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Intrinsic Sterol- and Phosphatidylcholine Transfer Activities of 17β-Hydroxysteroid Dehydrogenase Type IV

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Previous studies have shown that the 80 kDa 17β-hydroxysteroid dehydrogenase (17β-HSD) type IV comprises distinct domains, including an N-terminal region related to the short chain alcohol dehydrogenase multigene family and a C-terminal part related to the lipid transfer protein sterol carrier protein 2 (SCP2). In this study, we have investigated whether the SCP2-related part of the 80 kDa protein leads to an intrinsic sterol and phospholipid transfer activity, as shown earlier for the 60 kDa SCP2-related peroxisomal 3-ketoacyl CoA thiolase with intrinsic sterol and phospholipid transfer activity called sterol carrier protein x (SCPx). Our results indicate that a fraction rich in the 80 kDa form of 17β-HSD type IV exhibits high transfer activities for 7-dehydrocholesterol and phosphatidylcholine. In addition, a purified recombinant peptide derived from the SCP2-related domain of the 17β-HSD type IV has about 30% of the transfer activities for 7-dehydrocholesterol and phosphatidylcholine seen with purified recombinant human SCP2. We conclude that the 80 kDa type IV 17β-HSD represents a potentially multifunctional protein with intrinsic *in vitro* sterol and phospholipid transfer activity in addition to its enzymatic activity.

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INTRODUCTION

Intracellular sterol traffic is a poorly understood process that may involve sterol-rich vesicles, intracellular lipoprotein-like particles, and sterol carrier or sterol transfer proteins [reviewed in 1]. *In vitro* studies have led to the hypothesis that sterol transfer proteins may contribute to target-specific intracellular transport of cholesterol, required for fundamental metabolic pathways like steroidogenesis, bile acid biosynthesis, cholesterol esterification and cholesterol efflux. Likely candidates in this respect are two sterol transfer proteins, named sterol carrier protein 2 (SCP2) and sterol carrier protein x (SCPx). SCP2 and SCPx are expressed from a common gene via alternative transcription initiation and/or alternative splicing [2]. SCP2 is a 13.5 kDa basic sterol- and phospholipid transfer protein, also named non-specific lipid transfer protein, consisting of 123 amino acids (aa). Results obtained from experiments performed *in vitro* led to the assumption that the protein may mediate the chronic activation of steroidogenesis by catalyzing the transfer of cholesterol to the outer mitochondrial membrane in adrenals and ovaries [3–6]. In addition, its high level of expression in liver, cholesterol-loaded macrophages and intestine suggested a function in intracellular cholesterol transfer in these tissues. Consistent with the hypothesis, SCP2 promotes the exchange of a wide variety of phospholipids, glycolipids and sterols between membranes *in vitro* [7–10]. In addition, SCP2 activates the *in vitro* enzymatic conversion of 7-dehydrocholesterol to cholesterol by microsomal sterol Δ7-reductase [11], the esterification of cholesterol catalyzed by acyl-CoA:cholesterol acyl transferase *in vitro* [12–14], and the introduction of the less-polar substrates in bile acid biosynthesis to the membrane bound enzymes *in vitro* [15, 16]. However, experimen-

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Abbreviations: aa, amino acids; 7-DHC, 7-dehydrocholesterol; GST, glutathione *S*-transferase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; PCR, polymerase chain reaction; PC, phosphatidylcholine; SCAD, short chain alcohol dehydrogenase; SCP2, human sterol carrier protein 2; SCPx, human sterol carrier protein x; SCP2*, porcine C-terminal domain similar to SCP2; VHF, very hydrophobic fraction rich in 17β-HSD.

tal proof for such a role of SCP2 *in vivo* has not been obtained to date.

We earlier identified SCPx as peroxisomal 3-ketoacyl-CoA thiolase with intrinsic sterol and phospholipid transfer activity consisting of 547 aa [17]. The C-terminal 143 aa of SCPx are completely identical with the precursor of SCP2 (pre-SCP2) [2] and it is assumed that the C-terminal domain is responsible for the intrinsic lipid transfer activity of SCPx [17]. 17β-hydroxysteroid dehydrogenase (type IV) [17β-HSD (IV)] is translated as an 80 kDa protein which consists of distinct domains, including an N-terminal domain related to the SCAD multigene family and a C-terminal domain related to SCP2 [18]. In this study, we have investigated whether presence of the SCP2-related domain in 17β-HSD IV leads to sterol and phospholipid transfer activities of this 80 kDa protein.

