University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
November 2020

The Role of a Gα12-Interacting Domain in p114RhoGEF Signaling Function

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Abstract

P114RhoGEF/ARHGEF18 is a Rho-activating guanine nucleotide exchange factor (RhoGEF) important in the activation of RhoA GTPases. This signaling pathway induces the formation of actin stress fibers, which are crucial to the structure of contractile actomyosin bundles found in non-muscle cells, and also drives cell proliferation via SRF (serum response factor)-mediated gene transcription. Heterotrimeric G proteins of the G12/13 subfamily, consisting of $G\alpha_{12}$ and $G\alpha_{13}$, stimulate specialized RhoGEFs through interaction with their RGS-homology (RH) domain. Despite lacking a RH domain, p114RhoGEF is bound to $G\alpha_{12}$ through a unique structural region that evolved more recently than other components of p114RhoGEF. This specialized region, when examined evolutionarily, appears to be absent in jawless fishes such as lampreys, yet is present in other vertebrates including cartilaginous fishes such as sharks. This bioinformatic analysis suggests the $G\alpha_{12}$-interacting region evolved as a relatively recent domain within p114RhoGEF. To understand the significance of this region in the function of p114RhoGEF, three mutations dissecting this region were engineered. The primary mutant construct removed the entire 106-residue $G\alpha_{12}$ binding region, and two other mutations converted crucial glutamic acid residues identified in a previous study (Martin et al., 2016) to positively charged arginine residues. Luciferase assays were used to measure SRF-mediated transcriptional activation. Luminometric readings suggest that, in comparison to normal p114RhoGEF, the mutant missing this essential binding region gradually tapers to weaker signaling results at lower DNA concentrations. Future work should include examining the SRF signaling results of the point mutants, thus examining their structural significance in the $G\alpha_{12}$ binding region as a whole. These results demonstrate a role of this binding region in modulating or stabilizing the signaling function of p114RhoGEF. This finding improves our understanding of the fine details of a system of interactions that drives Gα12-mediated Rho signaling.

1. Introduction

Heterotrimeric G proteins hold an expansive capital on regulating cell homeostasis. By coordinating signaling between a vast number of G-protein coupled receptors (GPCRs), ~750 or so within the human genome, and a small grouping of effector enzymes and channels in the cell, they control processes that sustain life. These processes include muscle contractility, glycogen metabolism, neurotransmission, cell polarity, proliferation, migration and invasion, cytoskeletal rearrangements, and countless other cellular pathways and physiologic events[1,2]. Many of these responses are initiated at the cell surface by external stimuli that bind seven-transmembrane-span receptors, which themselves have physically coupled to the heterotrimeric guanine nucleotide-binding proteins (G-proteins) on the cytoplasmic face of the plasma membrane.The profound impact this group of proteins has on nearly all cellular processes and their therapeutic potential has rendered G-proteins and their effectors one of the most intensely studied signal transduction mechanisms of molecular biology to this day[1].

Cells respond to changes in their environment through a complex interplay of intercellular signaling proteins. When receptor activation triggers the α subunit of the G-protein to release GDP and bind GTP, this event triggers the generation of two signaling entities, the active GTP-bound α subunit and the stable dimer of β and ɣ subunits. The

activated α subunit plays an essential role as it stimulates a vast number of downstream effectors that include kinases, phosphatases, second messenger generation, ion channels, and transcriptional regulatory factors [2]. There are four groupings of the α subunit of G-proteins: Gs, Gi, Gq, and G12/13. The Gα12/13 subfamily in mammalian cells has been implicated in pathways crucial to homeostatic regulation[3]. Many G12/13 mediated responses require the downstream activation of Rho, a small GTPase that, when activated by the exchange of bound GDP for GTP, drives signaling through effector proteins [4,5].

The bridge between G12/13 and Rho is a small sub-class of Rho-mediated guanine nucleotide exchange factors (RhoGEFs), including p115RhoGEF leukemia-associated RhoGEF (LARG) and PDZ-RhoGEF5. Most RhoGEFs contain a standard domain structure consisting of tandem DH (Dbl homology) and PH (pleckstrin homology) domains. The DH domain is directly involved in the catalytic activity of GDP–GTP exchange[6]. Another common feature of the G12/13 responsive RhoGEFs is an RGS-homology (RH) domain, similar in sequence to a hallmark structural region in G-protein signaling regulators (RGS) that bind activated α subunits and accelerate the GTP hydrolysis to inactivated states [7]. Rapid advancement in the understanding of G12/13 signaling through RH-RhoGEFs continues to occur, but there are many less understood pathways that utilize this G-protein subfamily in ways that subvert current understanding of these signaling routes. Gα12 and Gα13, despite having 67% amino acid identity, have diverse binding partners that do not overlap. Although the interactions between Gα12/13 to Rho is well studied, the structural determinants of Ga12/13 and the RhoGEFs that facilitate these interactions are less understood.

