Salus University

Annual Progress Report: 2010 Formula Grant

Reporting Period

July 1, 2011 – April 30, 2012

Formula Grant Overview

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.

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/Clrfl36 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

The purpose of this study was to test the role the of RD3 in regulation of one of the key enzymes in photoreceptor signal transduction and survival – guanylyl cyclase – and the effects of mutations found in patients with LCA12 and other retinal disorders. This research project developed according to the original plan and all specific Aims have been completed as follows.

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

Aim 1 has been accomplished. We have completed characterization of RD3 effects on enzymatic and regulatory activities of retinal guanylyl cyclase isozymes 1 and 2 (RetGC1 and RetGC2, respectively) and published the main portion of the findings. We have demonstrated that RD3 inhibits both RetGC isozymes isolated from native mouse retinas (Peshenko et al., 2011a) in a complex fashion. The effect of RD3 on RetGC regulation is dual: (i) it reduces the catalytic activity of the enzyme as a non-competitive inhibitor, (ii) it also competes with guanylyl cyclase...
activating proteins 1 and 2 (GCAP1 and GCAP2), thus eliminating them from their complex with the native RetGC1 and RetGC2 isozymes in photoreceptor membranes as well as with recombinant RetGC (Peshenko et al., 2011b) (Fig.1 and 2).

We have determined that when the two proteins are co-expressed in HEK293 cells, RD3 is able to associate with wild type RetGC1 but fails to associate with RetGC affected by mutations found in patients with LCA type 1 described in previous publication (Peshenko et al., 2010) (Fig. 3).

Based on our findings, we formulated a new hypothesis, that RD3 is required for blocking the activity of RetGC1 in the inner segment in order to prevent premature activation of cGMP synthesis in a wrong compartment of the cell. These findings were published in Biochemistry (Peshenko et al., 2011b)

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.

Aim 2 has also been accomplished. We expressed in E. coli BLR (DE3) strain and purified the following recombinant forms of RD3 protein reported in patients with congenital visual disorders. All these mutations -Trp6Arg/Glu23Asp, Gly35Arg, Gly57Val, Arg68Trp, Phe100ter, and Lys130Met, have been tested for their ability to suppress activation of retinal photoreceptor guanylyl cyclase (RetGC1) by GCAP1 (Fig. 4). We found that Phe100ter mutation co-segregated with LCA12 completely eliminates the RD3 ability to block interaction of RetGC1 with GCAP1, while three other mutations, Arg68Trp, Gly57Val, and Lys130Met, significantly decrease that ability. The results have been published (Peshenko et al., 2011b).

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.