MATERIALS AND METHODS

Materials

All lipid chemicals were purchased from Sigma (St Louis, U.S.A.) and were of the highest quality available. A culture of *Bacillus megaterium* was kindly provided by Professor F. Meinhardt (University of Münster, Germany). The pGEX vector was purchased from Pharmacia (Uppsala, Sweden).

Protein expression and purification

A DNA fragment encoding amino acids 597–737 (SCP2*) of porcine 17β-HSD IV was obtained from the cDNA [18] by PCR amplification. The 5′-primer introduced an in frame EcoRI site (TTTGAATTCACTGTCATTTCAAATGCATACGTGG, position 1858–1882) and the 3′-primer a KpnI site (TTTGGTACCTTAAATCTTGGCATAGTCTTTAA, position 2260–2283) for the ligation into the multicloning site of the pGEX pTL2 vector. *E. coli* strain JM107 containing the pGEX-SCP2* plasmid was grown in M9 minimal medium containing 50 μg/ml ampicillin. The cells were grown in a rotary shaker at 37°C to an $O.D._{600\,nm}$ of 0.6. Expression of GST-SCP2* was induced by adding isopropyl-D-thiogalactopyranoside to a final concentration of 1 mM, followed by further incubation at 37°C for 3 h. Preparation of the cell extract, and purification and cleavage of the GST fusion proteins were performed as described earlier [19].

The purification of porcine 17β-HSD IV from kidney resulted in two products [20]: a homogeneous 32 kDa protein representing the N-terminal SCAD domain fragment of the primary translation product and a fraction (addressed as VHF) consisting of the 17β-HSD IV SCAD peptide (32 kDa), actin (45 kDa) and two species of close to 80 kDa, representing the primary 80 kDa 17β-HSD IV translation product and a covalent dehydrogenase–actin complex. Quantitation of VHF proteins was performed after SDS–PAGE and Coomassie Blue staining.

Assays of in vitro sterol—and phosphatidylcholine transfer activities of 17β-HSD IV

Sterol and phospholipid transfer activities were measured in a direct lipid transfer assay by monitoring the net transfer of 7-dehydrocholesterol (7-DHC) and phosphatidylcholine (PC) from small unilamellar vesicles (SUVs) to *Bacillus megaterium* protoplasts as described in references [11, 19]. Briefly, SUVs containing egg yolk PC/7-DHC (65:35 mol%) were incubated with *Bacillus megaterium* protoplasts at 37°C for 30 min. Assays contained 2 mM of lipid, 2.5 mg of protoplast protein, and 1 nmol of protein in a total volume of 500 μl. Protoplasts were then separated from SUVs by centrifugation in an Eppendorf centrifuge for 4 min at 8000 rpm. The sedimented protoplasts were washed once with SPA (0.3 M sucrose, 0.3% (w/v) NaN_3, 0.06 M potassium phosphate, pH 6.2), resuspended with 300 μl SPA and then lysed by adding an equal volume of 15% ethanolic KOH. 7-DHC was extracted once with 1.2 ml of *n*-hexane (yield: 97.5%) and quantified by recording an UV-spectrum between 320 and 250 nm (absorptivity at 294 nm: 7200 M^{-1} cm^{-1}). Incubations containing purified SCP2, SCPx or SCP2-glutathione-*S* transferase (GST) fusion protein were used as positive controls, negative controls contained bovine serum albumin.

Protein- and DNA data bank comparisons

All sequences were obtained from the EMBL and SWISS-PROT data banks. Sequence analyses were performed with the program TREE present in the HUSAR 3.0 software package (DKFZ, Heidelberg, Germany). Blocks of significant consensus sequences were detected with the program MACAW 103 [21].

