FEBS Letters 580 (2006) 4200–4204

Impacts of two point mutations of RPE65 from Leber's congenital amaurosis on the stability, subcellular localization and isomerohydrolase activity of RPE65

Ying Chen, Gennadiy Moiseyev, Yusuke Takahashi, Jian-xing Ma*

The University of Oklahoma Health Sciences Center, Department of Medicine Endocrinology, Department of Cell Biology, 941 Stanton L. Young Blvd, BSEB 328B, Oklahoma City, OK 73104, United States

Received 12 May 2006; revised 20 June 2006; accepted 27 June 2006

Available online 5 July 2006

Edited by Gianni Cesareni

Abstract RPE65, a membrane-associated protein in the retinal pigment epithelium, is the isomerohydrolase essential for regenerating 11-*cis* retinal, the chromophore for visual pigments. RPE65 mutations are associated with inherited retinal dystrophies. Here we report that single point mutations of RPE65, Y144D and P363T, identified in patients with Leber's congenital amaurosis (LCA), significantly decreased the stability of RPE65. Moreover, these mutations altered subcellular localization of RPE65 and abolished its isomerohydrolase activity. These observations suggest that the decreased protein stability and altered subcellular localization of RPE65 may represent a mechanism for these mutations to lead to vision loss in LCA patients.

Keywords: LRAT; Retinoid; Retinal degeneration; RPE; RPE65; Visual cycle

1. Introduction

The phototransduction pathway initiates after the light-induced isomerization of 11-*cis* retinal, the chromophore for visual pigments of both rod and cone photoreceptors, to its all-*trans* isomer [1,2]. Recycling of 11-*cis* retinal through the retinoid visual cycle is essential for the regeneration of visual pigments and for normal vision [2–5]. A key step in the visual cycle is the conversion of all-*trans* retinyl ester to 11-*cis* retinol by a single enzyme, isomerohydrolase, in the retinal pigment epithelium (RPE) [3,6]. Recent studies have indicated that RPE65 is the isomerohydrolase which produces 11-*cis* retinol from all-*trans* retinyl ester [7–9]. Its isomerohydrolase activity requires lecithin retinol acyltransferase (LRAT) in the same membrane as a functional partner to provide all-*trans* retinyl ester, the hydrophobic substrate of RPE65 [10].

*Corresponding author. Fax: +1 405 271 3973.
E-mail address: jian-xing-ma@ouhsc.edu (J. Ma).

Abbreviations: CHX, cycloheximide; LCA, Leber's congenital amaurosis; LRAT, lecithin retinol acyltransferase; MOI, multiplicity of infection; RPE, retinal pigment epithelium; ER, endoplasmic reticulum

RPE65 is an abundant membrane-associated protein in the retinal pigment epithelium (RPE), associated with endoplasmic reticulum (ER) [11]. A number of mutations in the RPE65 gene which cause truncation of the protein or amino acid substitutions have been shown to associate with inherited retinal dystrophies in patients, such as retinitis pigmentosa (RP), Leber's congenital amaurosis (LCA) and early-onset severe retinal dystrophies [12–17]. Recent studies showed that some of the RPE65 mutations in patients impair the isomerohydrolase activity of RPE65 [7,18]. However, the molecular mechanisms for these mutations, especially the mis-sense mutations, to impair its isomerohydrolase activity and consequent retinal degeneration have not been elucidated.

In the present studies, we studied the impacts of two point mutations of RPE65, Y144D and P363T, identified in patients with LCA, on the stability, subcellular localization and enzymatic activity of RPE65.

2. Methods

2.1. Site-directed mutagenesis, expression and isomerohydrolase activity assay

Point mutations Y144D and P363T were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the wild-type (wt) human RPE65 cDNA as the template and confirmed by DNA sequencing from both strands [18]. Generation of recombinant adenoviruses, measurements of the expression and isomerohydrolase activities were as described [8].

2.2. Real-time RT-PCR

The cells were infected with adenoviruses expressing wt and mutant RPE65 for 18 h; then the cells were harvested and washed with RNase-free PBS twice on ice. The RNA was isolated using RNAeasy (Qiagen, Valencia, CA) according to manufacturer's protocol. RNA was treated with RNase-free DNase I (Invitrogen, Carsbad, CA) for 15 min to remove possible contamination from the virus or genomic DNA. The treated RNA was used for reverse transcription as described previously [18]. The generated cDNA was used as the template for real-time PCR using a pair of primers specific for human RPE65 cDNA. The 18s rRNA was used as a reference for normalization of the PCR products from the RPE65 mRNA. To exclude any possible amplification from trace viral DNA, a negative control which omits reverse transcriptase in the RT reaction was used for each RT-PCR.