We accomplished Aim 3 as well. We put together constructs for expression of RD3 in vivo in transgenic animals: Rho-Rd3GFP-mP1 and Rho-Rd3-mP1, containing a 4.2-kb rod opsin gene upstream fragment containing rod-specific promoter, linked to coding sequence of human RD3, with or without encoded C-terminal GFP, and followed by a fragment of the last exon of a mouse protamine 1 gene containing a polyadenylation signal. These constructs are now ready for delivery as transgenes to compensate for the truncated RD3 form in rd3/rd3 mice. The rd3/rd3 mouse line in a pigmented background has been maintained in Salus vivarium.
Figure 1. RD3 inhibits RetGC catalytic activity at submicromolar concentrations. A. Wild type mouse rod outer segment membranes were titrated with human recombinant RD3 in the presence of EGTA. *Inset*, Coomassie - stained gel of human RD3 isolated from *E. coli* (*left lane*) and molecular weight standards (*right lane*). B. Human recombinant RetGC1 activated by 1.5 µM GCAP1 in the presence of EGTA was assayed at different concentrations of the human recombinant RD3 expressed and purified from *E. coli*. C. Human recombinant RetGC1 was activated by 1.5 µM bovine GCAP1 in the presence of EGTA and titrated with protein extracts from HEK293 cells either expressing or not expressing RD3. *Inset*, immunoblotting of HEK293 cell extracts probed with anti-RD3 antibody 497, *left lane* - non-transfected cells; *right lane* – RD3 plasmid-transfected cells (notice that there is no endogenous RD3 expression in the non-transfected cells). The data in A-D are fitted by the equation, \( a = (a_{\text{max}} - a_{\text{min}})/(1+[\text{RD3}]/IC_{50}) + a_{\text{min}} \); where \( a_{\text{max}} \) and \( a_{\text{min}} \) are the maximal and minimal activity of guanylyl cyclase in the experiment, respectively, and the \( IC_{50} \) is the concentration of RD3 producing 50% inhibition. D-F. The effect of RD3 on guanylyl cyclase catalytic activity in ROS fractions measured in the absence of GCAPs. The RetGC activity in ROS fraction from GCAP1,2 -/ mouse retinas was titrated with the purified *E. coli*-expressed RD3 in the presence of EGTA. Michaelis plot of the non-stimulated RetGC catalytic activity in the absence (●) or in the presence of 30 nM (■) or 60 nM (▲) purified recombinant RD3. F. Lineweaver-Burke plot for data from panel E illustrates suppression of the \( V_{\text{max}} \) by RD3 without a major effect on \( K_{\text{M,GTP}} \). The \( K_{i} \), calculated using the equation for a noncompetitive inhibition, \( V_{i}=V_{\text{max}}(1+[\text{RD3}]/K_{i})^{-1} \), from three independent experiments was 19 nM ± 7 SD. The activity in assays containing retinal membranes is presented per rhodopsin content, in membranes expressing recombinant RetGC it is normalized by the maximal activity for each series of the membrane preparations (data from: Peshenko et al., 2011b).
Figure 2. RD3 inhibits RetGC activation through competition with GCAPs. A. The guanylyl cyclase activity in HEK293 homogenates containing RetGC1 expressed alone (●) or co-expressed with human RD3 (▲) was assayed in the presence of added recombinant GCAP1. The data were fitted assuming Michaelis hyperbolic function, $a=a_{\text{max}}[\text{GCAP}]/(K_{1/2}+(\text{GCAP}))$; equalized by RetGC1 content in both samples. B. Immunoblotting. The cells expressing RetGC1, either alone or co-transfected with RD3, from panel A were probed with anti-RetGC1 (left) or anti-RD3 polyclonal antibody 497 (right). Non-transfected HEK293 cells (leftmost in each panel) were used as a specificity control. C-F. Competition of RD3 with GCAP1 and GCAP2 in RetGC assay. C, D. RetGC1 expressed in HEK293 cells was activated by purified GCAP1 (C) or GCAP2 (D) in the absence (●) or in the presence of 3 nM RD3 (▲) or 9 nM RD3 (■). The data were fitted by Michaelis hyperbolic function. Maximal RetGC1 activity ($a_{\text{max}}$, mean ± SD) at 0 nm, 3 nM or 9 nM RD3 was 4.8 ± 0.13, 4.8 ± 0.11, and 4.6 ± 0.16 nmol/min/mg protein, respectively, when activated by GCAP1 and 2.1 ± 0.05, 2.6 ± 0.2 and 2.7 ± 0.1 nmol/min/mg when activated by GCAP2. The respective concentrations of GCAP producing half-maximal activation ($K_{1/2}$) were 1.1 ± 0.12, 4.1 ± 0.6, and 7.5 ± 0.5 µM (GCAP1) and 5.9 ± 0.6, 19 ± 2.6, and 36 ± 1.5 µM (GCAP2).
Figure 3. RD3 expressed in HEK293 cells co-localizes with wild type RetGC1 but fails to form a complex with RetGC mutants affected by mutations found in LCA patients (Peshenko et al., 2010). Left column, RD3-GFP was co-expressed in HEK293 cells with wild type RetGC1 and either D639Y or R768W RetGC1 mutants (Peshenko et al., 2010); notice that the GFP fluorescence has membrane-associated pattern in the presence of WT RetGC1, but spreads throughout the cell when co-expressed with D639Y and R768W mutants. Right three columns, non-tagged RD3 was co-expressed with RetGC and its mutants as described above, and the cells were stained with anti-RD3 (green) and anti-RetGC1 (red) antibody; RD3 co-localizes with the WT RetGC1, but not with the LCA-related mutants.

Figure 4. Effect of mutations in RD3 found in human patients with retinal diseases on RetGC activity in vitro. Inhibition of RetGC1 activity by the E. coli - expressed RD3 mutants. RetGC activity in independent assays was normalized per activity measured in the absence of RD3. Recombinant RetGC1 expressed in HEK293 cells was reconstituted with 1.5 µM GCAP1 at indicated concentrations of RD3 (○) or its mutants: W6R/E23D (●), G35R (▲), G57V (□), R68W (■), K130M (▲), and F100ter (●). The IC_{50} values (mean ± SE, n) for the inhibition were 4.6 ± 1.3 nM, 5 (WT); 6.4 ± 2 nM, 3 (W6R/E23D); 7.9 ± 1 nM, 3 (G35R); 68 ± 13 nM, 3 (G57V); 17.3 ± 3.8 nM, 3 (R68W); and 14.3 ± 2.4 nM, 3 (K130M); the IC_{50} for the F100ter was >>10 µM. The difference in the IC_{50} values from the wild type was significant (P in unpaired Student t-test assuming equal variance) for the G57V (<0.001), F100ter (<0.0001), R68W (0.012), and K130M (0.014) mutants (see Peshenko et al., 2011b, publication for other details).
PUBLICATIONS

One new paper was published: