mature SCP-2 (Fig. 7E), suggesting potential cell type-specific forms of regulation of SCP-x/pro-SCP-2 metabolism. These differences might account for the more striking response that we always obtained in H4 cells. In fact, CHO cells express high levels of the 15.4-kDa pro-SCP-2, whereas H4 cells express high levels of mature 12.9-kDa SCP-2 (Fig. 7E). Interestingly, the expression of endogenous 12.9-kDa SCP-2 seems to inversely correlate with those of pro-SCP-2 (Fig. 7E), supporting the overall conclusions obtained with the experiments involving transgenic pro-SCP-2 (Fig. 5). Because these results might have an important impact on γ secretase activity in the brain and on Alzheimer disease pathogenesis, we decided to analyze the expression pattern of SCP-2 in primary neurons and in neuroblastoma (SHEP) cells. As shown in Fig. 7F, both primary neurons and SHEP cells behaved very similarly to H4 cells. Finally, all the low molecular mass forms of SCP-2 (15.4-kDa pro-SCP-2, 15.0-kDa SCP-2, and mature 12.9-kDa SCP-2) could be observed in a cell extract from mouse brain cortex (Fig. 7G).

Interestingly, the expression levels of the mature and transcriptionally active SCP-2 directly correlated with those of endogenous CD147 (Fig. 7F), supporting the conclusions reached with transgenic pro-SCP-2 (Fig. 5).

Finally, the different transcriptional roles of SCP-x and pro-SCP-2 were analyzed with a dual luciferase activity assay. The promoter of CD147 (region 3 and part c of Fig. 4A) was subcloned into the promoterless fire fly luciferase reporter vector pGL3-basic. The luciferase activity of the CD147 promoter construct was measured after transient transfection in control,
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FIGURE 7. The transcriptionally active 12.9-kDa SCP-2 originates from SCP-x. A, H4-APPc105 cells were stably transfected with the 58-kDa SCP-x (lanes 3 and 4; compare with lane 1) and then analyzed by Western blot with the indicated antibodies. Lane 2 shows pro-SCP-2 transfected cells. B, H4-APPc105 cells were treated with antisense oligonucleotides designed against scp-x prior to Western blot analysis. The effects produced by the antisense were compared with a scp-x sense oligonucleotide. C, migration of the peroxisomal marker, catalase, on a 10–28% Nycodenz gradient. The gradient was performed as before (10). D, fractions 22 and 24, containing highly purified peroxisomal fractions were analyzed by Western blot with an anti-SCP-x/pro-SCP-2 antibody. E, Western blot analysis of endogenous SCP-2 in CHO (lane 1) and H4 (lane 2) cells. As control, lane 3 shows H4 cells stably transfected with pro-SCP-2. F, Western blot analysis of endogenous SCP-2 and CD147. Lane 1, CHO cells; lane 2, H4 (human neuroglioma) cells; lane 3 (primary neurons; day 6 of culture); lane 4, brain cortex; lane 5, SHEP (human neuroblastoma) cells. G, longer exposure of F.

FIGURE 8. Luciferase reporter activity of pro-SCP-2 and SCP-x. A and B, the promoter of CD147 was subcloned into the promoterless fire fly luciferase reporter vector pGL3-basic. The luciferase activity of the CD147 promoter constructs was measured after transfection in control, pro-SCP-2-expressing, and SCP-x-expressing H4 cells. Fig. 8 indicates that the luciferase activity of the CD147 promoter was dramatically affected by the expression of pro-SCP-2 and SCP-x. Specifically, pro-SCP-2 decreased, whereas SCP-x increased the luciferase activity, when compared with control cells. These results are consistent with the fact that pro-SCP-2 blocks (Fig. 5), whereas SCP-x stimulates (Fig. 7) the generation of the mature and transcriptionally active 12.9-kDa SCP-2.

DISCUSSION

The most salient conclusion of this study is that the SCP-x/pro-SCP-2 gene has both transcriptional and transcription regulatory activity. The transcriptional activity resides in the mature 12.9-kDa SCP-2, which originates from SCP-x following post-translational processing at the peroxisomal surface and translocation to the nucleus (Fig. 9). Targeting of SCP-x to the peroxisomes is provided by a functionally active PTS1 at the C terminus; the removal of PTS1 seems a required step for the nuclear translocation of the 12.9-kDa SCP-2. In fact, only the mature 12.9-kDa SCP-2 (and not full-length pro-SCP-2) could be detected with a protein-DNA pull-down assay. This conclusion is also supported by the fact that mosquito (Aedes aegypti) SCP-2, which lacks the PTS1 sequence, can be found in the nucleus with classical immunoelectron microscopy (29). Interestingly, patients affected by Zellweger syndrome (which is characterized by the inability of the cell to assemble peroxisomes) are unable to import SCP-x into the peroxisomes and lack the mature 12.9-kDa SCP-2 form (1), further confirming that the processing of SCP-x occurs on the peroxisomal surface.

The DNA binding function of mature 12.9-kDa SCP-2 was proven by four independent approaches: ChIP (Fig. 4, A–C), protein-DNA pull-down (Fig. 4E), EMSA (Fig. 4, F and G), and luciferase reporter
amphipathic SCP-2 (Fig. 4). This regulatory activity requires both the C-terminal PTS1 sequence, which targets pro-SCP-2 to the peroxisomes, and the 20-amino acid leading amphipathic α-helix at the N terminus. In addition, pro-SCP-2 has cholesterol binding and cholesterol carrier properties, which do not require the N-terminal leading sequence or the C-terminal PTS1.

The generation of the transcriptionally active 12.9-kDa SCP-2 is tightly controlled by pro-SCP-2, most likely acting as a competitive regulator of the docking machinery (Fig. 9). This regulatory activity requires both the C-terminal PTS1, which targets pro-SCP-2 to the peroxisomes, and the 20-amino acid leading amphipathic α-helix at the N terminus. Therefore, the SCP-x/pro-SCP-2 gene not only generates a complex set of different proteins with distinct biochemical functions and local activity (Fig. 8). The EMSA was performed with affinity-purified 12.9-kDa SCP-2, indicating that the DNA binding activity resides on the SCP-2 fragment. These results were further supported by the fact that the transcriptionally active fragment is preferentially found in the nucleus (Fig. 4D).

The generation of the transcriptionally active 12.9-kDa SCP-2 is tightly controlled by pro-SCP-2, most likely acting as a competitive regulator of the docking machinery (Fig. 9). This regulatory activity requires both the C-terminal PTS1, which targets pro-SCP-2 to the peroxisomes, and the 20-amino acid leading amphipathic α-helix at the N terminus. Therefore, the SCP-x/pro-SCP-2 gene not only generates a complex set of different proteins with distinct biochemical functions and local activity but also provides a novel form of regulation for its own biological functions.

Our results produced with biochemical and genetic approaches seem to rule out an important contribution of pro-SCP-2 in the generation of the mature and transcriptionally active, 12.9-kDa SCP-2. Obviously, it is still possible that extremely high expression levels of pro-SCP-2 may also lead to its processing and consequent release of the 12.9-kDa product (1). This seems to be particularly evident in hepatoma cells where ~10-fold increase in the expression of pro-SCP-2 can lead to substantial processing (1). Similar processing appeared only partial in other peripheral cell lines (1). It is also possible that differences in the ability to resolve the different SCP-2 species on SDS-PAGE might explain some of the differences found in the literature (1). However, the fact that "knock-down" of SCP-x resulted in the complete absence of the transcriptionally active fragment (Fig. 7B) seems to exclude any physiological relevance. Indeed, the contribution of SCP-x to the generation of smaller products, migrating as mature SCP-2 on SDS-PAGE, is more consistent and easier to replicate in every cell type analyzed so far (1). Obviously, we always need to take in account possible cell type-specific behaviors determined by the different levels of the endogenous SCP-2 species (as shown in Fig. 7, E–G). Finally, the striking correlation between the results obtained by expressing transgenic pro-SCP-2 (Fig. 5) and those obtained by comparing the relative levels of pro-SCP-2 and mature (12.9-kDa) SCP-2 (Fig. 7, E–G) strongly support our conclusions.

3-Oxoacyl-CoA thiolase, cholesterol binding, and cholesterol carrier activities are among previously characterized functions of the SCP-x/pro-SCP-2 gene. With the exception of the thiolase activity, all of these functions (including the transcriptional and transcription regulatory) reside in the common pro-SCP-2 product of the fusion gene. The transcription regulatory function requires both the 20-amino acid leading sequence at the N terminus and the C-terminal PTS1 (present study), whereas the cholesterol binding and cholesterol carrier functions seem to reside in a stretch of ~50 amino acids that constitute the center of the SCP-2 structural module (26–28). In addition to cholesterol, this pocket can also bind different classes of fatty acids.

The common "pro-SCP-2 module" is found in several genes expressed in bacteria, archaea, and eukaryotes either as a fusion product with SCP-2 at the C terminus of another protein domain or as a nonfused protein (30). Even though the functions of pro-SCP-2 in these genes have not been completely determined, it is likely that the transcriptional activity (perhaps even the transcription regulatory one) has been conserved throughout evolution.

γ-Secretase is a multimeric protein complex that is responsible for the intramembrane proteolysis of a large number of type 1 membrane proteins (31), which are involved in many and diverse functions. These include calcium homeostasis, cell adhesion, vesicular and protein transport, apoptosis, and cell signaling. In addition, in many cases (i.e. APP, Notch, p75NL1, and DCC) the cytoplasmic tail resulting from the γ cleavage is able to translocate to the nucleus and act as a transcription factor. Therefore, in addition to Alzheimer disease neurobiol-
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ogy, the regulation of γ-secretase activity by SCP-x/pro-SCP-2 can potentially and dramatically impact on many different cellular, physiological, and pathological events.

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