Salus University

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2013 – April 30, 2014

Formula Grant Overview

Salus University received $40,030 in formula funds for the grant award period January 1, 2013 through April 30, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Interaction of Guanylyl Cyclase GUCY2D with its Regulatory Proteins: Effects of Mutations Causing Inherited Blindness – GUCY2D guanylyl cyclase is essential for retinal responses to light stimuli. Mutations in GUCY2D gene and in its regulatory proteins cause various forms of congenital blindness found in the North American population. Molecular mechanisms leading to GUCY2D malfunction are multiple and vary for different types of mutations. The purpose of this short-term pilot project is to obtain new knowledge of how principle mechanisms of GUCY2D interactions with its regulatory proteins become altered by the disease-causing mutations. This knowledge should be valuable for planning future studies on approaches to gene therapy of the GUCY2D-related blindness.

Duration of Project

1/1/2013 – 4/30/2014

Project Overview

The long-term research objective for the study initiated with this pilot project is to obtain new knowledge about specific molecular mechanisms in the retina that enable visual perception and, when altered, cause congenital blindness. Mutations in GUCY2D, both gain-of-function and loss-of-function, cause various types of severe congenital vision loss: cone-rod degeneration, central areolar choroidal dystrophy, progressive cone degeneration, Leber congenital amaurosis (LCA1), cone-rod dystrophy. Mutant GUCY2D alleles are known to circulate in population with a carrier frequency of 1/574 in North America, where they constitute the 4th major cause of LCA1, a severe early-onset recessive form of blindness. Application of gene therapy to treat loss-of-function mutations in GUCY2D has been proven effective in transgenic mouse models and currently even enters the trial phase for treatment of LCA1 human patients. With the gene therapy gradually getting grounds and perspectives for treatment of congenital blindness, the molecular mechanisms underlying the congenital disorders caused by mutations in GUCY2D become particularly important to identify in order to develop the most appropriate strategies (e.g., suppression of the abnormal gene or delivery of the missing activity). Preliminary

functional analysis of various LCA1 alleles of GUCY2D indicates that a variety of potential mechanisms can be involved in this abnormal function. Some mutations could inactivate catalytic activity of the enzyme without preventing its interaction with the regulatory proteins; other mutations could suppress GUCY2D ability to undergo activation by GCAPs, by blocking the GUCY2D/GCAP interactions. The purpose of this short-term pilot study is to further evaluate molecular mechanisms of GUCY2D interactions and their abnormalities in LCA1. The specific aims of this study follow:

Specific Aim 1 - To establish how mutations in GUCY2D gene linked to autosomal recessive LCA1 affect the ability of the retinal guanylyl cyclase to bind GCAP1 and Rd3. We hypothesize that mutations in GUCY2D can produce the loss-of-function phenotype through different mechanisms, either the loss of regulatory response from other proteins, or through the loss of catalytic activity (even if the ability to contact regulatory proteins remains). The objective of the study is to test this possibility using recombinant proteins produced in cultured cells.

Specific Aim 2 - To test how the mutation in GCAP2 causing retinitis pigmentosa associates with the GUCY2D guanylyl cyclase. We hypothesize that the disease-causing mutation, G157R, can alter GCAP2 interaction with the GUCY2D and/or competition with RD3 protein.

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

Congenital blindness caused by defects in the function of retinal proteins requires adequate approaches for treatment by genetic correction of the functions – either through suppression of the abnormally regulated activity or by restoring the missing function by delivering appropriate genetic constructs. This approach has demonstrated its feasibility in a variety of animal models mimicking human hereditary blindness and entered human clinical trials. However, in order to select the proper strategy, application of this method requires a profound understanding of the molecular mechanisms causing the diseases. Defects in retinal guanylyl cyclase GUCY2D not only is a cause of severe retinal dysfunctions but have also been proven to respond to correction by gene therapy in animals. Therefore, we expect that probing the protein interactions of GYCY2D affected by the blinding mutations will provide valuable new knowledge and understanding of the basic mechanisms of congenital blindness. The proposed short-term pilot study can help better evaluate basic mechanisms that become altered in disease and can be of importance in the future in designing their correction.

Summary of Research Completed

Specific Aim 1.

We produced several mutants of retinal guanylyl cyclase (RetGC1) coded by the human *GUCY2D* gene to probe for the effects of mutations in the guanylyl cyclase on its binding to the activator protein GCAP1 or inhibitor protein RD3. Fluorescently labeled with green fluorescent protein (GFP) RD3 and GCAP1 were co-expressed with GUCYD variants in HEK293 cell line and their co-localization was analyzed using confocal microscopy. We have demonstrated that a short form of GUCY2D in which the extracellular segment is lacking or substituted with a red fluorescent protein (mOrange) fully retains the ability to co-localize with Rd3 or GCAP1 (Fig. 1 and 2). This strongly indicates that LCA-linked mutations located in the extracellular portion of RetGC1 such as E970Q, E103K, W150R, E255K, T312P, T312M or R365W (Stone 2007) exert their adverse effect through mechanism(s) different from disruption of GUCY2D interactions with GCAP1 and RD3. Among the remaining possibilities there are incorrect intracellular trafficking of GUCY2D and/or altered intracellular stability of the mutated cyclase, which could only be addressed in vivo using transgenic models. The removal of the C-terminal portion of the cyclase downstream from Arg941 also did not prevent binding of GCAP1. Therefore, we can effectively rule out that such LCA-linked mutations as C984Y, P999S, S1007L, and M1009L located in the C-terminal portion of GUCY2D (Stone, 2007) act through disruption of GCAP1 binding with the cyclase. Most likely, these mutations directly affect catalytic activity of the cyclase, similarly to the R1091x mutation (Jacobson et al., 213). On the other hand, truncation of the C-terminal part of the cyclase hampers efficient binding of the inhibitory protein, RD3 (Fig. 1). Two LCA-linked mutations in the kinase-homology domain of GUCY2D, W708R and I734T, fail to bind GCAP1-GFP (Fig. 2). This opens a possibility that these residues are directly involved in forming the interface structure for GCAP1 on the cyclase.

Specific Aim 2

We have introduced the Gly→Arg substitution in position 4 (non-coordinating side chain) of the 12-amino acid calcium-binding loop of EF-hand 4 in bovine GCAP2 mimicking the G157R mutation reported in human patients with polymorphic retinitis pigmentosa (Sato et al., 2005) and tested its biochemical properties in regulation of the native guanylyl cyclase in isolated rod outer segment membranes purified from dark-adapted bovine retinas. The proper N-fatty acylation (myristoylation) of the recombinant protein was verified and confirmed by mass-spectrometry analysis. We compared both dose-dependence of the cyclase activation by wild type and mutant GCAP2 and its Ca^{2+}-sensitivity in native rod outer segment membranes. We find no indication that such substitution has any effect on either of these two major parameters (Fig. 3). While we would not expect a gain-of-function effect based on the recessive nature of the GCAP2-linked retinitis pigmentosa reported by Sato et al., we expected that the mutation would cause a loss-of-function effect – either lack of cyclase activation or abnormally high sensitivity to inhibition by Ca^{2+}. However, the activity of mutated GCAP2 did not change. This result leaves two possibilities - either the intracellular expression or stability of the mutant GCAP2 *in vivo* is affected by the mutation (this question cannot be addressed using in vitro biochemistry and can only be resolved by using animal models) or the reliability of the reported direct genetic link between GCAP2 and retinal degeneration needs to be revisited.

Figures.

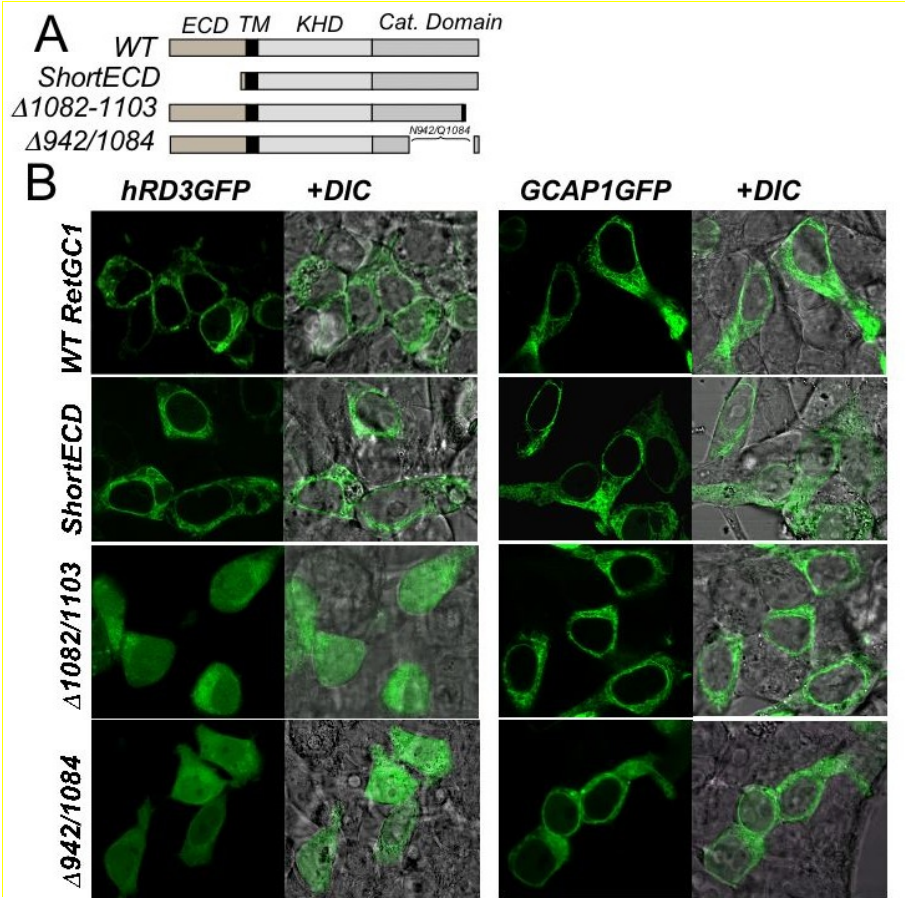

Figure 1. A. Schematic representation of wild type RetGC1 coded by GUCY2D gene and its variants. *Top to bottom*: wild type, 'Short ECD" lacking extracellular segment; carboxy-terminus truncations after resides 1081 or 942. B. human RD3-GFP (left column) or GCAP1-GFP (right column) pattern in HEK293 cells expressing wild type (first row from top), Short ECD RetGC1 (second row), truncation after residues 1081 (third row) and 941 (bottom row). Each fluorescence image is also shown superimposed on differential contrast image (DIC) in the right part of each column. Note membrane pattern of GCAP1GFP in all cases and diffused pattern of RD3 after carboxy-terminal deletions.

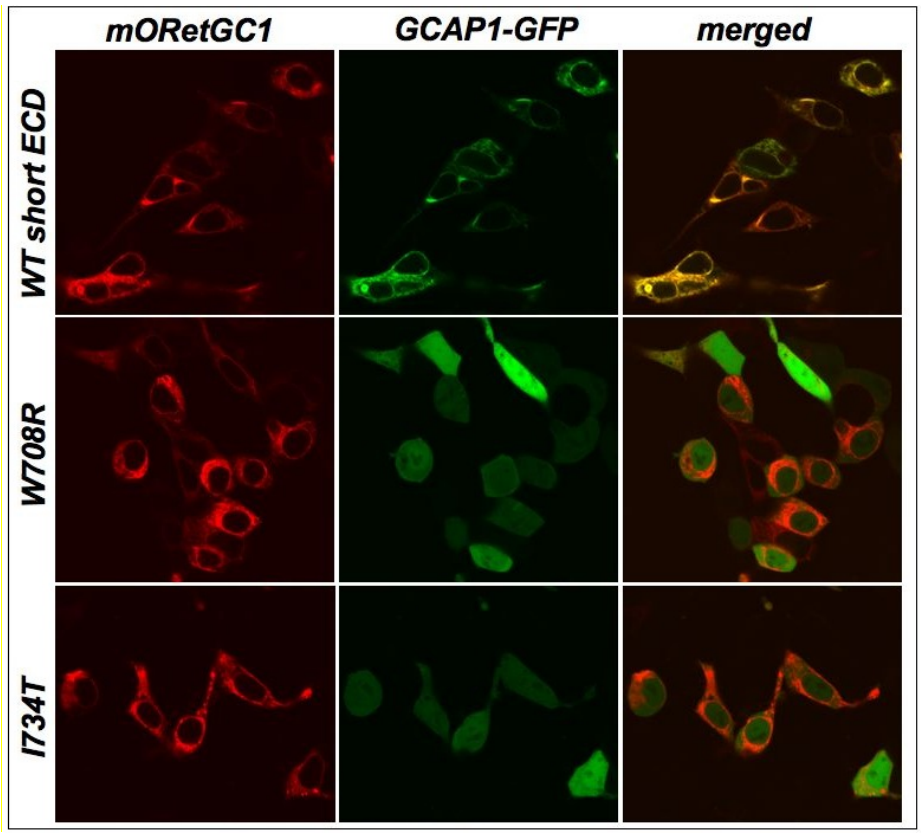

Figure 2. Co-expression of fluorescently labeled RetGC1 (coded by *GUCY2D* cDNA) and GCAP1. The extracellular segment of human GUCY2D was substituted with the mOrange red fluorescent protein tag positioned between the leader peptide and the transmembrane domain. Green fluorescent protein (GFP) tag was placed at the C-terminus of GCAP1. The mixture of mOrangeGUCY2D and GCAP1-GFP – coding plasmids was transfected into HEK293 cells using a Promega FuGene HD reagent. The confocal images were taken using Olympus FV1000 Spectral system. *Left to right*: mOrangeGUCY2D, GCAP1-GFP, both images merged. *Top to bottom*: mOrangeGucy2D containing wild type intracellular segment, mOrangeGucy2D containing W708R substitution in kinase homology domain, mOrangeGucy2D containing I734T substitution in kinase homology domain. While GCAP1 co-localizes with the wild type mOrangeGucy2D in membranes and, none of the LCA-linked mutations, W708R and I734T, is able to bind GCAP1-GFP, which makes GCAP1 uniformly spread through the cytoplasm and the nucleus.

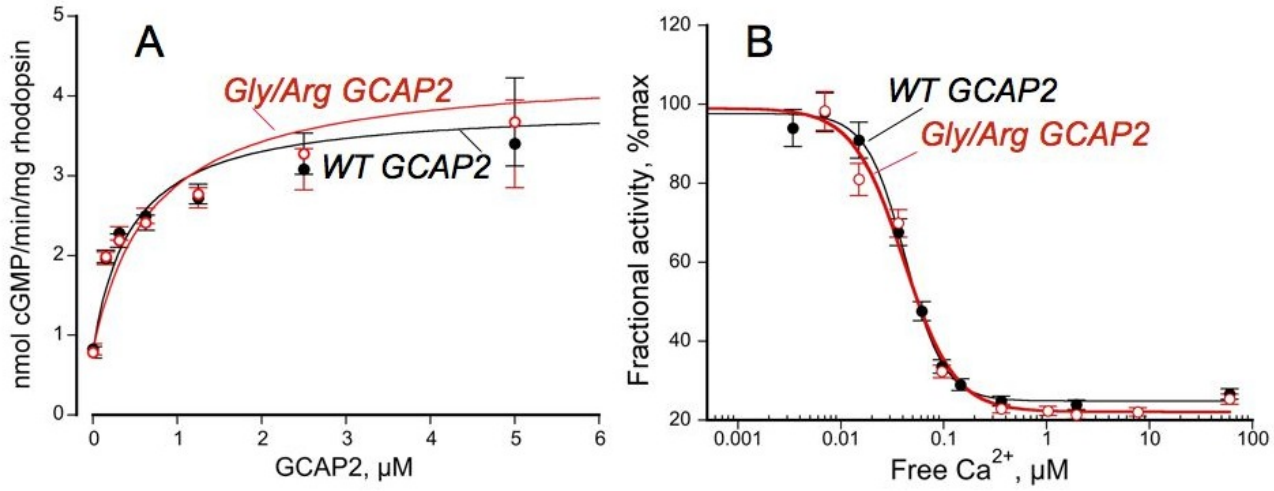

Figure 3. Regulatory properties of GCAP2 mimicking human G157R mutation. A: Dose dependence of guanylyl cyclase activation in native bovine rod outer segment (ROS). ROS membranes were isolated using density gradient centrifugation and hypotonically extracted to deplete the endogenous GCAPs. The washed membranes were reconstituted with wild type GCAP2 (black closed symbols) or GCAP2 harboring substitution Gly→Arg in the 4th position of EF-hand 4 calcium binding loop (red open symbols). The guanylyl cyclase activity was assayed under infrared illumination using [^{32}P-α]GTP as a substrate at zero free calcium concentration. B: Fractional activity of guanylyl cyclase in washed ROS membranes reconstituted with wild type (black) or mutant (red) GCAP2 in the presence of EGTA/Ca^{2+}/Mg^{2+} buffers varying free Ca^{2+} concentrations at a constant 1 mM free Mg^{2+}. The activity at each tested Ca^{2+} concentration was normalized per the maximal activity in each set. The mutation changed neither dose dependence nor Ca2+ sensitivity of cGMP synthesis by the cyclase in photoreceptor membranes.