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Characterization of Key Residues and Membrane Association Domains in RDH10

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SYNOPSIS

RDH10 was originally identified from the retinal pigment epithelium (RPE) and retinal Müller cells. It has retinoid oxidoreductase activity and is thought to play a role in the retinoid visual cycle. A recent study showed that RDH10 is essential for generating retinoic acid at early embryonic stages. The present study demonstrated that wild-type (wt) RDH10 catalyzed both oxidation of all-*trans* retinol and reduction of all-*trans* retinal in a cofactor-dependent manner *in vitro*. In cultured cells, however, oxidation is the favored reaction catalyzed by RDH10. Substitution of any of the predicted key residues in the catalytic center conserved in the retinol dehydrogenase (RDH) family abolished the enzymatic activity of RDH10 without affecting its protein level. Unlike other RDH members, however, substitution of Ser197, a key residue for stabilizing the substrate, by Gly and Ala did not abolish the enzymatic activity of RDH10, whereas RDH10 mutants Ser197Cys, Ser197Thr and Ser197Val completely lost the enzymatic activity. These results suggest that the size of the residue at position 197 is critical for the activity of RDH10. Mutations of the three glycine residues (Gly43, Gly47 and Gly49) in the predicted cofactor-binding motif (Gly-X^3-Gly-X-Gly) of RDH10 abolished its enzymatic activity, suggesting that the cofactor-binding motif is essential for its activity. Deletion of the two hydrophobic domains dissociated RDH10 from the membrane and abolished its activity. These studies identified the key residues for the activity of RDH10 and will contribute to the further elucidation of mechanism of this important enzyme.

Keywords

dehydrogenase; retinoic acid; RPE; vitamin A; visual cycle

INTRODUCTION

It is well known that retinoid (vitamin A derivatives) metabolism is essential not only for normal vision but also for embryonic development and growth. A number of enzymes, retinoid-binding proteins and transporters are involved in modification, conversion and circulation of retinoids [1–3]. Vitamin A (all-*trans* retinol, atROL) is bound by retinol-binding protein (RBP) and transported from the liver to ocular tissues and non-ocular tissues. 11-*cis* retinal (11cRAL) is covalently bound to opsin molecule and served as chromophore for rod and cone visual pigments. A single photon isomerizes 11cRAL to all-*trans* retinal (atRAL), which triggers the activation of the phototransduction cascade and generates vision [4,5]. Subsequently, 11cRAL is regenerated in the retinal pigment epithelium (RPE) through the retinoid visual cycle which involves a number of enzymes and retinoid binding proteins [6,7]. An intact visual cycle is essential for normal vision [8].

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Retinoid metabolism is also essential for the generation of retinoic acid which plays an important role in regulating development and cell differentiation [1,2]. In certain tissues, atROL is first oxidized to atRAL which is then oxidized to all-*trans* retinoic acid (atRA) by a known enzyme, retinaldehyde dehydrogenase (Raldh). AtRA is known to regulate cell differentiation, development and morphogenesis through the retinoic acid receptors or retinoid X receptors [1–3].

Retinol dehydrogenases (RDH) are the enzymes to catalyze oxidation of retinol into retinal or reduction of retinal into retinol in a cofactor dependent manner in both vision and retinoid signaling. The RDH activity is observed in two distinct classes of enzymes, microsomal retinol dehydrogenases (RoDH) in the short-chain dehydrogenase/reductase (SDR) family and cytosolic alcohol dehydrogenases (ADH) in the medium-chain dehydrogenase/reductase (MDR) family [9–15]. More than 20,000 sequences (including species variants) of SDR have been deposited in the database [15]. A number of studies have been carried out to identify the functionally important residues among the highly conserved residues in the family by evaluating the impacts of mutations of the residues using site-directed mutagenesis [9,16–23] and crystal structure analyses of the SDR family members such as drosophila alcohol dehydrogenase (DADH [24]), human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD-1 [25]) and bacterial 3β/17β-HSD [26]. Most of the mutations of the conserved residues abolish their enzymatic activities [9,16–23]. These studies elucidated that the highly conserved residues in SDRs are the key residues and suggested that a triad of Ser-Tyr-Lys residues form the catalytic site, in which Tyr functions as the catalytic base, whereas Ser stabilizes the substrate, and Lys interacts with cofactor and lowers the pK_a of the Tyr-OH. The conserved Asn residue interacts with the active site Lys via a water molecule to form a tetrad Asn-Ser-Tyr-Lys [10,17,27]. Although the SDR family members share low (approximately 15–30%) sequence identities, functionally important residues, such as catalytic sites (Asn-Ser-Tyr-Lys) and cofactor-binding motifs (Gly-X^3-Gly-X-Gly), are highly conserved in the SDR family [9–11,17]. The available 3-D structures of SDRs have revealed relatively similar α/β folding patterns of a Rossmann-fold [9,10,24,25,27]. Likewise, a number of studies had been carried out to investigate cofactor specificities of the SDR family. The residues between the first β-sheet and the third α-helix of SDRs are potentially important determinants of the specificity of cofactor toward either NADP(H) or NAD(H) [9,19,24,28–34]. Further, RoDHs (membrane-bound SDR) have been shown to possess hydrophobic domains at the N-terminal and/or C-terminal regions, which may anchor the proteins into the membrane [11,35–39].

RDH10 (retinol dehydrogenase class 10) was first cloned from bovine, mouse and human RPE [40]. The amino acid sequence homologies among RDH10 from different species are exceptionally high, with a 100% identity between bovine and human RDH10 and a 99% identity between mouse and human RDH10 at the amino acid level [40]. The highly conserved sequence of RDH10 across the evolution suggests its functional significance. We also showed that RDH10 is predominantly localized in the microsomal fraction, similar to other membrane-bound RDHs [40]. A recent study showed that RDH10 is highly expressed in many other non-ocular tissues during forelimb and hindlimb differentiation, whereas other RDHs (RDH1, 5, 6, 7 and 11) have significantly lower expression than RDH10 at this stage [41]. Moreover, mice carrying a miss-sense mutation of RDH10 have an embryonic lethal phenotype, indicating an important role of RDH10 in the early embryonic development [42]. Furthermore, the lethal phenotype of this RDH10 mutant mouse can be rescued by RA treatment of the pregnant mother, suggesting that RDH10 is essential for RA generation at early stages of embryonic development [42]. Although its exact function in RA generation is uncertain, RDH10 is likely to function as the enzyme to convert atROL to atRAL which is the substrate for Raldh2 to synthesize RA [2,11]. This assumption is supported by a recent study showing that RDH10 and Raldh colocalize in tissues during embryogenesis and organ differentiation [43].

To further study the structure and function of this important enzyme, the present study has characterized the conserved key residues in the predicted catalytic center, cofactor-binding site, and membrane association domain in RDH10.

EXPERIMENTAL

Alignment of amino acid sequences, hydropathy analysis and molecular modeling of RDH10

To predict key residues for the enzymatic activities of RDH10, amino acid sequences of human, mouse and rat RDH10 were aligned with human 17-β hydroxy steroid dehydrogenase type-1 (17β-HSD-1) using "Clustal-W" program in BioEdit (Ibis Therapeutics, Carlsbad, CA). In order to define hydrophobic domains of RDH10, the primary structure of human RDH10 was analyzed using the "Kyte-Doolittle scale" program in BioEdit. Based on the known crystal structure of human 17β-HSD-1 (PDB#1EQU), a 3-D structural model of human RDH10 was constructed using "Swiss Model optimize mode". Structural analyses were carried out using SwissPdb Viewer (www.expasy.ch/spdbv) and the resulting structures were displayed by a 3-D rendering application, POV-Ray version 3.61 (http://www.povray.org/).

