Biochemical Defects in 11-cis-Retinol Dehydrogenase Mutants Associated with Fundus Albipunctatus*

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Mutations in the gene encoding 11-cis-retinol dehydrogenase (RDH5; EC 1.1.1.105) are associated with fundus albipunctatus, an autosomal recessive eye disease characterized by stationary night blindness and accumulation of white spots in the retina. In addition, some mutated alleles are associated with development of cone dystrophy, especially in elderly patients. The numbers of identified RDH5 mutations linked to fundus albipunctatus have increased considerably during recent years. In this work, we have characterized the biochemical and cell biological properties of 11 mutants of RDH5 to understand the molecular pathology of the disease. All RDH5 mutants showed decreased protein stability and subcellular mislocalization and, in most cases, loss of enzymatic activity in vitro and in vivo. Surprisingly, mutant A294P displays significant enzymatic activity. Cross-linking studies and molecular modeling showed that RDH5 is dimeric, and co-expression analyses of wild-type and mutated alleles showed that the mutated enzymes, in a trans-dominant-negative manner, influenced the in vivo enzymatic properties of functional variants of the enzyme, particularly the A294P mutant. Thus, under certain conditions, nonfunctional alleles act in a dominant-negative way on functional but relatively unstable mutated alleles. However, in heterozygous individuals carrying one wild-type allele, the disease is recessive, probably due to the stability of the wild-type enzyme.

Mutations in several identified genes in the visual cycle result in hereditary forms of stationary night blindness, and there are still additional forms of stationary night blindness for which the underlying genetic defect is unknown (for a review, see Ref. 1). Fundus albipunctatus is a distinct form of stationary night blindness characterized by a delay in the regeneration of cone and rod photopigments and accumulation of white spots in the retina. The genetic basis for this rare eye disease has been assigned to mutations in the gene encoding 11-cis-retinol dehydrogenase (RDH5) (2–9). RDH5, an enzyme abundantly expressed in the retinal pigment epithelium, is a key enzyme in the oxidation of 11-cis-retinol (11cROL) into 11-cis-retinal (11cRAL), the ultimate chromophore of mammalian visual pigments (10). The first two identified mutations in RDH5, S73F and G238W, resulted in decreased protein stability and loss of enzymatic activity and provided compelling evidence that reduced formation of 11cRAL is the cause of the disease (2). In addition, 16 other mutations in the RDH5 gene have been reported to segregate with the disease, in most cases without evidence for impaired catalytic properties (summarized in Table I).

Fundus albipunctatus is a nonprogressive form of night blindness. Recently, it was reported that some patients with fundus albipunctatus, particularly the elderly, developed progressive cone dystrophy (5). However, it is not clear whether cone dystrophy is present in all patients with fundus albipunctatus, or whether it is found only in a subset of elderly patients.

In addition to metabolizing 11cROL, RDH5 also uses 9-cis-retinol (9cROL) as substrate in vitro. The substrate preferences and the extraocular tissue expression have led to the suggestion that RDH5 has dual and tissue-specific roles; in the eye, RDH5 generates 11cRAL, whereas in extracellular tissues, RDH5 may participate in the generation of 9-cis-retinoic acid (9cRA) (11–13). In this work, we describe some biochemical and cell biological properties of 11 RDH5 mutants associated with fundus albipunctatus. Using HPLC and a novel cell reporter-based assay, the catalytic activities of RDH5 mutants were explored in vitro and in vivo. Our data suggest that most RDH5 mutants are misfolded and unstable and that functional RDH5 dimers are required for efficient catalysis.

EXPERIMENTAL PROCEDURES

Construction of RDH5 Mutants—Human RDH5 cDNA (14) was subcloned into the eukaryotic expression vector pSG5 (15), and the expression vectors for mutant enzymes were generated by single-stranded mutagenesis (16, 17). Mutants S73F and G238W were generated as described previously (2). To construct the nine additional mutants, we used the following primers: 5'-GTCTTCATACACCGTGGACTCTACA- GCC (G35S), 5'-AGGAAGGCGATGTGTGTTGCTGTC (L105S), 5'-CTGACCGGACAACCTTACGCTGCTG (A429P), 5'-ACGACTACACGGCGAGGCGCTGGCCTGG (R280H), 5'-GGCGACGTCGTTCAAGGAGGTTTATC (V129A), 5'-GAGCTTAAAATCTCAGCGGATGCC (L310E).

Subconfluent COS-1 cells grown in 10-cm Petri dishes were transfected with the expression plasmids (7 μg/dish) encoding wild-type enzyme or mutant RDH5s. The cells were collected 48 h after transfection, and microsomes were prepared and resuspended in phosphate-buffered saline, as described previously (10). Protein concentrations were determined using Bradford analysis, and aliquots were stored at –80 °C. Steady-state expression levels of wild-type and mutant enzymes were detected by immunoblotting with the ECL system (Amersham Biosciences, Inc.), using a charge-coupled device camera for quantification.

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1 The abbreviations used are: RDH, retinol dehydrogenase; 9cRA, 9-cis-retinoic acid; 9cROL, 9-cis-retinol; 11cRAL, 11-cis-retinal; 11cROL, 11-cis-retinol; ER, endoplasmic reticulum; SANPAH, N-succinimidyl-6-(4-azido-2'-nitrophenylamino) hexaoxide; HPLC, high pressure liquid chromatography; SDR, short chain dehydrogenase/reductase.
TABLE I  Naturally occurring RDH5 mutations

| RDH5 mutation | Phenotype | Ref. no. |
|---------------|-----------|---------|
| G35S         | F. A.     | 5       |
| R42 (1 bp del) | F. A.    | 9       |
| S73F         | F. A.     | 7       |
| L105I        | F. A.     | 9       |
| G107R        | F. A., C. D. | 5     |
| D128N        | F. A.     | 3       |
| V132M        | F. A.     | 5       |
| R157W        | F. A.     | 7       |
| V177G        | F. A.     | 8       |
| V212 (4 bp del) | F. A. | 4       |
| G65SW        | F. A.     | 2 and 3 |
| A240 (frame shift) | F. A., C. D. | 5 |
| V264G        | F. A.     | 4       |
| C267Y        | F. A.     | 9       |
| R280H        | F. A.     | 3 and 5 |
| Y281H        | F. A., C. D. | 5     |
| A294P        | F. A.     | 5       |
| L310EV       | F. A., C. D. | 4–6  |

a F. A., fundus albipunctatus; C. D., cone dystrophy.
b T. P. Dryja, personal communication.

(LAS 1000; Fuji Film). Polyclonal rabbit anti-mouse RDH5 Iggs were generated as described previously (18).

Enzymatic Analysis in Vitro Using HPLC—Enzymatic analysis was carried out essentially as described previously using 11cRAL (50 μM) and NADH (500 μM) or 9cROL (50 μM) and NAD (500 μM) as substrates and cofactors, respectively (12). For wild-type RDH5, microsomes containing 3 μg of total protein were used. The amounts of mutant protein used were based on quantitative immunoblot analyses. Thus, to compensate for the lower expression level, the amounts used were 10 times that of wild-type for S73F, G238W, and L310EV and 5 times that of wild-type for A294P. 11cRAL and 9cROL were kind gifts of National Eye Institute (through Dr. R. Crouch, Medical University of South Carolina, Charleston, SC) and Dr. Michael Klaus (Hoffman-La Roche AG), respectively.

Enzymatic Analysis Using the in Vivo Cell Reporter System—The catalytic properties of wild-type and mutant RDHs were explored using the recently developed co-transfection assay as described previously (19), using the GAL4-retinoid X receptor reporter system in JEG-3 and 293 cells (12). Unless otherwise indicated, 100 ng of plasmids encoding wild-type or mutant forms of RDH5 and 100 ng of plasmid encoding retinaldehyde dehydrogenase were co-transfected together with the reporter plasmids. All transfections were done using calcium phosphate precipitation, and the amount of total DNA in each transfection was kept at 700 ng by adding empty CMX-PL1 plasmid. Six h after transfection, the JEG-3 or 293 cells were washed with phosphate-buffered saline and fresh medium containing 10% charcoal-stripped fetal calf serum, and 1 μM 9cROL was added. The cells were harvested after 24 h of incubation with the substrate. The cells were lysed, and the luciferase and β-galactosidase activities were measured and normalized as described previously (19).

