EFFECT OF DISTINCT REGULATOR OF G-PROTEIN SIGNALING 10 ISOFORMS ON CYTOKINE PRODUCTION.

by

BENJAMIN JACKWOOD

(Under the Direction of Shelley Hooks)

ABSTRACT

G-protein coupled receptors (GPCRs) mediate a wide variety of cellular functions related to cell proliferation and survival. Regulators of G-protein Signaling (RGS) proteins that are important negative regulators of both G-proteins and GPCR products. The focus of this thesis involves two human protein variants of RGS10 and their effects on cytokine levels. RGS proteins are GTPase Accelerating Proteins (GAPs) which can facilitate an increased rate of GTP hydrolysis to drive inactivation of GPCR signaling. Based on their ability to regulate GPCRs, RGS proteins are implicated in multiple disease states including cancer and neuro-inflammation. The aim of this study was to define the similarities or differences among RGS10 protein isoforms, and help understand their non-canonical function. Particularly, differences in primary sequence of RGS10 protein variants and their ability to mediate inflammatory cytokines in human embryonic kidney (HEK) cells was investigated.

INDEX WORDS: GPCR, RGS10, ISOFORM, TNF-α, INFLAMMATION, VARIANTS

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CHAPTER 1

PURPOSE OF THE STUDY AND EXPECTED RESULTS

The purpose of this study was to identify distinct functions among two RGS10 protein variants found in humans. RGS10 is implicated in a number of disease states such as cancer, chemoresistance, and neuroinflammation. By acting downstream of stimulatory lipopolysaccharide (LPS) endotoxin, RGS10 blunts inflammatory cytokine signaling (Lee *et al.* 2008). However, human RGS10 occurs in two isoforms, and the ability for each protein variant to regulate cytokine production has not yet been investigated. The main goal of this research is to determine whether cellular expression of two RGS10 protein variants show differences in intercellular pro-inflammatory cytokine transcript levels, particularly Tumor Necrosis Factor-alpha (TNF-α). A second aim of this study was characterizing the phosphorylation event and regulation of RGS10 at its serine residue 168. Protein phosphorylation is an important molecular regulator. The ability of RGS10 to be phosphorylated by PKA is evident (Burgon *et al.* 2001). Less characterized are the effects of RGS10 localization after phosphorylation. Possible conclusions from this work may lead to specific and important therapeutic drug targets for the development of medicine which treat diseases such as cancer or auto-immune disorders linked to RGS10 dysregulation.

It has not been published in academic literature how isoform RGS10-1 compares to RGS10-2 in their ability to mediate cellular levels of cytokines. The two predominant human isoforms of RGS10 differ in primary sequence at their N-terminus. Specifically,

RGS10-1 has 18 amino acids not found on the shorter RGS10-2 protein. This difference in primary structure may also play an important biological role in cells including regulation of RGS10 distribution intracellularly. The possibility that RGS10-1 and RGS10-2 mediate cytokine induced inflammation differently has not been investigated until this study. Our lab has previously shown that RGS10-1 is down regulated in chemotherapy resistant ovarian cancer cell lines (Ali M.W. *et al.* 2013). These results suggest an importance for cancer cells to suppress RGS10-1 mRNA transcripts before acquiring chemoresistance. Furthermore, the mechanism may be targeted to epigenetic silencing of a second unique promoter region seen on the RGS10 gene. The existence of two distinct RGS10 variants might be due to genetic variation alone, but difference in protein structure often changes a protein's function by altering binding. It is possible phosphorylation of RGS10 alters its binding profile too, but complete crystal structures are currently unavailable. As shown in this thesis, the extra 18 amino acids on the longer protein variant add a predicted Nuclear Localization Signal (NLS) not shared by shorter isoform RGS10-2. An extra NLS region would predict stronger affinity for karyopherins. Karyopherins are nuclear transportation proteins. Increased transport of RGS10-1 to the nucleus may regulate some non-canonical function by acting as a transcription factor in the nucleus. Since the longer variant has two NLS domains compared to only one on the shorter variant, we expect overexpression of RGS10-1 to blunt cytokine molecule TNF-α to a greater extent than RGS10-2 if it is acting through a non-canonical nuclear pathway. On the other hand, if RGS10 variants regulate TNF-α through GAP activity only, then nuclear localization will be inhibiting and RGS10-2 maybe more effective at reducing pro-inflammatory cytokine production.

CHAPTER 2

LITERATURE REVIEW: REGULATOR OF G-PROTEIN SIGNALING-10 PROTEIN

Multiple sclerosis (MS), Alzheimer's disease, and Parkinson's Disease are all thought to arise as result of chronic neuroinflammation and damage to the central nervous system (CNS). Neurodegenerative diseases like these often lead to cell death resulting in gradual onset of symptoms like dementia. MS is a particularly debilitating disease where myelin sheaths on nerve cells are damaged. Cognitive dysfunction is highly prevalent in MS, occurring in roughly 50% of patients (Heaton *et al.* 1995). Multiple Sclerosis is not an inherited disease at birth, but instead a gradual loss of functional neurons occurs over the course a lifetime. Age dependent factors and neuroinflammation underlie diagnosis which affects more than 2.1 million MS patients all over the world (Eftekharian *et al.* 2016). Many treatment measures have been proposed, including healthy lifestyle changes and medication. Although there are number of immune-based therapeutic drugs available for the treatment of MS, it is difficult for medical professionals to predict which drug might work best for an individual patient due to a lack of mechanistic information on the disease. Alzheimer's Disease is a type of progressive dementia for which no cure exists. Parkinson's Disease is also a central nervous system disorder that affects movement, often including tremors. While the causes of neurodegenerative disorders are not fully understood yet, chronic exposure to inflammatory mediators in the CNS microenvironment certainly play a role in onset of MS disease (Steinman *et al.* 2012).

G-protein Coupled Receptor (GPCR) signaling plays an important role in various aspects of neuroinflammation including: antigen presentation, cytokine/chemokine production, as well as T-cell differentiation, proliferation, or invasion (Du and Xie 2012). Many molecules regulate downstream effects of GPCRs. Regulator of G-protein Signaling 10 (RGS10) is a small protein responsible for regulation of GPCR deactivation. Additionally, RGS10 is an emerging molecular switch important for the toggling of microglial activation or deactivation cycles. Induced by stress responses and dopaminergic neuron sensitivity to inflammatory stimuli, neuroinflammation-induced loss of RGS10 is a risk factor for idiopathic Parkinson's Disease (Lee *et al.* 2008).

G-protein coupled receptors (GPCRs) regulate the functions of many signaling pathways in the human body. Cardiovascular, endocrine, immune, and nervous systems are all regulated to some degree by GPCR signaling. As essential cell signaling receptors found at the plasma membrane, abnormalities in GPCR signaling have been implicated in an array of diseases such as cancer, and nervous system disorders. Additionally, serious debilitating neurodegenerative and autoimmune diseases often result from improper regulation of GPCR activation/deactivation cycling. GPCRs are activated by a diverse range of ligands extending from photon excitation to peptides or hormones such as dopamine and amino-acid glutamate (Heng *et al*. 2013). Although a diverse family of ligands bind and influence GPCR conformation change, all GPCRs share common structural features. Each receptor consists of an extracellular N-terminus allowing binding of activator or inhibitory molecules. Between the N-terminus and C-terminus spans seven α-helical transmembrane domains which align in the plasma membrane.

