Biochemical and Biophysical Research Communications 391 (2010) 1757–1761

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Negative charge of the glutamic acid 417 residue is crucial for isomerohydrolase activity of RPE65

Olga Nikolaeva, Yusuke Takahashi, Gennadiy Moiseyev *, Jian-xing Ma

Department of Medicine Endocrinology, Harold Hamm Oklahoma Diabetes Center, The University of Oklahoma Health Sciences Center, 941 Stanton L. Young Blvd., BSEB302, Oklahoma City, OK 73104, United States

Department of Cell Biology, Harold Hamm Oklahoma Diabetes Center, The University of Oklahoma Health Sciences Center, 941 Stanton L. Young Blvd., BSEB302, Oklahoma City, OK 73104, United States

ARTICLE INFO

Article history:
Received 11 December 2009
Available online 30 December 2009

Keywords:
RPE65
Visual cycle
Isomerohydrolase
Leber congenital amaurosis
Retinoids

ABSTRACT

RPE65 is the isomerohydrolase essential for regeneration of 11-*cis* retinal, the chromophore of visual pigments. Here we compared the impacts of two mutations in RPE65, E417Q identified in patients with Leber congenital amaurosis (LCA), and E417D on isomerohydrolase activity. Although both mutations decreased the stability of RPE65 and altered its sub-cellular localization, E417Q abolished isomerohydrolase activity whereas the E417D mutant retained partial enzymatic activity suggesting that the negative charge of E417 is important for RPE65 catalytic activity. Loss of charge at this position may represent a mechanism by which the E417Q mutation causes blindness in LCA patients.

Introduction

RPE65, identified as an iron-dependent isomerohydrolase of the visual cycle, is a key enzyme for regeneration of 11-*cis* retinal, the chromophore of both rod and cone visual pigments [1–3]. RPE65 is predominantly expressed in the retinal pigment epithelium (RPE) and associated with the endoplasmic reticulum membrane [4]. Mutations in the *RPE65* gene cause severe congenital retinal dystrophies, such as juvenile severe retinitis pigmentosa and Leber congenital amaurosis (LCA), which lead to blindness [5–8]. Genetic analysis of LCA patients suggests that *RPE65* gene mutations account for 3% [9] to 16% [10] of total cases of LCA. However, the molecular mechanism by which *RPE65*–LCA mutations cause such severe visual deficiency remains elusive. Recent studies have shown that a number of RPE65 mutants associated with LCA cause either partial or total loss of its isomerohydrolase activity [3,11–13]. Here we focused on evaluation of the impacts of the mutation E417Q of RPE65 (associated with LCA [14]) on the stability, sub-cellular localization, and enzymatic activity of the protein. To investigate how the negative charge of residue E417 affects the enzymatic activity of RPE65, we constructed and studied the mutant E417D.

Materials and methods

Site-directed mutagenesis, expression and isomerohydrolase activity assay. Two point mutations E417Q and E417D in human RPE65 (hRPE65) were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the wild-type hRPE65 (wtRPE65) cDNA as the template and confirmed by DNA sequencing from both strands as described before [11]. Generation of recombinant adenoviruses (Ad-RPE65) was performed as described before [15]. Measurements of the expression and isomerohydrolase activities of wtRPE65 and its mutants were performed as published previously [11,15].

Sub-cellular fractionation and stability assay. QBI-293A cells (Qbiogene, Carlsbad, CA) stably expressing LRAT (293A-LRAT) [16] were infected with adenoviruses expressing wtRPE65 and its mutants at MOI of 100 and the sub-cellular localization of recombinant RPE65 was elucidated using FractPrep™ kit (BioVision, Mountain View, CA). The sub-cellular fractions were examined by Western blot analysis as described [11]. To estimate the half-lives of wtRPE65 and its mutants, 293A-LRAT cells were infected with the adenoviruses at MOI of 20 and stability assays were performed as described previously [11].

Modeling of the hRPE65 mutants structure. To analyze the 3D structure of hRPE65 and its mutants, a sequence homology-based program Swiss Model (http://swissmodel.expasy.org/) was

Abbreviations: Ad-RPE65, adenovirus expressing RPE65; LCA, Leber congenital amaurosis; MOI, multiplicity of infection; RPE, retinal pigment epithelium; CHX, cycloheximide.

* Corresponding author. Address: Department of Medicine Endocrinology, Harold Hamm Oklahoma Diabetes Center, The University of Oklahoma Health Sciences Center, 941 Stanton L. Young Blvd., BSEB302, Oklahoma City, OK 73104, United State. Fax: +1 405 271 3973.

E-mail address: gennadiy-moiseyev@ouhsc.edu (G. Moiseyev).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.12.149

employed utilizing bovine RPE65 crystal structure (3FSN, http://www.pdb.org/pdb/explore/explore.do?structureId=3FSN) [17] as a template.

Results

RPE65 mutations E417Q and E417D significantly alter intracellular localization

To determine the sub-cellular localization of wtRPE65 and its E417Q and E417D mutants, we employed cell lysate fractionation. Western blot analysis of sub-cellular fractions revealed that wtRPE65 was predominantly present in the membrane fraction and at a lower level in the cytosolic fraction (Fig. 1A). In contrast, both E417D and E417Q mutants showed considerably decreased levels in the membrane fraction (Fig. 1B). Moreover, unlike wtRPE65, both mutant proteins were predominantly localized in the cytoskeletal fraction containing detergent-resistant inclusion bodies (Fig. 1B). Amounts of both of the mutants in the cytosolic fractions were found to be similar to that of wtRPE65.

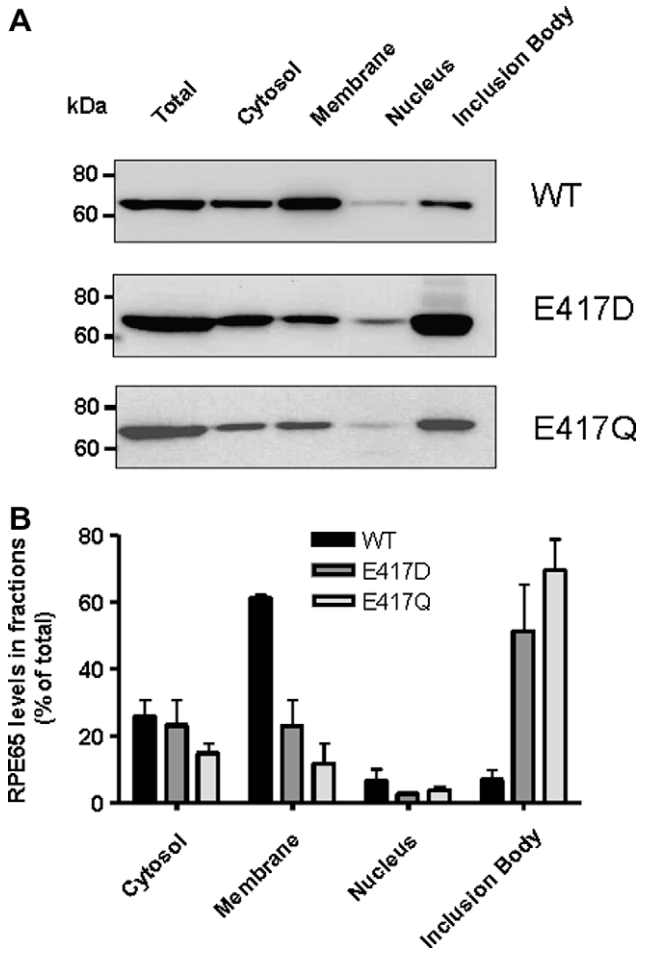

Fig. 1. Mutations in RPE65 alter its sub-cellular fractionation. The 293A-LRAT cells infected with Ad-wtRPE65, Ad-E417Q and Ad-E417D at MOI of 100 were harvested 18 h after infection, homogenized and fractionated. (A) Total cell lysate (32 μg), and equal amount of protein (8 μg) from the cytosolic, membrane, nuclear and cytoskeletal/including inclusion body fractions were analyzed by Western blot using the anti-RPE65 antibody. (B) Protein levels of wtRPE65 and the mutants were quantified by densitometry and presented as % of total protein levels of wtRPE65 or its mutants correspondingly (mean ± SD, $n = 3$).

