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# Characterization of human Short Chain Dehydrogenase/Reductase SDR16C family members related to Retinol Dehydrogenase 10

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## Abstract

All-*trans*-retinoic acid (RA) is a bioactive derivative of vitamin A that serves as an activating ligand for nuclear transcription factors, retinoic acid receptors. RA biosynthesis is initiated by the enzymes that oxidize retinol to retinaldehyde. It is well established that retinol dehydrogenase 10 (RDH10, SDR16C4), which belongs to the 16C family of the short chain dehydrogenase/reductase (SDR) superfamily of proteins, is the major enzyme responsible for the oxidation of retinol to retinaldehyde for RA biosynthesis during embryogenesis. However, several lines of evidence point towards the existence of additional retinol dehydrogenases that contribute to RA biosynthesis *in vivo*. In close proximity to *RDH10* gene on human chromosome 8 are located two genes that are phylogenetically related to *RDH10*. The predicted protein products of these genes, retinol dehydrogenase epidermal 2 (RDHE2, SDR16C5) and retinol dehydrogenase epidermal 2-similar (RDHE2S, SDR16C6), share 59% and 56% sequence similarity with RDH10, respectively. Previously, we showed that the single ortholog of the human RDHE2 and RDHE2S in frogs, *Xenopus laevis* rdhe2, oxidizes retinol to retinaldehyde and is essential for frog embryonic development. In this study, we explored the potential of each of the two human proteins to contribute to RA biosynthesis. The results of this study demonstrate that human RDHE2 exhibits a relatively low but reproducible activity when expressed in either HepG2 or HEK293 cells. Expression of the native RDHE2 is downregulated in the presence of elevated levels of RA. On the other hand, the protein encoded by the human *RDHE2S* gene is unstable when expressed in HEK293 cells. RDHE2S protein produced in Sf9 cells is stable but has no detectable catalytic activity towards retinol. We conclude that the human RDHE2S does not contribute to RA

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[3]*Abbreviations:* SDR, short-chain dehydrogenase/reductase; RDH, Retinol Dehydrogenase; RALDH, retinaldehyde dehydrogenase; RA, all-*trans*-retinoic acid; RAR, retinoic acid receptors; HEK 293 cells, human embryonic kidney 293 cells; CRBP1, cellular retinol binding protein 1.



*Conflict of Interest statement.* The authors declare that there are no conflicts of interest.

biosynthesis, whereas the low-activity RA-sensitive human RDHE2 may have a role in adjusting the cellular levels of RA in accord with specific physiological conditions.

**Keywords**

retinol; dehydrogenase; epidermal; human

## 1. Introduction

All-*trans*-Retinoic acid (RA)[3] is the major bioactive form of vitamin A that influences a broad spectrum of physiological processes during embryogenesis and adulthood [1-3]. Through interactions with nuclear transcription factors, retinoic acid receptors (RARs), RA regulates the expression of over 500 genes [4]. RA is synthesized from the alcohol form of vitamin A (all-*trans*-retinol) *via* a two-step process. First, all-*trans*-retinol is oxidized to all-*trans*-retinaldehyde by retinol dehydrogenases, and then all-*trans*-retinaldehyde is oxidized to RA by at least three different retinaldehyde dehydrogenases (RALDH 1-3) [reviewed in ref. 5].

Studies from several laboratories demonstrated that retinol dehydrogenase 10 (RDH10) is indispensable for RA biosynthesis during mouse embryogenesis [6-10]. Disruption of retinol oxidation through inactivation of RDH10 results in forelimb, craniofacial, neural, and heart defects, which cumulatively lead to mid-gestational lethality [6, 8]. While this phenotype indicates that RDH10 serves as the major retinol dehydrogenase during mid-embryogenesis, RDH10-null mice also provide evidence for the existence of additional sources of retinol dehydrogenase activity. Most notably, RA synthesis persists in the neural tube of these embryos at E9.5 and E10.5 [7, 10]. Furthermore, RDH10-null embryos can be rescued by supplementation of maternal diets with retinaldehyde when embryos are between developmental stages E7.5 and E9.5 [8]. In adulthood, *Rdh10* in Sertoli and/or germ cells appears to be dispensable for spermatogenesis [11]. These observations suggest that other retinol dehydrogenases besides RDH10 contribute to the oxidation of retinol to retinaldehyde for RA biosynthesis during later stages of development as well as in certain adult tissues. However, the molecular identities of these additional retinol dehydrogenases remain elusive.

RDH10 belongs to 16C family of the short-chain dehydrogenase/reductase (SDRs) superfamily of proteins [12, 13]. Notably, in human genome adjacent to the gene encoding RDH10 on chromosome 8 are located two other genes encoding members of the SDR16C family: retinol dehydrogenase epidermal 2 (RDHE2, SDR16C5) and retinol dehydrogenase epidermal 2-similar (RDHE2S, SDR16C6) [14, 15]. The deduced RDHE2 and RDHE2S proteins share the highest sequence similarity (~56-59%) with RDH10. Previously, we reported that frog rdhe2, which represents the single genomic equivalent of human RDHE2 and RDHE2S in *Xenopus laevis*, functions as a retinol dehydrogenase *in vivo* and is essential for embryonic development in frogs [14]. This finding suggests that the human orthologs of the frog rdhe2 may also have a role in RA biosynthesis in humans, except that this role has been split between two proteins, RDHE2 and RDHE2S. Two lines of evidence

support the notion that both RDHE2 and RDHE2S may complement RDH10 activity in mammals. First, our previous report demonstrated that the partially purified recombinant human RDHE2 expressed in Sf9 cells recognizes retinol as a substrate with $NAD^+$ as the preferred cofactor [16]. Second, while the human RDHE2S has never been characterized, we showed that its murine ortholog is highly active as a retinol dehydrogenase and contributes to RA biosynthesis when expressed in living human embryonic kidney (HEK) 293 cells [14]. The goal of the present study was to evaluate the properties of human RDHE2 and RDHE2S in order to assess their potential for contributing to RA biosynthesis in humans.