Some ligands bind the core region of the receptor within α-helices (Heng *et al*. 2013). Intracellularly, a C-terminus couples to a heterotrimeric G-protein complex. A heterotrimeric G-protein complex consists of guanine nucleotide-binding Gα subunit and Gβγ dimer (Venkatakrishnan *et al.* 2013). Upon agonist binding at the receptor's N-terminus a GPCR conformational change is induced. The change of structure allows guanine nucleotide exchange of GDP for GTP. Binding GTP intracellularly, the small G-protein Gα becomes dissociated from Gβγ dimer, and both G-proteins are considered activated at this point of the cycle. G-proteins β and γ stay together to go on triggering cell signal cascades. The canonical function of RGS proteins is thought to be a GAP activity on Gα shifting GPCR signal towards deactivation.

Variants exist for each alpha-subunit, and they are Gα-i, Gα-s, and Gα-q. A primary literature publication in *Nature* was the first to describe that RGS10 selectively deactivates the Gαi family of G-proteins (Hunt *et al.* 1996). Activated Gα-i inhibits adenylyl cyclase (AC), stunting enzymatic conversion of ATP to cyclic-adenosine monophosphate (cAMP) and decreasing the local intracellular cAMP concentrations. Intracellular levels of cAMP are important for the tight regulation of downstream signaling molecules such as Protein Kinase A (PKA). Phosphorylation, activation/deactivation of effector enzymes, and transcription factor production are all globally regulated by PKA. As RGS10 facilitates return of GPCR signaling to an inactive state it influences these downstream cellular processes. Nevertheless, RGS10 modulates sensitivity to inflammation-induced cell survival by interaction with the PKA/CREB pathway (Lee *et al*. 2012). This thesis will focus on RGS10 variants, and their ability to function as a GTPase Accelerating Proteins (GAPs) of the Gα-i subunit.

The Regulator of G-protein Signaling (RGS) protein family is a widely diverse group of proteins that regulate signaling pathways downstream of GPCRs. Since the middle 1990s, more than thirty functional RGS genes have been recognized and classified into eight subfamilies that are expressed throughout eukaryotic organisms, from simple fungi to animals including humans (Zheng *et al.* 1999). The main role of RGS proteins is to regulate the amplitude and duration of G-protein signaling. As shown in Figure 2.1, RGS proteins facilitate GTP hydrolysis and deactivation of signaling through their ability to function as GAPs for Gα subunits. RGS10 has selective GAP activity for Gα-i subunits which is induced upon binding (Hunt *et al.* 1996). Specifically, RGS proteins accelerate the deactivation of G-proteins through binding and stabilizing the Gα-GTP hydrolysis transition state. Binding results in optimal increase of GTP hydrolysis up to 1000-fold (Posner et al., 1999). Being regulators of evolutionarily conserved GPCRs, all RGS proteins contain a similar or homologous RGS domain. RGS domains consist of approximately 120 amino acids and are responsible for GAP activity. Interestingly, the RGS domain alone is sufficient for the RGS proteins' canonical GAP activity and interaction with Gα proteins (De Vries *et al.* 1995). Primary protein sequence outside of the highly conserved RGS domain, may be responsible for any non-canonical activity. In fact, RGS proteins contain a number of structural domains in addition to the RGS domain which regulate activity and determine binding affinities (Ross *et al* 2000). Non-RGS domain regions may provide distinct function from the inactivation of Gα subunits, such as binding G-protein coupled Inwardly-Rectifying potassium channels (GIRKs) or Adenylyl Cyclase (Abramow-Newerly *et al.* 2006).

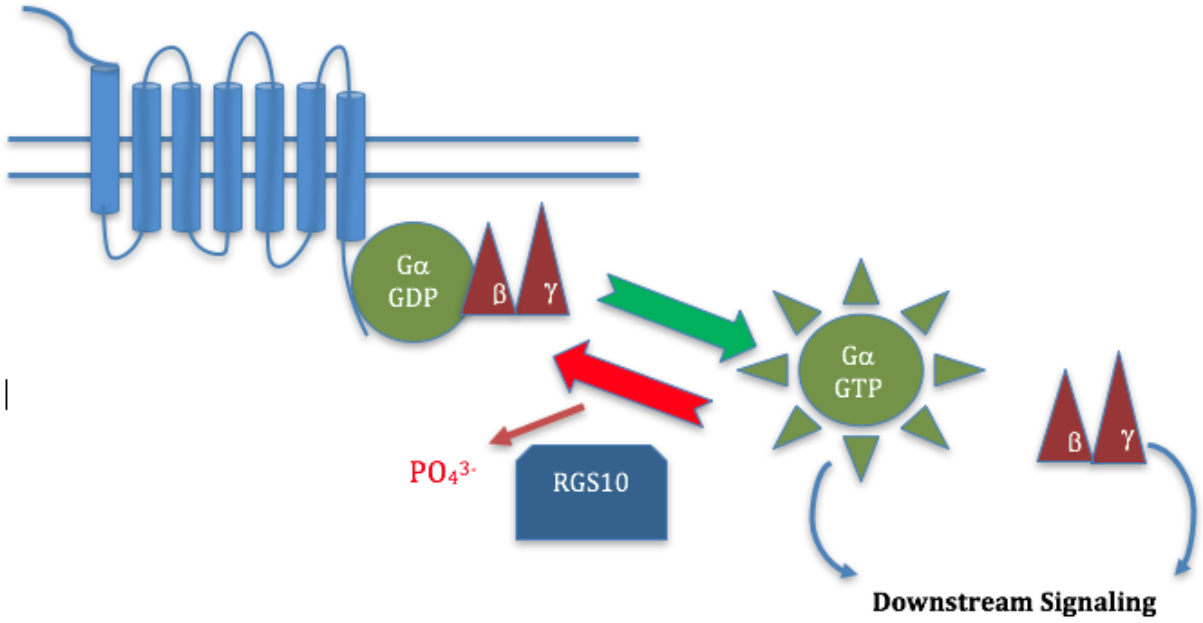

Figure 2.1: A Model Depicting Gα-Protein Activation/Deactivation Cycle. Activation upon agonist binding to GPCR N-termini results in release of GTP-bound Gα subunit and Gβγ dimer. Once dissociated, both G-protein subunits mediate downstream signaling pathways. RGS proteins facilitate deactivation of the cycle returning GPCRs to the inactive state by binding Gα-i and accelerating GTP hydrolysis rate.

RGS10 belongs to the R12/D subfamily. Smaller in size and length, RGS10 lacks the multiple regulatory domains that are found in the other larger RGS family members. Similar to its other family members however, RGS10 contains phosphorylation and palmitoylation sites that are important for its regulation. It was demonstrated that RGS10 can be phosphorylated at Serine-168 by the cAMP-dependent Protein Kinase A (PKA), and it is suggested that it may be required for its nuclear translocation (Burgon *et al*. 2001). Implicating a non-canonical role for RGS10, Chatterjee and Fisher reveal that RGS proteins reside in the nucleus of COS-7 cells (Chatterjee *et al.,* 2000). It is not certain what the role of RGS10 in the nucleus might be. What has been seen is some evidence of RGS10 translocation to the nucleus after lipopolysaccharide (LPS) treatment (Lee *et al.* 2008). RGS10 could translocate to the nucleus after subsequent phosphorylation. However, the possibility that cytosolic protein degradation of RGS10 by Toll-like receptor 4 (TLR-4) stimulation after LPS treatment, has not been ruled out. In the case of phosphorylation, RGS10 does not lose its ability to function as a GAP (Burgon *et al.* 2001). A better understanding of the normal function of RGS10 in the CNS may reveal its potential as a therapeutic target.

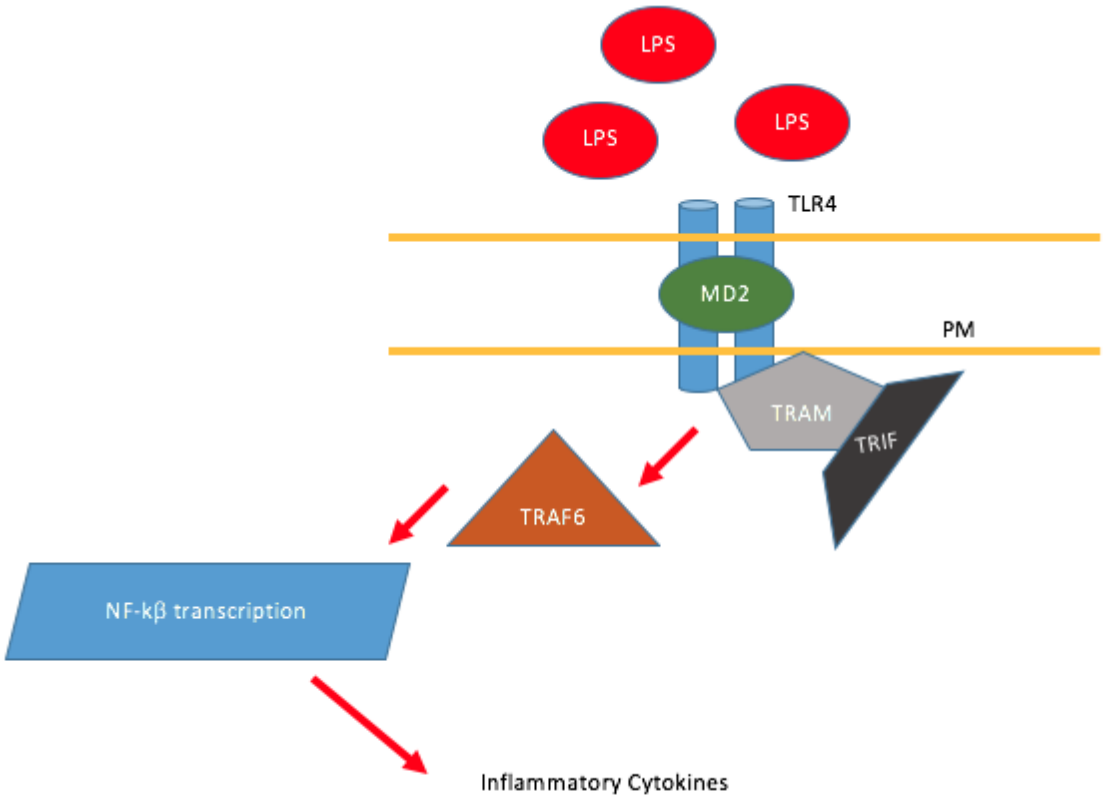

Figure 2.2: A Simple Pathway Model of LPS Stimulated Tumor Necrosis Factor-Alpha (TNF-α). Endotoxin lipopolysaccharide antagonizes toll-like receptor 4 which recruits adaptor molecules. TNF associated factor 6 (TRAF6) is then activated to ultimately allow nuclear factor transcription and production of TNF-α. Cytokine production from this pathway helps recruit the immune system to manage potentially harmful gram-negative bacteria containing LPS or other forms of chronic inflammation.

There is evidence RGS10 plays an important regulatory role at the CNS. RGS10 proteins are found at high levels in tissue associated with the immune system including brain, thymus, and lymph node (Larminie *et al*. 2004). It has been shown that RGS10 exerts an anti-inflammatory effect in microglia, the central nervous system's resident immune cells (Lee *et al*. 2008). Pro-inflammatory cytokines such as TNF-α and Interleukin 1-beta (IL-1β) are produced by activated microglia (Jack *et al.* 2005). Chronic inflammation from these agents leads to neuronal stress and often cell death. Additionally, these cytokines can induce indirect neuronal damage via increased sensitization of neurons to glutamate excitotoxicity (Pitt *et al*. 2000). By creating genetically modified mice models not containing functional alleles for RGS10 proteins, brain sections of knockout RGS10 mice have been studied for inflammation related dysregulation. It was observed RGS10- null microglia produced significantly higher levels of pro-inflammatory cytokines including TNF-α and IL-1β, than wild-type (WT) microglia when dosed with stimulating LPS biweekly (Lee *et al*. 2008). This suggests that normally, RGS10 suppresses pro-inflammatory cytokines in activated microglial cells. When microglial cells lacking RGS10 are activated, their production of cytokines goes unchecked (Lee *et al*. 2008).

Studying RGS10 variants allows for some insight to cellular function. Our lab has shown that RGS10 is epigenetically downregulated in chemoresistant ovarian cancer cells (Ali M.W. *et al.* 2013). Increased DNA methylation and DNA bound histone deacetylation are thought to be controlling factors of RGS10 gene silencing. It is possible RGS10 is a tumor suppressor gene. A gene that, when silenced, increases the selective growth advantage of the cancer cell and sustains tumorigenesis. It Is not

known whether RGS10 is acting directly, through canonical mechanism, to prevent sustained neuronal inflammation induced damage or indirectly by nuclear localization and binding partners. RGS10 proteins have been observed in both the cytoplasmic and the nuclear environment (Chatterjee *et al.* 2000). Cytoplasmic RGS10 has GAP activity, but some papers suggest nuclear localization is necessary for RGS10 to blunt inflammatory responses (Lee et al. 2008). It would be interesting to study the possible function RGS10 variants, containing different numbers of localization signals, have on TNF-α mediation. Also, why do cancer cells downregulate a particular RGS10 protein variant which contains multiple NLS domains? This is an important question as mechanisms that include changes in gene transcription may be used as therapeutic targets for blocking or delaying the progression of cancer and neuroinflammatory diseases.

CHAPTER 3

OVEREXPRESSION OF RGS10 ISOFORMS IN HUMAN EMBRYONIC KIDNEY (HEK) CELLS AND ITS EFFECT ON CYTOKINE PRODUCTION

3.1 Background

The National Center for Biotechnology is a U.S. government funded national resource which lists two main isoforms of human RGS10. A long variant RGS10-1 and a shorter variant RGS10-2 exist in human cells. The longer variant RGS10-1, is the predominant variant in ovarian cells (Ali M.W. *et al.* 2013). These isoforms share 91% amino acid sequence homology. Our lab previously linked the suppression of RGS10 to increased cell survival and chemoresistance in cultured ovarian cancer cell lines, and further showed that RGS10 knockdown increases cell growth and survival (Hooks *et al.* 2010). Expression profiles driven by two distinct promoters, which are regulated differently in cancer cells, suggests different functions. By understanding the similarities and differences between RGS10 variants we might learn more about the molecular mechanisms behind how RGS10 protein functions.

Current studies have performed a detailed analysis of the expression of RGS10 isoforms in normal and cancer-derived ovarian cells and determined the changes in epigenetic marks on RGS10 promoter DNA and histones in cells with different RGS10 expression levels predicted that the RGS10-1 promoter may be epigenetically regulated by DNA methylation for multiple reasons. First, silencing of tumor suppressors via DNA hypermethylation of their promoter regions is a major mechanism for cancer

progression (Tsou *et al.* 2002). Findings that the promoter of RGS10-1 was distinctly enriched in CpG dinucleotides and that inhibition of DNMT activity dramatically increased RGS10-1 expression supports the hypothesis that RGS10-1 transcription may be negatively regulated by DNA methylation (Ali M.W. *et al.* 2013). Loss of histone acetylation and gain of HDAC-1 binding at RGS10-1 promoters in ovarian cancer cells associates with low RGS10-1 expression.

3.2 Sequence Analysis of Unique RGS10 N-termini

Before experiments using RGS10 isoforms began, primary amino acid structure for each protein was examined. Both isoforms share sequence homology, but differ at their N-termini. An extra 18 amino acids exist on longer variant RGS10-1. Figure 3.1 models how the variants differ by having unique first exons, but they share four common exons. The longer transcript RGS10-1 gives rise to a 21 kilo Dalton (kDa) protein RGS10a containing 181 amino acids. The shorter transcript variant RGS10-2 gives rise to a 19.5 kDa protein RGS10b, comprised of 167 amino acids. Each variant has a number of predicted nuclear localization sequences. Predicted nuclear localization sequences from (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_ref.cgi) were generated by a software program based on a publication in the *National Academy of Sciences* journal (Kosugi *et al.* 2009). Interestingly, many scientific papers find serine phosphorylation on an NLS domain raises the binding affinity for karyopherin molecules such as importin-α in the paper cited (Jeong *et al.* 2015).

A.

Predicted NLSs in query sequence

```
MEHIHDSDGSSSSSHQSLKSTAKWAASLENLLEDPEGVKRFREFLKKEFS 50
EENVLFWLACEDFKKMQDKTQMQEKAKEIYMTFLSSKASSQVNVEGQSRL 100
NEKILEEPHPLMFQKLQDQIFNLMKYDSYSRFLKSDLFLKHKRTEEEEED 150
LPDAQTAAKRASRIYNT                                   167
```

B.

Predicted NLSs in query sequence

```
MFNRAVSRLSRKRPPSDIHDSDGSSSSSHQSLKSTAKWAASLENLLEDPE 50
GVKRFREFLKKEFSEENVLFWLACEDFKKMQDKTQMQEKAKEIYMTFLSS 100
KASSQVNVEGQSRLNEKILEEPHPLMFQKLQDQIFNLMKYDSYSRFLKSD 150
LFLKHKRTEEEEEDLPDAQTAAKRASRIYNT                     181
```

C.

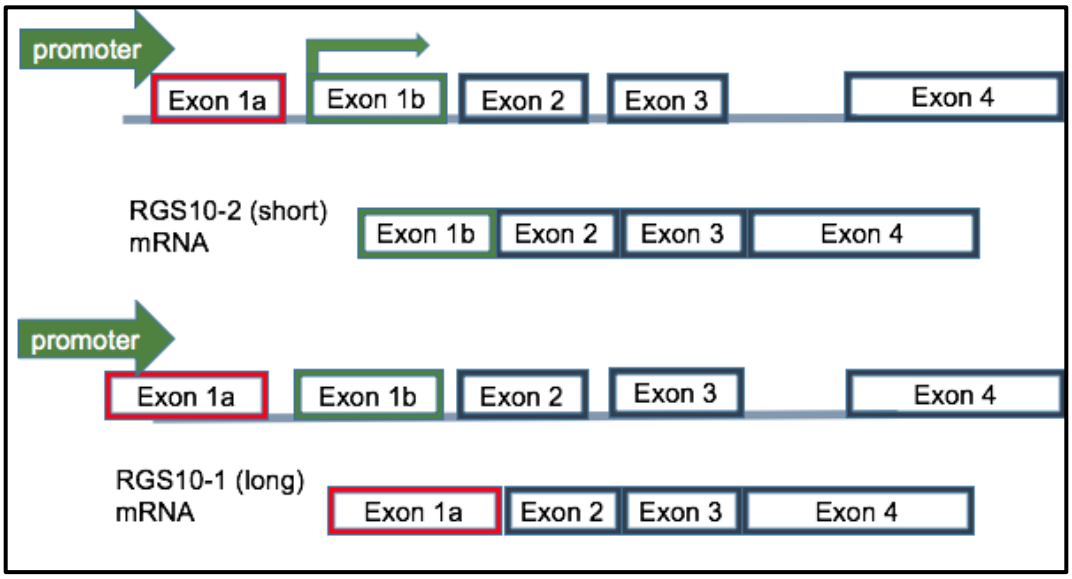

Figure 3.1: Classical Nuclear Localization Signal Sequences and Gene Structure. Predicted nuclear localization sequences from (http://nls-mapper.iab.cgi) shown in red. **(A)** Only one domain is predicted on RGS10-2. **(B)** RGS10-1 contains two predicted NLS domains. **(C)** A model depicting structure of mRNA encoded by the RGS10 gene. By having separate first exons but four common shared exons, isoforms are able to differentiate their number of NLS domains

A.

>gi|48146133|ref|NP_002916.1 [Homo sapiens] RGS10 regulator of G-protein signaling 10 isoform b

1 MEHIHDSDGS SSSSHQSLKS TAKWAASLEN LLEDPEGVKR FREFLKKEFS EENVLFWLAC EDFKKMQDK TQMQEKAKEIY

82 MTFLSSKASS QVNVEGQSRL NEKILEEPHP LMFQKLQDQI FNLMKYDSYS RFLKSDLFLK HKRTEEEEED LPDAQTAAKR ASRIYNT 167

>gi|52694755|ref|NP_00100539.1 [Homo sapiens] RGS10 regulator of G-protein signaling 10 isoform a

1 MFNRAVSRLS RKRPPSDIHD SDGSSSSSHQ SLKSTAKWAAS LENLLEDPE GVKRFREFLK KEFSEENVLF WLACEDFKKM

82 QDKTQMQEKA KEIYMTFLSS KASSQVNVEG QSRLNEKILE EPHPLMFQKL QDQIFNLMKY DSYSRFLKSD LFLKHKRTEE EEEDLPDAQT AAKRASRIYN T 181

B.