RESULTS AND DISCUSSION

DNA and protein data bank comparisons revealed high similarity scores between the sequences of 17β-HSD IV and SCP2 [18]. The relative homology between the C-terminal 141 amino acids (aa) of porcine 17β-HSD (SCP2*) and pre-SCP2 sequences from different species or PXP 18, representing the yeast homologue of SCP2, is shown in the dendrogram of Fig. 1. Since 17β-HSD SCP2* is placed in a common branch together with the pre-SCP2 cluster, well separated from PXP18, it appears that the relative homology between SCP2* and pre-SCP2 is in general higher than that between pre-SCP2 and the two known PXP18 sequences, which represent active lipid transfer proteins. The size of the 17β-HSD SCP2* region (~127 aa) corresponds well to that of SCP2 (123 aa, lacking the 20 amino acid leader sequence) and the matches cluster into four relatively short regions corresponding to pre-SCP2 aa positions 25–36, 44–55, 70–76

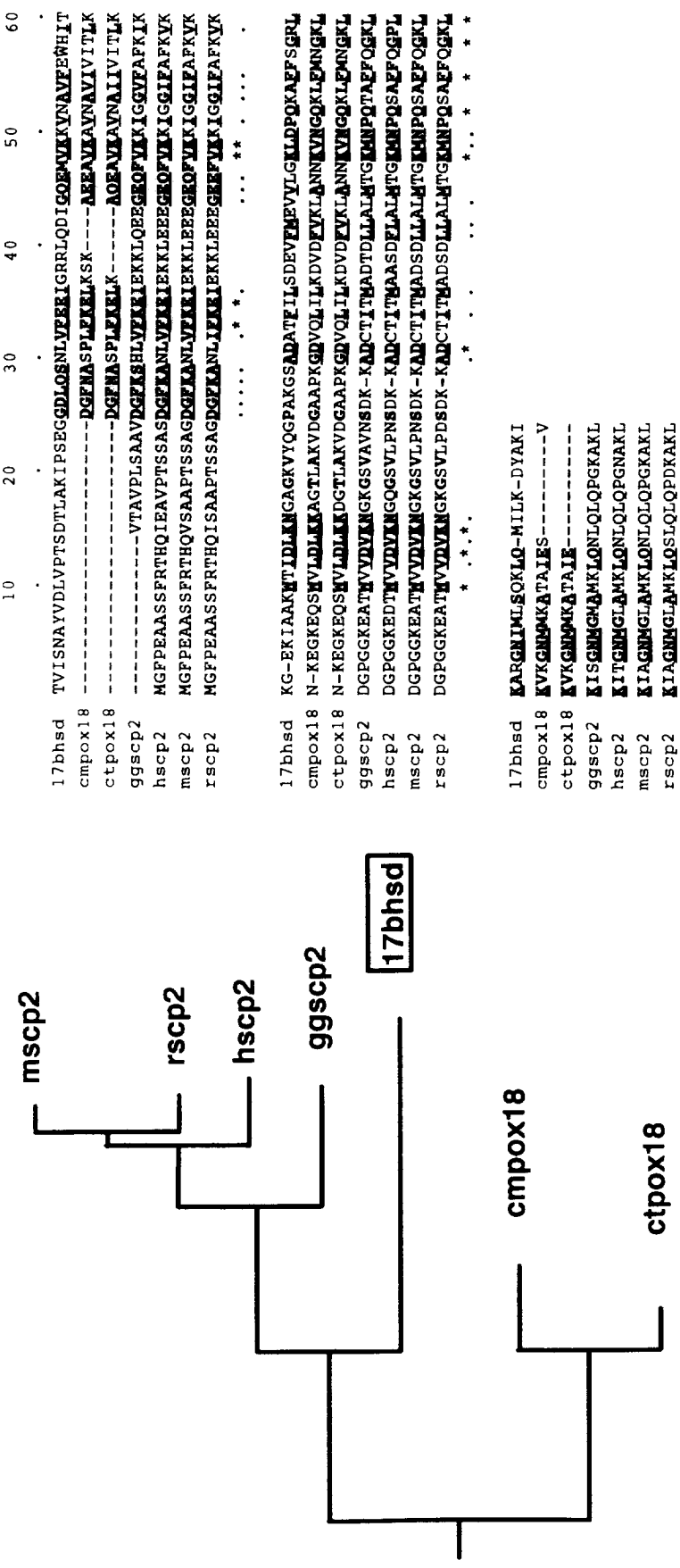

Fig. 1. Homology between the C-terminal region of pig 17β-HSD IV and several known SCP2 related sequences. The dendrogram shown in the left panel represents the relative homology between the displayed sequences. Asterisks below the sequences indicate identical residues, points indicate conserved residues. Bold letters indicate identical or residues with chemically similar side chains. 17*b*hsd, 17β-HSD IV; mscp2, murine SCP2; rscp2, rat SCP2, hscp2, human SCP2; ggscp2, *Gallus gallus* SCP2; cmpox18, *Candida maltosa* pox18; ctpox18, *Candida tropicalis* pox18.

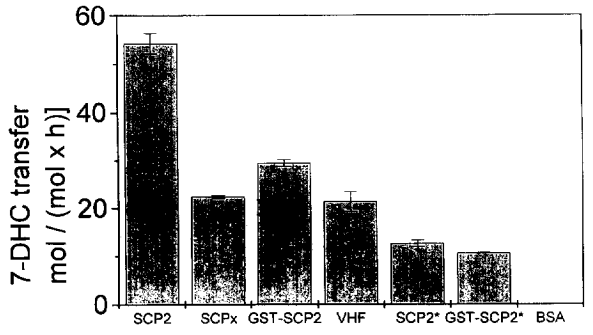

Fig. 2. *In vitro* transfer of 7-dehydrocholesterol from small unilamellar vesicles to *Bacillus megaterium* protoplasts. The bars represent mean values ± SD of assays performed in triplicates as described in Materials and Methods. The specific activity in case of the VHF fraction was calculated with the assumption that the activity was exclusively due to the 80 kDa precursor of 17β-HSD IV contained within the fraction. SCP2* refers to the SCP2-related peptide derived from the sequence of porcine 17β-HSD IV.