2.3. Sub-cellular fractionation

The infected QBI-293A cells were lysed. Total cell lysates were separated into cytosolic, membrane, nuclear, and cytoskeletal fractions with FractPrep™ (BioVision, Mountain View, CA), following

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.078

manufacture's protocol. The same amount of proteins (10 μg) from each fraction was applied to 8% gel for SDS–PAGE and Western blot analysis using an antibody specific for RPE65 [10,19].

2.4. Immunocytochemistry

The QBI-293A cells expressing wtRPE65 and its mutants were cultured on coverslip and then fixed with 4% paraformaldehyde. After blocking, the cells were incubated with a mouse anti-disulfide isomerase (an ER marker) antibody (Invitrogene, La Jolla, CA) and a rabbit anti-RPE65 antibody for 2 h. After three washes, the slides were incubated with a Texas Red-conjugated goat anti-rabbit antibody and an FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Lab. Inc., West Grove, PA) at a dilution of 1:150 for 30 min. Then, the slides were rinsed, counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield™ mounting media. The slides were viewed on a LSM 510 laser scanning confocal microscope (Carl Zeiss Meditec, Inc., Jena, Germany).

3. Results

3.1. Decreased protein levels of RPE65 mutants Y144D and P363T

Western blot analysis using the same amount of total protein from 293A-LRAT cells infected with the same titer (MOI 40) of Ad-wtRPE65, Ad-Y144D and Ad-P363T showed that both of the mutations significantly decreased RPE65 protein levels, when compared to that of wtRPE65 (Fig. 1A and B). Under the same culture condition, however, mRNA levels of these mutants had no significant difference from that of wtRPE65, as shown by quantitative real-time RT-PCR [18] (Fig. 1C), suggesting that the lower protein levels of the mutants cannot be ascribed to the decreased expression of RPE65. The RT-PCR without reverse transcriptase (negative control) did not produce any detectable PCR product, indicating that the PCR product of RPE65 was not amplified from virus DNA contamination (data not shown).

3.2. Mutations Y144D and P363T both abolish the enzymatic activity of RPE65

The enzymatic activities of mutants Y144D and P363T were compared with that of wtRPE65 expressed in 293A-LRAT cells, a stable cell line expressing LRAT, by the in vitro isomerohydrolase activity assay using all-*trans* [^{3}H] retinol as the substrate as described previously [8]. To exclude possible impacts of the decreased levels of the mutant proteins on their enzymatic activities, the mutants were over-expressed using a higher titer of the Ad-Y144D and Ad-P363T (MOI 400). As showed by Western blot analysis using the same amount of total cell lysate, protein levels of both the mutants, when expressed with MOI 400, were comparable to that of wtRPE65 expressed at MOI 20 (Fig. 2A). At the comparable protein levels, however, mutants Y144D and P363T did not generate any detectable 11-*cis* [^{3}H] retinol in 293A-LRAT cells although all-*trans* retinyl ester was produced by LRAT co-expressed in the cells (Fig. 2C and D). At a similar expression level, wtRPE65 (expressed with MOI 20) generated significant amount of 11-*cis* [^{3}H] retinol (Fig. 2B), suggesting that the impaired isomerohydrolase activities in the mutants are not ascribed to their decreased protein levels (Fig. 2).

3.3. Mutations Y144D and P363T decreased the protein stability of RPE65

In order to define the cause for the decreased RPE65 protein levels by the Y144D and P363 mutations, we have compared

