Salus University

Annual Progress Report: 2010 Formula Grant

Reporting Period

January 1, 2011 – June 30, 2011

Formula Grant Overview

The Salus University received $45,203 in formula funds for the grant award period January 1, 2011 through April 30, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Role of RD3 protein in Leber Congenital Amaurosis LCA12 – The purpose of this project is to characterize new protein interactions in photoreceptor cells to extend our knowledge about the processes leading to photoreceptor death triggered by abnormal regulation of one of the key photoreceptor enzymes, guanylyl cyclase. In particular, we will characterize the functional effects of mutations found in human homolog of the *RD3* (*Clorf36*) gene in patients with different retinal disorders and model in vitro its interactions with retinal guanylyl cyclase pertinent to congenital blindness, Leber congenital amaurosis LCA12.

Anticipated Duration of Project

1/1/2011 – 4/30/2012

Project Overview

The broad research objective of the proposed short-term pilot research is to improve the knowledge of mechanisms related to normal vision and blinding genetic disorders in humans. The specific objectives are to identify the newly emerged functions of a photoreceptor protein RD3 implicated in Leber congenital amaurosis (LCA12), a congenital blindness at childhood. We have obtained strong preliminary data pointing at the ability of the RD3 protein to suppress the activity of a key enzyme involved in signal transduction, retinal guanylyl cyclase (RetGC). We have designed the approach that will, through extensive in vitro study of the RD3/RetGC interactions, define the most important events in RetGC regulation affected by RD3 and the effect of mutations described in human RD3 gene in patients with congenital retinal disorders. The central idea of the planned research objectives is to provide, through accumulation of the critically important new findings, a better basis for the subsequent in-depth analysis of the processes involved in cyclic GMP metabolism leading to photoreceptor dysfunction.

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Other Participating Researchers

Andrey Savchenko, PhD – employed by Salus University

Expected Research Outcomes and Benefits

There are many types of congenital blindness caused by degeneration of the retina. One of the early-onset types of retinal dystrophy, Leber congenital amaurosis type 12 (LCA12), results in severe loss of vision in early childhood. Previously, other researchers have described that in *rd3* mouse genetic model of retinal degeneration and in human patients with LCA12, there is a similar damage in the *RD3/Clorf36* gene that results in abnormal mRNA splicing and a shortened RD3 protein product. A number of other mutations have been reported in this gene in patients with other retinal abnormalities, but the direct evidence for their biochemical link with the retinal diseases have not yet been revealed. More recently, another group of researchers have reported that the RD3 protein associates with retinal guanylyl cyclase (RetGC) and is essential for maintaining the normal levels of RetGC in rods and cones. In this project, we plan to study the functional effects of RD3 interaction with RetGC on the cyclase activity and the effects of mutations found in human patients with visual disorders. Characterization and evaluation of the potential role of these mutations in development of congenital blindness will provide a new knowledge that can be used for improving diagnostic methods and the strategy for their potential genetic therapy.

Summary of Research Completed

This research project developed according to the original plan and the progress toward the originally proposed Specific Aims has been achieved as follows.

THE FIRST SPECIFIC AIM is to characterize the effects of RD3 on human RetGC activity in vitro.

Progress: In accordance with the original Time Frame and Milestones of the project, in order to study the biochemical effects of RD3 on retinal guanylyl cyclase (RetGC), we have isolated fractions of rod outer segment (ROS) membranes from mouse retinas that lack regulatory proteins, GCAPs, and have only one of the RetGC isozyme present - either RetGC1 or RetGC2. This was accomplished by breeding mice with gene knockouts for GUCY2E or GUCY2F genes into a genetic background of a double-GCAP knockout ($GCA1A^{-/-}GUCA1B^{-/-}$). The ROS fractions from each triple-knockout line were isolated from dark-adapted retinas using density gradient centrifugation (Fig. 1). The methodology for isolation of these preparations has been

described in full detail in our recent publication that depicts enzymatic and regulatory properties of RetGC isozymes (Peshenko et al., 2011). We then conducted the preliminary analysis of the RD3 effect on individual RetGC1 isozymes in these membranes and demonstrated that RD3 with high affinity inhibits their basal activity in the absence of GCAPs (Fig. 2). In the membranes containing both native RetGC isozymes, RD3 effectively outcompetes both GCAP1 and GCAP2 (Fig. 2 C-D). Therefore, the effects we previously observed using recombinant RetGC have now been unambiguously confirmed for the native guanylyl cyclase.

THE SECOND SPECIFIC AIM is to replicate mutations found in human genes in recombinant proteins and to evaluate their biochemical impact on the RetGC regulation.

Progress. In accordance with the plan, we now expressed several mutants of RD3 described in patients with various forms of retinal diseases, W6R/E23D; G35R; G57V; R68W; F100ter; and K130M, and we have already purified them in sufficient quantities for studying the effect of these mutations on regulatory properties of RD3 (Fig. 3).

THE THIRD SPECIFIC AIM is to develop gene construct for the mutant RD3 expression in mouse rods in order to subsequently evaluate the effect of mutant RD3 in vivo.

Progress. The final decision on making the transgenic construct still depends on the outcome from the experiments on Specific Aim 2 (the mutants to be used for transgenic expression are subject of selection from these experiments). In the mean time, we are preparing the animal model that will be used in the subsequent study. The *rd3/rd3* mice have been procured from the Jackson Laboratories JAX deposit in the original albino background. We are currently breeding them into a pigmented background by crossing them with C57B6 strain and then back-crossing to a homozygous rd3/rd3 state. This background we chose for reduction of possible effects of light damage affecting the retinal phenotype in albino background. The rd3 mice are notoriously difficult breeders, but we have already produced 8 pigmented rd3/rd3 mice, sufficient for maintaining this colony in our vivarium.

PUBLICATIONS:

One manuscript acknowledging support from Pennsylvania Department of Health has been accepted for publication in Biochemistry:

Peshenko IV, Olshevskaya EV, Savchenko AB, Karan S, Palczewski K, Baehr W, Dizhoor AM. (2011) Enzymatic Properties and Regulation of the Native Isozymes of Retinal Membrane Guanylyl Cyclase (RetGC) from Mouse Photoreceptors. ***Biochemistry*** 50(25): 5590-600

One more manuscript has been submitted for publication and is currently under revision in the Journal of Biological Chemistry.