In RhoGEFs, the essential domain features such as the DH, PH, and RH domains act as critical features in establishing signaling pathways, yet there are some unique exceptions to this schematic. AKAP-Lbc and p114RhoGEF both lack an RH domain. In p115RhoGEF, LARG, and PDZ-RhoGEF, the RH domain provides a surface for G12/13 α subunits to bind the protein and stimulate the activity of the RhoGEF,[5,7]. Mechanistic studies yielded confirmation that in the absence of an RH domain, the G12/13 subunit will use differential mechanisms to stimulate activity towards Rho. P114RhoGEF and AKAP-Lbc both harbor a binding region with a specific biased affinity towards Gα12 within the G12/13 subunit, which further sets them apart from the model used in RH-RhoGEFs, which show preference to Gα13. Understanding the finer details of the mechanism to which these RhoGEFs can communicate with their binding partners is vital due to how many physiological events these proteins influence and mediate. Small RhoGTPases direct cell shape changes and movements during tissue morphogenesis; their activities are tightly regulated in space and time to specify the desired pattern of actomyosin contractility that supports tissue morphogenesis. P114RhoGEF t precisely activates RhoA and has been noted to play crucial roles centered around its localization in tight cell junctions [8,9]. Critical aspects of cell mechanics are governed by the spatial-temporal control over Rho activity, which makes the drivers of this activity a subject of much interest. Malfunctions in the regulation of RhoGEFs such as p114RhoGEF can lead to the decline in the cell's junctional integrity during key events in morphogenesis and general homeostasis [10]. The commutative pathways between the Gα12/13, RhoGEFs, and subsequently, Rho is vast and has countless unknown intricacies. Because the signaling routes are so vast, singling out a group of signaling cascades can garner a lot of information useful to medicine development. Even the slightest of faults within systems as tightly organized and regulated as RhoGEFs can lead to significant health complications such as tumor formation, loss of epithelial integrity, and cancer metastasis progression. Upregulation and underexpression of a small RhoGEF such as p114RhoGEF can create a domino effect of chaos that negatively impacts the body[11].

In this study, the Gα12 binding surface of P114RhoGEF is dissected to understand this region's importance in the protein's ability to conduct signal transduction. When examined evolutionarily, this specialized region appears to be absent in jawless fishes such as lampreys yet is present in other vertebrates, including cartilaginous fishes, i.e., sharks. This bioinformatic analysis suggests the Gα12-interacting region evolved as a relatively recent domain within p114RhoGEF. To further understand this region's significance in the function of p114RhoGEF, three mutations dissecting this region were engineered. The primary mutant construct removed the entire 106-residue Gα12 binding region, and two other mutations converted crucial glutamic acid residues identified in a previous study[7] to positively charged arginine residues. Luciferase assays were used to measure SRF-mediated transcriptional activation of both an unaltered p114RhoGEF protein and the mutant construct lacking the Gα12 binding site. Luminometric readings suggest that, compared to normal p114RhoGEF, the mutant missing this essential binding region gradually tapers to weaker signaling results at lower DNA concentrations. Further research in the signaling effects of the point mutations on p114RhoGEF SRF activation in comparison to an unaltered version is needed to understand this Gα12 binding site of the RhoGEF in more detail, as well as other assays examining signal transduction of the primary deletion mutant.

2. Materials and Methods

2.1 PCR-Based Mutagenesis

Two point mutations at residues 695, 789, and the deletion of the Gα12 binding site on p114RhoGEF mutant were constructed using PCR-based mutagenesis. They were designed to have a 22 base-pair overlap with the adjacent amplimer. Primary PCR products were agarose gel-extracted. Products were then subjected to a second round of PCR using end primers containing 5'-end restriction sites for the KPN1-High Fidelity and Xho-1 restriction enzymes for cloning into the mammalian expression plasmid pcDNA3.1. Following this all mutant plasmid constructs were purified and then verified by sequencing (Genewiz, NJ).

