Salus University

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – April 30, 2011

Formula Grant Overview

The Salus University received $59,678 in formula funds for the grant award period January 1, 2010 through April 30, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Mechanisms of Signal Transduction and Degenerative Diseases in the Retina – The general long-term purpose of this high-risk pilot project is to obtain new knowledge for the subsequent in-depth study of the intricate mechanisms triggering photoreceptor death. Human congenital retinal diseases causing blindness often arise from deregulated signal transduction mechanisms in rods and cones. Our main purpose is to evaluate how intracellular signal transduction messengers, cAMP and cGMP, contribute to retinal degeneration in a mammalian retina. The specific aim in the planned pilot study is to develop a new, presently unavailable, transgenic mouse model that would produce cAMP through a controlled light-sensitive mechanism in vivo. The outcome from this proof-of-concept project can then be used for leveraging funds to further expand the study on a larger scale.

Duration of Project

5/1/2010 – 4/30/2011

Project Overview

The broad objective of the study is to evaluate how different cyclic nucleotides contribute to the degenerative processes in retinal rods and cones. The leading cause for many types of congenital blindness, such as retinitis pigmentosa, Leber congenital amaurosis, dominant rod and cone dystrophies, is the death of photoreceptors triggered by mutations in the proteins that constitute normal signaling pathways. Deregulation of signal transduction in these cases affects intracellular concentrations of cyclic nucleotides, cAMP and cGMP. While a number of studies, including several in vivo and in vitro models reported by the PI, clearly confirm the involvement of an abnormal cGMP synthesis as a culprit in some of the human disease-causing mutations, the role of cAMP is much less clear, despite the indication of its possible involvement. There is biochemical evidence that cAMP concentrations change in some types of degenerating retinas, but the main question remains whether this is a feed-back response caused to mitigate the degenerative process or a part of the cell destruction mechanism itself. One of the main problems for the evaluation of the cAMP as the potential trigger of the photoreceptor cell death lies with

the complexity of the cAMP versus cGMP analysis in vivo. The cGMP synthesis is extremely powerful in rods and cones compared to that of cAMP, while the destruction of both nucleotides is sensitive to light and can be catalyzed by the same enzyme, PDE6. We reason that in order to specifically evaluate the possible role of cAMP in photoreceptor signaling and death, it is necessary to manipulate with the cAMP concentrations in living mouse photoreceptors without interference from the cGMP synthesis. Therefore, we plan in the specific aims of this project to express in mouse rods a mutated form of guanylyl cyclase turned into adenylate cyclase (RetGC1AC). In a genetic background lacking the normal endogenous photoreceptor guanylyl cyclase (RetGC), this would specifically allow for regulation of cAMP synthesis in rods by the same light- and calcium-sensitive mechanisms that would normally control cGMP synthesis.

Principal Investigator

Alexander M. Dizhoor, PhD
Professor
Salus University
8360 Old York Road
Elkins Park, PA 19027

Other Participating Researchers

Andrey Savchenko, PhD – employed by Salus University

Expected Research Outcomes and Benefits

At the end-point of the pilot study initiated with this high-risk application, we expect to produce a new genetic mouse model suitable for in-depth study of the intricate role of cyclic nucleotides disease-related retinal degenerative processes. The use of such a model should have a two-fold benefit: i) it may help to answer questions about the specific role of cAMP in photoreceptor signaling and death affected by mutations found in human patients; ii) it will likely create a new, presently unavailable, research tool for manipulating the cAMP concentrations in living photoreceptor neurons at will.

Summary of Research Completed

During the last year of the project, we accomplished the main portion of the research plan (the entire Aim 1 and most of the Aim 2): we constructed the transgenic vector, verified the biochemical phenotype of the cyclase mutant encoded by this construct in a series of transfection experiments that involved other mutants of the same cyclase as controls for specificity, injected the transgene in mouse eggs and selected 10 positive F0 founders out of 53 mice born from the injected eggs. We are currently already breeding them into RetGC1 knockout background, as planned. The purpose of the breeding in this background is to quantify the expression of the transgene in different lines and select the best expressing line(s) by immunoblotting for the subsequent in-depth study. The detailed description of the results follows.

i) Progress toward Aim. 1. DNA construct and adenylyl cyclase activity verification.