Chemical Cross-linking of RDH5—Chemical cross-linking was performed separately using the UV-activated heterobifunctional cross-linker N-succinimidyl-4-(4′-azido-2′-nitrophenylamino) hexanoate (SANPAH; Pierce) and the homobifunctional non-UV-activated cross-linker bis(sulfosuccinimidyl) suberate (Pierce). Microsomal membrane fractions from COS-1 cells (5 μg total protein/30 μl incubation) expressing human RDH5 were incubated with 0.5 mM SANPAH in phosphate-buffered saline for 30 min at room temperature in the dark. Free reactive groups were quenched in 20 mM Tris-HCl buffer (pH 7.5) for 15 min at room temperature. Photosactivation was then performed using 366 nm UV light, for 10 min. The reactions were analyzed by SDS-PAGE and immunoblotting using the anti-RDH5 Ig followed by ECL+ detection. Similarly, recombinant microsomal mouse RDH5 protein (8 μg total protein/30 μl incubation) generated in baculovirus-infected Sf9 cells (12) was incubated with 1 mM bis(sulfosuccinimidyl) suberate in 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid/ phosphate-buffered saline for 30 min at room temperature. Reactions were quenched as described above, and aliquots of the incubations were analyzed by immunoblotting.

Immunofluorescence Staining of RDH5—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum, 1% penicillin/streptomycin, and 1% l-glutamine. The day before transfection, cells were seeded in 6-well dishes on coverslips (8 × 10^5 cells/well). Cells were transfected with 1–6 μg of plasmid DNA using LipofectAMINE PLUS (Life Technologies, Inc.). The cells were incubated for 24 h and then prepared for indirect immunofluorescence microscopy. The localization of RDH5 was determined using fluorescein isothiocyanate-conjugated anti-rabbit IgG or tetramethylrhodamine isothiocyanate-conjugated anti-goat IgG (17). The ER was visualized using an anti-calnexin antibody (Santa Cruz Biotechnology), and the Golgi complex was visualized using Texas Red-conjugated wheat germ agglutinin (Molecular Probes). Immunofluorescence was detected using a Zeiss fluorescence microscope.

Molecular Modeling of RDH5—The amino acid sequence of human RDH5 (14) was modeled into the known three-dimensional structure of 17β-hydroxysteroid dehydrogenase (Protein Data Bank code 1bhs, Ref. 21) using the program ICM, version 2.7 (Molsoft Inc., San Diego, CA). The details of the modeling procedure, including model relaxation for possible sterical overlaps of the side chains and energy minimization, were as outlined previously (22). Homologous retinol dehydrogenases were extracted from the KIND data base (23) using FASTA3 (24) and aligned using ClustalW (25).

RESULTS

Expression Levels of RDH5 Mutants in COS-1 Cells—COS-1 cells were transfected with expression plasmids encoding wild-type and 11 mutants of RDH5 (Fig. 1A), and microsomes were subsequently prepared from the cells and analyzed by immunoblotting using anti-RDH5 Ig. The expression levels of the 11 mutants varied from almost undetectable (mutants A294P, L105I, and L310EV) compared with wild-type RDH5. In mutant V212(4bp del), a premature stop codon is introduced following amino residue 245, thus generating a shorter protein.

In Vitro Enzymatic Activities of Several RDH5 Mutants—Isolated microsomes from COS-1 cells expressing wild-type RDH5 and 2 of the 11 RDH5 mutants (A294P and L310EV)
were analyzed by HPLC for the ability to reduce 11cRAL into 11cROL. We further analyzed the ability of wild-type RDH5 and four RDH5 mutants (S73F, G238W, A294P, and L310EV) to oxidize 9cROL into 9-cis-retinal. These results, combined with our previous data on the S73F and G238W mutants (2), demonstrate that the enzymatic activities of S73F, G238W, and L310EV were greatly reduced compared with the wild-type enzyme. G238W was not detected in the untreated samples. Chemical cross-linking generated a noncovalent homodimer.