FIGURES

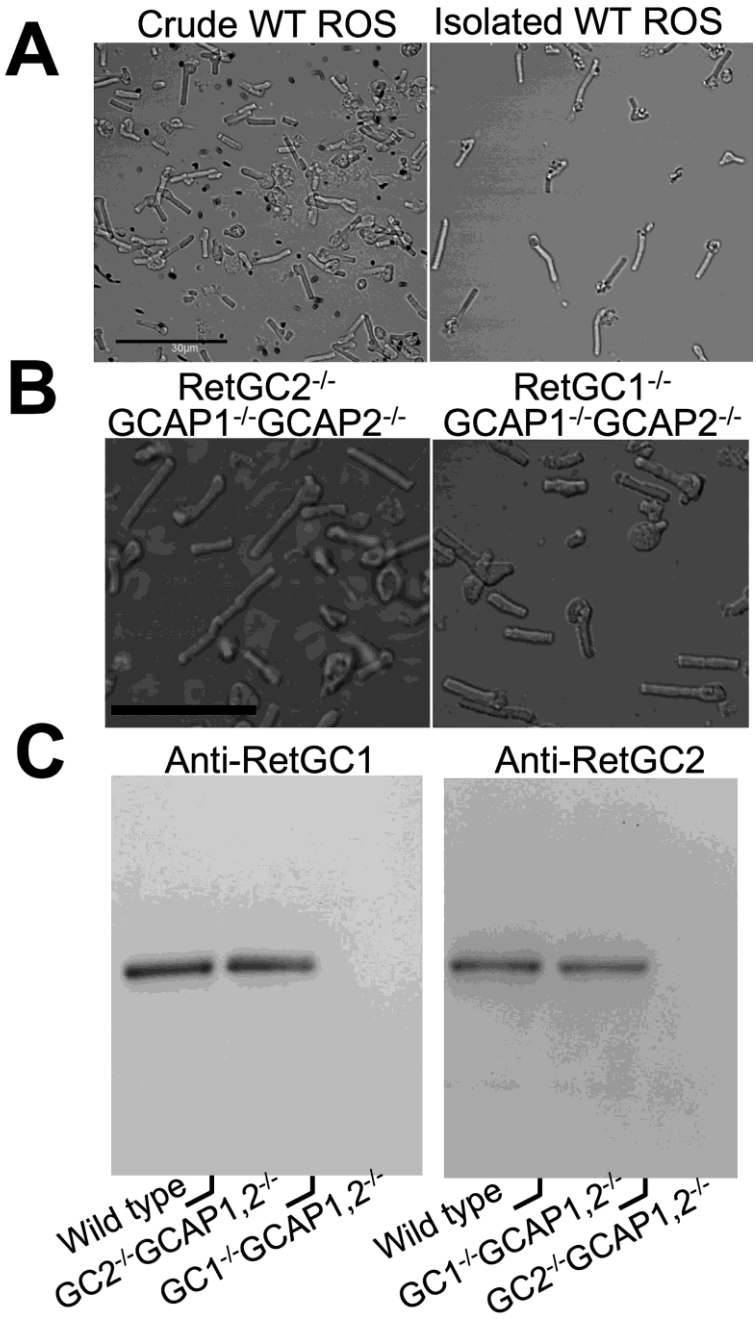

Fig. 1. ROS fractions isolated from mouse retinas contain individual native isozymes of RetGC. ***A***. Mouse ROS preparations before (*left*) and after (*right*) density gradient centrifugation; bar size: 30 µm. ***B.*** Gradient-purified fractions from RetGC2$^{-/-}$GCAP1$^{-/-}$GCAP2$^{-/-}$ (*left*) or RetGC1$^{-/-}$GCAP1$^{-/-}$GCAP2$^{-/-}$ (*right*) retinas containing highly enriched ROS; bar size: 20 µm. ***C***. Only one RetGC isozyme remains in each triple-knockout mouse ROS fraction. The ROS fractions from WT and the RetGC1$^{-/-}$GCAP1$^{-/-}$GCAP2$^{-/-}$ or RetGC2$^{-/-}$GCAP1$^{-/-}$GCAP2$^{-/-}$ mice equalized by rhodopsin content (10 µg or rhodopsin per lane, *left*, or 20 µg of rhodopsin per lane, *right*) were tested after SDS-PAGE by immunoblotting probed with anti-human RetGC1KHD (*left panel*) or anti-mouse RetGC2KHD (*right panel*) antibodies (*Peshenko et al., 2011*).