and 107–126 (Fig. 1). Although the overall homology between SCP2 and SCP2* is relatively low (~35%), all residues which have been earlier shown by site-directed mutagenesis to be crucial for the sterol and phospholipid transfer activity of human SCP2 [19], are conserved in SCP2*. In addition, the clusters of similarity between SCP2 and SCP2* are restricted to the core region of SCP2 that has been shown by deletion mutagenesis to harbour the part of the molecule involved in its lipid transfer activities [19]. This region of SCP2 consists of a unique structure consisting of three amphipatic α-helices and a five-standard β-sheet [22]. Secondary structure analysis and superimposing the two sequences (not shown) suggested that, despite the relatively low degree of sequence identity between SCP2 and SCP2*, evident from Fig. 1, the two structures most likely reveal considerable structural conservation, particularly with respect to the N-terminal α-helical and the following β-sheet structures. Taken together, these results suggested a high probability that SCP2* might act as an active sterol- and phospholipid transfer domain in the 80 kDa precursor of 17β-HSD IV.

Since the 80 kDa 17β-HSD IV is present within the VHF-fraction isolated from pig endometrium [20], we first investigated whether this fraction contained activity to transfer PC and 7-DHC from donor SUVs to acceptor *Bacillus megaterium* protoplasts, as was shown earlier for SCP2 and SCPx [19]. Figures 2 and 3 show that high levels of transfer activities for both PC and 7-DHC are associated with the VHF-fraction. Under the assumption that this activity was exclusively due to the 80 kDa precursor of 17β-HSD IV, contained within the fraction, we evaluated that this protein transferred 65.2 mol PC/(Mol prot. × h) representing 44% of the molar activity of SCP2 (149.3 mol/[mol prot. × h]). The transfer activity for 7-DHC was 21.4 mol/(mol prot. × h) representing 39% of the value for SCP2 (54.3 mol/[mol prot. × h]) (Fig. 2). The specific PC and 7-DHC transfer activities calculated for the 80 kDa precursor of 17β-HSD IV were almost identical as compared with SCPx (Figs 2 and 3).