Construction of human RDH10 expression vector and site-directed mutagenesis

The human RDH10 cDNA was subcloned into the pGEM-T easy vector (Promega, Madison, WI). The site-directed mutants of predicted key residues in the catalytic center, N169A, N169D, S197A, S197C, S197G, S197T, S197V, Y210A, Y210F, K214A and K214R, a triple mutant (G43A+G47A+G49A) of the three glycine residues in the predicted cofactor-binding motif (Gly-X^3-Gly-X-Gly), and the deletion mutants of two potential hydrophobic domains, Δ2-23, Δ293-329 and a double deletion mutant in which both of the hydrophobic domains were deleted, were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the protocol recommended by the manufacturer. Primers used for the mutagenesis are summarized in Table 1. The full-length cDNA sequences of the mutants were confirmed from both directions using ABI-3730 DNA sequencer (Applied Biosystems, Foster City, CA) and subcloned into pcDNA6.1 vector (Invitrogen, Carlsbad, CA). As a negative control, a red fluorescence protein, RFP (dsRed, Clonetech, Mountain View, CA) was amplified with additional restriction enzyme sites (Not I in a forward primer and Hind III in a reverse primer) and subcloned into the pGEM-T easy vector. The sequence of the cloned RFP cDNA was confirmed from both directions and subcloned into pcDNA3.1(−) (Invitrogen, Carlsbad, CA). Following the sequence confirmations, the expression constructs were purified by QIAfilter Maxi Prep kit (Qiagen, Valencia, CA). Furthermore, the purified expression constructs were transiently transfected into COS-1 cells using Fugene6 transfection reagent (Roche, Indianapolis, IN). At 48 hr post transfection, cells were harvested and the gene expression was confirmed by Western blot analysis using an anti-RDH10 antibody [40].

Western blot analysis

Protein concentrations were measured by Bradford assay [44]. For Western blot analyses, proteins were resolved with SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 5% (wt/vol) non-fat dry milk in TBST (Tris-buffered saline with Tween-20) for 30 min and subsequently incubated overnight at 4°C with 1:1000 dilution of an anti-RDH10 polyclonal antibody [40]. After three washes with TBST, the membrane was incubated for 1 hr with 1:6700 dilutions of an HRP-conjugated anti-rabbit IgG antibody (Millipore, Billerica, MA) in TBST containing 1% non-fat dry milk. After four washes with TBST, the bands were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Science, Boston, MA) or Super Signal West Dura Extended Duration Substrate (Pirce, Rockford, IL). As needed, the membrane was stripped by the stripping buffer (Pirce, Rockford, IL) and re-blotted with an antibody specific for β-actin for

loading control. The bands (intensity × area) were semi-quantified by densitometry using GeneTools (SynGene, Frederick, MD), averaged from at least 3 independent experiments.

In vitro RDH activity assay

RDH activity assays were performed under dim red light. AtROL and atRAL (Sigma, St. Louis, MO) were dissolved in dimethyl formamide (DMF). For each assay, 31–125 μg of total membrane proteins of COS-1 cells expressing RDH10 and its mutants were added into 200 μL reaction buffer (100 mM sodium phosphate, pH 7.4, containing 0.5% BSA and 1 mM NADP or NADPH). The reaction was started by addition of atROL or atRAL in 2 μl of DMF. Final concentration of atROL was 0.7–11 μM and that of atRAL was 40–620 μM. After 30 min incubation, the reaction was stopped by addition of 300 μl ice-cold methanol. Retinoids were extracted and separated by normal phase HPLC as described previously [45]. The peak of each retinoid isomer was identified based on the retention time of retinoid standards. For the activity assays of the deletion mutants of RDH10, the total cell lysates were incubated with NADP and all-*trans* [11,12-^{3}H]-retinol (1 mCi/ml, 45.5 Ci/mmol, American Radiolabeled Chemical, Inc, St. Louis, MO). The oxidizing activities of RDH10 mutants were calculated from the area of the atRAL peak using Radiomatic 610TR software (Perkin Elmer, Boston, MA). Elution peaks were identified by spiking with pure retinoid standards. The enzymatic activity was calculated from the area of the generated atRAL or atROL peaks using synthetic atRAL and atROL as standards for calibration. Kinetic parameters were calculated using EnzFitter program (Biosoft, Cambridge, UK).

Sub-cellular fractionation of cultured cells

The COS-1 cells expressing wtRDH10 or the deletion mutants were harvested and washed twice with ice-cold PBS. Subsequently, cells were fractionated into cytosolic, membrane, nuclear, and cytoskeletal (containing inclusion body) fractions using FractPrep™ (BioVision, Mountain View, CA) following manufacturer's protocol. The same amount of proteins (0.5 μg for wt and 1 μg for mutants) from each fraction was resolved by 10% SDS-PAGE and analyzed by Western blot analyses using the antibody for RDH10 [40] to identify the sub-cellular localization of RDH10 or its mutants.

Determination of the favored reaction catalyzed by RDH10 in intact cells

The expression plasmids of RFP (negative control) and wtRDH10 were separately transfected into COS-1 cells. In order to eliminate the variation of transfection efficiency, the cells transfected with the same plasmid were trypsinized, pooled, seeded into the culture dishes at 18 hr post-transfection and cultured for 24 hr. Under the dim-red light, the cells were treated with 5 μM atRAL or 2 μM atROL in fresh DMEM media containing 10% FBS. The cells were harvested at 0, 1, 2, 3, 6, 12 and 24 hr of the treatment, washed twice with ice-cold PBS. The cell pellets were lysed by sonication in 300 μL of the extraction buffer containing 50% ethanol and 50 mM 3-(N-Morpholino)propanesulfonic acid (MOPS, pH. 6.0). Retinoids were extracted by 300 μL of hexane and analyzed by HPLC as described above.

RESULTS

Substitutions of conserved key residues in the predicted catalytic center and the cofactor-binding motif do not significantly alter expression levels of RDH10

Human RDH10 was aligned with other well-characterized RDHs (Fig. 1A), and potential key residues in the catalytic center were predicted as Asn169, Ser197, Tyr210 and Lys214, based on sequence homology. Similarly, the conserved cofactor-binding motif was identified as Gly43, Gly47 and Gly49. The structural model of RDH10 was predicted using homology modeling with 17β-HSD as a template. The predicted 3-D structure retains the structural

features of Rossman-fold consisting of seven parallel β-sheets surrounded by six parallel α-helixes (Fig. 1B). Conserved key residues of RDH10 for the catalytic activity and cofactor binding were predicted at positions almost identical to those in 17β-HSD (Fig. 1C). To confirm the roles of the predicted key residues, these residues were replaced with alanine or another residue with a side chain structure similar to the native residue. To analyze the impact of these point mutations on expression levels of the mutant proteins, the expression constructs of wtRDH10 and its mutants were transiently transfected into COS-1 cells, and expression levels were measured by Western blot analyses (Fig. 1D). The conserved Ser197 has been proposed to be a key residue responsible for stabilizing substrate in the catalytic center of the RDH family [10,17]. We substituted Ser197 with five different residues with different sizes of the side chain (S197A, S197C, S197G, S197T and S197V) to study the impacts of the side chain size on the enzymatic activity. None of these mutants showed significantly changes in the protein expression levels, compared to that of wtRDH10 after normalized by β-actin levels from the same blots (Fig. 1D).

The enzymatic activity of RDH10 is abolished by most mutations of the predicted key residues in the catalytic center and cofactor-binding motif

WtRDH10 and its mutants were transiently expressed in COS-1 cells. The membrane fraction was isolated to eliminate endogenous cofactors and other cytosolic dehydrogenases possibly expressed in the cell line. The membrane fractions containing similar levels of RDH10 or its mutants (Fig. 2A) were incubated with atROL and cofactor NADP, and the produced retinoids were analyzed by HPLC. The membrane containing wtRDH10 showed a substantially higher atRAL production than that of negative control (Fig. 2B–C). Most of the site-directed mutants did not show detectable enzymatic activities (except for Ser197 mutants see Supplemental Fig. 1). Surprisingly, S197G and S197A mutants retained significant enzymatic activities, although lower than that of wtRDH10, whereas substitutions of Ser197 by Cys, Thr and Val abolished the enzymatic activity of RDH10 (Fig. 2D–H). Calculated kinetic constants, K_m (μM) and V_{max} (nmol/mg protein/hr) for atROL substrate were 4.1 and 3.35 for wtRDH10, 0.18 and 0.22 for S197A and 1.1 and 0.74 for S197G mutants (Table 2). The relative activities or catalytic efficiencies (V_{max}/K_m) of S197A and S197G were 1.22 and 0.673, respectively, comparable to that of wtRDH10 (0.817), suggesting that these mutations did not result in significant changes in the RDH10 enzymatic activity.

Sequence domains responsible for membrane association of RDH10

Most of RDHs possess at least one hydrophobic domain responsible for their membrane association [11,12,35–37]. Hydropathy analysis of the RDH10 sequence identified two potential hydrophobic domains: one at the N-terminus (residues 2 to 23) and the other near the C-terminus (residues 293–329) in human RDH10 (Fig. 3A). The N-terminal domain was predicted as a transmembrane domain, while the C-terminal one may be a membrane association domain, based on their hydrophobicities (Fig. 3B). To delineate which of these two regions is responsible for the membrane association of RDH10, these two regions were separately deleted to generate mutants Δ2-23, Δ293-329, and the double mutant lacking both of these regions. The proportions of these mutants in the membrane fraction were measured by cell fractionation analysis. Different subcellular fractions were analyzed by Western blotting using an antibody specific for RDH10, and RDH10 levels were semi-quantified by densitometry (Fig. 3C–G). WtRDH10 was predominantly localized in the membrane fraction (except for those in inclusion bodies) (Fig. 3C), consistent with our previous report [40]. The amounts of protein of the single deletion mutants, Δ2-23 and Δ293-329 in the membrane fraction were significantly decreased, compared to that of wtRDH10 (Fig. 3B–D). However, in each of the single deletion mutants, there were still detectable proteins in the membrane, suggesting that deletion of one of the hydrophobic domains did not completely dissociate the

protein from the membrane (Fig. 3C, 3D). In contrast, the double deletion mutant was completely dissociated from the membrane (Fig. 3E, 3F).

Since a significant amount of Δ2-23 and double deletion mutant proteins exist in the cytosolic fraction, *in vitro* activity assays were carried out using total cell lysates. None of the deletion mutants showed any detectable enzymatic activities, whereas wtRDH10 exhibited significant oxidation of atROL to atRAL (Fig. 3H–L).

In vitro measurement of the atRAL-reducing activity of wtRDH10 and its affinity for nicotinamide adenine dinucleotide cofactors

We examined if wtRDH10 also catalyzes the reduction of atRAL, similar to many other RDHs. The membrane fraction of COS-1 cells expressing wtRDH10 was incubated with atRAL and NADPH. The membrane fraction from the cells expressing RFP did not show detectable reducing activity of atRAL, whereas a substantial amount of atROL was generated from atRAL substrate by RDH10 (Fig. 4A and B), with K_m and V_{max} for atRAL of 570 μM and 65 nmol/mg protein/hr, respectively (Table 3). This result indicates that RDH10 is an oxidoreductase, similar to other RDHs and ADHs. Moreover, we performed another set of activity assays with various concentrations of potential cofactors, NADP, NADPH, NAD and NADH, to calculate their affinities (K_m) to wtRDH10. The calculated K_m were 27 ± 1, 15 ± 2, 36 ± 4 and 110 ± 30 μM for cofactors NADP, NADPH, NAD and NADH, respectively (see Table 3).

Determination of the favored reaction catalyzed by RDH10 in intact cells

Although the *in vitro* assays showed that RDH10 catalyzed both oxidation of atROL and reduction of atRAL (Fig. 2 and Fig. 4), it is unclear which is the favored reaction catalyzed by RDH10 under physiological conditions. To address this question, we performed further analysis to determine the reaction direction catalyzed by RDH10 in intact cells. The COS-1 cells expressing wtRDH10 and those expressing RFP (negative control) were incubated with atROL or atRAL separately and harvested at various time points. The retinoids generated in the treated cells were analyzed by HPLC. As shown in Figure 5A, both of the cells expressing RDH10 and the negative control cells expressing RFP generated substantial amounts of atROL from the atRAL substrate, suggesting existence of endogenous reductases. Actually, the cells expressing RDH10 showed a slightly lower activity in reducing atRAL into atROL, compared to the negative control ($P<0.05$ at 12 and 24 hr of incubation), suggesting that expression of RDH10 did not enhance the reduction in the cells. In contrast, the ells expressing RDH10 oxidized significantly higher amounts of atROL into atRAL in an incubation time-dependent manner ($P<0.05$ at all time points), while the negative control cells showed almost no detectable oxidation activity, suggesting RDH10 prefers oxidation in intact cells as well as under *in vitro* assay condition (Fig. 5B).

DISCUSSION

RDH10 is an enzyme essential for generation of RA during embryonic development [42]. The primary structure of RDH10 is highly conserved across species, suggesting that it may have significant physiological functions [40]. However, the structure and function of this important enzyme have not been well investigated. The present study for the first time identified key residues in RDH10 essential for its enzymatic activity and sequence domains responsible for its membrane association.

RDH10 belongs to the SDR family [40,45]. The highly conserved four residues (Asn, Ser, Tyr and Lys) in the catalytic center and three Gly in the cofactor-binding motif (Gly-X^3-Gly-X-Gly) have been shown to be essential for enzymatic activities in other members of the RDH family [9,10,17]. Sequence alignment with these known enzymes predicted that Asn169,

Ser197, Tyr210 and Lys214 in RDH10 are potential key residues in the catalytic center, and that Gly43, Gly47 and Gly49 may constitute the cofactor-binding motif. To confirm the role of the potential key residues predicted based on the sequence alignment, these predicted key residues were mutated using site-directed mutagenesis. Although these mutants did not show any significant change in RDH10 protein levels, the enzymatic activity assay demonstrated that single point mutations at Asn169, Ser197, Tyr210, Lys214 and the triple Gly mutations of the cofactor-binding motif abolished the enzymatic activity of RDH10, indicating that these residues are indeed essential for its enzymatic activity, but not for the protein stability. Current findings regarding enzymatic activity of RDH10 indicate that RDH10 retains all the key residues of the catalytic center conserved in the RDH family, and these residues are directly involved in the catalytic reaction of RDH10.

Filling and coworkers proposed that Asn111 (Asn169 in RDH10) is a part of catalytic site highly conserved in SDRs, and substitution of the Asn by Leu in 3β-HSD abolished its activity [17]. Our results with the Asn169 mutants are consistent with this observation from 3β-HSD. In the 3-D model of RDH10, however, Asn169 is unlikely to have direct interactions with the other three residues in the catalytic center. The role of Asn169 in the enzymatic activity of RDH10 may be through interactions with the well-conserved Ser213 (see Fig. 1A) which could serve as a bridge to connect Asn169 to the catalytic center (Ser-Tyr-Lys).

Ser197 in RDH10 is considered important for stabilizing the substrate, based on sequence alignment with other SDR family members (Ser139 in DADH, Ser142 in 17β-HSD-1 and Ser138 in 3β-HSD) [10,17]. It was reported that mutants of DADH with Ser139 substituted by Ala and Cys showed no detectable catalytic activities [16]. Similarly, substitution of the Ser at the corresponding positions in 17β-HSD-1 and 3β-HSD by Cys, Gly and Ala abolished their catalytic activities [19,22], whereas mutant S138T in 3β-HSD showing activity similar to that of the wt enzyme [17,18]. Unlike previous studies, however, our results showed that the S197A mutant retained enzymatic activity of RDH10, while the S197T mutant completely lost its activity. This disparity suggests that RDH10 is different from 3β-HSD in the structure of the substrate-binding pocket and substrate itself. Indeed, the S139A mutation in DADH abolished its enzymatic activity, whereas the S142A mutant of 17β-HSD-1 did not completely lose its catalytic activity [22]. This result supports our hypothesis that the mutations at the corresponding position may have slightly different impacts on the catalytic activity in different enzymes. The phenomenon that substitutions of Ser197 by Ala or Thr generate different impacts on its activity suggests that the size of the side chain of the amino acid at this position is important for its enzymatic activity. Moreover, it was reported that there are three water molecules in hydrogen bond distance to the side chain of the catalytic triad; one of them, wat107, is present in accessible distance to OH group of Ser138. These water molecules are possibly significant for the proton release steps in DADH catalysis, [24]. Likewise, a water molecule present near the NH group of Ser142 in 17β-HSD-1 might play roles similar to wat107 in DADH [25]. The cavity generated by substitution of Ser197 by the residues with a smaller side chain (e.g. Gly and Ala) might be filled by an internal water molecule (wat107) or other external water molecules from the outside of protein. Consistent with the 3-D model analyses, our hypothesis is supported by the results that Ser197 can be replaced by an amino acid with a single methyl in the side chain without abolishing the activity of RDH10, while replacement of Ser197 by residues with a larger side chain, such as Cys, Thr and Val, may result in structural disturbance in the catalytic center, and thus, affect the enzymatic activity of RDH10.

A number of studies have shown that the residues between the first β-sheet and the third α-helix of SDRs are important for its cofactor specificity [9,24,28–34]. (i) Positively charged residues are present at the junction between the first β-strand (Asp7-Ala12 in DADH) and the first α-helix (Gly16-Lys27) (glycine-rich motif) and/or at the beginning of the third α-helix (Val68-Leu82) [9,24,28]. (ii) Site-directed mutants D38N and A46R in DADH increased its

cofactor specificity for NADP [29,30]. (iii) The enzymes preferring NAD(H) possess an Asp while the NADP(H)-prefering enzymes have a positive-charged residue at the end of the second β-strand (position Asp37 and Val38 in 3α,20β-hydroxysteroid dehydrogenase) [31–34]. (iv) Site-directed mutants, S12K and L36D of 17β-HSD-1 altered the cofactor specificity, as the S12K mutant increased NADP(H) preference, whereas L36D changed its cofactor preference from NADP(H) to NAD(H) [19]. Based on these criteria, RDH10 seems to be an NAD-prefering enzyme, since RDH10 does not possess positively charged residues around the glycine-rich motif, and an Asp residue presents at the corresponding position 37 (Asp61 in RDH10). However, the present study showed that there is no significant difference on the binding constant of cofactors in RDH10, whereas the binding constant of NAD(H) and NADP (H) in the other strictly cofactor-dependent RDH enzymes showed more than 1000-fold higher than that of its preferred cofactors (RalR1 (RDH11), 2700-fold [46]; RDH12, 1800-fold [47]; RDH13, 4000-fold [38]). In general, the RDHs preferring NAD(H) are most likely to catalyze oxidations *in vivo* and those preferring NADP(H) are most likely to catalyze reductions *in vivo* [12,13]. However, some microsomal retinol dehydrogenases class-1 (RoDH1) from the rat liver [48,49] and human retinol dehydrogenase in the epidermis (hRoDH-E2) [50] prefer NADP as a cofactor to catalyze oxidation, same as RDH10 [12]. Similarly, another recent study showed that amphioxus retinol dehydrogenase-2 (BfRDH2) catalyzes an NADH-dependent reduction of atRAL to atROL [39]. These studies support the contention that the cofactor preference does not necessarily constrain the direction of the reaction in RDH10 and some SDR. When the conserved three Gly in the glycine-rich motif were replaced by Ala, the triple Gly mutant completely lost its catalytic activity of RDH10, most likely due to the loss of the cofactor from the binding pocket. This indicates that the conformation of the glycine-rich motif itself is important for the cofactor binding. Substitution of Gly14 in DADH by Val virtually inactivated the enzyme, whereas replacement by Ala retained 69% of the wt DADH activity [51]. This result suggests that the structure of the glycine-rich motif is essential for its catalytic activity and any structural disturbance can alter the enzymatic activity. Our current results revealed the significance of the glycine-rich motif in the co-factor-binding site.

Another common feature of RDHs is that most of the enzymes in this family are membrane-associated proteins [11,35–37]. It was reported that RDHs are associated with the smooth endoplasmic reticulum (ER) and/or the microsomal membrane via hydrophobic anchors or transmembrane domains in the N- or C-terminus of RDHs [11,35–37]. Previously, we showed that RDH10 is predominantly expressed in the microsomal fraction of the RPE [40]. Sequence hydropathy analysis predicts two hydrophobic domains in RDH10, one at the N-terminus and the other near the C-terminus. Based on the hydropathy plot, the N-terminal hydrophobic domain might form a transmembrane domain. To identify the mechanism for the membrane association of RDH10, these hydrophobic domains were deleted, and the deletion mutants expressed in COS-1 cells. The subcellular localizations of the deletion mutants were compared with that of wtRDH10 by cell fractionation and Western blotting using an RDH10-specific antibody [40]. The subcellular fractionation showed that the deletion of a single hydrophobic domain did not completely abolish its membrane association, whereas deletion of both of these domains completely dissociated the protein from the membrane. These results suggest that both the hydrophobic domains of RDH10 contribute to its membrane-anchoring. Dissociation of RDH10 from the membrane abolishes its enzymatic activity, although the total protein levels are similar to that of wtRDH10. This result suggests that membrane association is essential for the enzymatic activity in RDH10 or for maintaining its native structure.

Similar to other RDH enzymes, our *in vitro* assay showed that RDH10 can catalyze reversible interconversion of retinoids *in vitro*, i.e. oxidation of atROL and reduction of atRAL. In our previous studies, we did not detect significant reduction of atRAL to atROL catalyzed by RDH10 [40]. The possible reason is that the concentration of the substrate used for the assay, ^{3}H-labeled atRAL, was substantially lower (0.1 μM levels) in our previous studies,

compared to its K_m which is 570 μM, calculated from the present study. The present study showed that affinity of RDH10 for atROL (K_m=4.1 μM) is 140-fold higher than its affinity for atRAL (K_m=570 μM), suggesting that RDH10 favors oxidation at lower concentration of substrate. Moreover, calculated catalytic efficiency of RDH10 for atROL is 7-fold higher than that of atRAL. This is a further support that the RDH10 preferred oxidation. This suggests that atRAL is unlikely to serve as a substrate for RDH10 *in vivo*, since its K_m is well above physiological concentrations of atRAL in tissues.

Here, we showed that the favored reaction of RDH10 were oxidation both *in vitro* and in intact cells. Oxidation of atROL in COS-1 cell expressing RDH10 was detectable and significantly higher than that of COS-1 cell expressing RFP as negative control (see Fig. 5), although the RDH10-expressing cells showed significantly lower interconversion of atRAL into atROL. It strongly suggests that RDH10 did not contribute to the reducing activity of atRAL in intact cells. Moreover, RDH10 catalyzed oxidation in this assay condition, while K_m of RDH10 for atRAL to catalyze the reduction was 114-fold higher than the treated concentration (5.0 μM). Therefore, we conclude that the favored reaction of RDH10 is oxidation rather than reduction. Based on these results, it is possible that other RDHs reduce toxic atRAL into atROL under the physiological conditions, and when necessary, atROL can be oxidized by RDH10 to generate atRAL which is further oxidized to atRA by Raldh for retinoid signaling.