2.2 Luminometry Assays

Human embryonic kidney cells (HEK293) grown to approximately 80% confluence in 12-well plates were transfected with 200 ng of Serum Response Element-luciferase plasmid and 20 ng of the internal standard luciferase plasmid harboring the cDNA for Renilla. The first five samples utilized an unaltered p114RhoGEF DNA at masses of 2, 5, 20, 50, and 200 ng. The next five wells contained the Gα12 binding site deletion mutants with the same DNA masses as the previous grouping. Well 11 contained no DNA masses from either p114 sample and acted as a control. This was done again under the same conditions with lower DNA concentrations of the functional p114 positive control and the Gα12 binding site deletion mutant. The lower concentrations are as follows: 0.2, 05, 1.0, 2.0, and 5.0 ng of DNA. Lysates were cleared for 1 minute at 16000 g, then the spernatants were analyzed for SRE-driven firefly luciferase expression and an internal control of Renilla luciferase expression using a dual-luciferase assay system and GloMax 20/20 luminometer (Promega).

2.3 LALIGN Evolutionary Bioinformatics

Taking the Gα12 binding sequence found in human P114RhoGEF as well as the entire human p114RhoGEF protein select representative species of early vertebrates were examined for their own homolog of the RhoGEF. The protein in its entirety was compared to sequences resembling it in representative organisms found using the NCBI database. To examine the sequences in comparison to one another, the bioinformatic sequence analysis program, LALIGN[12], a tool of ExPASy, was utilized to examine p114RhoGEF sequences.

3. Results

3.1 LALIGN Evolutionary Bioinformatics with Gα12 Binding Region of p114RhoGEF and AKAP-Lbc versus Extant Sister Vertebrate Clades.

Previous studies indicate that invertebrate versions of p114RhoGEF are drastically different from the version of this protein we are familiar with. The role it plays in the cells of other organisms is fundamentally the same, but the sequence and makeup of the protein is much different. Notably, the region of mammalian p114RhoGEF that governs Gα12 binding is absent in invertebrate RhoGEFs. How this interaction occurs in vertebrates is unknown, and it draws us to question the development of that crucial region in mammalian cells. This unique region acts as the crucial binding surface for Gα12 interactions but it has not always been present. Seeking to understand the functional history of this region in the protein, bioinformatic examinations were undertaken looking at early vertebrate species and comparing their representative p114RhoGEFs to the mammalian human RhoGEF. (Figure 1).

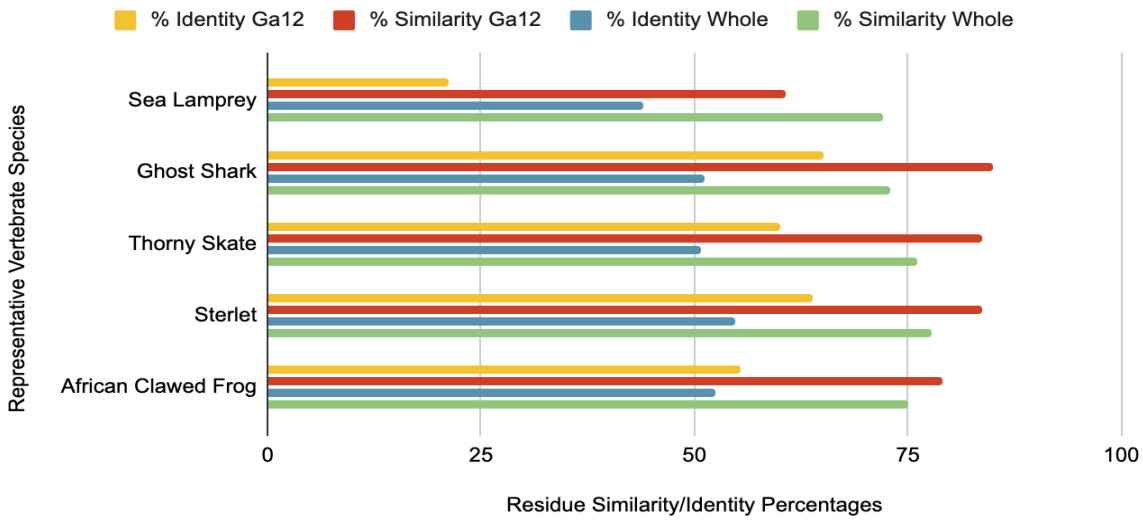

Figure 1. LALIGN Results by Percentage of Similar & Identical Amino Acid Residues

Figure 1. LALIGN amino acid residue similarity and identity percentage results. The 106 amino acid spanning Gα12-binding region of p114 or the entire p114 RhoGEF protein (mammalian-human) was evaluated in comparison to proteins present in representative early vertebrate species that act as each species version of p114 RhoGEF. This follows a progression of early vertebrate fishes from the agnatha to the gnathostomes. African Clawed Frog was included in this data as a later representation of vertebrate species to show in comparison to the otherwise chronological progression from the lamprey to the sterlet.

Bioinformatic analysis revealed the jawless fish *Petromyzon marinus (*Sea Lamprey) to lack the Gα12 binding region. Yet, when examining the next evolutionary member, cartilaginous fish *Callorhinchus milii* (Australian Ghost Shark) the region we identify as the Gα12 binding surface defines itself with a spike of 51.1% identity to the mammalian residues, and 73% similarity. Following this trend through *Amblyraja radiata* (Thorny Skate) and *Acipenser ruthenus* (Sterlet) a gradual but noticeable sharpening of this region, getting closer in identity and similarity to the mammalian version was observed. This bioinformatic analysis suggests the Gα12-interacting region evolved as a relatively recent domain within p114RhoGEF since predating vertebrates, the region is not present in the sequence, as seen with Dp114RhoGEF in *Drosophila* (Fruit Fly). The closest human protein to match the unique Gα12 binding region is, in fact, the Gα12 binding region hosted within the AKAP-lbc RhoGEF. AKAP-lbc's binding site is 47.6% identical to the one found on p114RhoGEF which drove inquiries into whether the same trend of the Gα12 binding site developing in early vertebrates was present here also. The trend seen in p114RhoGEF's bioinformatic data was also observed in AKAP-lbc sequence comparisons.

3.2. Point Mutations and P114RhoGEF Gα12 Binding Site Deletion Mutant Development

To understand the role of the Gα12 binding surface of p114RhoGEF, PCR based mutations within this binding site were engineered. Illustrations of the general structure of p114RhoGEF, including important regional features as well as the mutant constructs are represented in Figure 2. The primary mutation used for this experiment was one that removed the 106 amino acid long Gα12 binding site in its entirety, leaving the protein significantly shorter in total length. This massive region being removed from the protein made piecing the N terminal fragment and the C terminal fragment difficult in comparison to the point mutations that were also created. In order to successfully engineer the desired product the amplimers used had to have a larger overlapping section than 18 base pairs. For this mutation the overlap between adjacent amplimers had to have a 22 base pair overlap. In conjunction with the large deletion mutant two point mutations were designed to change the glutamic acid residues identified in a previous study to have crucial roles within the binding surface[7] to similar, but charge reversal residues being arginine. The first of the two mutants, E695R, following PCR and purification was able to be sequenced but the second, E789R continues in the PCR process. Seeing that many alterations to the protocol of splicing the fragments of the mutated regions together was required in

order to get the large deletion mutant and the first point mutant past PCR steps, further research is required in order to sequence the third mutation.

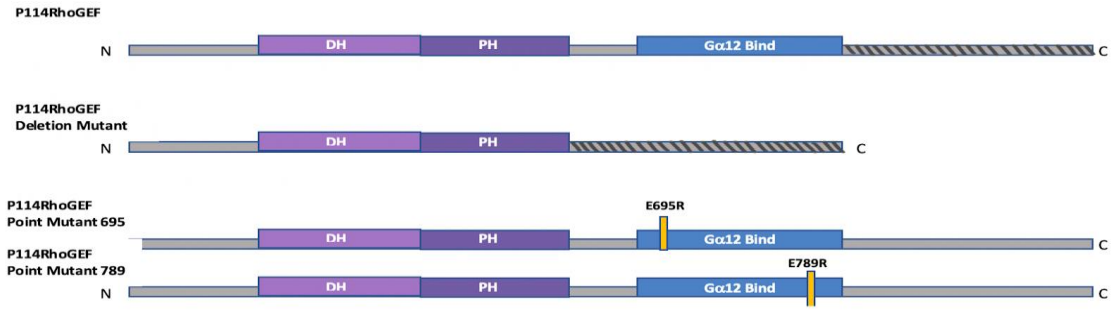

Figure 2. Illustrated Representations of Deletion and Point Mutants within the Gα12 Binding Site of p114RhoGEF

Figure 2. The plasmid pcDNA 3.1 containing a functional, myc-tagged p114RhoGEF created by a past lab member, Ally Brandon, was utilized to splice in three distinct mutant p114RhoGEF constructs. The primary mutant used in this experiment removes the entirety of the Gα12 binding site of the RhoGEF, leaving it 106 amino acids shorter than the native p114RhoGEF. The two other mutations converted crucial glutamic acid residues identified in a previous study[7] to positively charged arginine residues.

