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Cardiac Protein RGS2: Structural and Functional Determinants

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CARDIAC PROTEIN RGS2: STRUCTURAL AND FUNCTIONAL DETERMINANTS

by

Stephanie Jan Chambers

Thesis submitted in partial fulfillment of the requirements for the degree

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Approved:

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Logan, UT

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CARDIAC PROTEIN RGS2: STRUCTURAL AND FUNCTIONAL DETERMINANTS

Advisor—Dr. Brett Adams, Department of Biology, Utah State University

INTRODUCTION:

In this study, I explored the structure-function relationships of a cardiac RGS (regulator of G-protein signaling) protein, RGS2.

Contractions of the heart are triggered by calcium ions (Ca) entering heart cells through L-type Ca channels (1a). The activity of L-type channels (and thereby Ca influx) is modulated by cell surface receptors that couple to intracellular G proteins. G proteins in turn, are regulated by a newly-discovered family of proteins called RGS. RGS proteins speed the hydrolysis of GTP by G proteins, and thereby control the strength and duration of G protein-mediated signals. Stimulation of G proteins, which are coupled to receptors, can alter the rate of heart contractions (2).

Contraction rate of heart cells is important for several reasons. Namely, a slower, controlled contraction rate, or heartbeat, pumps oxygenated blood throughout the body more efficiently than a fast contraction rate. It has been shown that inadequate blood flow through the heart is directly correlated with deficiency of calcium channel stimulation (1b). Several RGS proteins are expressed in heart, but their physiological significance and ability to affect influx of calcium ions through calcium channels has been largely unexplored. This study reveals new insights into the structure/function relationships of RGS2. These insights may be helpful in understanding the molecular basis of certain cardiovascular diseases.

DETAILED EXPLANATION:

Proteins in the RGS2 family are composed of a highly conserved core region and variable terminal regions (3). My major objective was to determine the functional importance of the RGS2 non-core regions (N-terminus and C-terminus). I used the polymerase chain reaction (PCR) to construct mutant RGS2 proteins lacking the amino-terminus, the carboxyl-terminus, or both. These mutant RGS2 proteins were expressed in a cell line and their abilities to influence L-type Ca channel activity were determined via electrophysiological experiments. RGS proteins speed up the rate of GTP hydrolysis by 100-fold, the inhibition of extracellular calcium should decrease the rate of heart contraction. Thus, we expected their presence to inhibit the influx of extracellular calcium into the cell.

Cell culture methods were employed to maintain mammalian HEK293 cells for transfection with normal RGS2 and RGS2 core. Cardiac calcium current was measured via patch-clamp recording. Patch-clamp recording was used to measure whole-cell Ca channel currents and current inhibition during activation of coexpressed M1 muscarinic acetylcholine receptors. Through the use of specifically designed oligonucleotide primers, we amplified only the core region of RGS2. The primers contained restriction enzyme sites to facilitate insertion of the RGS2 core into an expression vector. The PCR product was gel purified and digested with restriction enzymes corresponding to the primer enzyme sites. The digested PCR product was then ligated into the bicistronic mammalian expression vector pIRES-2, which independently expresses green fluorescent protein as a transfection marker. Alternatively, transfected cells were identified by cotransfection with an expression plasmid encoding the cell surface antigen CD8. We found that RGS2 prevents channel inhibition by M1 receptors, and that the RGS2 core

does not prevent channel inhibition. Thus, structural regions flanking RGS2 core are required for proper function of RGS2. The functional importance of other structural regions of RGS2 are presently being analyzed.

REFERENCES:

1. Krukenkamp IB, Levitsky S. *Physiological and Cellular Mechanisms of Myocardial Protection*. Am. J. of Surg.; 172: 79-84.
2. Neer, EJ. *Heterotrimeric G Proteins: Organizers of Transmembrane Signals*. Cell 1995; 80:249-257.
3. Berman DM, Gilman AG. *Mammalian RGS Proteins: Barbarians at the Gate*. J. Biol. Chem. 1998; 273(3): 1269-1272.
4. Melliti K, Meza U, Fisher R, Adams B. *Regulators of G protein signaling (RGS) proteins attenuate the G protein-mediated inhibition of N-type Ca channels*. J. Gen. Physiol. 1999; 113:97-109.