## 2. Materials and Methods

### 2.1. Construction of expression vectors

All primer sequences with corresponding restriction sites used for generation of constructs are listed in Table I. The construct encoding FLAG-tagged human RDHE2 in pCMV-Tag 4A vector described previously [16] was PCR-amplified and cloned into BamHI and XbaI sites of pCS105 vector for expression in mammalian cells.

The full-length sequence of the cDNA encoding human RDHE2S has not been reported. Based on the gene structure, two open reading frames encoding 316 or 323 amino acid polypeptide can be predicted for the human RDHE2S depending on which splice junction sites were used to connect putative exons 3 and 4. A cDNA encoding the shorter 316-amino acid version of predicted human RDHE2S (RDHE2Ss) was synthesized and cloned into pCR-Blunt plasmid by Retrogen (San Diego, CA). To generate FLAG-tagged human RDHE2Ss expression construct, the corresponding cDNA was cloned into EcoRI and XhoI sites of pCMV-Tag 4A vector in frame with the C-terminal FLAG tag. A cDNA encoding the longer 323-amino acid version of human RDHE2S (RDHE2Sl) was generated through mutagenesis using the FLAG-tagged RDHE2Ss in pCMV-Tag 4A vector as a template. For expression in mammalian cells, the long and short versions of FLAG-tagged RDHE2S were PCR-amplified using primers specified in Table I and cloned into EcoRI and XbaI sites of pCS105 vector. In addition, the FLAG-tagged *RDHE2S* cDNA variants were cloned into BamHI and XbaI sites of pVL1393 vector for expression in Sf9 cells.

Human CRBP1 cDNA was cloned into EcoRI and XbaI sites of pTRACER$^{TM}$-CMV2 vector (Thermo Fisher Scientific, Waltham, MA USA). Human HA-tagged RALDH1 expression construct in pcDNA3.1-neo (CMV promoter) was a generous gift of Dr. Sylvie Mader in the Department of Biochemistry, University of Montreal, Canada. All expression constructs and plasmids were verified by sequencing.

### 2.2. Cell culture models

For activity assays in whole cells, SDR expression constructs were transfected into HEK 293 or HepG2 cells using Lipofectamine (Invitrogen, Carlsbad, CA) according to the previously published protocol [17, 18]. For the analysis of retinoid metabolism by the cells, culture medium was supplemented with 10 μM all-*trans*-retinol and cells were cultured for additional 12 h. Retinoids were extracted from the cells and analyzed by normal phase HPLC as described previously [18]. All assays were performed in triplicates.

For activity assays using cellular fractions, RDHE2S short and long variants were expressed in Sf9 insect cells using pVL1393 transfer vector and BaculoGold Baculovirus Expression System (BD Biosciences, San Jose, CA) as described for other SDRs [19]. The subcellular fractions were isolated by differential centrifugation as described previously [19]. The membrane pellets were resuspended in 90 mM $KH_2PO_4$, 40 mM KCl (pH 7.4) and 20% glycerol (w/v).

The effect of retinoids on expression of SDRs was analyzed using human organotypic skin raft cultures prepared as described in [20]. Fully grown skin rafts were incubated overnight with either 2 μM all-*trans*-retinol, 0.1 μM RA, or vehicle supplied in culture medium. Each treatment group included four rafts. Gene expression in skin rafts was analyzed by qPCR [20]. The discarded unidentifiable human foreskin tissue used for isolation of keratinocytes was classified as exempt by the University of Alabama at Birmingham Institutional Review Board for Human Use.

As described previously in [17], HEK293 cells with silenced expression of *DHRS3* gene were generated using pLKO.1 vector carrying *DHRS3* shRNA. The DNA oligonucleotide sequences encoding shRNA targeting *DHRS3* were as follows: 5′-CCG GCA CCT GCA TGA ACA CTT TCA ACT CGA GTT GAA AGT GTT CAT GCA GGT GTT TTT G-3′ (forward) and 5′-AAT TCA AAA ACA CCT GCA TGA ACA CTT TCA ACT CGA GTT GAA AGT GTT CAT GCA GGT G-3′ (reverse). Stably transfected clones were selected using puromycin (3 μg/ml).

### 2.3. In vitro activity assays and immunoblotting

Activity assays using subcellular fractions of Sf9 cells were performed as described previously [18]. Briefly, 1-10 μg of mitochondrial or microsomal fractions were incubated with 5 μM all-*trans*-retinol and 1 mM $NAD^+$. Final volumes of reactions were adjusted to 500 μL with 90 mM $KH_2PO_4$, 40 mM KCl (pH 7.4) and 20% glycerol (w/v) and samples were incubated at 37° C for 15 min. Retinoids were extracted and quantified using normal phase HPLC as described in [18].