Mutations E417Q and E417D impair the protein stability of RPE65

To evaluate the impact of mutations of glutamic acid at position 417 on the protein stability, we measured the protein degradation rate after the blockade of translation by cycloheximide (CHX) in 293A-LRAT cells. The cells were separately infected with adenoviruses at MOI of 20 and incubated for 18 h, followed by the addition of 25 μg/ml of CHX. WtRPE65 and its mutant protein levels were measured by Western blot analysis at 0, 2, 6, and 10 h after the CHX addition (Fig. 2A) and semi-quantified by densitometry (Fig. 2B). WtRPE65 was found to have high stability, with an apparent half-life longer than 10 h. In contrast, both mutants showed dramatically accelerated rates of protein degradation with half-lives of less than 2 h (Fig. 2B).

The enzymatic activity of RPE65 is abolished in the E417Q mutant, but partially retained in the E417D mutant

Both E417Q and E417D mutants were expressed in 293A-LRAT cells using adenovirus with MOI of 100. Both mutants had protein expression levels of approximately 70% of wtRPE65 (Fig. 3A and B). Enzymatic activities of the mutants were compared with that of wtRPE65 expressed in 293A-LRAT cells at the same MOI of 100. At the comparable protein levels, wtRPE65 generated significant amounts of 11-*cis* retinol (Fig. 3C); the E417D mutant produced a decreased but detectable amount of 11-*cis* retinol, while the E417Q mutant did not show any detectable isomerohydrolase activity under the same assay conditions (Fig. 3D and E). Further, to determine the effect of RPE65 abundance in the cell lysate on isomerohydrolase activity, the cells were infected separately with Ad-wtRPE65 and Ad-E417D at different MOIs (from 10 to 100). The 11-*cis* retinol generated in these reactions increased proportionally with increasing MOI for both wtRPE65 and E417D (Fig. 3F). Nevertheless, at all tested MOIs, the amount of 11-*cis* retinol generated by E417D was approximately 5-fold lower than the amount generated by wtRPE65 (Fig. 3F).

Discussion

RPE65 protein is the only known enzyme in the visual cycle with the ability to catalyze the conversion of all-*trans* retinyl esters to 11-*cis* retinol [1–3]. Mutations in the *RPE65* gene are known to cause inherited retinal dystrophies such as LCA [14,18]. However, the mechanism by which RPE65 mutations leading to the disease is still unknown. In this study, we investigated the E417Q variant of RPE65 which has been identified in LCA patients [14] and compared its activity with that of E417D. The results showed that E417Q completely lost enzymatic activity, while E417D retained partial activity. This result suggests that the negative charge at residue E417 is crucial for the enzymatic activity of RPE65 and the visual defect in patients with E417Q may be ascribed to the loss of the negative charge at E417.

In the present study, the majority of both E417Q and E417D mutant proteins were localized in the cytoskeletal/inclusion body fraction in contrast with the predominantly membrane-associated wtRPE65 protein. This mislocalization may contribute to the accelerated degradation rate of the mutant proteins. The half-life of wtRPE65 was longer than 10 h, which is in agreement with that of a previously published study [11,12], whereas the half-life of each mutant was found to be shorter than 2 h. The lower stability of both E417Q and E417D mutants of RPE65 indicates that their protein folding is disturbed. Unlike previously reported RPE65 mutations [11,12,19], both E417D and E417Q demonstrated expression levels comparable with that of wtRPE65 in 293A-LRAT cells. Therefore, the lack of enzymatic activity in the E417Q mutant