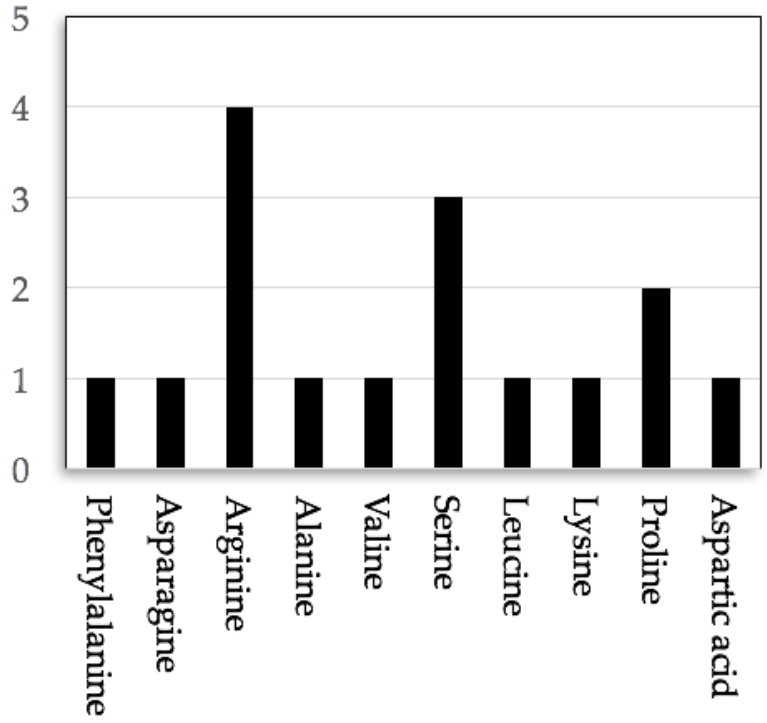

Figure 3.2: Positive Charged Identification and Histogram Analysis of Amino Acid Residues Unique to RGS10-1 variant N-Terminus. (A) Four arginine residues and one lysine residue are responsible for evenly distributed positively charged side chains

which associate with nuclear import by karyopherin proteins (red). The underlined region corresponds to the conserved RGS domain responsible for its GAP activity. **(B)** A histogram depicting number of individual residues found in the 18 amino acids unique to RGS10-1.

The side chains of lysine and arginine are positively charged at physiological pH. Positive charge leads to nuclear import via binding karyopherin molecule importin-alpha (Köhler *et al.* 1999). Karyopherins are proteins which recognize positive charge and hydrophobic regions to shuttle molecules bi-directionally across a nuclear envelope. RGS10-1 may bind importin-alpha and tract to the nucleus passing through a nuclear pore complex. Nuclear pore complexes are large proteins allowing transport of molecules across the nuclear envelope. The upper size limit for these complexes nears 60 kilo Daltons (Winey *et al.* 1997). RGS10 is a smaller protein with size nearing 20 kilo Daltons. In theory, RGS10 will easily diffuse through a nuclear pore complex.

Determining whether cellular expression of different RGS10 isoforms affects cytokine production is the initial aim of this study. By obtaining plasmid vector constructs of separate RGS10 protein isoforms, overexpression of these genes *in vivo* using HEK cells is possible. Cell culture treatment of LPS at 10 ng/mL, followed by mRNA isolation, will allow a way to access and measure cytokine production via qualitative Real Time-Polymerase Chain Reactions (RT- PCR). Previous data suggests that the longer protein variant, containing two NLS domains, seems to be important for chemoresistant cancer to negatively regulate or remove (Ali M.W. *et al.* 2013). If RGS10 exerts its anti-inflammatory effect through nuclear activity, then RGS10-1 may also block cytokine production to a greater extent than shorter isoform RGS10-2.

CHAPTER 4

MATERIALS AND METHODS

4.1: Cells and Reagents

Human Embryonic Kidney (HEK293) cells were chosen for these experiments because they have a fast growth rate, low endogenous RGS10 expression, and can be transfected with high efficiency. As such, they provide a great low noise model, for studying the biology and physiology of RGS10. The HEK293 cell system used in these experiments expresses Toll-like Receptor 4 (TLR4). Activation of TLR4 receptors by lipopolysaccharide (LPS) leads to activation of NFKB, which results in the transcription of inflammatory cytokines produced during an immune response.

All cells were grown in media containing 5mM penicillin-streptomycin at 37^{o} Celsius with 5% carbon dioxide. HEK cells were purchased from American Type Culture Collection (ATCC) and maintained in McCoy 5A Dulbecco's modified media (DMEM). The media was additionally supplemented with 10% fetal bovine serum (FBS) from PAA Laboratories. Transiently transfected cells were treated with LPS at 10 ng/mL followed by an exposure period of 24 hours. RGS10-2 gene was obtained from cDNA Resource Center (www.cdna.org). A plasmid with RGS10-1 insert containing Myc-DDK epitope tags on the N- terminal side was purchased from Origene. Together, the Myc and DDK tag spans 18 amino acids on the N-terminal end before the RGS10 gene sequence starts at the methionine codon.

4.2: Cloning Experiments

In an attempt to clone each gene into similar expression vectors, restriction enzymes were employed to remove RGS10-1 from pcMV6 entry vector. DNA and PCR products were analyzed with 1% DNA-agarose gels and purified using Qiagen Quick Gel Extraction kit and PCR Purification Kit (Invitrogen). Bam HI and Xho 1 restriction enzymes were used to double digest pcMV6 plasmid at 37° Celsius for 4 hours. The purified products were ligated into cut plasmids using T4 ligase enzyme (Agilent Technologies) which were then transformed into competent XL-Blue *Escherichia coli* (E.coli) bacteria. 10 individual colonies were isolated from Carbenicillin LB-agar plates and out-grown. QIAprep Spin Miniprep Kit (Qiagen Sample & Assay Technologies) was used to purify the plasmids from each colony at a set absorbance range, which were then sent for sequencing using T7 promoter sequencing primers at nearby UGA Genomics Facility. Clone sequences were subjected to screens for quality and complete conversion. However, no positive identification of a successful ligation reaction by Sanger sequencing at UGA Genomic Facility was made; likely due to low DNA yield or purity. Plasmid transfection into HEK cells was instead performed using separate vector constructs as described in section 4.4.

4.3: Western Blot Analysis

To evaluate RGS10 expression in HEK cells, cell lysates were obtained in 100µl lysis buffer (2M Tris-HCL, 4% SDS, 10% glycerol, Bromophenol blue, and 0.5% 2-Mercaptoethanol) capable of denaturation and solubilizing proteins completely. After vortex mixing and centrifugation, soluble proteins were run on 10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with RGS10 antibody. Before loading,

lysates were boiled for five minutes and then analyzed using SDS-PAGE. Membranes were incubated with RGS10 primary antibodies (Santa Cruz Biotechnology, Inc.) and HRP-conjugated rabbit secondary antibodies (Pierce) at a (1:1000) ratio. Membranes were visualized using ECL reagents (Pierce). Membranes were additionally blotted with GAPDH antibodies conjugated to mouse (Life Technologies) as a loading control.

4.4: Plasmid Transfection

Transient transfections were performed using Lipofectamine Plus reagent (Roche), according to manufacturer's instructions. HEK293 cells were plated in 24-well plates at 100,000 cells per well, and transfected with 500ng RGS10-1 Myc and DDK tagged plasmid DNA, 500 ng HA-tagged RGS10-2 plasmid DNA, or empty vector. Additionally, RGS10 SA mutant was transfected into cells with 500ng DNA per well. Assays were performed 48 hours after transient transfection, and total protein was processed for immunoblotting to confirm protein expression with antibodies.

4.5: Qualitative Real-time PCR

After 48 hours of incubation in transfection media, cells were harvested in TRIzol reagent (Invitrogen), and mRNA was isolated. cDNA was synthesized from 2 µg of total RNA using a High Capacity Reverse Transcriptase cDNA kit (Qiagen). Quantitative real-time polymerase chain reaction was performed using Superscript III kit for RT-PCR (Invitrogen) and Power SYBR Green reagent (Applied Biosystems). Reactions were normalized using the housekeeping gene GAPDH and calculations were performed according to the $2^{-\Delta\Delta CT}$ method. As described in the manufacturer's protocol, cells were lysed in Trizol and agitated on a vortex rotor for 2 minutes. 200µl of chloroform was added and was incubated for five minutes at room temperature. Samples were

centrifuged at 12,000 G for 20 minutes and the aqueous phase (400µl) was transferred to a 1.5 mL Eppendorf tube. 400µl of isopropanol was added and was incubated for 10 minutes at room temperature. Following centrifugation, pellets were washed with 1mL of cold 75% ethanol, centrifuged and resuspended in 50µl Diethyl pyrocarbonate (DEPC) water. As mentioned earlier, RNA was quantified by a spectrophotometer and cDNA was generated from 2µg of total extracted RNA using a Reverse Transcription Kit (Qiagen). Following cDNA synthesis, quantitative real-time polymerase chain reaction was performed using TaqMan Universal PCR Master Mix (Roche) and specific primers and probes targeting RGS10, TNF-α, or GAPDH coding regions.

Primers used were based on gene bank sequences of RGS10 variants from the NCBI gene bank (https://www.ncbi.nlm.nih.gov/genbank/). Primers used included: RGS10 forward: 5’ GAC CCA GAA GGC GTG AAA AGA 3’, RGS10 reverse: 5’ GCT GGA CAG AAA GGT CAT GTA 3’, GAPDH forward: 5' GGA AGC TCA CTG GCA TGG 3', GAPDH reverse: 5' TAG ACG GCA GGT CAG GTC 3', TNF-α forward 5’- TAC TGA ACT TCG GGG TGA TTG GTC 3’, and reverse 5’ CAG CCT TGT CCC TTG AAG AGA ACC 3’. Reactions were normalized against GAPDH expression.

CHAPTER 5

RESULTS AND DISCUSSION

As seen in Figure 5.1, pcDNA 3.1 plasmid containing RGS10-2 gene were able to be transiently transfected into HEK cells expressing TLR-4 receptor. The amount of overexpressed plasmid can be measured distinct from endogenous levels by use of a human influenza hemagglutinin (HA) epitope marker. Overexpression worked best when HEK cells were harvested 48 hours after transfection with plasmid and lipofectamine. Figure 5.1 shows that RGS10-2 is likely the only variant endogenously expressed in HEK cells. No RGS10-1 is seen. The larger molecular weight of the overexpressed plasmid comes as a results of 18 additional amino acids from the HA tag. RGS10-1 also has an 18 amino acid difference from RGS10-2, but a second band on the membrane was not seen. In conclusion, RGS10-2 is the lower band in Figure 5.1 and HA tagged RGS10-2 is the higher band.

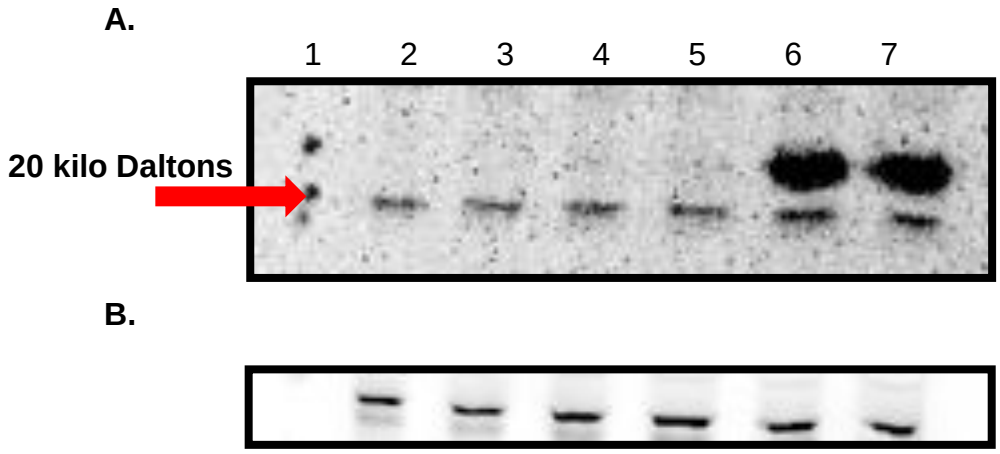

Figure 5.1: Immunoblot Showing Overexpression of RGS10-2 HA tagged plasmid in HEK cells. (A) Lane 1 contains 5μL reference protein size marker imaged by ELC reagents mentioned in 3.4. Lanes 2-7 contain whole cell lysates run on an SDS-page gel, transferred to nitrocellulose membrane, and immunoblotted with RGS10 specific antibodies. Lanes 6 and 7 contain cell lysates from transfection. The lower band indicates endogenous level of RGS10. The overexpressed plasmid containing RGS10-2 gene is tagged with HA epitope marker making the protein run higher on the SDS-page gel. **(B)** Immunofluorescence of GAPDH from the same whole cell lysates as an experimental control.

Several attempts to ligate the RGS10-2 gene into pcDNA 3.1, containing carbenecillin resistance gene, were conducted. Colonies transformed with the resulting ligation product grew on agar plates containing 100μg/μL carbenecillin antibiotic. Subsequent colonies were screened via visualizing RGS10 PCR product from overnight cultures (data not shown). Possible ligation products were sent for sequencing. Unfortunately, DNA Sanger sequence analysis by UGA Genomics Facility did not find a match. A single site mutation change, from serine to alanine, was obtained in RGS10-3 gene and confirmed by Sanger sequencing. The resulting plasmid containing a single site mutation on RGS10 gene was able to be overexpressed in HEK cells in excess of 600 fold above endogenous levels.

5.1: Limitations and Caveats

It is possible such robust overexpression of RGS10 overwhelms cellular machinery. Saturation of the mechanism by which RGS10 mediates TNF-α is one possible reason why no difference between variants was observed. In this case, RGS10 attenuation of cytokine production would be maxed. Another caveat of this experiment includes the molecular tags used. Epitope markers typically do not interfere with bioactivity or biodistribution. However, the Myc-DDK tag on RGD10-1 is present at the N-terminus before the gene starts. Since RGS10-1 differs from RGS10-2 at the N-terminus it is possible the Myc-DDK epitope marker interferes with NLS activity. A solution to this limitation would be cloning genes into a plasmid lacking epitope tags.

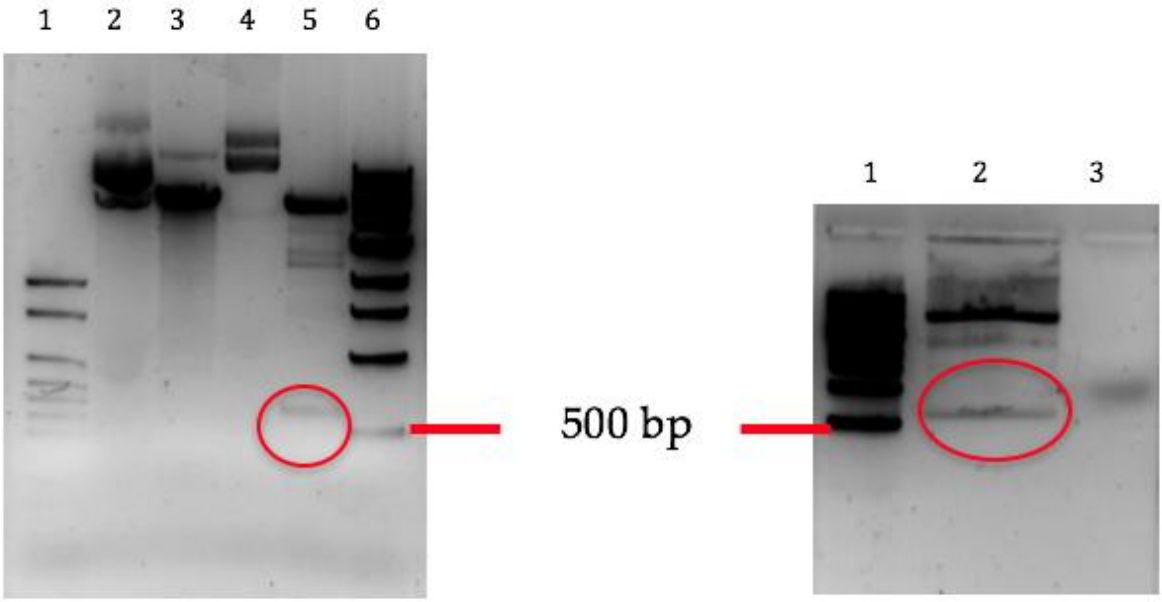

Figure 5.2: 1% Agarose Gel Visualized With UV Light and SYBR® Safe Reagent Containing Linear RGS10-1 Gene of Interest. Digestion of RGS10-1 with restriction enzymes Bam HI and Xho1 results in a 546 base pair product. RGS10 gene inserts are shown circled in red. **(A)** 1: Molecular weight marker. 2: Uncut pcDNA 3.1 vector. 3: Digested pcDNA 3.1 vector. 4: Uncut pcMV6 entry vector 5: Digested pcMV6 entry vector containing RGS10-1 gene. 6: Molecular weight marker. Notes: Digestion of empty pcDNA 3.1 vector with restriction enzymes Bam HI and Xho1 results in a 56 base pair product not visible on this gel. **(B)** 1: DNA size reference maker. 2: Enzyme digested RGS10-2 plasmid. 3: Loading dye.

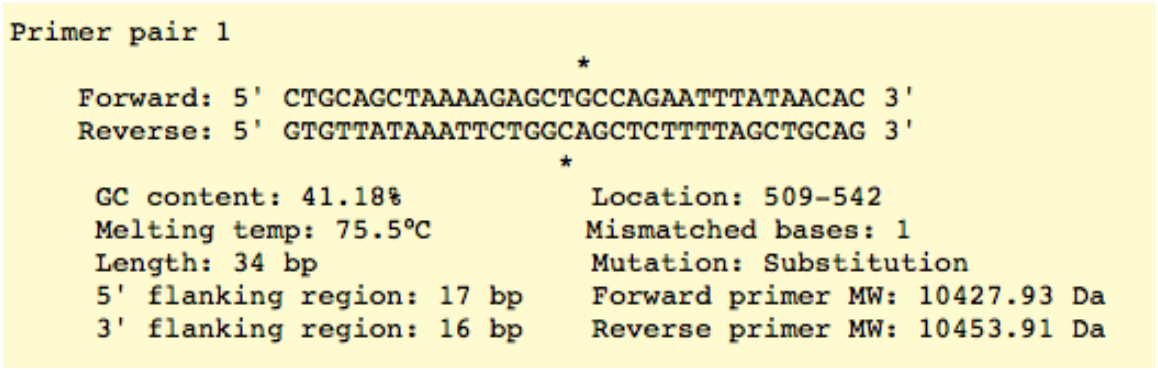

Figure 5.3: Forward and Reverse Primers For RGS10 SA Mutant Polymerase Chain Reaction. The (*) symbols represent mismatched base pairs. Above are the primer designs used in a PCR method experiment for creating and amplifying gene containing a mutated serine residue codon resulting in alanine codon substitution.

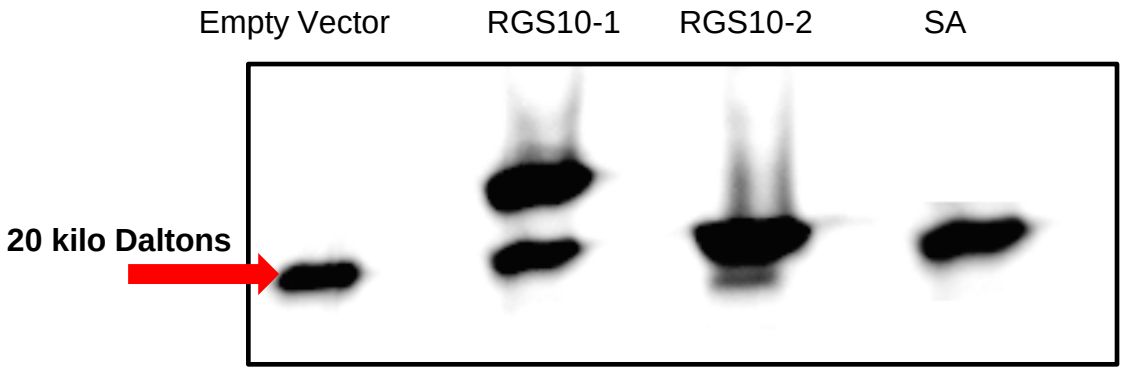

Figure 5.4: A Western Blot of RGS10 Variant Overexpression in HEK293 cells.

Each lane contains 15μL whole cell lysate. Samples were boiled for 5 minutes before loading. Empty vector overexpression is used as a control to show endogenous levels of RGS10-2. Overexpression of RGS10-1, RGS10-2, and SA mutant RGS10 results in similar protein expression levels. The larger molecular weight of RGS10-1 over endogenous comes from its unique N-terminus and Myc-DKK tag. The larger molecular weight of RGS10-2 over endogenous comes from its HA tag.

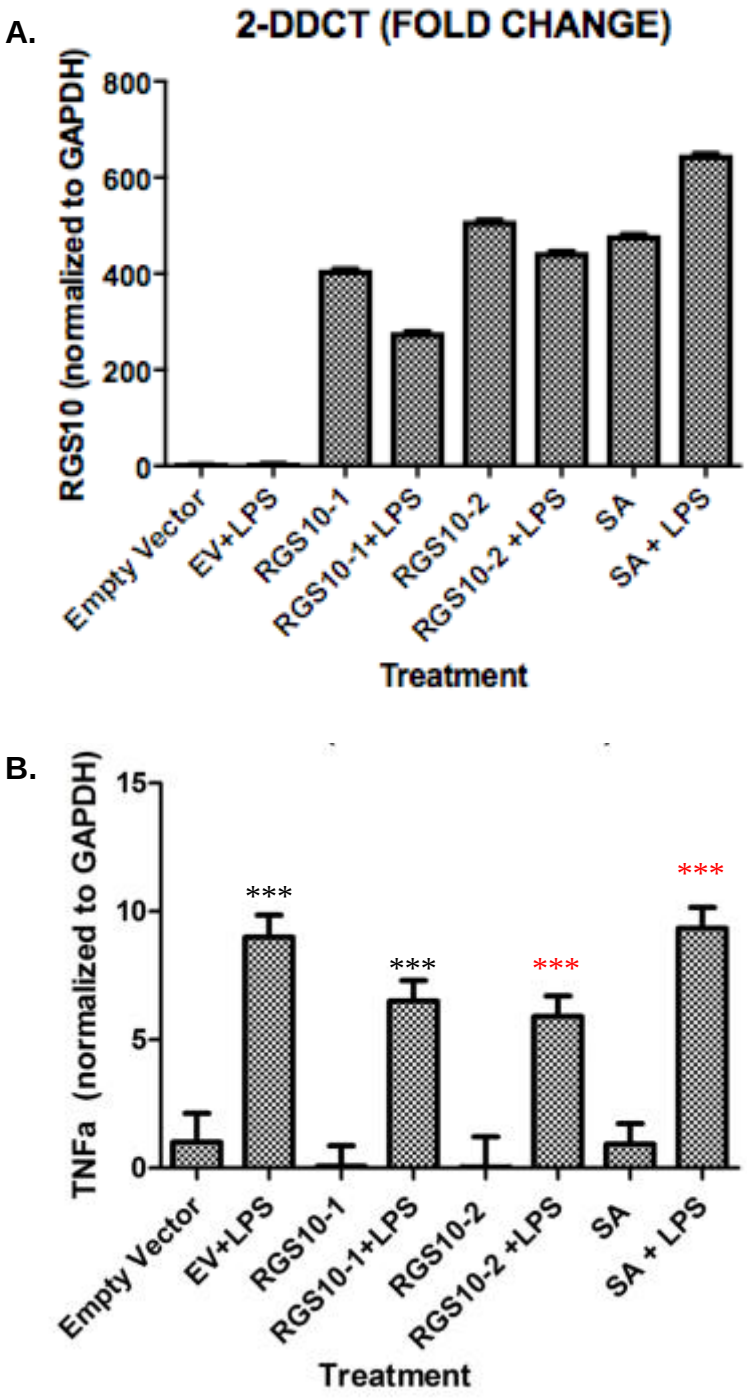

Figure 5.5: Real-time PCR Data. (A) RGS10 mRNA transcription levels for empty vector (EV), wildtype RGS10-1, PKA-deficient mutant RGS10 (SA), or wildtype RGS10-2. **(B)** The effect of empty vector (EV), wildtype RGS10-1, PKA-deficient mutant RGS10

(SA), or wildtype RGS10-2 transfection on TNF-α mRNA expression after treatment of either vehicle or 10ng/mL LPS for 24 hours. The significant decrease in TNF-α expression that is observed with both isoforms overexpressing RGS10 is lost in cells transfected with SA mutant.

5.2: Real-time PCR Analysis

As expected, wildtype RGS10 attenuates TNF-α expression after 24 hours of exposure to 10 ng/mL LPS (P-value = 0.0476). Figure 5.4 shows that both wild-type RGS10 isoforms reduce TNF-α compared to empty vector. Although TNF-α values were not significant between variants, both reduced TNF-α when overexpressed. Results from Figure 5.1 (B) were observed twice from two independent experiments. A two-tailed t-test was used to determine significance (P-value = 0.1653). Therefore, we fail to reject the null hypothesis that RGS10 isoforms mediate TNF-α equally. Errors bars are generated by calculating the standard error from the formula:

$$\frac{s}{\sqrt{n}}$$

where (s) is the standard deviation of the sample mean, and (n) is the size of the sample. The data above suggests similar TNF-α regulation between RGS10-1 and RGS10-2. Although the reason for multiple NLS sequences within a single molecule remains unclear, some studies have demonstrated that multiple NLSs can function cooperatively to enhance nuclear accumulation (Bruce *et al.* 1987). Theoretically, separate NLS domains may display binding specificities recognized by different karyopherin variants. From a genetic diversity perspective, multiple NLS domains on RGS10-1 may be of some importance in maintaining functionality if mutations cause a loss of one NLS region.

Our study is the first to demonstrate the presence of multiple NLS within RGS10, and suggests that these NLS domains may function cooperatively for more proficient nuclear translocation. However, data from this study indicates multiple NLS sequences do not improve RGS10-1's ability to block TNF-α mRNA compared to one sequence on RGS10-2. This experiment should to be repeated using a transiently transfected wildtype RGS10 lacking epitope markers, or simply conducted with identical entry plasmids. This change would help limit variation and remove uncertainty for a more sensitive comparison of variants.

Supporting what has been reported previously, SA mutant RGS10 loses the ability to reduce TNF-α expression after LPS treatment (P-value = 0.0413), suggesting that serine phosphorylation at site 168 on RGS10 is critical for its regulation of TNF-α expression. SA mutant mRNA transcript levels are increased when treated with LPS compared to SA mutant alone. LPS silences endogenous RGS10 at 48 hours, but overexpression is carried out by a transfected plasmid. It is likely differences in transfection efficiency are responsible for change in this trend.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Understanding the mechanism by which RGS10 reduces pro-inflammatory cytokine production in microglia could help design novel therapeutics for neuroinflammatory and neurodegenerative diseases. Here, we investigated the extent to which an inflammatory cytokine is regulated in presence of abundant RGS10 variants. The real goal of measuring cytokine mRNA transcripts in response to isoform overexpression was to help elucidate a role for RGS10 in mediating inflammatory cytokines. Specifically, transcriptional differences in TNF-α between two distinct primary amino acid sequences of RGS10 were measured. No significant difference was observed. There is a difference in number of NLS domains between variants, but data from this study revealed no change in cytokine regulation by RGS10 isoforms with different numbers of NLS motifs. For future work, other cytokine molecules could be tested, but a more sensitive test is needed to compare how number of NLS domains correlate to non-canonical RGS10 function.

Interestingly, SA mutant transcript levels are increased when treated with LPS compared to SA mutant alone. Increased SA mutant protein expression compared to the untreated SA group may be a result of unequal transformation efficiencies, but it also hints that phosphorylation of RGS10 may be important for its negative regulation. Previous studies revealed soluble TNF-α to be a critical mediator of 6-hydroxydopamine-induced nigral DA neuron death in vivo and in vitro (McCoy et al.,

2006). By affecting transcriptional levels of TNF-α, RGS10 provides some neuroprotective and anti-inflammatory effects. The significant reduction in TNF-α expression that results from RGS10 overexpression has been shown to be GAP independent (Burgon *et al.* 2001). Because RGS10 can be found in the nucleus, combined with our preliminary work showing that RGS10 variants contain predicted nuclear localization sequences, differing in number depending on the variant, it suggests RGS10 acts non-canonically from within the nucleus. It is unknown if RGS10 simply gets sequestered to the nucleus to prevent global GPCR deactivation. Furthermore, RGS10 acting through its canonical GAP activity can have an effect on PKA activity which contributes to its own anti-inflammatory effects (Lee *et al*. 2012).

It is thought that phosphorylation of a serine residue in RGS10 results in its subsequent nuclear localization (Burgon *et al.* 2001). Through employing a PKA-deficient mutant variant of RGS10, the importance of RGS10's serine 168 residue was probed for its ability to regulate transcription. The serine 168 residue appears to be essential in the GAP-independent anti-inflammatory function of RGS10. Typical RGS10 mediated reduction of TNF-α messenger RNA transcripts was not seen with the phosphorylation mutant. Further experiments need to be conducted to confirm that wildtype RGS10 is indeed sent to the nucleus upon phosphorylation. The finding of a small RGS protein possessing a non-canonical, GAP independent role within the nucleus of cells would be a significant discovery in the field of RGS proteins. Elucidating the mechanism behind RGS10's neuroprotective, anti-inflammatory effects would provide additional therapeutic treatment options to explore for devastating diseases such as Parkinson's Disease, Alzheimer's Disease, and Multiple Sclerosis.

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