For the construction of RetGC1AC cDNA, we incorporated mutations originally described by Tucker et al., in 1999, E925K and C995D, into a human RetGC1 cDNA using a conventional "splicing by overlap extension" technique. The sequence of the mutated cDNA fragment was verified by automated DNA sequencing and then DNA was subcloned into the NarI/NheI restriction sites of the human RetGC1 cDNA-containing modified pRCCMV plasmid, thus replacing the portion of the wild type human RetGC1 coding for catalytic domain of the cyclase. The resultant RetGC1AC DNA in a commercial pRCCMV vector was verified by DNA sequence of the entire cDNA on both strands. We then subcloned the cDNA from that vector to assemble the transgene construct in a pBluescript vector placing RetGC1AC cDNA under control of a 4.2-kb rhodopsin promoter that we previously used for targeting protein expression to mouse rods. The only difference from the original plan was the use of polyadenylation signal – we chose to replace protamine 1 gene fragment by a standard BGH polyadenylation signal from a pQBI plasmid, as most of our expression verification experiments conducted in HEK293 cells utilized that control element. The construct was verified by restriction nuclease digest and sequencing, amplified in E. coli, purified, and the transgenic insert was excised from the plasmid by NotI/PvuI digest as shown in the Fig. 1, after which the transgene fragment was gel-purified for injection into mouse eggs.

Before we proceeded with the injections, we needed to further verify the biochemical phenotype of the RetGC1AC mutant in our hands. The same cDNA fragment coding for RetGC1AC was expressed from pRCCMV vector under control of a CMV promoter using a Promega Profection protocol for transfection. The expression of the modified cyclase was confirmed by immunoblotting and the RetGC1AC activity was then assayed in membrane fraction of HEK293 cells with [α-^{32}P]ATP as a substrate instead of [α-^{32}P]GTP that we normally use for wild type RetGC assays. The products of the reaction were separated by thin-layer chromatography on fluorescent plastic-backed polyethyleneimine cellulose plates and [^{32}P]cAMP was quantified from the excised spots on chromatogram by radioactive scintillation counting (Fig. 2).

We have confirmed that the mutant RetGC1AC encoded by the transgene construct indeed demonstrated specific guanylyl cyclase activating protein (GCAP)-dependent stimulation of cAMP synthesis instead of cGMP. The assay was conducted in the presence of EGTA (0 free Ca2+) at 10 mM free Mg2+ concentrations, conditions optimized previously for studying wild type recombinant RetGC activation by GCAPs. While there was little adenylyl cyclase activity detected in the absence of GCAP1, in the presence of 10 µM GCAP1 cAMP activity in the same membrane preparation was sharply increased and reached over 7 pmol cAMP/mg protein/min (Fig. 2, panel a). We did not observe this effect in several control preparations expressing RetGC1AC (panels b-d): when the catalytic activity of the recombinant cyclase was inactivated by additional mutation that blocked Mg^{2+} binding in the active site, which completely prevents the binding of the Mg/ATP as a substrate (Fig. 2b); when RetGC1AC contained a mutation, D639Y (Fig. 2 c) which blocks GCAP1 binding to RetGC1; when we expressed wild type RetGC1, Fig. 2 d (the wild type RetGC possesses only guanylyl cyclase activity and does not utilize ATP as a substrate). Making these additional mutants required additional effort, but we thus ensured that the transgene we were going to deliver to the retina would impart the exact activity we expected. We can rely on the results shown in Fig. 2 that the RetGC1AC in the transgenic construct would impart to mouse rods the unique ability to accelerate cAMP synthesis

in the presence of GCAP, which would normally regulate only cGMP synthesis in photoreceptors.

ii) Progress toward Aim 2. Production of transgenic mice.

From the eggs injected with the purified transgene fragment, there were 53 mice born, which we screened by polymerase chain reaction (PCR) of their tail DNA samples using specific primers designed for the unique human RetGC1 DNA sequence, not present in mouse RetGC1 gene (in addition to that, the transgene is a cDNA, without multiple introns present in the endogenous RetGC1 gene). The specificity of the screening is shown in Fig. 3 (upper panel), where littermates either did or did not carry the transgene. We have identified 11 positive mice (one is not shown in Fig. 3), which we consider a reasonably confident number for selection of proper expressor line. However, in order to compare the expressing lines by immunoblot of retinal proteins, we need first to transfer the transgene into a genetic background lacking the endogenous RetGC1 expression. We received such a line from Dr. Garber's laboratory in U. of Texas several years ago and have maintained it since that time in our vivarium at Salus University. We already set up the breeding of the F0 RetGC1AC mice with the RetGC1 knockout line for initial comparison of the transgene expression levels.

FIGURES (see following pages)

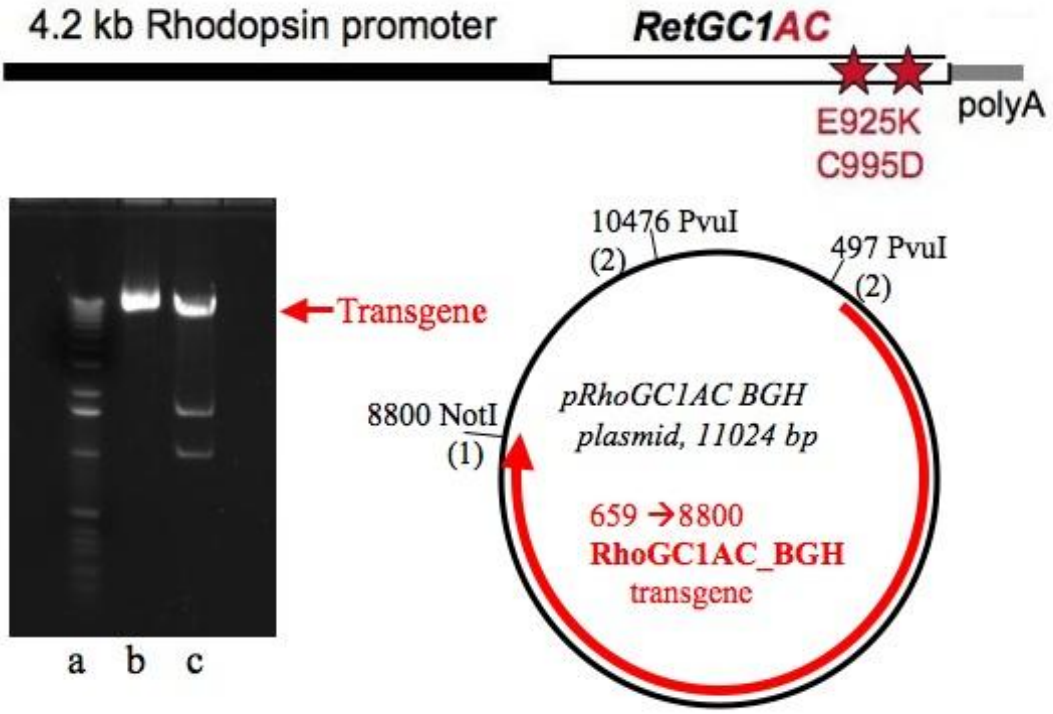

Fig. 1. The schematics of the transgene construct for RetGC1AC expression in mouse rods. The transgenic construct containing a 4.2 kb 5'-upstream fragment of mouse rod opsin gene (Rho), 3-kb RetGC1AC cDNA, and BGH polyadenylation fragment was assembled in a modified pBluescript vector and excised using NotI/PvuI digest releasing 8.2 transgene insert (gel: a – 1-kb ladder marker; b- uncut plasmid; c – digested plasmid). The insert purified from gel was injected in fertilized mouse eggs.