Although deficiency in some RDHs, such as RDH5 or RDH12, caused certain retinal dystrophies in human [8,52,53], knockout of most RDHs did not show significant phenotypes such as developmental defects or impaired vision in mice [54–57]. This strongly suggests that these RDH members have functional overlaps and can substitute each other's functions. In contrast, RDH10 deficiency causes embryonic lethality in the mouse, suggesting that other RDH members cannot substitute the function of RDH10 during the embryonic development. An important difference between RDH10 and other RDHs is its extremely high sequence homology across evolution, which support that RDH10 is functionally more significant than other RDH enzymes. RDH10 is likely to play an essential role in producing atRAL which is the substrate for synthesis of RA at early developmental stages. The time course and regional transcriptional regulation of RDH10 expression has not been investigated. It is possible that RDH10 has an earliest expression among all of RDH enzymes and/or is expressed at the critical stages when the development and differentiation of the major organs occur.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

11cRAL, 11-*cis* retinal
ADH, alcohol dehydrogenases
atRA, all-*trans* retinoic acid
atRAL, all-*trans* retinal
atROL, all-*trans* retinol
HSD, hydroxysteroid dehydrogenase
NADH, nicotinamide adenine dinucleotide
NADPH, nicotinamide adenine dinucleotide phosphate
Raldh, retinaldehyde dehydrogenase
RDH, retinol dehydrogenase
RPE, retinal pigment epithelium
SDR, short-chain dehydrogenases/reductases

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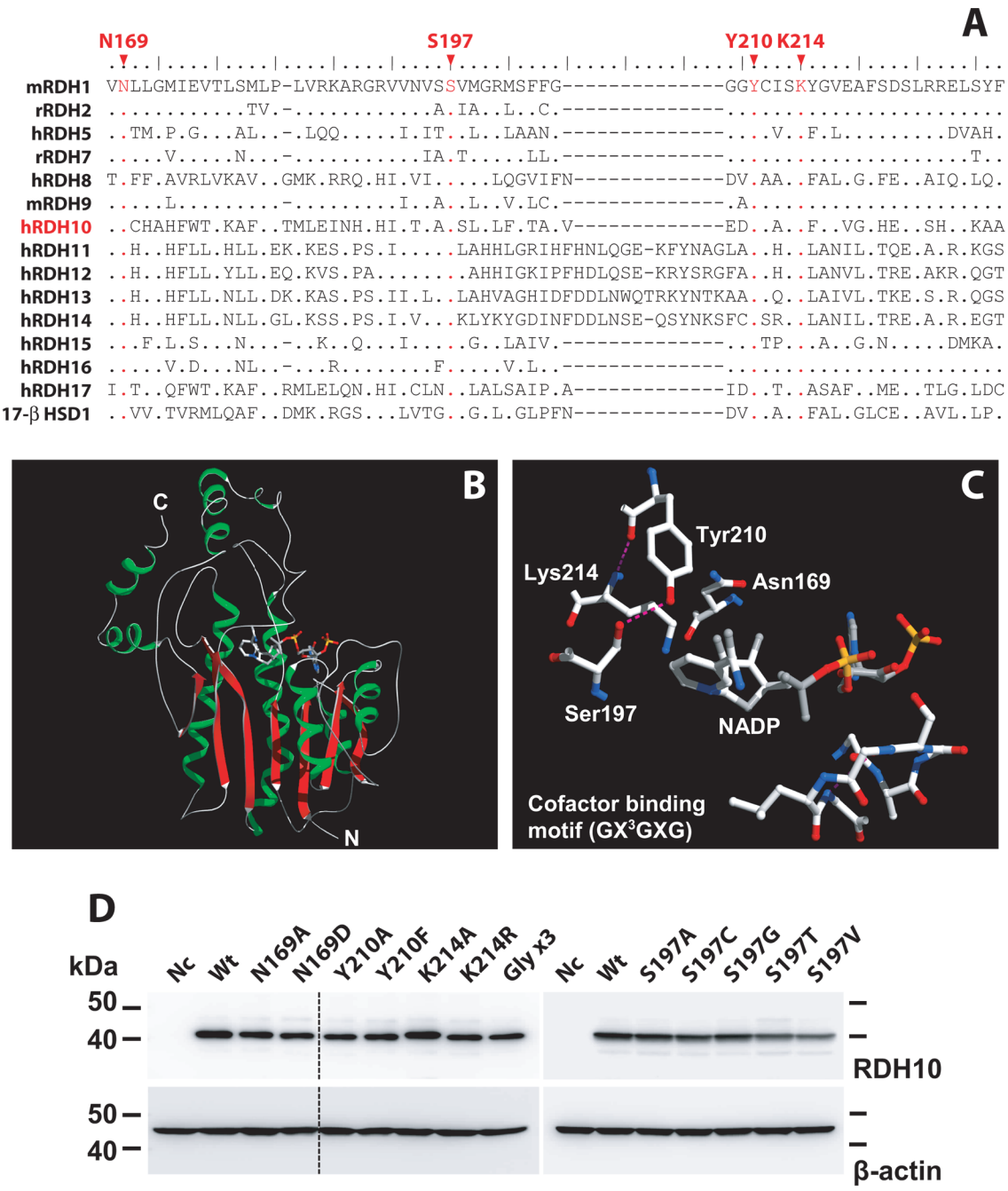

Figure 1. Structural analysis of RDH10 and impact of site-directed mutations of the predicted key residues on RDH10 levels

(**A**) Sequence alignment of the catalytic center of RDH family members and 17β-HSD1. The potential key residues (D169, S197, Y210 and K214, numbering is based on the sequence of human RDH10) in the catalytic center are completely conserved among the RDH family members. In the sequence alignment, m, r and h indicate mouse, rat and human, respectively. The protein accession numbers are mRDH1; NP_536684, rRDH2; NP_954678, hRDH5; NP_002896, rRDH7; NP_598227, hRDH8; NP_056540, mRDH9; NP_694773, hRDH10; NP_742034, hRDH11; NP_057110, hRDH12; NP_689656, hRDH13; NP_612421, hRDH14; NP_065956, hRDH15; NP_005762, hRDH16; NP_003699, hRDH17; NP_004744, 17β-

HSD1; 1EQU_A, respectively. (**B**) A structural model of RDH10 predicted based on sequence homology with human 17β-HSD1. Predicted ribbon structure forms Rosmann-fold, consisting of seven β-sheets (red colored) flanked by α-helices (green colored). (**C**) Predicted 3-D structure of the catalytic center; the perfectly conserved 4 residues (Asn169, Ser197, Tyr210 and Lys214), 3 glycine (Gly43, Gly47 and Gly49) residues in cofactor binding motif, and cofactor, NADP are shown. (**D**) The same amount of total proteins (20 μg) from cells expressing each mutant was applied. Nc, negative control (cells expressing RFP); Gly ×3, the triple Gly mutant. The membrane was blotted with a polyclonal anti-RDH10 antibody, then stripped and re-blotted with an antibody specific for β-actin for loading control.

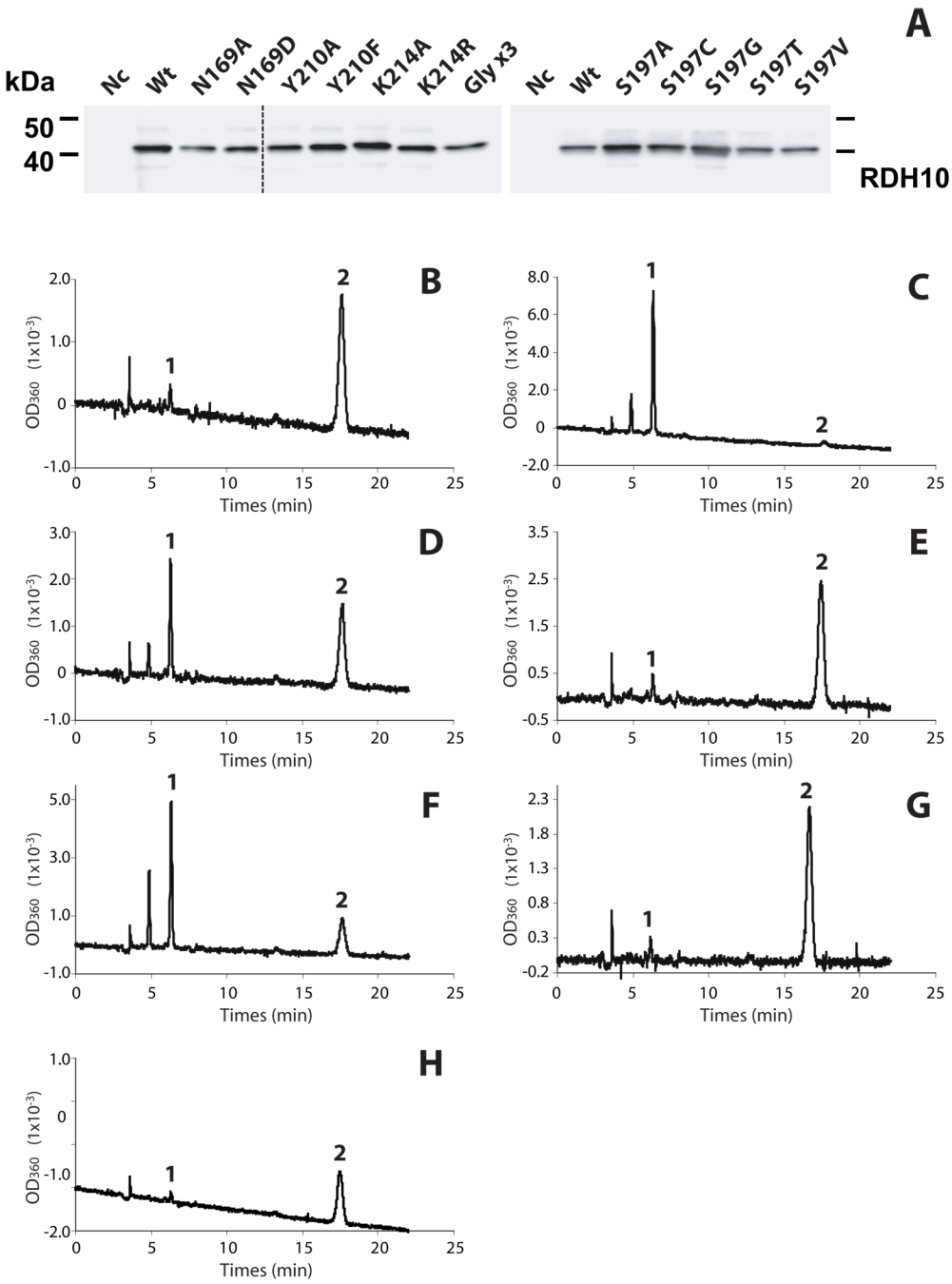

Figure 2. Impacts of site-directed mutations of the predicted key residues on RDH10 enzymatic activities

(A) The same amount of membrane proteins (5 μg) of each mutant was applied for Western blot analysis to confirm the expression of RDH10. (**B–H**) The same membrane preparations as those used in (**A**) were applied for the RDH10 activity assay. Membrane proteins (31 μg in cell expressing wtRDH10 and 125 μg in the mutants) were incubated with atROL and NADP at 37°C for 30 min. The produced retinoids were then extracted from the reactions and analyzed by HPLC. (**B–H**) Representative HPLC profile of retinoids from the negative control (**B**), wtRDH10 (**C**), S197A (**D**), S197C (**E**), S197G (**F**), S197T (**G**) and S197V (**H**). Peaks 1 and 2 indicate atRAL and atROL, respectively.

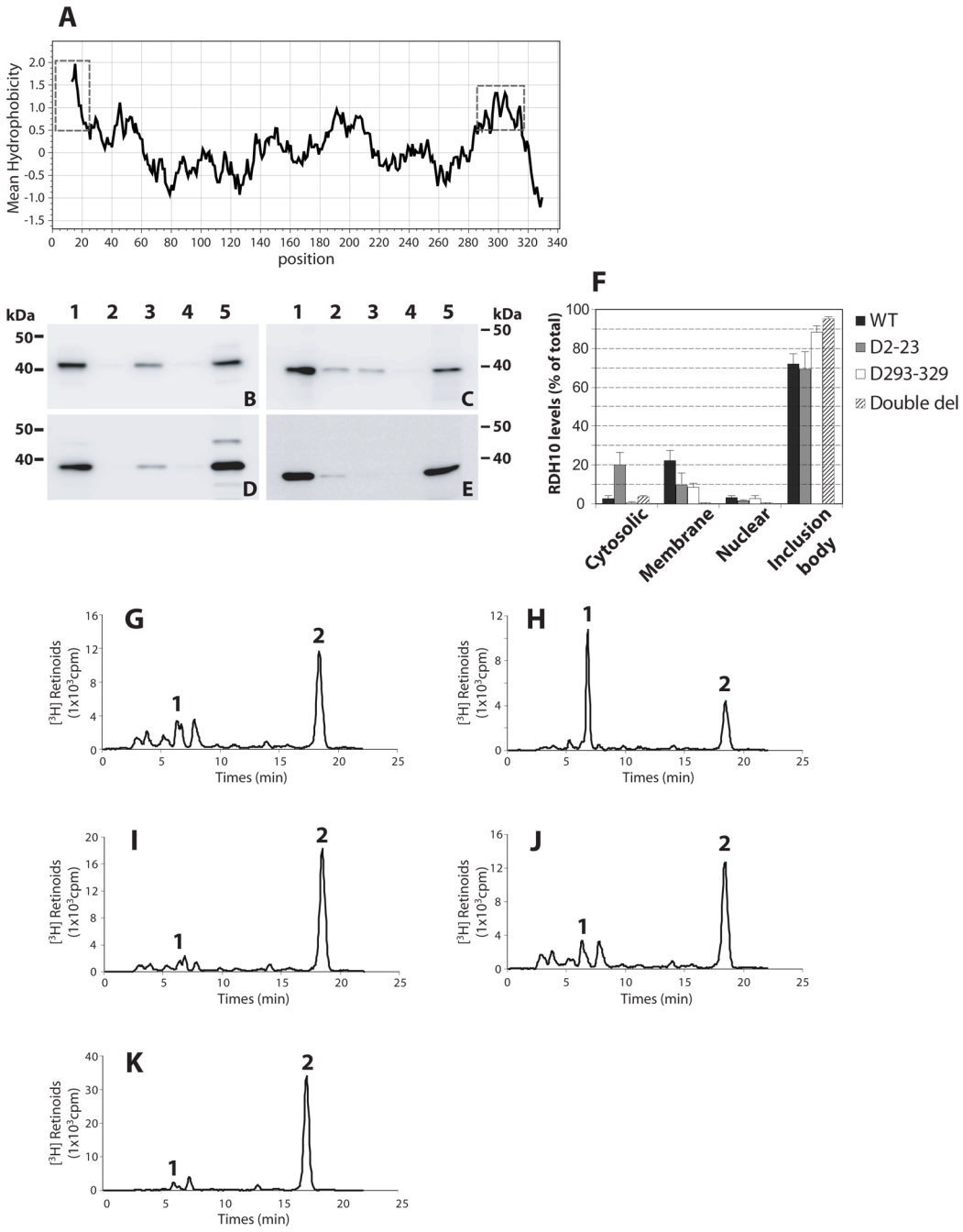

Figure 3. Enzymatic activity and membrane association of RDH10 mutants

(**A**) Hydropathy analysis using "Kyte-Doolittle scale" program predicted two hydrophobic domains (boxed with broken line). (**B–E**) Subcellular fractionation of the deletion mutants. COS-1 cells expressing RDH10 deletion mutants were fractionated, and the equal amounts of proteins (1 μg of wtRDH10 and 5 μg of mutants) from each cell lysate and the same amounts of subcellular fractions (0.5 μg of wt and 1 μg of mutants) were analyzed by Western blot analysis. (**B**) wtRDH10, (**C**) Δ2-23, (**D**) Δ293-329, (**E**) Double deletion mutant. Lane **1**, total cell lysate (before fractionation); **2**, cytosolic; **3**, membrane; **4**, Nuclear fraction, and **5**, cytosokeltal fraction containing inclusion body. (**F**) Relative RDH10 levels in each fraction were semi-quantified by densitometry, averaged from 3 independent experiments and

expressed as % of the total RDH10 level (mean ± SD, n=3). (**G–K**) *In vitro* RDH10 activity assays. ^{3}H-labeld atROL and NADP were incubated with 125 μg of total cell lysates from cells expressing (**G**) RFP, (**H**) wtRDH10, (**I**) Δ2-23, (**J**) Δ293-329 and (**K**) the double deletion mutant. Peaks 1 and 2 indicate atRAL and atROL, respectively.

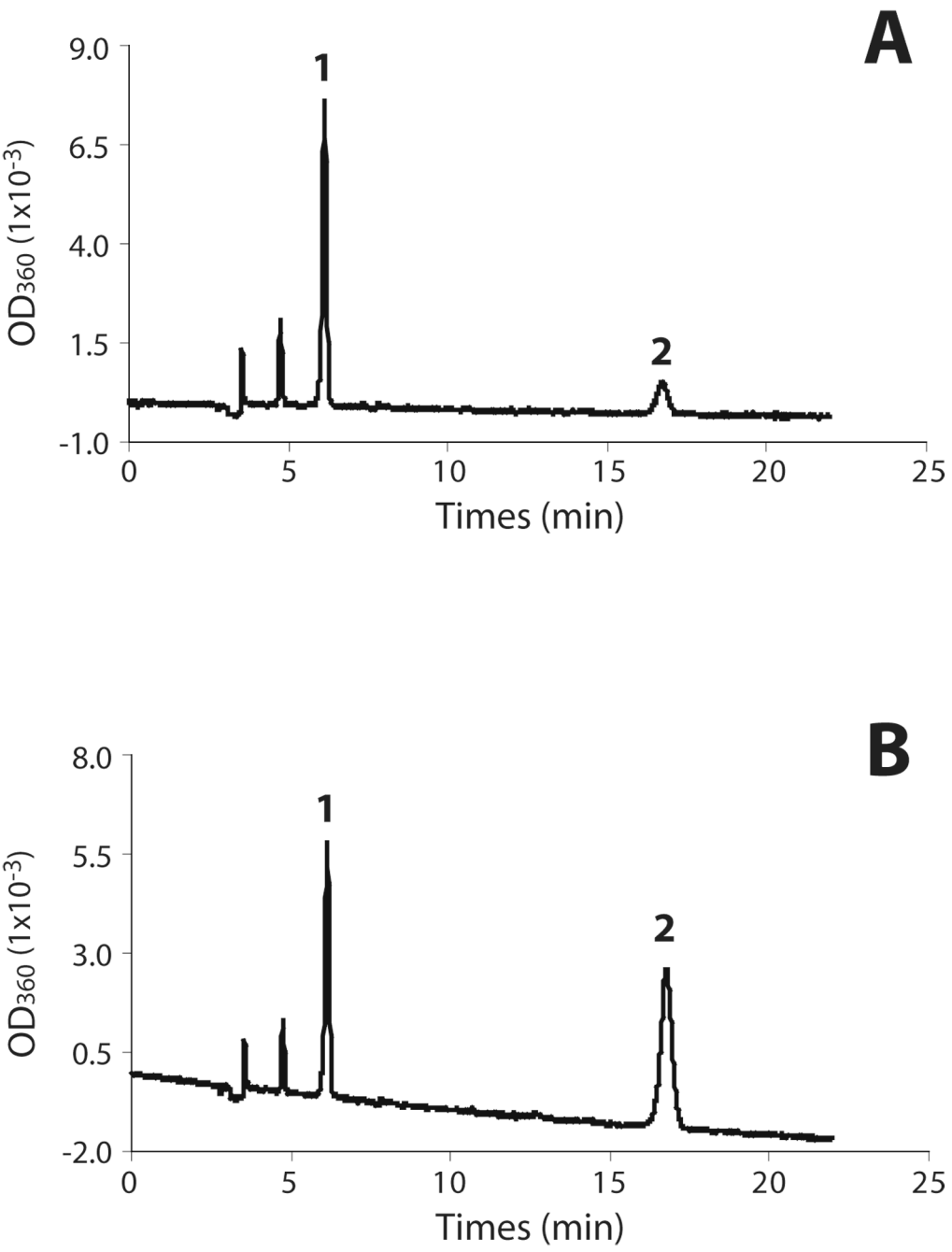

Figure 4. Reduction of atRAL to atROL by wtRDH10

The total membrane proteins (16 μg) from the cells expressing wtRDH10 were incubated with atRAL and NADPH at 37°C for 30 min. The produced retinoids were extracted and analyzed by HPLC. (**A**) Negative control cells expressing RFP; (**B**) cells expressing wtRDH10. Peaks 1 and 2 indicate atRAL and atROL, respectively.