To obtain more direct experimental evidence for the involvement of the C-terminal SCP2-related domain contained within the 80 kDa precursor of 17β-HSD IV in the lipid transfer activity of the VHF-fraction, we next investigated the sterol and PC transfer activities of a recombinant peptide consisting of aa 597 to the C-terminus of the 17β-HSD IV 80 kDa precursor. The purified peptide stimulated the spontaneous transfer of PC and 7-DHC from donors to acceptors, measured in the presence of bovine serum albumin, 147- and 157-fold, respectively (Figs 2 and 3). Relatively high activities were also measured with the GST-SCP2* fusion protein (Figs 2 and 3) suggesting that SCP2* was also active if attached to the C-terminus of a non-related protein. As is obvious from Figs 2 and 3, SCP2* thus behaved similarly as SCP2 in this respect. Although the specific lipid transfer activities of the recombinant SCP2-related peptide were lower than the activities of SCP2, these results nevertheless provided convincing evidence for high levels of 7-DHC and PC transfer activities associated with the SCP2-related domain peptide derived from the 80 kDa precursor of 17β-HSD IV. One possible reason for the lower activity measured with purified recombinant SCP2* compared with SCP2 is that recombinant SCP2* may lack some N-terminal amino acids necessary for its full activity. It is known for SCP2 that deletion of only 5 aa from the N-terminus results in more than 50% inactivation [19].

Posttranslational processing of the 80 kDa 17β-HSD IV precursor leads to the 32 kDa enzymatically active mature 17β-HSD IV and a 48 kDa peptide containing SCP2* and an N-terminal peptide homologous to acyl-CoA hydratase/dehydrogenase bifunctional enzymes (Adamski, in preparation), assumed to participate in peroxisomal β-oxidation of various fatty acids.

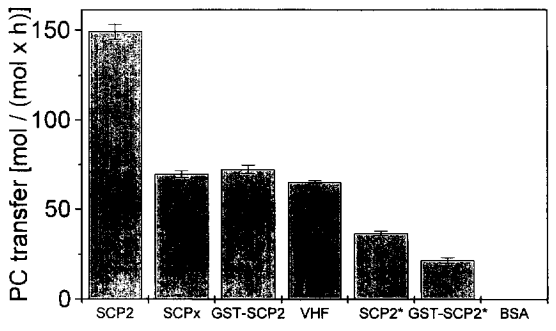

Fig. 3. *In vitro* transfer of phosphatidylcholine (PC) from small unilamellar vesicles to *Bacillus megaterium* protoplasts. The bars represent mean values ± SD of assays performed in triplicates as described in Materials and Methods. The specific activity in case of the VHF fraction was calculated with the assumption that the activity was exclusively due to the 80 kDa precursor of 17β-HSD IV contained within the fraction. SCP2* refers to the SCP2-related peptide derived from the sequence of 17β-HSD IV.

Currently it is not clear, whether the 48 kDa peptide is further processed to yield free SCP2*, as is the case for SCP2 and SCPx. We have recently obtained evidence for proteolytic processing of SCPx in peroxisomes *in vivo* resulting in cleavage at the junction between the thiolase-like domain (residue 1–424) and the SCP2 domain (residues 425–547) (Seedorf, in preparation). In addition to alternative transcription initiation and/or alternative splicing, this mechanism adds further complexity to the way in which SCP2 and SCPx are alternatively expressed from a single gene. Finding SCP2* now, acting as an active sterol- and phospholipid transfer protein *in vitro*, as part of a peptide related to a protein potentially involved in peroxisomal β-oxidation of fatty acids is particularly intriguing, because SCPx has been earlier identified as peroxisomal 3-ketoacyl-CoA thiolase with intrinsic sterol- and phospholipid transfer activity, also assumed to participate in this important peroxisomal metabolic pathway [17]. Although experimental evidence is still lacking, it is tempting to speculate that the enzyme activities of the two peroxisomal proteins may act consecutively in a so far unidentified pathway of peroxisomal β-oxidation.

We conclude that evolution has independently created two metabolically related enzymes activities comprising a remarkably similar molecular architecture with each containing highly homologous sterol- and phospholipid transfer domains at their C-termini. Since this cannot be expected to happen simply by chance, our finding suggests functional importance of the SCP2-domains for the roles of these proteins *in vivo*. Clearly, future investigations should focus on elucidating this role that has remained unclear to date.

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