Antibodies used for Western blot analysis were as follows. Rabbit polyclonal antibodies against FLAG-epitope were from Sigma Aldrich (St. Louis, MO); against β-actin - from Abcam (Cambridge, UK); and against human DHRS3 - from Proteintech (Rosemont, IL). Rabbit polyclonal antiserum against human cellular retinol binding protein 1 (CRBP1) was custom made by Cocalico Biologicals (Reamstown, PA). To increase the antigenicity of CRBP1, the protein was expressed in *E. coli* ER2566 as a fusion to intein and chitin binding region (74 kDa protein) using pKYB1 expression vector (New England BioLabs, Ipswich, MA) [21]. The antiserum underwent affinity purification with CRBP1 immobilized on nitrocellulose. Mouse monoclonal antibody against HA-epitope was a gift from Dr. Hengbin Wang (Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham School of Medicine).

Protein samples were separated in 12% polyacrylamide gels in the presence of sodium dodecyl sulfate and transferred to Amersham Hybond P PVDF membranes (GE Healthcare, Little Chalfont, UK). Following transfer, membranes were blocked with 4% bovine serum

albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) and incubated with rabbit polyclonal or mouse monoclonal antibodies diluted with 4% BSA in TBST overnight at 4°C. Dilutions of primary antibodies are indicated in figure legends. Membranes were rinsed with TBST and incubated for 1 h at room temperature in goat anti-rabbit antibody or goat anti-mouse antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), both of which were diluted 1:10,000 with 4% BSA in TBST. Protein visualization was achieved with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

### 2.4. Northern Blot Analysis

RNA was isolated using Trizol Reagent (Invitrogen). For northern blot, 8 μg of total RNA from HEK293 cells expressing *RDHE2Ss* or *RDHE2Sl* were separated in a 1.5% agarose and formaldehyde gel and transferred to Amersham Hybond-XL membrane (GE Healthcare). Membranes were incubated with [$^{32}$P]-labeled probes synthesized using linearized *RDHE2Ss* in pCMV-Tag 4A vector and Random Primed DNA Labeling Kit (Roche, Mannheim, Germany).

### 2.5. Statistical Analysis

Statistical analysis was performed using two-tailed unpaired Student's *t* test.

## 3. Results

### 3.1. Recombinant human RDHE2 contributes to RA biosynthesis in intact cells

Our earlier study showed that human RDHE2 expressed in Sf9 insect cells as a fusion to the C-terminal $His_6$-tag and purified using $Ni^{2+}$-affinity chromatography recognized all-*trans*-retinol and all-*trans*-retinaldehyde as substrates and exhibited a strong preference for NAD(H) as cofactor [16]. However, our previous attempts to detect the activity of RDHE2 in intact cells were unsuccessful, likely due to low levels of RDHE2 expression. Here, we re-examined RDHE2 activity in intact cells using the expression vector pCS105, which produced higher amounts of RDHE2 protein. As shown in Fig. 1, FLAG-tagged RDHE2 was easily detectable by immunoblotting with FLAG antibodies when expressed in HepG2 cells (Fig. 1 A). Activity assays showed that overexpression of RDHE2 in HepG2 cells resulted in a small but reproducible increase in the conversion of retinol to retinaldehyde and further to RA as compared to the cells transfected with empty vector (Fig. 1 B). While the increase in RA alone in RDHE2-transfected cells did not quite reach statistical significance ($p$=0.06), the increase in the sum of both products produced by RDHE2-overexpressing cells from retinol (retinaldehyde plus RA) was statistically significant (2.2 ± 0.17 versus 1.67 ± 0.24 nmoles/mg, $p$=0.035).

Recently, we reported that RDH10 interacts with another member of the SDR16C superfamily, dehydrogenase/reductase 3 (DHRS3, SDR16C1), and this interaction enhances the activities of both enzymes [17]. To test whether DHRS3 interacts with RDHE2, we co-expressed the two proteins in HEK293 cells. No changes in activity of either enzyme were detected upon incubation of cells with retinol or retinaldehyde (data not shown). Thus, unlike RDH10, RDHE2 does not appear to partner with DHRS3.

Another factor that could influence the activity of RDHE2 in intact cells is the cellular retinol binding protein type 1 (CRBP1). CRBP1 promotes the uptake of retinol into cells [22]; moreover, retinol bound to CRBP1 was proposed to serve as the true substrate for retinol dehydrogenases [23]. To test whether CRBP1 enhanced the activity of human RDHE2, we co-expressed the two proteins in HEK293 cells (Fig. 2 A). As shown in Fig. 2 B, the increased level of CRBP1 had no effect on RDHE2 activity. On the other hand, co-expression with RALDH1 enhanced the conversion of retinol to RA in the cells overexpressing RDHE2 above the level observed with RALDH1 alone (Figs. 2 A and B). Collectively, these studies demonstrated that RDHE2 consistently exhibits a low but reproducible retinol dehydrogenase activity in both HepG2 and HEK293 cells.

### 3.2. Expression of native RDHE2 is downregulated by RA

Human *RDHE2* was originally identified as a transcript highly upregulated in psoriasis [24]. *Psoriasis vulgaris* is a chronic autoimmune inflammatory skin disease characterized by hyperproliferation of keratinocytes [25]. It is well established that hyperproliferation of keratinocytes can be induced by elevated levels of RA [26]. To determine whether *RDHE2* is regulated by RA in skin, we used an organotypic skin raft culture model of stratified human epidermis [20]. Gene expression and regulation in this model closely resembles that in normal human skin [27]. Remarkably, treatment of human skin rafts with 2 μM retinol or 0.1 μM RA resulted in a 4-fold and 9-fold decrease in *RDHE2* expression, respectively (Fig. 3 A). In comparison, *AKR1B10*, a cytosolic aldo keto reductase that was proposed to function as a retinaldehyde reductase [reviewed in ref. 28] was slightly upregulated.