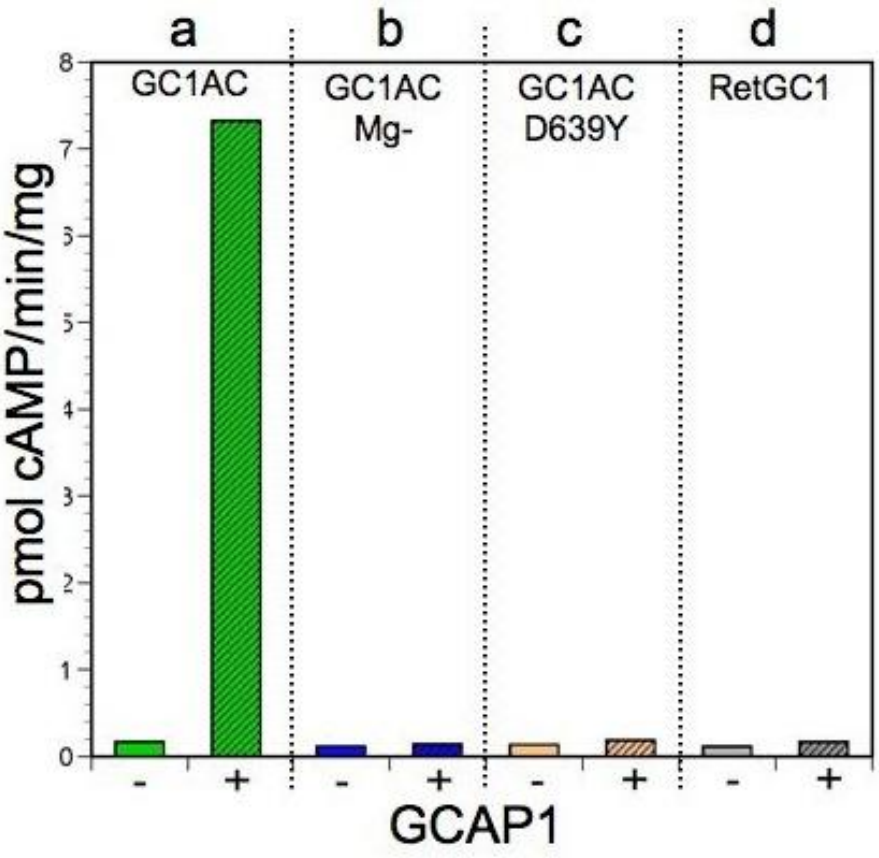

Fig. 2. RetGC1 mutants expressed in HEK 293 cells were assayed in the presence of 2 mM EGTA and 10 mM $MgCl_2$ in the absence (-) or in the presence (+) of 10 µM purified recombinant GCAP1; a- RetGC1AC, b-RetGC1AC with the Mg2+-binding site of the catalytic domain inactivated, c – RetGC1AC with the GCAP1 binding domain inactivated, d - wild type RetGC1. The assay was conducted using radiolabeled ATP as a substrate.

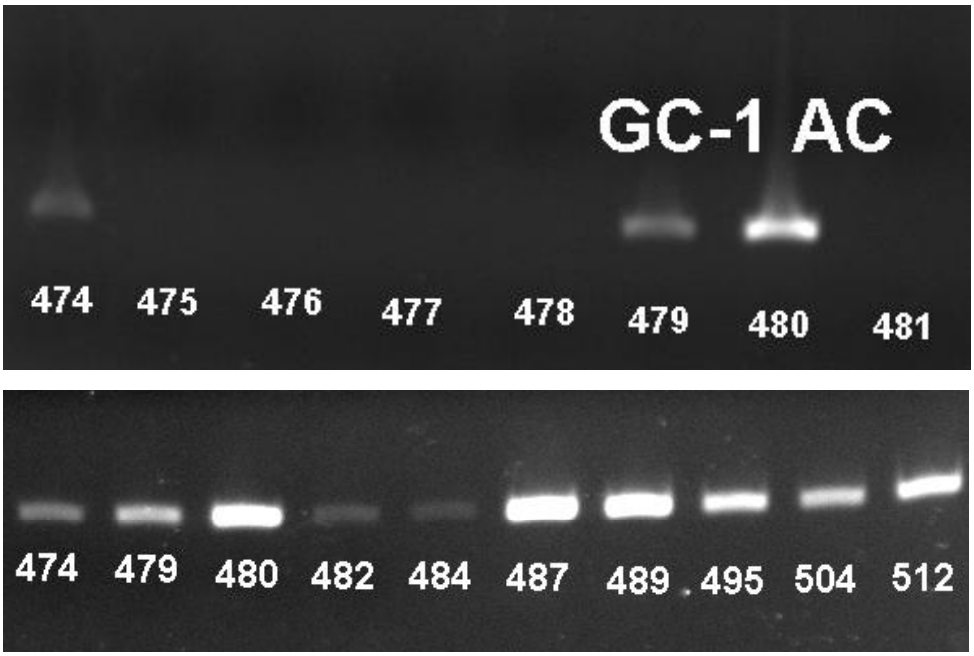

Fig. 3. PCR detection of the RetGC1AC transgene in mouse tail DNA samples screened by PCR using primers specific for the human RetGC1AC mutant. Top panel: an example of the transgene detection in a single litter. Bottom panel: re-testing of all positives selected from screening of 52 F0 mice.