To analyze whether the mutant enzymes displayed a trans-dominant-negative effect on the wild-type RDH5 and A294P mutant, increasing amounts of mutants S73F, G238W, and R280H were co-expressed with fixed amounts of wild-type RDH5 and A294P mutant. The ability to generate 9cRA from 9cROL was then monitored using the coupled enzyme/reporter system (19). The results showed that expression of all mutant enzymes reduced the activity of the wild-type enzyme in a dose-dependent manner and that substantial overexpression of the mutants was needed to reduce the activity of the wild-type enzyme to background levels. Co-expression of the wild-type enzyme and the A294P mutant also resulted in dose-dependent reduction of the reporter activity. Interestingly, co-expression of the A294P mutant with the S73F, G238W, and R280H mutants resulted in significantly reduced levels of reporter activity at the lowest expression levels of the inactive enzymes (Fig. 3C). Transfections with increasing amounts of plasmid encoding the wild-type enzyme did not suppress the reporter activity, suggesting that the overexpression per se did not inhibit the reporter activity. We conclude that the A294P mutant is exquisitely sensitive to the presence of the nonfunctional mutant enzymes in this in vivo system. On the contrary, the wild-type enzyme remains largely functional in the presence of lower levels of the mutant enzymes.

**Subcellular Localization of the Mutant RDH5 Enzymes**—The previous observations that expression of mutant proteins in the visual system may perturb the structure and function of the mutant protein-expressing cells, leading to degenerative diseases (26, 27), prompted us to explore the structure of cells expressing the RDH5 mutants and determine the subcellular localization of the mutant enzymes. The subcellular localization of the wild-type and mutant enzymes was determined using indirect immunofluorescence microscopy in transfected COS-1 cells. Wild-type RDH5 showed a staining pattern typical of the ER (Fig. 4, A and C), as demonstrated by the co-localization with the ER marker calnexin (Fig. 4, B and C). In contrast, all analyzed mutants (S73F, G238W, A294P, and L310EV) displayed a different staining pattern with a strong perinuclear localization, and the cells were generally enlarged compared with cells expressing the wild-type enzyme (Fig. 4, D, G, and J–L). Co-localization using Texas Red-conjugated wheat germ agglutinin as a marker of the Golgi complex (Fig. 4E) and...
calnexin as a marker of the ER (Fig. 4, B and H) revealed that both these markers co-localized with the mutant RDH5 enzymes in the perinuclear area (Fig. 4, F and I). These results suggest that expression of the mutant RDH5 enzymes causes the ER marker to redistribute, indicating that the ER compartment becomes perturbed compared with normal cells. A similar difference in the localization of the wild-type and mutant enzymes was also seen in transfected Chinese hamster ovary cells (data not shown).

Molecular Modeling of RDH5—To visualize the gross features of the catalytic ectodomain of RDH5, we generated a molecular model of the enzyme based on the known three-dimensional structure of 17β-hydroxysteroid dehydrogenase (21), which shows 30.5% identity to RDH5 in an optimal amino acid sequence alignment. The modeling showed that the globular domain of human RDH5 was compatible with the typical SDR fold. Consistent with the biochemical cross-linking studies (see above), a subunit interface allowing dimerization of the RDH5 monomers was present. This is further supported by the fact that most of the residues strictly conserved through the eight different retinol dehydrogenases are found at structurally important regions. These include the central β-sheet, the two long α-helices at the subunit interface, the active site triad (Y175, K179, and S163), and the TGXXGXXXG pattern close to the coenzyme fold, typical of all SDR enzymes (Fig. 5). The N-terminal membrane signal anchor and the C-terminal transmembrane-spanning region with the short cytosolic tail were excluded from the modeling because these regions are not confined to the conserved SDR domain (28).

The consequences of the mutations were examined in the RDH5 model. Most mutations were predicted to affect the folding and stability of the ectodomain in the enzyme with consequences for catalytic efficiency and expression level (the results are summarized in Table II).