OTHER IMPORTANT WORK WITH DR. BRETT ADAMS:

From a summer research program during high school, I was able to gain a familiarity with basic lab procedures. This allowed me to begin meaningful work as an assistant in various projects once I began in Dr. Adams' lab as a freshman at Utah State University. My work included animal dissection of adult and neonatal organs (i.e., heart ventricle and dorsal root ganglia) and establishing primary cell cultures from these organs. My work also entailed various basic procedures such as purifying DNA, growing bacterial cultures, etc.

PROTEIN II TRANSCRIPTION FACTOR OF APOLIPOPROTEIN B

Advisor—Dr. Joan Hevel, Department of Chemistry & Biochemistry, Utah State University

INTRODUCTION:

Protein involvement in human disease is on the forefront of proteomic research. Proteins are involved in a multitude of mammalian cellular processes. Structure-function relationships govern the responsibilities of both structural and enzymatic proteins. Advancement in proteomic research over the past five years has enabled researchers to begin ascribing function to the extensive array of human proteins. Understanding these functions is critical to determining variation in protein function when the cellular environment is in a diseased, stressed, or cancerous state. It is especially important to determine what causes proteins themselves to trigger disease.

Medical researchers are now implicating specific proteins in leading to cardiovascular disease (CVD) (1). CVD has become an umbrella-term for the myriad of problems that develop in the vascular system due to its domino-effect of decreasing the efficiency of other organs. This makes treatment very difficult as the disease progresses and is one reason CVD has become an epidemic in the United States. Cholesterol deposition is one main cause of CVD in humans. LDL (Low-Density Lipoprotein) is one of the main components involved in causing cholesterol deposition. My interest has been in studying LDL, whose main component is ApoB (Apoliprotein B). Transcription of ApoB takes place when transcription factors bind to DNA encoding the specific gene. These transcription factors are protein molecules which possess consensus sequences for binding to DNA. It is hoped that understanding protein-DNA interactions that trigger synthesis of harmful molecules will lead to measures of therapeutic intervention. Because ApoB can only be synthesized after the action of sequence-specific DNA-binding proteins, my specific goal has been to identify the second of three binding proteins necessary for transcription of ApoB. (The first and third binding proteins are already known.) This project is being currently being pursued under the supervision of Dr. Joan Hevel, Department of Chemistry and Biochemistry, Utah State University. Hopefully, therapeutic strategies may be developed in response to present research in proteomics.

DETAILED EXPLANATION:

DLD (Low-Density Lipoprotein) transports cholesterol in the blood; high levels of blood LDL are correlated to risk of heart disease (2). LDL's lipid/protein structure contains a major component—Apolipoprotein B (ApoB). Because ApoB is a precursor to LDL synthesis, it is confirmed that blood levels of ApoB are a better predictor of cardiovascular disease (CVD) risk than LDL blood levels (for which millions of Americans are currently screened every year) (3).

Production of ApoB requires transcription of the ApoB gene. Transcription, like many biological processes, involves the action of sequence-specific DNA-binding proteins. In the case of ApoB, there are three binding proteins; two known (HNF 1-alpha, C/EBP) and one unknown (called "Protein II"). The binding sites for these three proteins are located on the core enhancer of the ApoB gene. Essentially, these proteins act as gatekeepers to beginning the synthesis of ApoB. They must be bound to their respective binding sites on the enhancer for transcription of ApoB to occur. It has been shown that binding HNF-1-alpha is necessary for full activity of the ApoB enhancer in HepG2 cells; when HNF 1-alpha binding does not occur, activity of the core enhancer decreases by 80% (4). It was also shown that binding of C/EBP is necessary for full

activity of the enhancer. Mutagenesis of HNF-1 alpha and/or C/EBP binding sites shows that binding of both these proteins is essential for full ApoB gene expression (5). The third component necessary for full activity of the core enhancer, Protein II, remains relatively unexplored. Disruption of Protein II binding also diminishes the capacity of enhancer activity. Although work has been done to identify both proteins HNF-1alpha and C/EBP, the identity of Protein II is yet to be discovered. Currently, my work seeks to 1) design an affinity resin based on the sequence that binds Protein II (via DNA affinity chromatography); 2) purify protein II using the designed affinity resin; and, 3) characterize Protein II by mass spectrometry to discern the identity and perhaps the specific function of Protein II in enhancing transcription of ApoB. Using the DNA sequence of the Protein II binding site, we will design a sequence-based affinity resin using an established method (6). The designed affinity resin will allow us to isolate Protein II from a mixture of several proteins in HepG2 cells. Protein II can then be enzymatically digested to obtain its peptide components and characterized using mass spectrometry. Data from the mass spectral analysis will be used to perform database searchers to identify Protein II. After the identity of Protein II is known, we will classify the functional regions of the protein in order to discern how its binding to the enhancer region of ApoB increases transcription of ApoB. Because transcription of the ApoB gene s regulated in a tissue-specific manner, this project will also provide insights into the regulation of tissue-specific gene expression.

My results will increase understanding of structure-function relationships in order to eventually control synthesis of ApoB through the use of specifically designed antagonist molecules. These results will also increase our understanding of how levels of LDL are regulated in the body, leading to better understanding of what causes proteins to initiate synthesis of harmful molecules.

Through continued research into protein biochemistry, I hope to contribute to our current knowledge of cardiovascular health.

REFERENCES:

1. Munter P., L.L. Hamm, J.W. Kusek, J. Chen, P.K. Whelton, J. Hed. The Prevalence of Non-traditional Risk Factors for Coronary Heart Disease In Patients.
2. Ross, J.L., M.A. Manuszak, J.E. Wachs. Identification and Management of Cardiovascular Disease Risk: Beyond Low-Density Lipoprotein Cholesterol. American Association of Occupational Health Nurses. 2003. 51(12):521-31.
3. Williams, K., A.D. Sniderman, N. Satter, R. Jr. K'Agostino, L.E. Wagenknecht, S.M. Haffner. Comparison of the Associations of Apolipoprotein B and Low-Density Lipoprotein in Cholesterol With Other Cardiovascular Risk Factors In The Insulin Resistance Atherosclerosis Study (IRAS). Circulation. 2003. 108(19):2132-2316.
4. Brooks, A.R., B.P. Nagy, S. Taylor, W.S. Simonet, J.M. Taylor, B. Levy-Wilson. Sequences Containing the Second-Intron Enhancer Are Essential for Transcription of the Human Apolipoprotein B Gene in the Livers of Transgenic Mice. Molecular and Cellular Biology. 1994. 12(4):2243-2256.
5. Brooks, A.R., B. Levy-Wilson. Hepatocyte Nuclear Factor 1 and C/EBP are Essential for Human Apolipoprotein B in the Activity of Second Intron Enhancer. Molecular and Cellular Biology. 1992. 12(3):1134-1148.
6. Kadonaga, J.T. Purification of Sequence-Specific Binding Proteins by DNA Affinity Chromatography. Methods in Enzymology. 1991. 208:10-23.

OTHER IMPORTANT WORK WITH DR. JOAN HEVEL:

I have established a room for tissue culture within our research facility. My work has involved each step of the process—from design layout and ordering equipment to the actual culturing of cells. This working facility will soon serve five researchers in projects involving A549 lung cells in tissue culture experiments.

Recently, my work with Dr. Hevel has explored the role of PRMT6 (Protein Arginine Methylation) in apoptosis induction via RNAi experiments. This work is detailed in the following poster presentation (Student Showcase 2005).

Cardiac RGS Protein Structural Determinants and Function

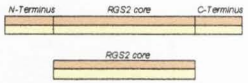
Stephanie Chambers, Roger Bannister and Brett Adams
Department of Biology, Utah State University, Logan, UT 84322

Introduction

Contractions of the heart are triggered by calcium ions (Ca^{2+}) entering heart cells through L-type Ca channels. The activity of L-type channels (and thereby Ca influx) is modulated by cell surface receptors that couple to intracellular molecular switches known as "G proteins" (for GTP-binding protein). G proteins, in turn, are regulated by a newly-discovered family of proteins called RGS (for regulator of G protein signaling). RGS proteins speed the hydrolysis of GTP by G proteins, and thereby control the strength and duration of G protein-mediated signals. Several RGS proteins are expressed in heart, but their physiological significance is currently unknown. In the present study, we explored the structure/function relationships of a cardiac RGS protein, RGS2. We used the polymerase chain reaction (PCR) to construct mutant RGS2 proteins lacking the amino-terminus, the carboxyl-terminus, or both. These mutant RGS2 proteins were expressed in a cell line and their abilities to influence L-type Ca channel activity were determined in electrophysiological experiments. This study reveals new insights into the structure/function relationships of RGS2. These insights may be helpful in understanding the molecular basis of certain cardiovascular diseases.

Objective

To determine the functional importance of the RGS2 non-core regions (N-terminus and C-terminus)



Materials & Methods

- Oligonucleotide primers were designed to amplify only the core region of RGS2. The primers contained restriction enzyme sites to facilitate insertion of the RGS2 core into an expression vector.

- The polymerase chain reaction (PCR) was used to amplify the RGS2 core region.

- The PCR product was gel purified and digested with restriction enzymes corresponding to the primer enzyme sites. The digested PCR product was then ligated into the bicistronic mammalian expression vector pIRES-2 (Clontech), which independently expresses green fluorescent protein as a transfection marker. Alternatively, transfected cells were identified by cotransfection with an expression plasmid encoding the cell surface antigen CD8 (e.g. Fig. 4)

- Cell culture methods were employed to maintain mammalian HEK293 cells for transfection with RGS2 and RGS2 core.

- Patch-clamp recording was used to measure whole-cell Ca channel currents and current inhibition during activation of coexpressed M1 muscarinic acetylcholine receptors.

Figure 1. Polymerase Chain Reaction

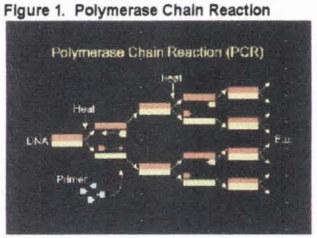


Figure 2. Gel Purification of PCR product

PCR cycle parameters:

Each of 30 cycles consisted of 1 minute at 95 °C to allow separation of DNA strands, followed by 1 minute at 55 °C to allow primers to anneal to template, followed by 1 minute at 72 °C to allow extension of new DNA strands.

Gel conditions:

0.7% agarose gel with ethidium bromide staining was used to confirm that the PCR product was the expected length. A standard DNA ladder composed of DNA fragments of known length (far right) was used for comparison with the PCR product (middle).

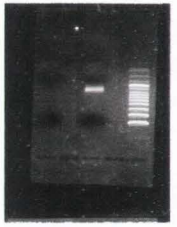


Figure 3. G protein cycle & signaling

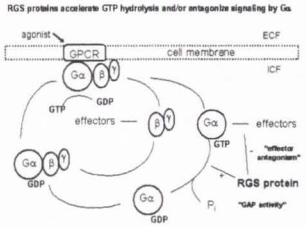
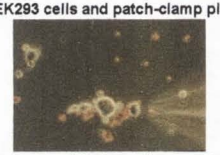
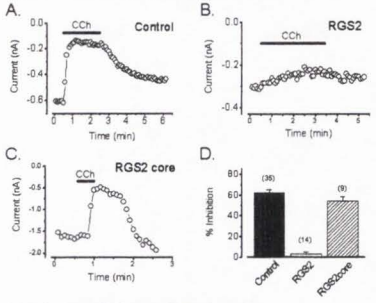


Figure 4. HEK293 cells and patch-clamp pipette



4 micron beads (brown) are coated with anti-CD8 antibody and stick to cells expressing cell-surface CD8 protein as a transfection marker.

Figure 5. Electrophysiological Results



Conclusions & Future Experiments

- RGS2 prevents channel inhibition by M1 receptors.
- RGS2 core does not prevent channel inhibition.
- Thus, structural regions flanking RGS2 core are required for proper function of RGS2.
- The functional importance of other structural regions of RGS2 are presently being analyzed.

Reference

Powledge, TM. Faseb. http://www.faseb.org/opar/bloodsupply/pcr.html

Utah State University

Biochemistry Classifieds: Stealthy RNAi Seeking Attractive PRMT6

Stephanie J. Chambers, Department of Biology, Utah State University
Joan M. Hevel, Department of Chemistry & Biochemistry, Utah State University

Introduction

The central dogma of science (one gene encodes information for one protein, one protein performs one function) is changing as we clarify the role of post-translational modifications (PTMs). PTMs are changes to protein structure after assembly by the ribosome. PTMs allow an organism to expand its functional proteome; i.e., create more function from a single gene. PTM-dependent change in protein function can dictate molecular messages in cellular signaling pathways. One class of enzymes that performs PTMs is PRMT (protein arginine methyltransferase), which catalyzes the methylation of specific peptidyl arginine residues in select proteins. Our laboratory is currently studying the methylarginine proteome of human A549 lung epithelial cells in order to understand how each of the 8 human PRMTs may be involved in cellular communication.

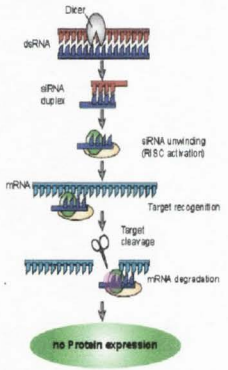
Objective

Knock-down expression of PRMT6 protein using RNA interference in human lung A549 epithelial cells. Cell lysates will be assessed for a) the presence of PRMT6 mRNA, b) PRMT6 protein and c) any altered change in the pattern of protein arginine methylation. We hope to identify which proteins are methylated by PRMT6 by comparing the methylarginine proteome of control and RNAi-treated cells.

Methods

In our research, we performed an RNAi (ribonucleic acid interference) experiment to knock-down PRMT6 expression in human A549 epithelial lung cells (See Figure 3). Introduction of double-stranded RNA (red/blue strand) into cells can suppress gene expression by targeting specific mRNA sequences for degradation. The general mechanism by which this occurs is shown in Figure 1 at left.

Figure 1. RNAi Mechanism



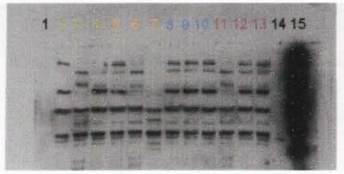
http://www.bioteach.ubc.ca/MolecularBiology/AntisenseRNA/siRNA.gif

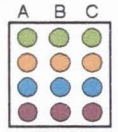
StealthRNAi molecules (Invitrogen) were designed against PRMT6 based on previous protocols and use of Invitrogen's on-line design program. Lipofectamine 2000 was used as a vehicle to suspend the RNAi molecules and to aid in the uptake of DNA into the cells. Antibodies to PRMT6 were purchased from Immunigenex. Human A549 lung epithelial cells were cultured in a 12-well plate. Cells at 50-60% confluency were treated with either media, Lipofectamine 2000, Stealth RNAi or a scrambled Stealth RNAi molecule as a negative control as depicted in Figure 2. Cells were harvested after 72 hours of treatment, a soluble protein lysate prepared, and the presence of PRMT6 assessed by Western analysis.

Results

Figure 2. Western Blot Results from 12-Well Experiment

Probed with anti-PRMT6

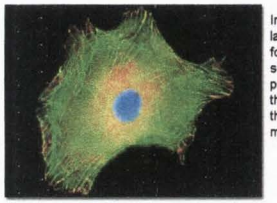




12-Well Plate

1. **Empty Lane**
2. Control A – Media Only
3. Control B – Media Only
4. Control C – Media Only
5. Vehicle A – Media + RNA Vehicle
6. Vehicle B – Media + RNA Vehicle
7. Vehicle C – Media + RNA Vehicle
8. RNAi A – Media + RNA Vehicle + RNAi
9. RNAi B – Media + RNA Vehicle + RNAi
10. RNAi C – Media + RNA Vehicle + RNAi
11. Scrambled A – Media + RNA Vehicle + Scrambled RNAi
12. Scrambled B – Media + RNA Vehicle + Scrambled RNAi
13. Scrambled C – Media + RNA Vehicle + Scrambled RNAi
14. **Control D – Cytoplasmic lysate**
15. **Marker**

Figure 3. Human Lung Carcinoma A549 Cell



Immunofluorescent labeling shows cell focal adhesion sites (green), protein complexes that anchor cells to the extracellular matrix.

micro.magnet.fsu.edu/

Results & Conclusions

- Given the current conditions, PRMT6 protein levels did not appear to change in A549 cells.

Future Experiments

- Asses the ability of the current conditions to decrease levels of PRMT6 mRNA.
- Optimize conditions for effective PRMT6 protein knock-down.
- Assess changes in methylation patterns in the cell after RNAi knockdown experiments.
- Assess phenotypic changes of PRMT6 knockdowns.

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