A strong downregulation of *RDHE2* expression was also observed in another model of elevated RA levels. This model was generated by stably silencing the expression of retinaldehyde reductase *DHRS3* in HEK293 cells (Fig. 3 B, upper panel) [17]. QPCR analysis showed that *RDHE2* transcript was downregulated by 11-fold in this model of RA excess (Fig. 3 B, lower panel). In contrast, *RDH10* transcript was upregulated by 1.5-fold (Fig. 3 B). These experiments demonstrated that in human organotypic skin raft culture and in human HEK293 cells, *RDHE2* was highly sensitive to the cellular levels of RA and responded in the direction opposite to that of *RDH10*.

### 3.3. Characterization of predicted human RDHE2S protein

The gene encoding RDHE2S protein is conserved in both human and mouse genome [14]. Previously, we reported that the murine RDHE2S exhibits a retinol dehydrogenase activity and contributes to RA biosynthesis when expressed in living HEK293 cells [14]. In this study, we examined whether the high retinol dehydrogenase activity associated with the murine RDHE2S enzyme is conserved by its human ortholog.

Thus far, no transcripts encoding the full-length human RDHE2S have been deposited in the GenBank Expressed Sequence Tags database. However, analysis of *RDHE2S* gene structure shows that it encodes an open reading frame that contains all of the components essential for enzymatic activity, such as the residues characteristic of SDR catalytic and cofactor binding sites (Fig. 4). Two different open reading frames can be predicted based on the structure of *RDHE2S* gene, depending on which donor splice junction site is utilized to connect putative

exons 3 and 4. One open reading frame encodes a 323-amino acid protein, as deduced from NCBI sequence XR_171732.1, and contains a 7-amino acid insertion (EYFVSPY) in front of the conserved catalytic Tyr not found in RDHE2S from mouse, rat or cow (Fig. 4). Alternatively, a shorter 316-amino acid protein may be generated if splicing occurs at the same junctions as in mouse, rat, and bovine cDNAs. Since no ESTs are available to support either variant of the cDNA, we generated two *RDHE2S* cDNAs, one encoding the 316-amino acid protein, referred to herein as hRDHE2Ss, and the other encoding the longer 323-amino acid protein, RDHE2Sl. *RDHE2S*s cDNA was synthesized by Retrogen, and was then used as a template for site-directed mutagenesis to generate the longer *RDHE2S*l cDNA.

When expressed in HEK293 cells, the shorter RDHE2Ss protein was observed at very low levels (Fig. 5 A), whereas the RDHE2Sl version of the protein was undetectable (data not shown). This could be due to either low levels of the encoding mRNAs or instability of the proteins. Northern blot analysis showed that the expression constructs encoding the short and long variants of RDHE2S both produced abundant mRNAs (Fig. 5 B). Thus, the low levels of RDHE2S variants appeared to be due to instability of these proteins in human cells.

To circumvent this problem, we expressed the RDHE2Ss and RDHE2Sl variants in Sf9 cells using the baculovirus expression system. Interestingly, both proteins proved stable in insect cells. Subcellular fractionation of Sf9 cells showed that the two variants were associated with mitochondrial (10,000*g* pellet) and microsomal membranes (105,000*g* pellet) (Fig. 5 C). The availability of stable proteins allowed us to utilize the corresponding subcellular fractions for assays of enzymatic activity. These assays revealed that neither mitochondria nor microsomes containing either RDHE2Ss or RDHE2Sl exhibited a detectable activity towards retinol or retinaldehyde in the presence of NAD(H) (data not shown). Thus, unlike the murine *RDHE2S* gene, human *RDHE2S* gene did not appear to encode a catalytically active retinol dehydrogenase. This finding suggested that in humans, as in frogs, RDHE2 may fulfill the functions of both RDHE2 and RDHE2S.

## 4. Discussion

While RDH10 is the major enzyme responsible for the biosynthesis of RA during embryogenesis, it does not account for all of retinaldehyde synthesis for RA production *in vivo*. Among members of the SDR superfamily, RDH10 is most closely related to RDHE2 and RDHE2S [14, 15]. Phylogenetic analysis places RDHE2, RDHE2S, and RDH10 into the same clade within SDR16C family, separately from other members, thus confirming their common origin [15]. This observation suggests that RDHE2 and RDHE2S may represent the missing retinol dehydrogenases that support the residual RA biosynthesis during late embryogenesis and in adult tissues.

The results of this study demonstrate that RDHE2 may, in fact, serve as a functional retinol dehydrogenase in certain human tissues. Based on the GenBank EST database, transcripts encoding human *RDHE2* are found in brain, esophagus, heart, intestine, lung, lymph node, mammary gland, mouth, pancreas, pharynx, stomach, and trachea, suggesting a broad impact on human physiology. Remarkably, the expression of human *RDHE2* is very sensitive to RA levels. The strong downregulation of *RDHE2* expression in response to RA

in skin epidermis observed using the organotypic skin raft culture may have several implications. First, because *RDHE2* is suppressed by RA, the upregulation of RDHE2 in psoriasis might signal that the steady-state levels of RA in psoriatic skin are actually reduced compared to healthy skin; and hence, proliferation of keratinocytes in psoriasis is not caused by overproduction of RA, as initially suggested [24, 29]. There is a possibility that the upregulation of RDHE2 occurs in response to RA insufficiency in order to normalize RA levels in psoriatic epidermis. We speculate that human RDHE2 functions as a highly inducible low-activity retinol dehydrogenase, which provides the means for adjusting the rate of RA production depending on physiological status of the tissue.

