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In search for function of two human orphan SDR enzymes: Hydroxysteroid dehydrogenase like 2 (HSDL2) and short-chain dehydrogenase/reductase-orphan (SDR-O)

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ABSTRACT

The protein superfamily of short-chain dehydrogenases/reductases (SDRs) today comprises over 20,000 members found in pro- and eukaryotes. Despite low amino acid sequence identity (only 15–30%), they share several similar characteristics in conformational structures, the N-terminal cofactor (NAD(P)/NAD(P)H) binding region being the most conserved. The enzymes catalyze oxido-reductive reactions and have a broad spectrum of substrates. Not all recently identified SDRs have been analyzed in detail yet, and we therefore characterized two rudimentarily annotated human SDR candidates: an orphan SDR (SDR-O) and hydroxysteroid dehydrogenase like 2 (HSDL2). We analyzed the amino acid sequence for cofactor preference, performed subcellular localization studies, and a screening for substrates of the enzymes, including steroid hormones and retinoids. None of both tested proteins showed a significant conversion of steroid hormones. However, the peroxisomal localization of human HSDL2 may suggest an involvement in fatty acid metabolism. For SDR-O a weak conversion of retinal into retinol was detectable in the presence of the cofactor NADH.

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1. Introduction

Members of the protein superfamily of short-chain dehydrogenases/reductases (SDRs) form a group of oxidoreductases that have been identified in all three domains of life. Of the more than 20,000 members identified so far more than 70 are found in humans. While the sequence identity between SDRs is relatively low (15–30%), three-dimensional structures, especially of the N-terminal part, are very similar. The N-terminus contains the Rossmann-fold, a structural element that forms the cofactor binding region of SDRs. Additionally, several sequence motifs are typical for these enzymes, such as the cofactor binding motif TGxxxGxG and the active center motif YxxxK [1].

SDRs are capable of catalyzing oxidations and reductions of a wide variety of substrates including sugars, steroids, retinoids, fatty acids, and xenobiotics. Several members of this protein family are not restricted to one substrate but exhibit a considerable multifunctionality. Peroxisomal 17β-hydroxysteroid dehydrogenase type 4, for example, catalyzes the oxidation not only of very long chain fatty acids and bile acid intermediates, but also of sex steroids [2]. Due to their pivotal roles in the regulation of different metabolic and signalling pathways, the dysfunction of SDR enzymes can lead to several diseases like Alzheimer's disease, cancer and obesity related medical conditions [3–5]. Therefore, SDRs constitute an enzyme class that may be an interesting target for drug development.

The metabolic functions of many members of the SDR superfamily are still unknown. Two partially characterized members of this enzyme family are orphan SDR (SDR-O or SDR9C7-1) and hydroxysteroid dehydrogenase like 2 (HSDL2 or SDR13C1-1). Human SDR-O is a protein that seems to be exclusively expressed in liver and shows high similarity to retinoid metabolizing enzymes [6]. Recent characterization of murine SDR-O showed that it was not capable of catalyzing the conversion of steroids or retinoids [6]. Human HSDL2 is a ubiquitously expressed enzyme consisting of an N-terminal SDR domain and a C-terminal SCP2-like domain, containing a peroxisomal targeting signal (ARL) [7]. The substrate specificity of this enzyme is unknown.

To gain an insight into the metabolic function of human SDR-O and HSDL2, we expressed them in mammalian and bacterial expression systems and performed a substrate screening. Furthermore, we analyzed the subcellular localization of the enzymes.

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2. Materials and methods

2.1. Cloning of expression constructs

Human HSDL2 full length coding cDNA sequence consistent with NCBI data bank entry NM_032303 was obtained from HepG2 cells by RT-PCR. HSDL2 cDNA was subcloned into the vectors pcDNA3, pcDNA3_N′Flag and pcDNA3_N′Myc (modified pcDNA3), pcDNA4_C′MycHisB (Invitrogen) and modified pGex 2T [8] for eukaryotic and prokaryotic expression by use of sequence specific primers with modified ends for restriction enzyme binding (Table 1).

Human SDR-O cDNA was ordered from RZPD DNA library in a pReceiver-M09 vector (GeneCopoeia, #Ex-T7021-M09). The SDR-O sequence occurred to have two point mutations (T141A and F177L) compared to NCBI database entries (NM_148897 and five different human SDR-O ESTs). Therefore, mutations were repaired by Quikchange mutagenesis (Stratagene), and finally the sequence was consistent with NCBI data bank entry NM_148897. SDR-O cDNA was then subcloned into the same expression vectors as HSDL2 by use of sequence specific primers with modified ends for restriction enzyme binding (Table 1). All resulting plasmids were subjected to sequence verification.

2.2. Eukaryotic expression for enzymatic assays

Human HEK293 cells were grown in DMEM medium supplemented with 10% FBS and 1% Pen/Strep (Invitrogen) in humidified conditions (37 °C, 5% CO_2). For transient transfection 8 μg of pcDNA3_SDR-O, pcDNA3_HSDL2, pcDNA3_N′Flag-HSDL2, pcDNA3_N′Flag-SDR-O or controls pcDNA3 and pcDNA3_N′Flag were introduced into cells using 24 μl of FuGENE 6 transfection reagent (Roche). Transfected cells were harvested 24 h after transfection and pelleted into aliquots of 4×10^6 cells each. One aliquot was taken for mRNA expression control immediately, and pellets were stored at −80 °C until use. Total protein content was measured with Bradford reagent (Bio-Rad).

For expression control RNA was isolated from 2 million transfected HEK293 cells using the RNeasy mini kit (Qiagen). One microgram total RNA was reverse transcribed into cDNA using the Revert Aid^{TM} First Strand cDNA Synthesis Kit (Fermentas) and oligo $(dT)_{18}$ primers. The following program was used for PCR with sequence specific primers (see Table 1, lines 1, 2, 5, 6, 23 and 24): 1 cycle 3′ 95 °C, 35 cycles 45″ 95 °C, 30″ 50 °C, 1′ 30″ 72 °C.

For expression control by Western blotting 20 μg total protein from lysed cells were applied to a 10% Tris/Tricine polyacrylamide gel, separated and transferred to a PVDF membrane (Pall) using a Trans-Blot apparatus (Bio-Rad). The blot was incubated with an mouse anti-Flag antibody (Sigma) followed by a HRP-labeled anti-mouse antibody (Dianova). Flag-tagged proteins were visualized by ECL-detection (PerkinElmer).

2.3. Bacterial expression

E. coli BL21DE3 Codon Plus RP or Rosetta2 DE3 pLysS were transformed with pGex_HSDL2, pGex_SDR-O or pGex as control and grown in LB media supplemented with ampicillin or ampicillin/chloramphenicol at 37 °C with continuous shaking. Expression of recombinant proteins was induced by IPTG (0.25 mM). Bacteria were harvested by centrifugation 4 h after induction and stored at −20 °C until use.

For expression control, aliquots of induced or not induced bacteria were analyzed by SDS-PAGE [9]. For this, bacteria were suspended in PBS and lysed by four freeze–thaw cycles in the presence of lysozyme. After centrifugation of the lysate for 30 min at 14,000 × *g*, equal volumes of supernatants and re-suspended pellets were applied to the gel.

For expression control by Western blotting proteins were transferred to a PVDF membrane (Pall) using a Trans-Blot apparatus (Bio-Rad). The blot was incubated with a mouse anti-GST antibody (Zymed) followed by a HRP-labeled anti-mouse antibody (Dianova). GST (-tagged) proteins were visualized by diaminobenzidine color reaction.

Protein content was determined by a modified Lowry method [10].

2.4. Enzymatic screening assays

For substrate screening, several steroids and retinoids were tested with human SDR-O and HSDL2 in oxidation or reduction

Table 1
Sequence specific primers used for cloning of HSDL2 and SDR-O into respective expression plasmids or expression control by RT-PCR.

Construct name	Primer sequence[a]	Restrict. enzyme
pcDNA3_HSDL2 (for)	5′-TTT**GGATCC**ATGTTACCCAACACCGGGAG-3′	BamHI
pcDNA3_HSDL2 (rev)	5′-TTT**CTCGAG**TCACAGTCTGGCATTCATCTGATTCAT-3′	XhoI
pGex_N′GST_HSDL2 (for)	5′-TTT**GGATCC**TTACCCAACACCGGGAGGCTG-3′	BamHI
pGex_N′GST_HSDL2 (rev)	5′-TTT**CTCGAG**TCACAGTCTGGCATTCATCTGATTCAT-3′	XhoI
pcDNA3_SDR-O (for)	5′-TTT**GAATTC**ATGGCGGCCCTCACAGACCTC-3′	EcoRI
pcDNA3_SDR-O (rev)	5′-TTT**CTCGAG**TTAGACACTGTCCGCTGGCCT-3′	XhoI
pGex_N′GST_SDR-O (for)	5′-TTT**GAATTC**GCGGCCCTCACAGACCTCTCATTTAT-3′	EcoRI
pGex_N′GST_SDR-O (rev)	5′-TTT**CTCGAG**TTAGACACTGTCCGCTGGCCT-3′	XhoI
pcDNA3_N′Flag_SDR-O (for)	5′-TTT**GAATTC**TGGCGGCCCTCACAGACCTCTCATTTA-3′	EcoRI
pcDNA3_N′Flag_SDR-O (rev)	5′-TTT**CTCGAG**TTAGACACTGTCCGCTGGCCT-3′	XhoI
pcDNA3_N′Myc_SDR-O (for)	5′-TTT**GAATTC**GCGGCCCTCACAGACCTCTCATTTAT-3′	EcoRI
pcDNA3_N′Myc_SDR-O (rev)	5′-TTT**CTCGAG**TTAGACACTGTCCGCTGGCCTTGGA-3′	XhoI
pcDNA3_C′Flag_SDR-O (for)	5′-TTT**GAATTC**ATGGCGGCCCTCACAGACCTC-3′	EcoRI
pcDNA3_C′Flag_SDR-O (rev)	5′-TTT**CTCGAG**GACACTGTCCGCTGGCCTTGG-3′	XhoI
pcDNA4_C′MycHis_SDR-O (for)	5′-TTT**GAATTC**ATGGCGGCCCTCACAGACCTC-3′	EcoRI
pcDNA4_C′MycHis_SDR-O (rev)	5′-TT**CCGCGG**GACACTGTCCGCTGGC-3′	SacII
pcDNA3_N′Flag_HSDL2 (for)	5′-TTT**GGATCC**TTACCCAACACCGGGAGGCTG-3′	BamHI
pcDNA3_N′Flag_HSDL2 (rev)	5′-TTT**CTCGAG**TCACAGTCTGGCATTCATCTGATTCAT-3′	XhoI
pcDNA3_N′Myc_HSDL2 (for)	5′-TTT**GCGGCC**GCAATTACCCAACACCGG-3′	NotI
pcDNA3_N′Myc_HSDL2 (rev)	5′-TTT**CTCGAG**TCACAGTCTGGCATTCATCTGATTCATTAGC-3′	XhoI
pcDNA4_C′MycHis_HSDL2 (for)	5′-TTT**GGATCC**ATGTTACCCAACACCGGGAG-3′	BamHI
pcDNA4_C′MycHis_HSDL2 (rev)	5′-TTT**CCGCGG**CAGTCTGGCATTCATCTG-3′	SacII
β-Actin for	5′-GGATTCCTATGTGGGCGACGAGG-3′	
β-Actin rev	5′-CACGGAGTACTTGCGCTCAGGAGG-3′	