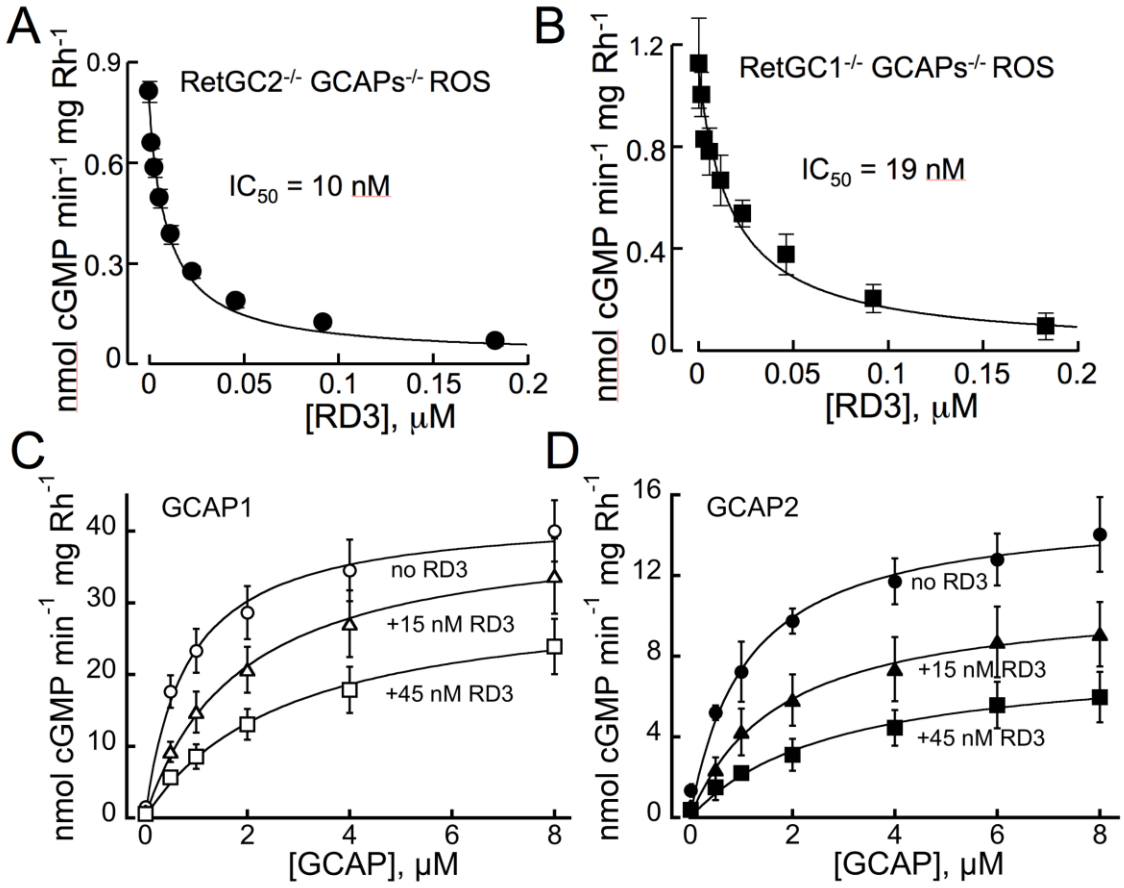

Fig. 2. A-B. RD3 inhibits native RetGC isozymes in native ROS membranes lacking both GCAP isoforms and containing only RetGC1 (A) or RetGC2 (B) isozymes of the cyclase. The membranes isolated from mouse retinas described in Fig. 1 were reconstituted with purified recombinant mouse RD3 and assayed for guanylyl cyclase activity. C-D. RD3 competes with GCAPs for the native RetGC. The native ROS membranes containing both RetGC1 and RetGC2 isolated from GCAPs-/- retinas were reconstituted with mouse GCAP1 (C) or GCAP2 (D) in the presence or in the absence of RD3 as indicated. The data (mean ± SD) were fitted assuming Michaelis hyperbolic function, $a=a_{max}[GCAP]/(K_{1/2}+[GCAP])$.

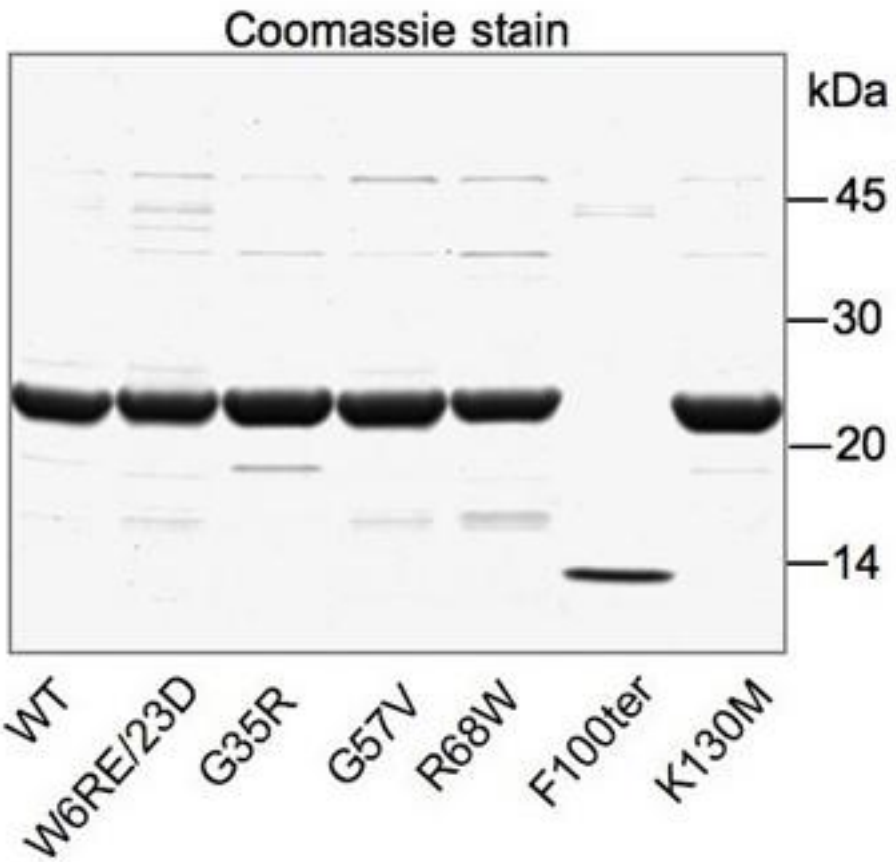

Fig. 3. Recombinant RD3 expressed in E. coli with mutations found in human patients with retinal diseases, stained by Brilliant Blue R-250 after SDS PAGE in 15% gel.