A surprising finding of this study is that human *RDHE2S* gene does not encode a functional protein, despite the full conservation of the primary structure and catalytic residues required for activity. The two putative variants of human RDHE2S protein are correctly targeted to the microsomal and mitochondrial membranes when expressed in insect Sf9 cells and both protein variants are stable in these cells. Nevertheless, neither variant exhibits any detectable activity towards retinol. Taking into account that no ESTs encoding human RDHE2S have been reported thus far, and that neither version of the protein is stable in HEK293 cells, it appears that the human ortholog of murine *Rdhe2s* gene lost its functional significance and represents a pseudogene, and that in humans, as in frogs, a single RDHE2 enzyme fulfills the functions of both enzymes.

Interestingly, genome-wide association studies have linked the chromosomal region harboring *RDHE2* (SDR16C5) and seven other genes to stature and growth in cattle, humans, and pigs [30-37], and beak deformity in chickens [38]. The most recent study specifically identified *RDHE2* as the important candidate gene in pig growth trait by an integrative genomic approach [39]. The results of our study suggest that this phenotype may be related to its activity as a retinol dehydrogenase.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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- The role of proteins RDHE2 and RDHE2S in retinoic acid synthesis was characterized.
- RDHE2 encoded by SDR16C5 gene functions as a low activity retinol dehydrogenase.
- Expression of endogenous RDHE2 is downregulated by treatment with retinoic acid.
- RDHE2S encoded by SDR16C6 gene is unstable and inactive towards retinol.
- In humans, RDHE2S is a pseudogene, but RDHE2 is a functional retinol dehydrogenase.

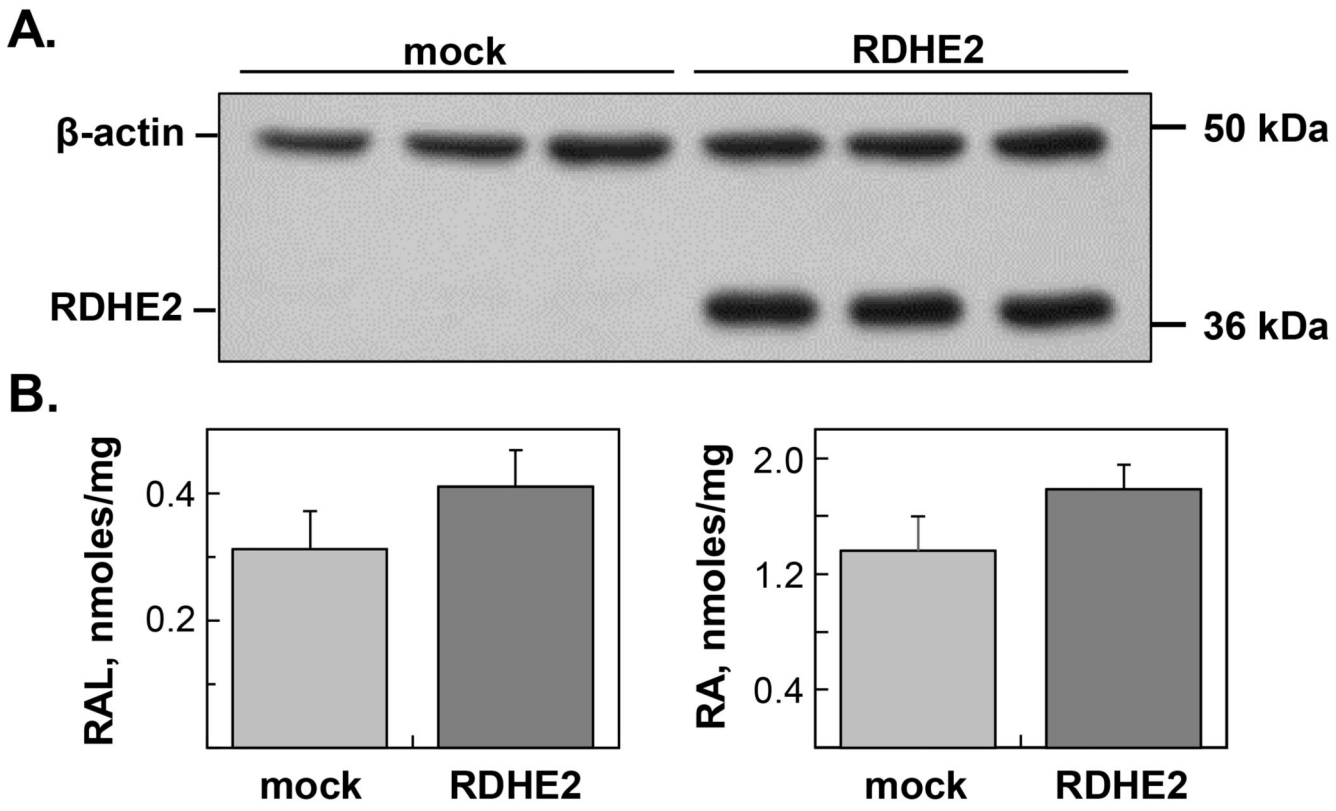


**Figure 1. Overexpression and activity of human RDHE2 in HepG2 cells**

(A) Expression of FLAG-tagged RDHE2 in HepG2 cell lysates (20 μg, $n$=3) was analyzed by western blotting using ANTI-FLAG antibody at a 1:3,000 dilution. β-Actin served as a loading control (1:2,000 dilution of antibodies). (B) Retinaldehyde (RAL) and RA production from retinol (10 μM) was quantified by HPLC. Mock, cells transfected with empty vector.

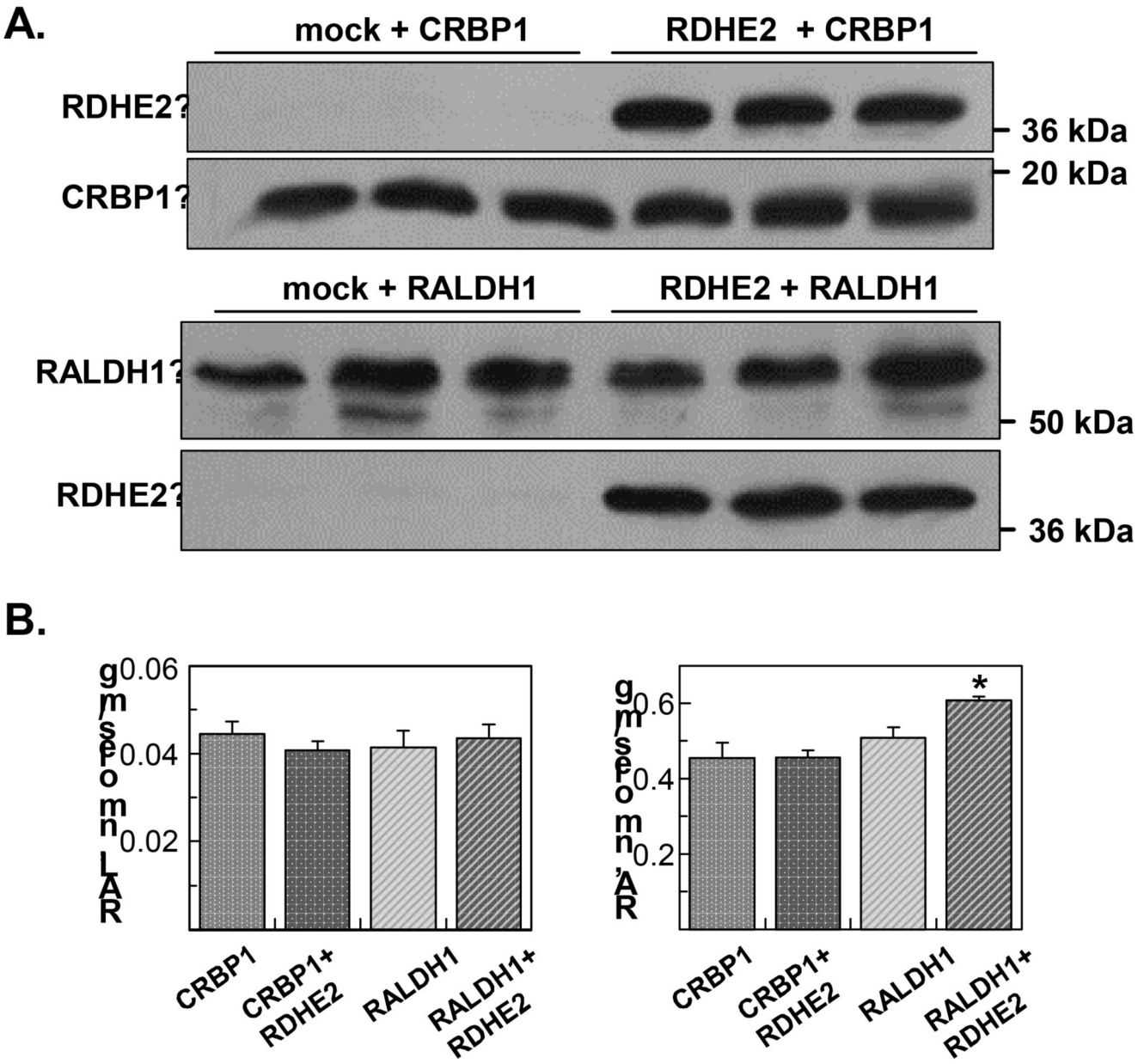


**Figure 2. Activity of human RDHE2 co-expressed in HEK293 cells with CRBP1 or RALDH1**

(A) Expression of FLAG-tagged RDHE2 in HEK 293 cell lysates (20 μg, $n$=3) was analyzed by western blotting as described in Fig. 1. Control cells were transfected with empty vector. CRBP1 (*upper panel*) was detected using 1:3,000 dilution of custom made CRBP1 antiserum; HA-tagged RALDH1 (*lower panel*) was detected using HA antibodies at a 1:50 dilution. The consistency in staining for CRBP1 ($n$=6) and HA-tag ($n$=6) confirms reproducibility of transfection efficiency and loading of samples. (B) Retinaldehyde (RAL) and RA production from retinol (10 μM) was quantified by HPLC; *$p$<0.05, mean ± S.D., $n$=3.