[a] Restriction sites in primers marked bold.

Table 2
Screen for substrate specificity with steroids and retinoids.

Activity		Substrate and expected product	SDR-O in HEK293	SDR-O in *E. coli*	HSDL2 in HEK293	HSDL2 in *E. coli*
11-oxo	Ox	Hydrocortisone (cortisol) → cortisone	NAD/NADP		NAD/NADP	
11β-red	Red	Cortisone → cortisol	NADH/NADPH		NADH/NADPH	
20-oxo	Ox	20α-Hydroxypregn 4-ene-3-one → progesterone	NAD/NADP		NAD/NADP	
20α-red	Red	Progesterone → 20α-hydroxypregn-4-ene-3-one	NADH/NADP		NADH/NADP	
3-oxo	Ox	Androsterone → 5α-androstane-3,17-dione	NAD		–	
3-oxo	Red	Dihydrotestosterone (DHT) → 5α-androstane 3α,17β-diol	NADH	NADH	NADPH	NADPH
3-oxo	Ox	Dehydroepiandrosterone (DHEA) → 5α-androst-5-ene-3,17-dione	NAD		NADP	
17-oxo	Ox	Estradiol → estrone	NAD		NADP	
17-oxo	Ox	Testosterone to androst-4-ene-3,17-dione	NAD		NADP	
17-oxo	Ox	5α-Androstane 3α,17β-diol → androsterone	NAD		NADP	
17-oxo	Ox	Dihydrotestosterone (DHT) → 5α-androstane-3,17-dione	NAD		NADP	
17β-red	Red	Estrone → estradiol	NADH		NADPH	
17β-red	Red	Androst-4-ene-dione → testosterone	NADH		NADPH	
17β-red	Red	Dehydroepiandrosterone (DHEA) → 5α-androst-5-ene-3β,17β-diol	NADH		NADPH	
	Ox	All-trans-retinol → all-trans-retinal	NAD/NADP	NAD/NADP	NAD/NADP	NAD/NADP
	Red	All-trans-retinal → all-trans-retinol	NADH/NADPH	NADH/NADPH	NADH/NADPH	NADH/NADPH

reactions (Table 2). Steroids were purchased from PerkinElmer or American Radiochemicals, all-trans-retinol and all-trans-retinal from Sigma.

2.4.1. Steroid screen

For steroid screening, transfected HEK293 cell pellet aliquots (4×10^6 cells) or bacteria (normalized according to $OD_{600\,nm}$) were re-suspended in 450 μl reaction buffer of 100 mM NaPi, pH 7.4, 0.05% BSA, 1 mM EDTA. Appropriate ^{3}H-labeled steroids (25 nM final) were added and the reaction was started by addition of cofactor (0.5 mg/ml final), NAD(P) for oxidation or NAD(P)H for reduction. Reaction was stopped after 90 min of continuous shaking at 37 °C with 0.21 M ascorbic acid in methanol:acetic acid 99:1 (v:v). Products and substrates were extracted from reaction mixture by use of Strata-C18-E SPE columns (Phenomenex), eluted with methanol, and analyzed by HPLC (Beckman-Coulter) using a Luna 5 μm C18(2), 125 mm × 4.00 mm column (Phenomenex). The solvents used were acetonitrile:water 43:57 (v:v) for androgens and estrogens, progestins, and methanol:water 43:57 (v:v) for corticosteroids at a flow rate of 1 ml/min. Radioactivity was detected with an online scintillation counter (Berthold LB506D) after mixing with ReadyFlow III (Beckman).

2.4.2. Retinoid screen

For retinoid screening, transfected HEK293 cell pellets (60 μg total protein) or bacteria (172–196 μg total protein) were suspended in a reaction buffer of 100 mM NaPi, pH 7.4, 1 mM EDTA. Appropriate retinoids (10 μM final, dissolved in DMF, Sigma) were added and the reaction was started with the addition of cofactor (0.5 mg/ml final), NAD(P) for oxidation or NAD(P)H for reduction. Reaction was stopped after 10–15 min of continuous shaking at 37 °C by addition of 0.21 M ascorbic acid in methanol:acetic acid 99:1 (v:v). The extraction procedure was modified from Chetyrkin et al. [11]. Products and substrates were extracted from reac-

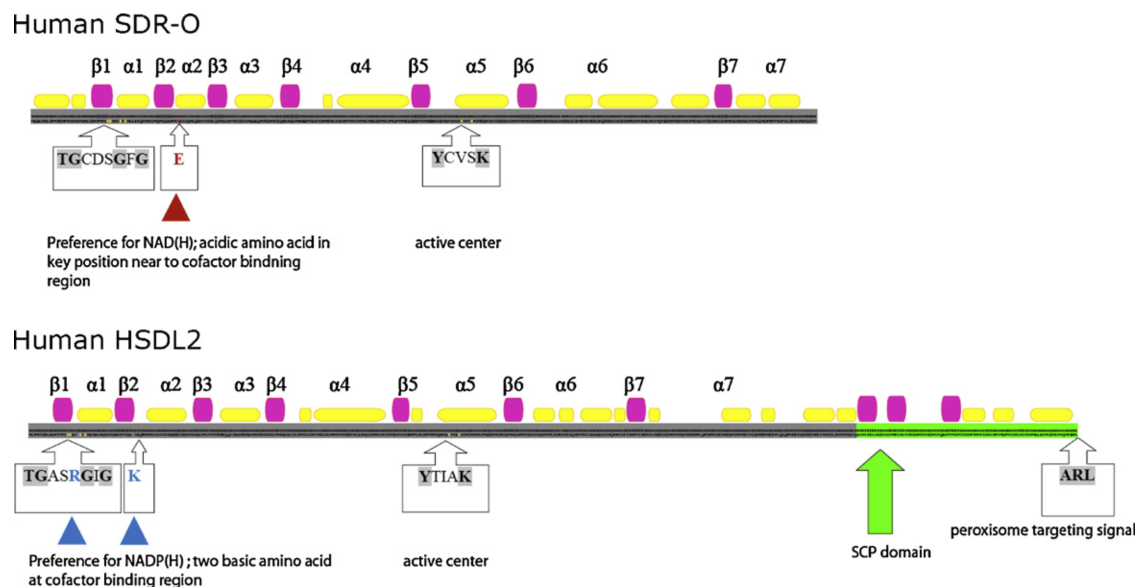

Fig. 1. Secondary structure analysis. The secondary structures were deduced from amino acid sequences by algorithms available at http://zeus.es.nl.

tion mixture by use of Strata-C18-E SPE columns (Phenomenex), eluted with acetonitrile, and analyzed by HPLC (Beckman-Coulter) using a Synergi 4 μ Fusion RP18, 150 mm × 4.6 mm column (Phenomenex) and the solvent acetonitrile:water 92:8 (v:v) at a flow rate of 1 ml/min. Retinoids were detected by absorption at 345 nm. Retinoids were handled in the dark, to prevent isomerization.

2.5. Subcellular localization studies

2.5.1. Transfection of HeLa cells for subcellular localization studies

For subcellular localization studies, human HeLa cells were seeded on 6-well plates containing glass coverslips and incubated at 37 °C and 5% CO_2 in MEM medium, supplemented with 10% FBS, 1% Pen/Strep (Invitrogen). Transient transfection was performed with 1 μg of plasmid DNA in 3 μl of FuGENE 6 transfection reagent (Roche). 24 h after transfection cells were stained for visualization of proteins.

The following plasmids were transfected: pcDNA3_N′Flag_SDR-O, pcDNA3_N′Myc_SDR-O, pcDNA3_C′Flag_SDR-O, pcDNA4_C′MycHisB_SDR_O, pcDNA3_N′Flag_HSDL2, pcDNA3_N′Myc_HSDL2, pcDNA4_C′MycHisB_HSDL2. For negative controls the respective vectors without insert were used. Organelle counterstaining was done either by co-transfection: pDSRed2-Peroxi (Clontech) for peroxisomes, pDsRed_ER (Clontech) for endoplasmic reticulum, and Organelle lights Endosomes-GFP (Invitrogen) for early endosomes, or by counterstaining with dye: MitoTracker Orange (Invitrogen) for mitochondria.

2.5.2. Visualization of proteins in HeLa cells

24 h after transfection, HeLa cells designated for mitochondria visualization were incubated for 30 min with fresh staining medium (MEM with 10% FBS containing 300 nM MitoTracker Orange). After removal of medium, cells were washed twice with PBS and fixed with 3.7% formaldehyde for 10 min at 37 °C. To enable binding of specific antibodies, fixed cells were permeabilized with 0.5% TritonX100 for 5 min at room temperature (RT). To prevent unspecific binding of proteins, blocking with 3% BSA in PBS was done for 30 min at RT. Incubation of primary antibodies, anti-Flag and anti-Myc (Sigma), respectively, in 200 μl solution (1:1000 in 3% BSA/PBS) for 1 h at RT was followed by two washing steps and incubation with secondary antibodies conjugated to fluorescent marker (1:2000 in 3% BSA/PBS). Antibodies were labeled either with Alexa Fluor 488 (green), Alexa Fluor 568 (red), or Cy3 (red) (Molecular Probes or Dianova). After another washing step, cells were incubated with a solution of Hoechst 33342 (Invitrogen) diluted 1:5000 in PBS for 2 min at RT to stain the nuclei. After washing twice with PBS, the cover slips with the cells were fixed on glass slides on a drop of Vectashield (Vectalabs). Fluorescence was detected with an Axiophot microscope (Zeiss) using appropriate filters.

3. Results

3.1. Bioinformatic prediction of cofactor specificity of SDR-O and HSDL2

Amino acid sequences of human SDR-O and HSDL2 show the characteristic motifs for SDR proteins [1], i.e. the Rossmann fold, the glycine rich motif TGxxxGxG necessary for cofactor binding, and the active site YxxxK (Fig. 1). While SDR-O is a single domain protein, HSDL2 additionally carries an SCP2-like domain with the peroxisomal targeting signal ARL at its C-terminus.

Not much is known about the substrate specificities and cofactor preferences of both enzymes. At least concerning the cofactor preference, amino acid sequence analysis can give valuable hints [12]. SDR-O possesses an acidic amino acid (E59) in a key position not far away from the cofactor binding region suggesting a preference for NAD(H) over NADP(H).

HSDL2 contains two basic amino acids that indicate a preference for NADP(H). Similar to other NADP(H) binding SDRs, an arginine is located at position 20 in the glycine rich TGxxxGxG motif. Furthermore, a lysine is found at position 42 adjacent to the second β-strand, another indicator for NADP(H) binding [13].

3.2. Substrate screening

3.2.1. Recombinant expression of SDR-O and HSDL2 in E. coli and HEK293 cells

Cloning and expression of SDR-O and HSDL2 were prerequisites for activity assays. In transiently transfected HEK293 cells (pcDNA3 constructs), expression was checked on mRNA level by RT-PCR. Transcripts of SDR-O and HSDL2 mRNAs were detectable 24 h after transfection, i.e. at the time when cells were harvested for activity measurements (Fig. 2A). Control with mock transfected cells

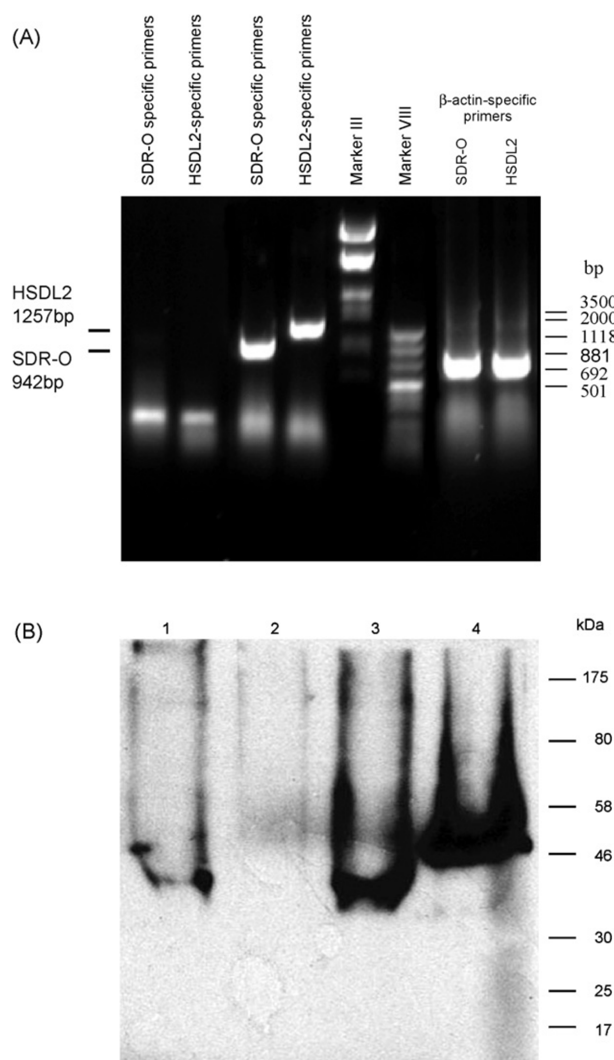

Fig. 2. Expression control for SDR-O and HSDL2 expressed in HEK293 cells. (A) mRNA expression control for SDR-O and HSDL2 after transfection in HEK293. PCR with SDR-O or HSDL2 specific primers: HEK293 mock-transfected with empty pcDNA3 (lanes 1 and 2), HEK 293 transiently transfected with pcDNA3-SDR-O (lane 3) and pcDNA3_HSDL2 (lane 4); PCR with β-actin specific primers: HEK293 transiently transfected with pcDNA3_SDR-O (lane 7) or pcDNA3_HSDL2 (lane 8). (B) Expression of SDR-O and HSDL2 detected by Western blot. Cells transfected with pcDNA3_N′Flag_prRDH as positive control (lane 1), cells transfected with empty pcDNA3_N′Flag as negative control (lane 2), cells transfected with pcDNA3_N′Flag_SDR-O and pcDNA3_N′Flag_HSDL2 (lanes 3 and 4, respectively).

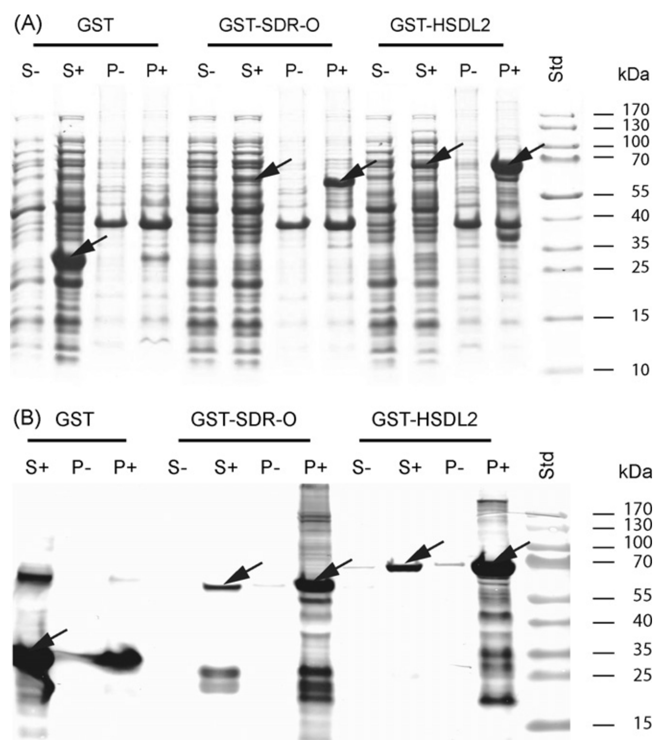

Fig. 3. Recombinant expression in *E. coli*. SDS-PAGE (A) and Western blot (B) indicate the distribution of the recombinant proteins between pellet and supernatant from lysed bacteria. P: pellet, S: supernatant, +: IPTG induced, −: not induced. Arrows indicate expressed proteins.

indicated no detectable endogenous SDR-O or HSDL2 transcripts. A Western blot with Flag-tagged constructs of SDR-O and HSDL2 in transiently transfected HEK293 cells showed that not only transcripts were present but also the proteins were expressed (Fig. 2B). Human SDR-O and HSDL2 were also expressed in *E. coli* BL21 DE3 Codon Plus RP or Rosetta2 DE3 pLysS as Glutathion-S-transferase (GST) fusion proteins. Since enzyme targeting is not influenced by an N-terminal affinity-tag (see Section 3.3), the GST-tag was chosen to facilitate solubility and affinity purification. SDS-PAGE showed that both GST-fused enzymes were readily expressed. Lysis of bacteria and subsequent analysis of supernatant and pellet fractions revealed that GST-SDR-O was nearly insoluble while GST-HSDL2 was partially soluble (Fig. 3). The proteins were anyhow used in activity assays, since observation of insolubility of proteins does not necessarily mean that the proteins are inactive as demonstrated in Deluca et al. who used only bacterial suspension with insoluble but highly enzymatically active 17β-HSD1 protein in activity assays [14].

3.2.2. Steroid and retinoid screening

Human SDR-O and HSDL2, expressed in HEK293 cells, were tested for enzymatic activity with several steroids and retinoid substrates (Table 2). Assays were performed in the presence of cofactors, NAD+ or NADP+ in case of oxidation, and NADH or NADPH for reduction reactions. No 3α-, 3β-, 17β-, 20α-, and 11β-HSD activity with the used steroids could be detected for both enzymes. The only activity observed in the screen was catalysis of the reduction of all-trans-retinal to all-trans-retinol by human SDR-O in the presence of NADH (Fig. 4). Only when SDR-O was expressed in the bacterial system an activity well above background conversion was detectable. We observed a rate of 16 pmol/min/mg total protein in our assay.

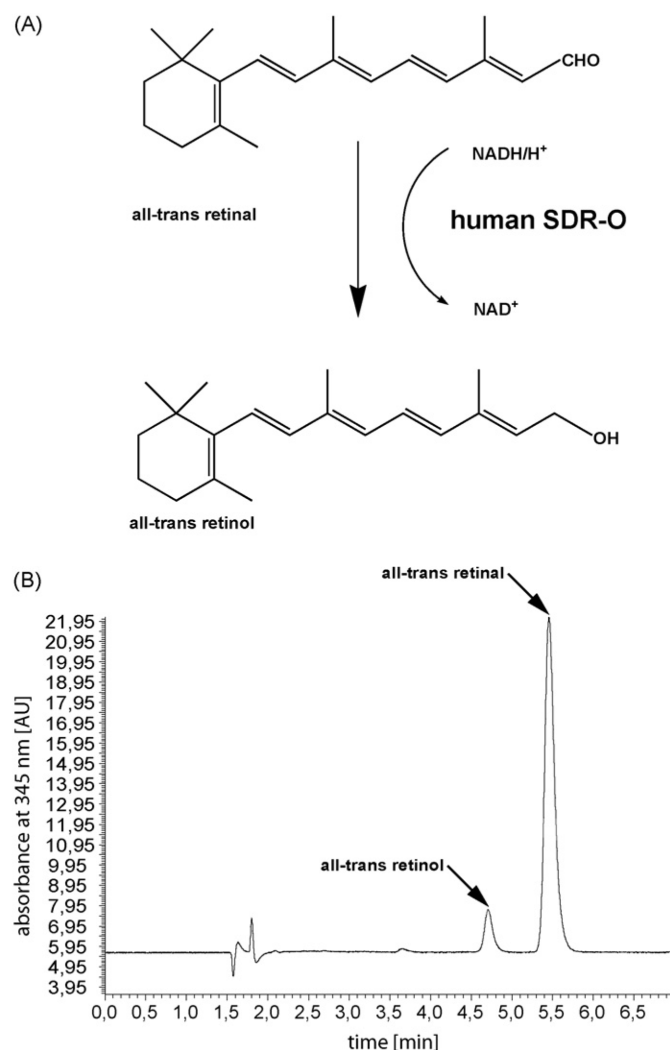

Fig. 4. Conversion of retinal to retinol by SDR-O. (A) Scheme of retinal to retinol reduction catalyzed by human SDR-O. (B) HPLC chromatogram showing the detection of all-trans-retinal reduction to all-trans-retinol catalyzed by human SDR-O.