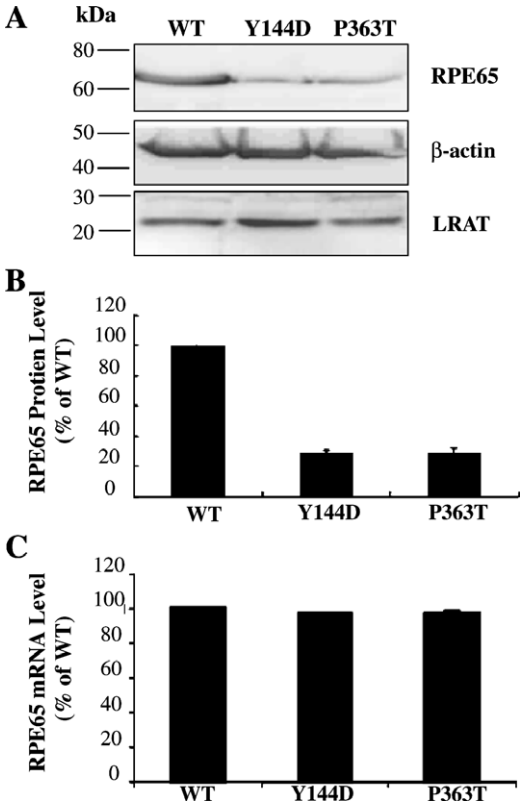

Fig. 1. Decreased protein levels but not the mRNA levels of Y144D and P363T. (A) Eighteen hours after the viral infection, 50 μg of total proteins from cells infected with the same titer (MOI 40) of Ad-wtRPE65, Ad-Y144D and Ad-P363T were blotted with the anti-RPE65 antibody. (B) RPE65 protein levels were semi-quantified by densitometry, normalized by β-actin levels and expressed as % of wtRPE65 (mean ± S.D., $n = 3$). (C) mRNA levels of wtRPE65, Y144D and P363T were quantified by real-time RT-PCR and expressed as % of that of wtRPE65 (mean ± S.D., $n = 3$).

protein stabilities of these two mutants with wtRPE65 as described previously [18]. QBI-293A cells were infected separately with Ad-wtRPE65, Ad-Y144D and Ad-P363T at MOI 10. The protein expression was allowed for 18 h and then stopped by addition of 25 μg/ml of cycloheximide (CHX) to the culture medium. The cells were harvested at 0, 2, 6 and 10 h after the addition of CHX, and the protein levels of the wtRPE65 and its mutants were measured by Western blot analysis and semi-quantified using densitometry. WtRPE65 showed a high stability, with a half-life more than 10 h (Fig. 3). Under the same conditions, the Y144D and P363T mutants exhibited significantly accelerated degradations, with apparent half-lives less than 4 and 2 h, respectively (Fig. 3), suggesting that these single point mutations destabilize the RPE65 protein.

3.4. Mutations Y144D and P363T alter subcellular localization of RPE65

To compare the sub-cellular localization of the RPE65 mutants with wtRPE65, we have performed both immunocytochemistry and cell lysate fractionation. QBI-293A cells expressing the wtRPE65 and the two mutants were homogenized and fractionated using the FractionPREP™ kit. RPE65 abundance in the cytosolic, membrane, nuclear, and cytoskeletal