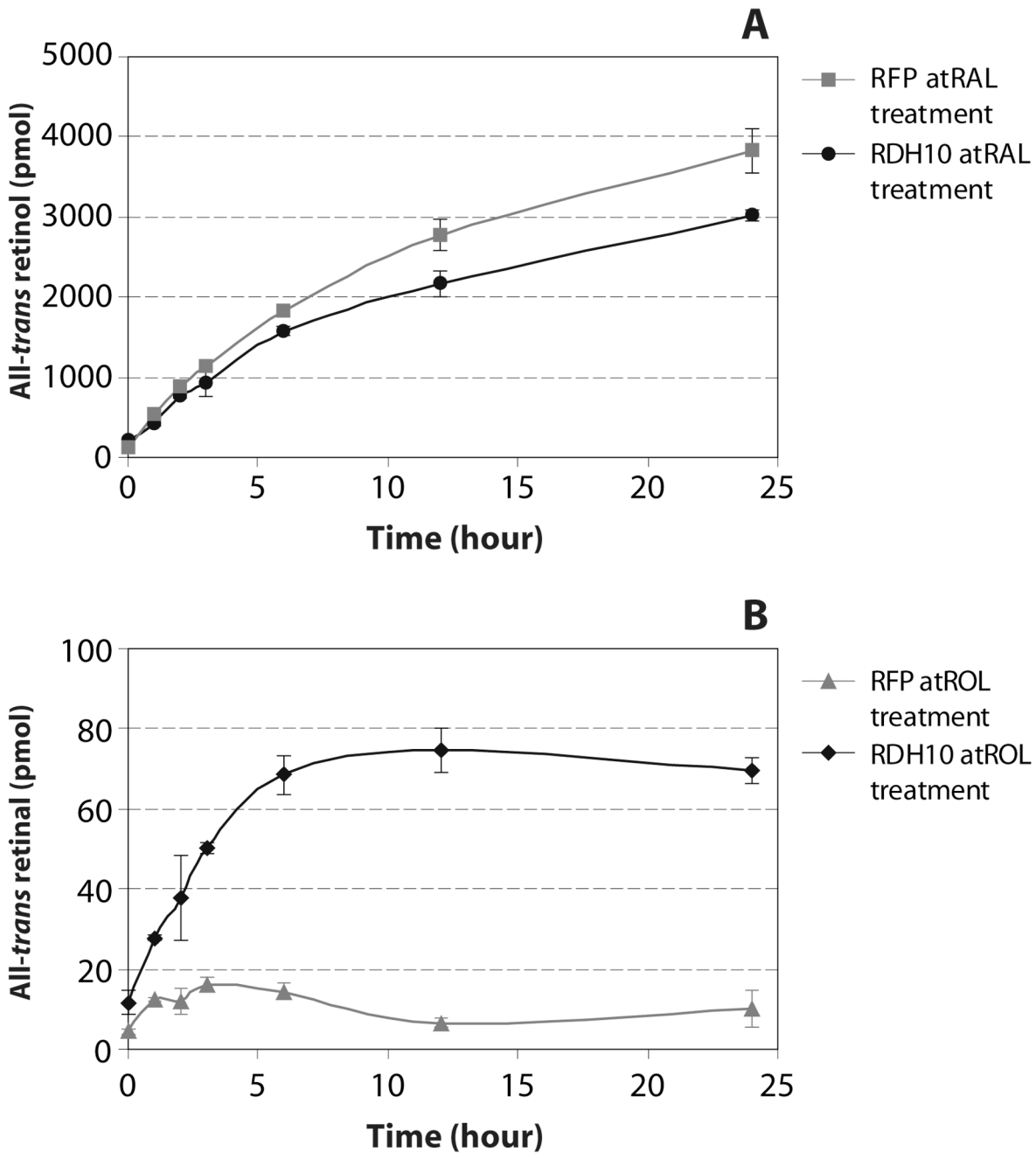

Figure 5. Enzymatic activity assay in cultured cells

COS-1 cells were transfected with plasmids expressing RFP (negative control) or with that expressing wtRDH10. The cells were incubated with 5 μM atRAL (A) or 2 μM atROL (B) for 0, 1, 2, 3, 6, 12 and 24 hr. The cells were harvested at the indicated time points, and the retinoids generated in the cells were quantified by HPLC. (**A**) Reduction of atRAL to atROL in the intact COS-1 cells. (**B**) Oxidation of atROL to atRAL in the cells. The generated retinoids in the cells were presented as pico-mole (mean±SEM, n=3).

Table 1

Primers for cloning and site-directed mutagenesis

RDH10-wt Fwd	**GAATTCGCCACCATGAACATCGTGGTGGAGTT**
RDH10-wt Rev	**GCGGCCGCTTAGATTCCATTTTTTGCTTCA**
N169A-Fwd	CCATGATGGTCGCTTGCCATGCACAC
N169A-Rev	GTGTGCATGGCAAGCGACCATCATGG
N169D-Fwd	CCATGATGGTCGATTGCCATGCACAC
N169D-Rev	GTGTGCATGGCAATCGACCATCATGG
S197A-Fwd	GGTCATATTGTGACAGTTGCAGCTTCCTTGGGATTGTTC
S197A-Rev	GAACAATCCCAAGGAAGCTGCAACTGTCACAATATGACC
S197C-Fwd	GGTCATATTGTGACAGTTGCATGTTCCTTGGGATTGTTC
S197C-Rev	GAACAATCCCAAGGAACATGCAACTGTCACAATATGACC
S197G-Fwd	GGTCATATTGTGACAGTTGCAGGTTCCTTGGGATTGTTC
S197G-Rev	GAACAATCCCAAGGAACCTGCAACTGTCACAATATGACC
S197T-Fwd	GGTCATATTGTGACAGTTGCAACTTCCTTGGGATTGTTC
S197T-Rev	GAACAATCCCAAGGAAGTTGCAACTGTCACAATATGACC
S197V-Fwd	GGTCATATTGTGACAGTTGCAGTTTCCTTGGGATTGTTC
S197V-Rev	GAACAATCCCAAGGAAACTGCAACTGTCACAATATGACC
Y210A-Fwd	GCCGGAGTTGAGGATGCCTGTGCCAGTAAATTTGG
Y210A-Rev	CCAAATTTACTGGCACAGGCATCCTCAACTCCGGC
Y210F-Fwd	GCCGGAGTTGAGGATTTCTGTGCCAGTAAATTTGG
Y210F-Rev	CCAAATTTACTGGCACAGAAATCCTCAACTCCGGC
K214A-Fwd	GGATTACTGTGCCAGTGCATTTGGAGTTGTGGGTTTTC
K214A-Rev	GAAAACCCACAACTCCAAATGCACTGGCACAGTAATCC
K214R-Fwd	GGATTACTGTGCCAGTAGATTTGGAGTTGTGGGTTTTC
K214R-Rev	GAAAACCCACAACTCCAAATCTACTGGCACAGTAATCC
G47+49A-Fwd	GCGCCGGCAGCGCCCTGGCCCGCCTCTTCGCG
G47+49A-Rev	CGCGAAGAGGCGGGCCAGGGCGCTGCCGGCGC
3×Gly-Fwd	GCCTCATCACCGCCGCCGGCAGCGCCCTG
3×Gly-Rev	CAGGGCGCTGCCGGCGGCGGTGATGAGGC
Δ2-29-Fwd	GATTGAATTCGCCACCATGCGCTGGCTGGTGCGGCCC
Δ2-29-Rev	GGGCCGCACCAGCCAGCGCATGGTGGCGAATTCAATC
Δ293-329-Fwd	GATCTGCACTCCCCGCCTCTGTATGTACCCCTTTATTGC
Δ293-329-Rev	GCAATAAAGGGGTACATACAGAGGCGGGGAGTGCAGATC

Table 2

Specific activities of wt RDH10 and mutants

Name	K_m	V_{max}	V_{max}/K_m	Name	K_m	V_{max}	V_{max}/K_m
Nc	N.D.	N.D.	N.D.	**S197T**	N.D.	N.D.	N.D.
WT	4.1 ± 0.3	3.35 ± 0.12	0.817	**S197V**	N.D.	N.D.	N.D.
D169A	N.D.	N.D.	N.D.	**Y210A**	N.D.	N.D.	N.D.
D169N	N.D.	N.D.	N.D.	**Y210F**	N.D.	N.D.	N.D.
S197A	0.18 ± 0.07	0.22 ± 0.04	1.22	**K214A**	N.D.	N.D.	N.D.
S197C	N.D.	N.D.	N.D.	**K214R**	N.D.	N.D.	N.D.
S197G	1.1 ± 0.3	0.74 ± 0.05	0.673	**Gly×3**	N.D.	N.D.	N.D.

N.D. Non Detectable, K_m (μM), V_{max} (nmol/mg/hr)

Table 3

Kinetic constants of wt RDH10

Substrate/co-factor	K_m (μM)	V_{max} (nmol/mg/hr)
All-*trans* retinol	4.1 ± 0.3	3.35 ± 0.12
All-*trans* retinal	570 ± 100	65 ± 7
NADP	27 ± 1.0	—
NADPH	15 ± 2.0	—
NAD	36 ± 4.0	—
NADH	110 ± 30	—