3.3. Subcellular localization

Subcellular localization studies can give hints to the involvement of an enzyme in a certain metabolic pathway. We therefore expressed N- and C-terminally tagged SDR-O and HSDL2 in HeLa cells and looked for intracellular distribution.

In case of human SDR-O both, N- and C-terminally tagged fusion constructs, showed a similar granular pattern (Fig. 5A). Counterstaining experiments showed neither peroxisomal, early endosome, endoplasmic reticulum (ER), cytoplasmic, nuclear nor mitochondrial co-localization (not shown).

For human HSDL2 distribution is more defined. The N-tagged HSDL2 protein was found in peroxisomes as expected for the enzyme since it displays a peroxisomal targeting signal at its C-terminus (Fig. 5B). On the other hand, the C-terminally tagged HSDL2 appeared in mitochondria very likely due to masking of the peroxisomal targeting signal (Fig. 5C).

4. Discussion

In this study, we characterized the human proteins SDR-O and HSDL2, enzymes barely annotated up to now. After cloning, proteins were expressed in bacteria and human cell lines.

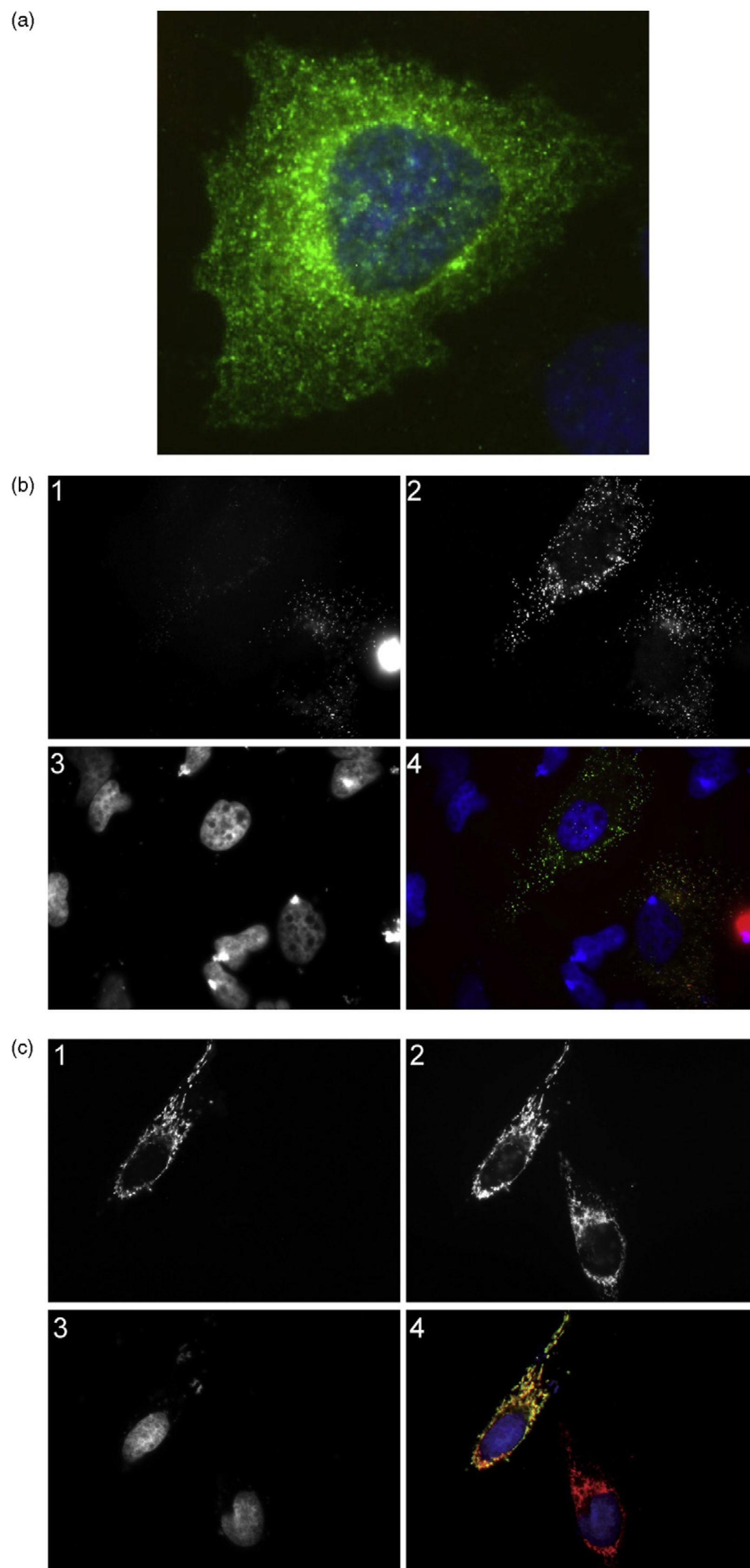

Fig. 5. Subcellular localization of SDR-O (A) and HSDL2 (B and C) in HeLa cells. (A) Representative subcellular distribution of SDR-O expressed as N- or C-tagged protein, (B) (1) HSDL2 expressed from vector pcDNA3_N′Flag_HSDL2, detected by anti-Flag primary and Alexa Flour 488 labeled secondary antibody (green); (2) peroxisomal staining by coexpression of pDsRed-Peroxi (red); (3) Hoechst staining of nuclei; (4) merge; (C) (1) HSDL2 expressed from vector pcDNA4_C′MycHis_HSDL2, detected by anti-Myc primary and Alexa Flour 488 labeled secondary antibody (green); (2) mitochondrial staining with MitoTracker Orange (red); (3) Hoechst staining of nuclei; (4) merge. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The gene for human SDR-O is localized on chromosome 12 in a cluster with RoDH4 and 3α-RoDH-like (3α-HSD/HSD17B6). In other mammalian genomes SDR-O is coded in the same position and orientation [15]. Phylogenetic analyses reveal a strong relationship between SDR-O and other human SDRs belonging to the retinol dehydrogenase like (RoDH-like) subfamily such as retinol dehydrogenase type 4 (RODH-4), RoDH-like (3α-HSD), RDHL, and 11-*cis*-RDH [15]. Similar to RoDH-like proteins human SDR-O possesses a characteristic motif (PD002736) on its C-terminus and shares all key amino acids of RoDH4 (Y238, V264, A271, P277, Y281, G284 and P298). Homology of SDR-O to retinol dehydrogenases may therefore be suggestive of a retinoid converting activity.

We analyzed the substrate specificity of human SDR-O with steroids and retinoids. No substantial 3α-, 17β-, 20α- and 11β-HSD activity was detectable with steroid substrates. On the other hand, a weak but significant conversion of all-trans-retinal to all-trans-retinol was observed for human SDR-O. Unexpectedly, this activity was detectable only when SDR-O was expressed in the bacterial system. In an earlier screen for substrate specificity of murine SDR-O, neither activity towards steroids nor towards retinoids was observed [6]. In the same report SDR-O was only expressed in mammalian cells but not in bacteria. The retinal conversion we observed in our hands may therefore be explained by the fact that much higher protein concentrations can be achieved when proteins are expressed in *E. coli* rather than in mammalian cells.