DISCUSSION

Patients with fundus albipunctatus have a reduced rate of rhodopsin regeneration, which is explained by the fact that RDH5 is the key enzyme for production of 11cRAL. However, the photoreceptor cells ultimately do recover, suggesting that mutant forms of the enzyme have residual activities or that alternative enzymes or biochemical pathways may supply 11cRAL at a slower rate than normal. In fact, evidence for the existence of an alternative enzymatic pathway comes from recent studies on RDH5-deficient mice (29). These mice have a normal dark adaptation and show no signs of fundus albipunctatus, and delayed dark adaptation can be detected only at high bleaching levels. Presumably, another enzyme is responsible for the production of 11cRAL in these mice. For instance, a NADP-dependent oxidizing system present in the retinal pigment epithelium was described recently (30).
To monitor the catalytic properties of 11 RDH5 mutants, we have used both 11cRAL and 9cROL as substrates. Previous data and data presented in this work suggest that 9cROL is a good substrate for the enzyme and that it can be used to monitor the catalytic activities of the mutant RDH5 alleles in a faithful manner. Furthermore, using 9cROL as substrate, we have introduced a novel in vivo reporter assay to monitor the catalytic properties of the RDH5 mutants in transfected cells.

Of the 18 identified mutations in RDH5, 16 are located in the catalytic ectodomain inside the lumen of the ER (Fig. 1), whereas mutants A294P and L310EV are located in the C-terminal trans-membrane region. Generally, the RDH5 mutants are unstable, probably due to misfolding of the enzyme, and are expressed at lower levels than wild-type RDH5. With one exception, the catalytic properties of the mutants, which were assayed both in vitro using HPLC and in vivo using the reporter assay, are greatly impaired. The A294P mutation results in a minor decrease in the enzymatic activity of the enzyme. This mutation affects the C-terminal membrane-spanning region, possibly leading to improper membrane insertion of the enzyme because the introduced proline residue might cause a bend in the transmembrane helix (31). A possible consequence of this would be that the C-terminal tail is not properly located in the cytosol or that the transmembrane region forms a hairpin structure in the membrane, leaving the C-terminal tail in the lumen. The observation that RH5 is homodimeric and that the A294P mutant exerts a dominant-negative effect on the wild-type enzyme suggests that the subunit interactions may extend into the transmembrane segment or that the altered conformation of the C-terminal tail would affect other molecular interactions involving this region. In other studies we have found that intact C-terminal tails in RDH5 and CRAD1, a closely related retinol dehydrogenase, are necessary for proper subcellular localization and function of the enzyme in transfected cells (19). In contrast, the L310EV mutation, which also affects the C-terminal transmembrane region, gives rise to a nonfunctional enzyme. A comparison of the protein stability of L310EV and A294P shows that mutant L310EV is less stable than A294P (10% and 22%, respectively; see Table II). However, this difference in expression level is unlikely to explain the differences in catalytic properties, suggesting that the L310EV mutant is inactive due to improper folding of the ectodomain.

The association of the A294P mutant with fundus albipunctatus is surprising, given that it displays a prominent activity comparable with wild-type RDH5 in both activity assays. These results are intriguing because the mutation was identified in a disease-affected heterozygote patient carrying the A294P allele in combination with the inactive R280H mutation. The combined expression of the two mutant alleles in the cell reporter
the formation of nonfunctional dimers of the enzyme. In part, the sensitivity of the A294P mutant, compared with the wild-type enzyme, may be explained by the lower steady-state expression level of the enzyme. This suggestion is supported by the fact that all of the tested mutations affect the functional properties of the wild-type enzyme in a dominant-negative fashion. However, under physiological conditions, the dominant-negative effect of the nonfunctional mutants is not observed in heterozygous individuals due to the instability and low steady-state levels of these mutants compared with wild-type RDH5. We hypothesize that under certain very rare conditions involving catalytically active but less stable mutants of RDH5, fundus albipunctatus may display a dominant inheritance.

All tested RDH5 mutants, including A294P, showed an abnormal perinuclear localization in transfected cells and induced a redistribution of the ER marker calnexin, suggesting that the structure of the ER is perturbed. The A294P mutant remains functional in this location, indicating that the abnormal ER structure per se does not render the enzymes inactive in the transfected cells. In part, this may be due to the redistribution of ER and Golgi components into the perinuclear structure. However, the abnormal cellular structures induced by the mutants may affect the general functionality of the cells, particularly the function of the exocytic pathway. This suggests that some of the long-term effects of the mutations in RDH5 may impair the function of the retinal pigment epithelium. It remains to be shown whether the development of cone dystrophy observed in some fundus albipunctatus patients, especially among elderly, is related to such nonspecific effects on the function of the retinal pigment epithelium or whether it is a direct consequence of the decreased supply of 11-cis-retinal to the cones.

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