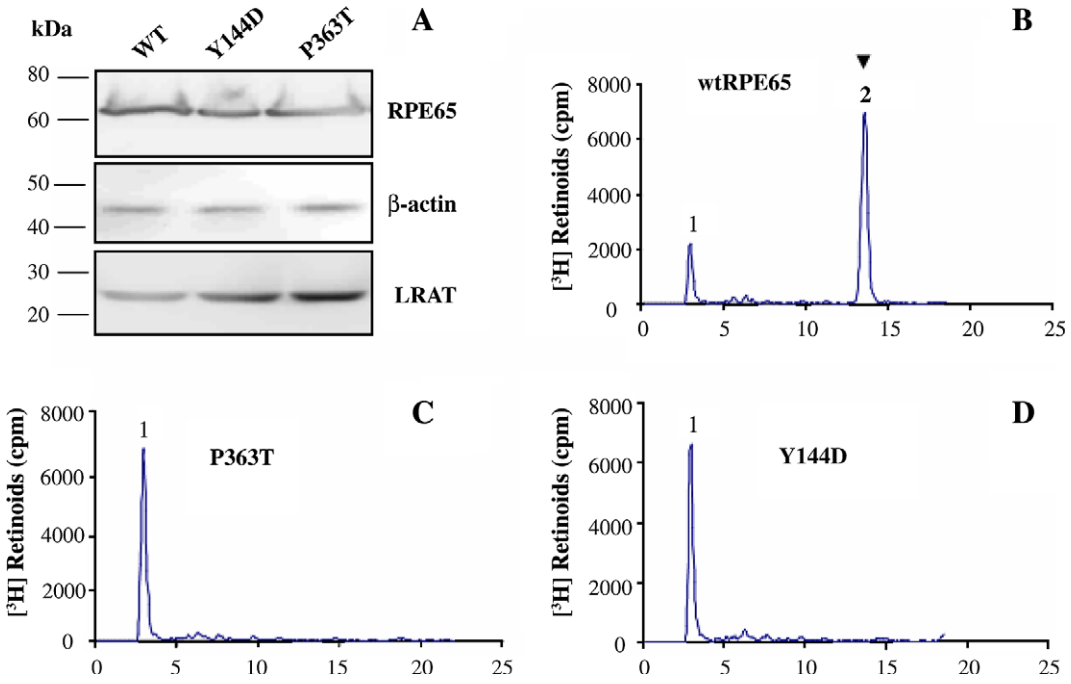

Fig. 2. Lack of isomerohydrolase activity in RPE65 mutants Y144D and P363T. The 293A-LRAT cells were infected with Ad-wtRPE65 (MOI 20), Ad-Y144D (MOI 400) and Ad-P363T (MOI 400) to achieve the mutant protein levels comparable to that of wtRPE65. (A) Twenty-two hours after the infection, 50 μg of total proteins were blotted with the antibody for RPE65. (B–D) Two hundred and fifty micrograms of total proteins from each sample were used for in vitro isomerohydrolase activity assay, with all-*trans* [^{3}H] retinol as the substrate, and the generated retinoids analyzed by HPLC. (B) Cells infected with Ad-wtRPE65. (C) and (D) Cells infected with Ad-Y144D and Ad-P363T, respectively. Peak 1, retinyl esters; 2, 11-*cis* retinol.

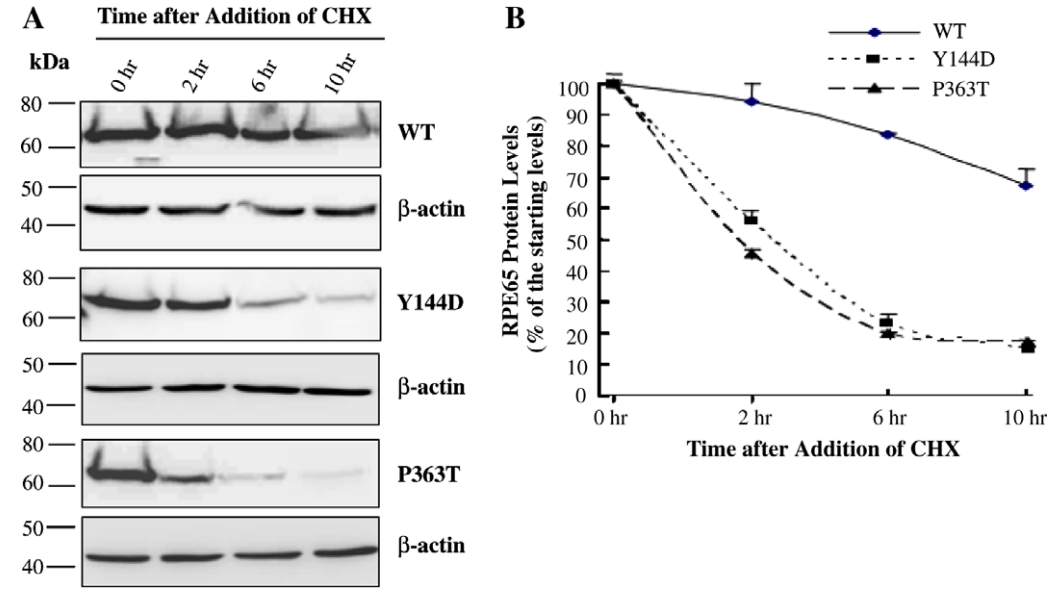

Fig. 3. Decreased protein stability of mutants Y144D and P363T. (A) QBI-293A cells were separately infected with Ad-wtRPE65, Ad-Y144D and Ad-P363T (MOI 10). The protein synthesis was stopped by the addition of CHX 18 h after the virus infection. Cells were harvested at 0, 2, 6 and 10 h following the addition of CHX. RPE65 levels were measured by Western blot analysis with the anti-RPE65 antibody, followed by stripping and reblotting with an anti-β-actin antibody. (B) RPE65 protein levels were semi-quantified by densitometry, normalized by β-actin levels and expressed as % of that before the addition of CHX (mean ± S.D., $n = 4$).

(including inclusion body) fractions were compared using Western blot analysis. Unlike wtRPE65 which was present at high levels in the membrane fraction and at relatively low levels in the cytosolic fraction, mutants Y144D and P363T showed increased levels in the cytosolic fraction while decreased levels in the membrane fraction (Fig. 4A). Both of the mutant proteins were also detected in the cytoskeletal/inclusion body fraction (Fig. 4A).

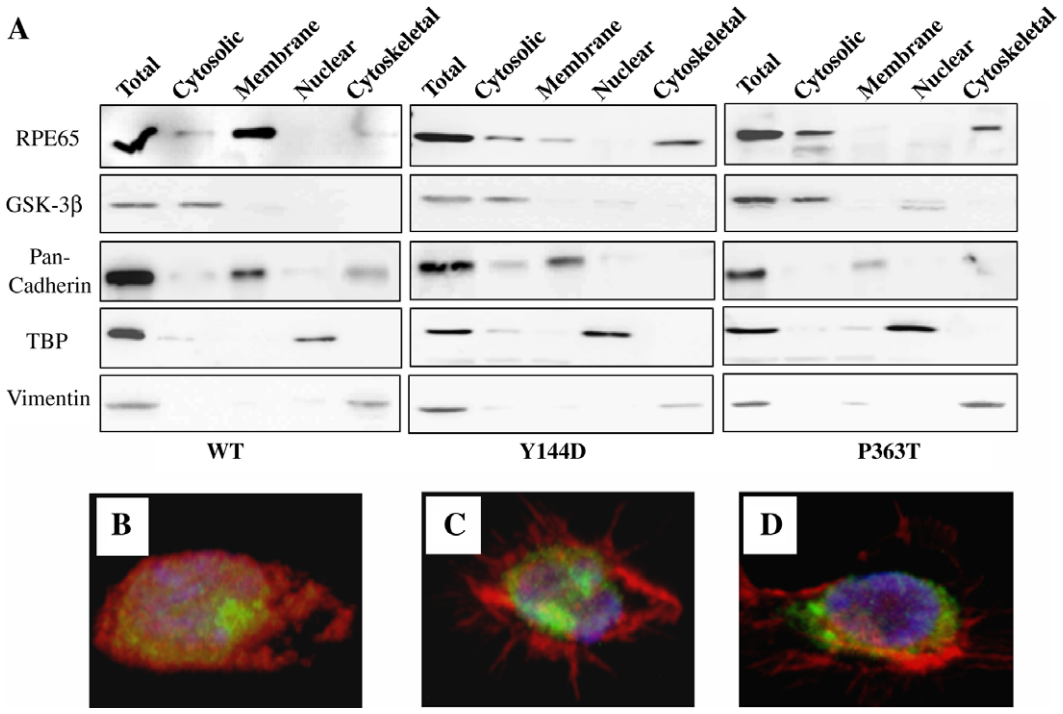

Fig. 4. Subcellular localization and fractionation of wtRPE65 and its mutants. (A) Eighteen hours after the infection with Ad-wtRPE65, AdY144D and Ad-P363T, QBI-293A cells were homogenized and fractionated. Equal amount of protein (10 μg) from the cytosolic, membrane, nuclear and cytoskeletal (including inclusion body) fractions were blotted with the anti-RPE65 antibody and with antibodies for GSK-3β (cytosol marker), pan-cadherin (membrane marker), TATA-binding protein (TBP, nucleus marker) and vimentin (cytoskeletal marker). (B–D) The cells infected with Ad-wtRPE65 (B), Ad-Y144D (C) and Ad-P363T (D) were double-stained with the anti-RPE65 antibody (red) and an antibody specific for an ER marker (green). The nucleus was counter-stained with DAPI (blue).

To further determine if these RPE65 mutants are associated with ER, the QBI-293A cells expressing wtRPE65, Y144D and P363T were double stained with an antibody specific for an ER marker and the anti-RPE65 antibody. The wtRPE65 signal was detected in both the ER and plasma membrane (Fig. 4B), while the Y144D and P363T mutants were detected mainly in the plasma membrane, but not co-localized with the ER marker (Fig. 4C and D), demonstrating altered subcellular distributions of the mutants.

4. Discussion

RPE65 is a membrane-associated protein predominantly expressed in the RPE [11]. Homozygous RPE65 gene knockout (*Rpe65*$^{-/-}$) mice are deficient in isomerohydrolase activity and lack 11-*cis* retinoids, suggesting an interrupted visual cycle [20]. Furthermore, we and others have recently reported that RPE65 is the isomerohydrolase converting all-*trans* retinyl ester to 11-*cis* retinol, a key step in the visual cycle [8,9]. Although multiple mutations in the RPE65 gene have been identified to associate with inherited retinal dystrophies such as RP, LCA and early-onset severe retinal dystrophies, the impacts of these point mutations on the structure and function of RPE65 have not been well investigated [12–15]. The present study reports for the first time that two point mutations of RPE65, Y144D and P363T, identified in patients with LCA, both significantly decrease RPE65 protein stability and altered its subcellular localization. These changes may be responsible for the abolished isomerohydrolase activity of the mutants.

RPE65 is a relatively stable protein with a half-life longer than 10 h, which may be responsible for its abundance in the RPE. In contrast, the point mutations Y144D and P363T significantly shortened the half-lives of RPE65 protein to less than 4 and 2 h. In contrast, the mRNA levels of these two mutants are similar to that of wtRPE65, suggesting that their transcription rates are not altered by the mutations. This result suggests that the accelerated RPE65 protein degradation rates, possibly due to disturbed protein folding introduced by the mutations, are responsible for the lower protein levels of the mutant proteins.

In vitro isomerohydrolase activity assay showed that wtRPE65 has a robust isomerohydrolase activity when co-expressed with LRAT. In contrast, mutants Y144D and P363T lack any detectable enzymatic activity. The abolished enzymatic activity cannot be ascribed to the lower protein levels of the mutants, as even at the levels comparable to that wtRPE65, which were achieved by over-expression of these mutants using high titers of the viruses, these RPE65 mutants still lack any isomerohydrolase activity. Therefore, it is likely that possible structure distortions introduced by the point mutations are responsible for the loss of the enzymatic activity.

Immunocytochemistry using double labeling showed that wtRPE65 is predominantly associated with the ER membrane, consistent with previous studies [21]. Unlike wtRPE65, both of the mutants are not localized in ER. Our recent studies suggested that co-localization of RPE65 with LRAT in the same membrane is essential for its isomerohydrolase activity [8]. The altered subcellular localization of the mutant proteins, may contribute to their decreased stabilities and abolished

enzymatic activities. Although the structural changes of RPE65 resulting from these two mutations remain to be investigated, the present study demonstrates that instability and mis-localization of RPE65 introduced by mutations Y144D and P363T may be responsible for the retinal degeneration in LCA patients. As there is no clinically satisfactory treatment for LCA and its pathogenesis is not completely understood, LCA remains a significant cause of inherited blindness. The present study has provided clues for further investigation of the pathogenesis of LCA and may contribute to the development of new therapies.

Acknowledgements: This study was supported by NIH Grants EY012231 and EY015650, Grants from OCAST, ADA and JDRF, and a COBRE Grant to the vision program at OUHSC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.078.

References

[1] Baylor, D. (1996) How photons start vision. Proc. Natl. Acad. Sci. USA 93, 560–565.
[2] McBee, J.K., Palczewski, K., Baehr, W. and Pepperberg, D.R. (2001) Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. Prog. Retin. Eye Res. 20, 469–529.
[3] Rando, R.R. (2001) The biochemistry of the visual cycle. Chem. Rev. 101, 1881–1896.
[4] Rando, R.R. (1992) Molecular mechanisms in visual pigment regeneration. Photochem. Photobiol. 56, 1145–1156.
[5] Lamb, T.D. and Pugh Jr., E.N. (2004) Dark adaptation and the retinoid cycle of vision. Prog. Retin. Eye Res. 23, 307–380.
[6] Rando, R.R. (1991) Membrane phospholipids as an energy source in the operation of the visual cycle. Biochemistry 30, 595–602.
[7] Redmond, T.M., Poliakov, E., Yu, S., Tsai, J.Y., Lu, Z. and Gentleman, S. (2005) Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. Proc. Natl. Acad. Sci. USA 102, 13658–13663.
[8] Moiseyev, G., Chen, Y., Takahashi, Y., Wu, B.X. and Ma, J.X. (2005) RPE65 is the isomerohydrolase in the retinoid visual cycle. Proc. Natl. Acad. Sci. USA 102, 12413–12418.
[9] Jin, M., Li, S., Moghrabi, W.N., Sun, H. and Travis, G.H. (2005) Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. Cell 122, 449–459.
[10] Moiseyev, G., Crouch, R.K., Goletz, P., Oatis Jr., J., Redmond, T.M. and Ma, J.X. (2003) Retinyl esters are the substrate for isomerohydrolase. Biochemistry 42, 2229–2238.
[11] Hamel, C.P., Tsilou, E., Pfeffer, B.A., Hooks, J.J., Detrick, B. and Redmond, T.M. (1993) Molecular cloning and expression of RPE65, a novel retinal pigment epithelium-specific microsomal protein that is post-transcriptionally regulated in vitro. J. Biol. Chem. 268, 15751–15757.
[12] Morimura, H., Fishman, G.A., Grover, S.A., Fulton, A.B., Berson, E.L. and Dryja, T.P. (1998) Mutations in the RPE65 gene in patients with autosomal recessive retinitis pigmentosa or leber congenital amaurosis. Proc. Natl. Acad. Sci. USA 95, 3088–3093.
[13] Gu, S.M., Thompson, D.A., Srikumari, C.R., Lorenz, B., Finckh, U., Nicoletti, A., Murthy, K.R., Rathmann, M., Kumaramanickavel, G., Denton, M.J. and Gal, A. (1997) Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. Nat. Genet. 17, 194–197.
[14] Marlhens, F., Bareil, C., Griffoin, J.M., Zrenner, E., Amalric, P., Eliaou, C., Liu, S.Y., Harris, E., Redmond, T.M., Arnaud, B., Claustres, M. and Hamel, C.P. (1997) Mutations in RPE65 cause Leber's congenital amaurosis [letter]. Nat. Genet. 17, 139–141.
[15] Thompson, D.A., Gyurus, P., Fleischer, L.L., Bingham, E.L., McHenry, C.L., Apfelstedt-Sylla, E., Zrenner, E., Lorenz, B., Richards, J.E., Jacobson, S.G., Sieving, P.A. and Gal, A. (2000) Genetics and phenotypes of RPE65 mutations in inherited retinal degeneration. Invest. Ophthalmol. Vis. Sci. 41, 4293–4299.
[16] Veske, A., Nilsson, S.E., Narfstrom, K. and Gal, A. (1999) Retinal dystrophy of Swedish briard/briard-beagle dogs is due to a 4-bp deletion in RPE65. Genomics 57, 57–61.
[17] Yzer, S., van den Born, L.I., Schuil, J., Kroes, H.Y., van Genderen, M.M., Boonstra, F.N., van den Helm, B., Brunner, H.G., Koenekoop, R.K. and Cremers, F.P. (2003) A Tyr368His RPE65 founder mutation is associated with variable expression and progression of early onset retinal dystrophy in 10 families of a genetically isolated population. J. Med. Genet. 40, 709–713.
[18] Takahashi, Y., Chen, Y., Moiseyev, G. and Ma, J.X. (2006) Two point mutations of RPE65 from patients with retinal dystrophies decrease the stability of RPE65 protein and abolish its isomerohydrolase activity. J. Biol. Chem. In press.
[19] Ma, J., Zhang, J., Othersen, K.L., Moiseyev, G., Ablonczy, Z., Redmond, T.M., Chen, Y. and Crouch, R.K. (2001) Expression, purification, and MALDI analysis of RPE65. Invest. Ophthalmol. Vis. Sci. 42, 1429–1435.
[20] Redmond, T.M., Yu, S., Lee, E., Bok, D., Hamasaki, D., Chen, N., Goletz, P., Ma, J.X., Crouch, R.K. and Pfeifer, K. (1998) Rpe65 is necessary for production of 11-*cis*-vitamin A in the retinal visual cycle. Nat. Genet. 20, 344–351.
[21] Tsilou, E., Hamel, C.P., Yu, S. and Redmond, T.M. (1997) RPE65, the major retinal pigment epithelium microsomal membrane protein, associates with phospholipid liposomes. Arch. Biochem. Biophys. 346, 21–27.