The reduction of all-trans-retinal to all-trans-retinol by SDR-O was only detectable in the presence of NADH but not with NADPH. The preference for NADH fits well with the *in silico* prediction of cofactor preference. It is notable, that enzymes preferring NAD(H) rather act as oxidases *in vivo*, while enzymes that use NADP(H) are reductases [16].

It was previously shown in our laboratory that mouse SDR-O is localized in mitochondria [17], while phylogeny suggested an ER localization [15]. The expression pattern we observed for human SDR-O was neither mitochondrial nor did it match to that of the endoplasmic reticulum (ER). Furthermore, no co-localization with peroxisomal, early endosomal or cytoplasmic markers was detectable in experiments with C- or N-terminally tagged SDR-O proteins. The distribution is rather granular and spread out over the whole cell, being denser in the perinuclear region (Fig. 4) and in vicinity of the cell membrane (not shown).

The poor catalytic activity of SDR-O in combination with its unusual cofactor preference suggests that the conversion of all-trans-retinal to all-trans-retinol is not the main function of the enzyme. Several SDR enzymes are known for their promiscuous activities [18] and the catalysis of retinoid conversion by SDR-O may represent a remnant of its retinol dehydrogenase ancestry.

Different to human SDR-O, human HSDL2 showed no catalytic activity with the tested steroid and retinoid substrates. Therefore, we were not able to experimentally confirm the predicted preference for NADP(H) and it still remains to be clarified if the enzyme is able to support catalysis. In SDR proteins, the amino acids Tyr, Lys, and a Ser located 12 amino acids upstream of the Tyr form the catalytic triad in the substrate binding site. In HSDL2 proteins, the respective Ser can be found 16 amino acids upstream of Tyr and this might influence its catalytic activity. While HSDL1 is natively inactive due to an amino acid exchange in its active site [19], in HSDL2 all SDR-specific motifs seem to be intact for proper enzymatic function.

As outlined below, human HSDL2 was found in peroxisomes or mitochondria, and from this localization, involvement in fatty acid metabolism may be postulated. Support for the hypothesis comes from the modular assembly of the enzyme, which consists of two domains, an SDR and an SCP2-like domain. Other enzymes carrying the SCP2-like domain are known to play a role in fatty acid metabolism, as e.g. 17β-HSD4 or SCPx [20]. Additionally, a recent publication reports the involvement of HSDL2 in cholesterol metabolism and homeostasis [21].

Intracellular localization studies showed mitochondrial localization for C-terminally tagged HSDL2 whereas the N-terminally tagged enzyme was found in peroxisomes. Human HSDL2 contains a peroxisomal targeting signal 1 (PTS1) at its C-terminus, which suggests peroxisomal import for the protein. Indeed, when this signal sequence is not masked, transport to peroxisomes was observed. Dai et al. reported that mouse HSDL2 N-terminally tagged with GFP localized in the cytoplasm and not in peroxisomes, as would be expected considering its peroxisomal targeting signal (RKL) [22]. Comparing amino acid sequences of human and mouse HSDL2, an additional peptide sequence of around 60 amino acids with low complexity (only containing amino acids Q, E, P, L, and K) can be found inside the mouse protein. This peptide stretch is integrated in the SCP2-like protein domain and might have an impact on the subcellular targeting process.

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