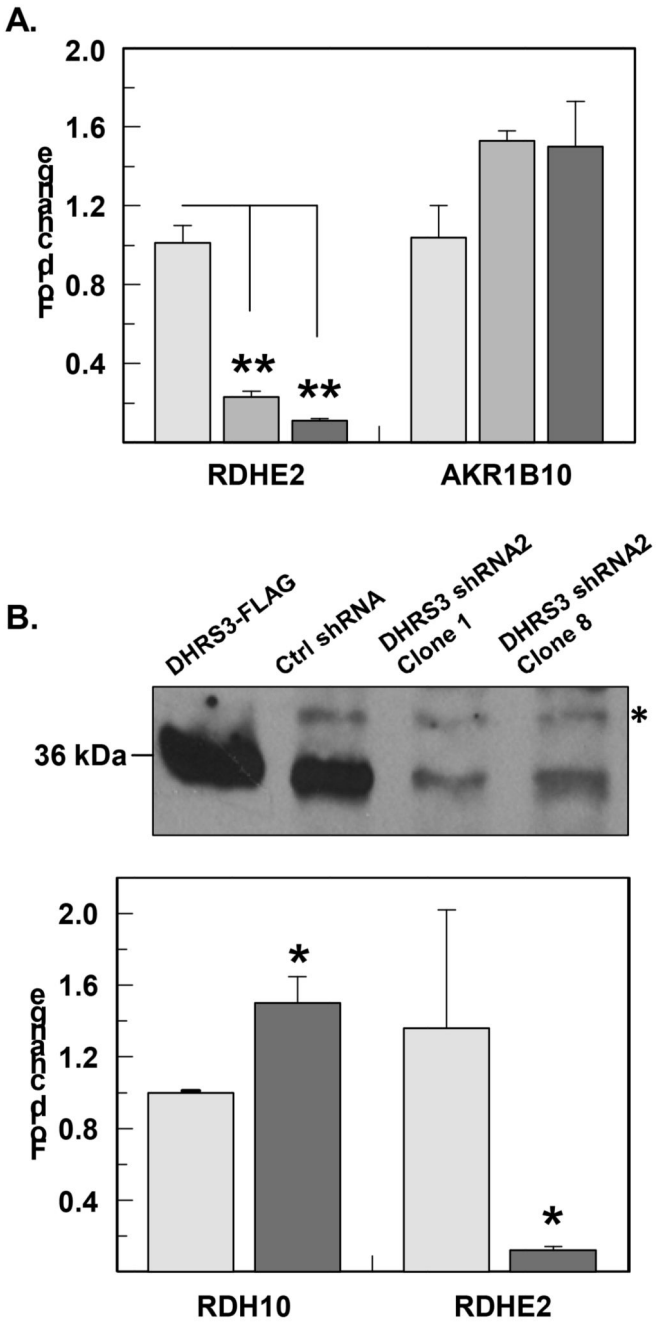


**Figure 3. Downregulation of RDHE2 expression by RA**

(A) Three individual rafts were used for treatment with vehicle (light gray), 2 μM retinol (medium gray), or 0.1 μM RA (dark gray). QPCR analysis of RDHE2 (RDHE2) and aldo keto reductase 1B10 (AKR1B10) expression was done in triplicates for each raft. Data were normalized per peptidylprolyl isomerase A (PPIA, cyclophilin A) and represent mean ± S.E.M.; $^{**}p<0.002$, $n=3$. (B) *Upper panel*, Western blot analysis of DHRS3 expression in HEK293 cells (50 μg) stably transfected with scrambled shRNA (Ctrl) or DHRS3 shRNA2 (clones 1 and 8). Note the reduced levels of DHRS3 protein in two separate clones

transfected with shRNA2. DHRS3-FLAG protein produced in Sf9 cells was loaded as a positive control (5 μg). Asterisk (*) indicates a non-specific protein band recognized by rabbit polyclonal antibodies against human DHRS3 that serves as a loading control. *Lower panel*, QPCR analysis of RDH10 and RDHE2 expression levels in HEK293 cells stably transfected with DHRS3 shRNA2 (dark gray) or 10 scrambled shRNA (light gray). QPCR was performed as in A; *$p<0.03$, $n=3$.


**Figure 4. Alignment of deduced human RDHE2S protein variants with known sequences of RDHE2S and RDHE2 proteins**

Two forms of *RDHE2S* cDNA were predicted based on alternative exon/intron splice junctions. The first cDNA encodes a 323-amino acid protein (human RDHE2Sl). The second cDNA encodes a 316-amino acid protein (RDHE2Ss). Sequences of known proteins were 11 obtained from NCBI. Accession numbers are as follows: mouse RDHE2S, NP_001074179.1; rat RDHE2S, NP_001102826.1; bovine RDHE2S, NP_001093177.1; human RDHE2, NP_620419.2; mouse RDHE2, NP_871789.1. Alignment was created using Clustal Omega. Conserved residues required for cofactor binding (GxxxGxG) and catalytic activity (YxxxK) are highlighted.

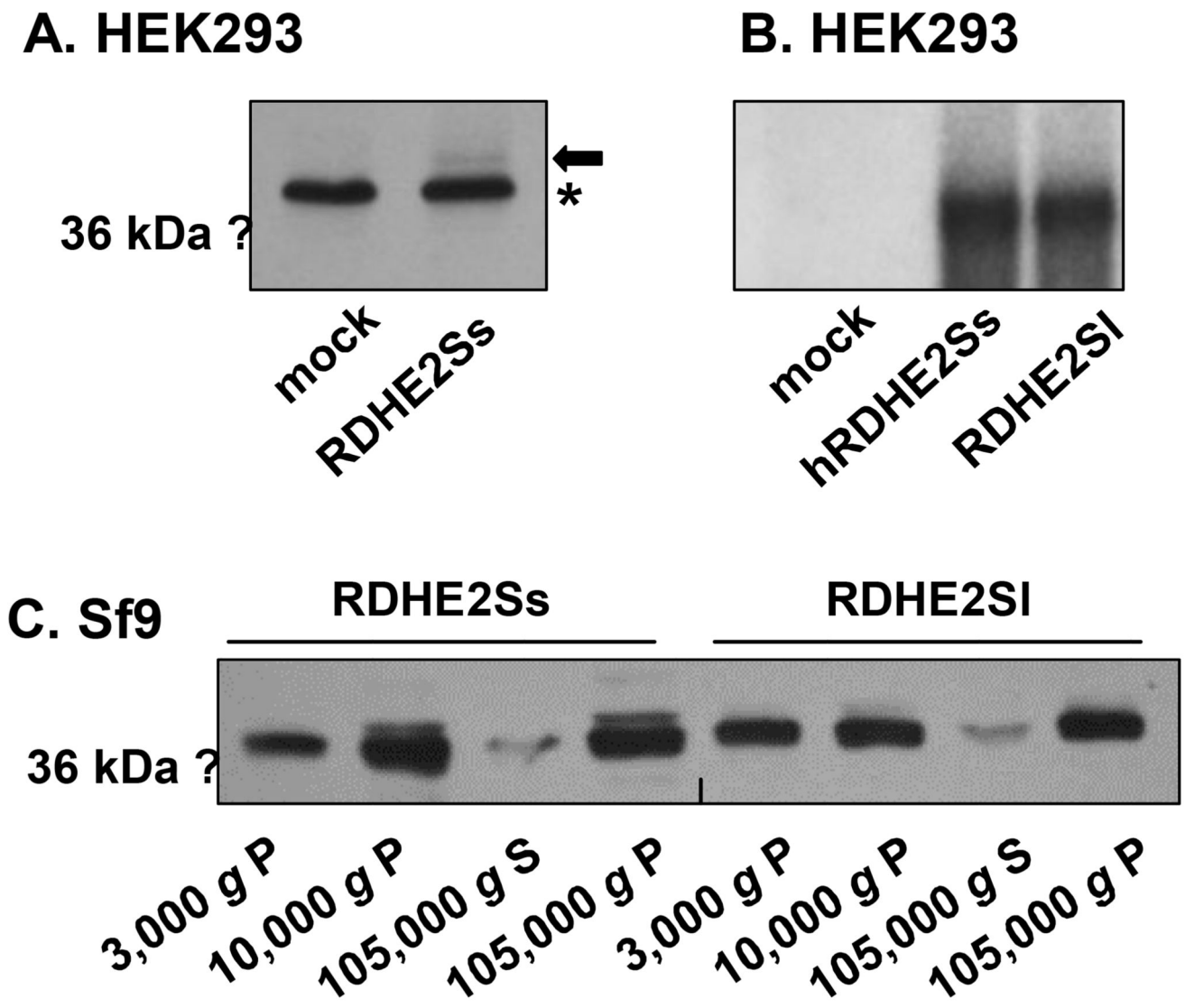


**Figure 5. Expression and characterization of human RDHE2S variants**

(A) Western blot analysis of human RDHE2Ss in HEK 293 cell lysates (25 μg). The corresponding protein was detected using FLAG antibodies at a 1:3,000 dilution and is indicated by an arrow; *non-specific band serves as a loading control. (B) Northern blot analysis of mRNA levels produced by expression vectors for the short (RDHE2Ss) and long (RDHE2Sl) forms of human RDHE2S; mock, HEK293 cells transfected with empty vector. (C) Western blot analysis of subcellular fractions isolated from Sf9 cells expressing FLAG-tagged RDHE2Ss and RDHE2Sl. P, pellet; S, supernatant. For 3,000*g* P, 10,000*g* P, and 105,000*g* S, 40 μg of protein was loaded. For 105,000*g* P, 10 μg of protein was loaded. The data are representative of at least 3 independent experiments.

**Table 1**

**Primers used for preparation of expression constructs**

| Construct | Primer name | Sequence |
|---|---|---|
| hRDHE2Ss-FLAG/pCS105; hRDHE2Sl-FLAG/pCS105; hRDHE2-FLAG/pCS105 | FLAG_XbaI_R | 5′-TACTCTAGACTACTTATCGTCGTCATC-3′ |
| hRDHE2-FLAG/pCS105 | hE2_BamHI_F | 5′-CTAGGATCCATGTCTTTCAACCTGCAATCAT-3′ |
| hRDHE2Ss-FLAG/pCMV-Tag4A; hRDHE2Ss-FLAG/pCS105; hRDHE2Sl-FLAG/pCS105 | hE2S_EcoRI_F | 5′-AATGAATTCATGGATGTGATTGCAGA-3′ |
| hRDHE2Ss-FLAG/pCMV-Tag4A | hE2S_XhoI_R | 5′-AATCTCGAGCTGGTGTTTCCCTTCAG-3′ |
| hRDHE2Ss-FLAG/pCMV-Tag4A | 21bp_insert_F | 5′-TTCACCATATTATTCTGCAAGTAAATTTGCAGCCTTTG-3′ |
| hRDHE2Sl-FLAG/pCMV-Tag4A | 21bp_insert_R | 5′-ACAAAGTACTCATCTGATAGTCCATTAATACCAACTAC-3′ |