3.3 Luminometric Assays Reveal SRE Signal Transductive Decline in Mutant Missing Gα12 Binding Site

To understand the impact that removing the Gα12 binding surface has on p114RhoGEF's ability to perform signal transduction, SRE-mediated signaling assays were conducted.

SRF, Serum Response Factor, is a transcription factor that binds the DNA element SRE in the promoters of multiple genes important to cell growth. Once it is bound with SRE it will stimulate the activation of Gα12/13 via RhoGEFs. RhoGEFs will stimulate Rho which then causes MRFTA to enter the nucleus of a cell. Once in the nucleus MRFTA will bind to SRE and will then drive transcription. In the cells, if an upstream protein such as Gα12/13 or p114RhoGEF drives a signalling pathway SRF will bind to the SRE within the luciferase plasmid and result in major transcription of the gene encoding the firefly luciferase protein. The more signalling that occurs, the greater the light readout on the luminometer. Simply this allows us to begin observing the differences between an unaltered p114RhoGEF and the mutant missing the Gα12 binding site. This difference in signalling ability was examined using two gradients, one spanning from 2ng of DNA per transfection to 200ng (Figure 3) and one at a lower concentration gradient of 0.2ng per transfection to 5ng of DNA.

In regards to luminometric output, we can begin to grasp how that binding site impacts signalling ability for the protein.

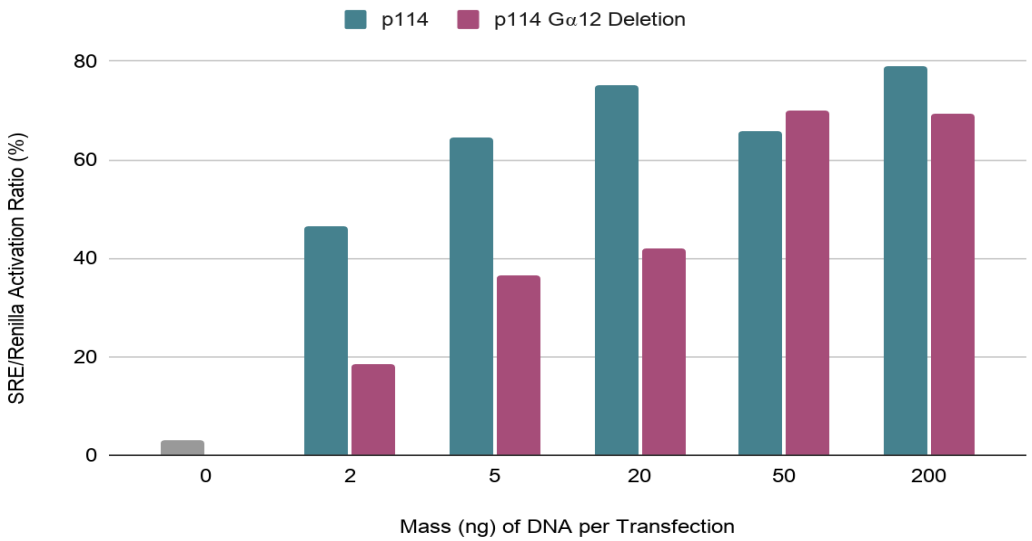

Figure 3 SRE/Renilla Activation Percentage for Gα12-Binding Site Deletion Mutant Versus Unaltered p114RhoGEF along DNA Concentration Gradient

Figure 3. Luciferase assays were used to measure SRF-mediated transcriptional activation. Luminometric readings were evaluated based on the mutant missing this essential Gα12 binding region in comparison to an unaltered p114RhoGEF as the positive control. The control where no DNA from either p114RhoGEF sample was transfected is indicated in grey. The results of this assay prompted a second experiment looking at a lower concentration gradient of DNA per transfection as the difference in signalling was most extreme at the lowest values presented here.

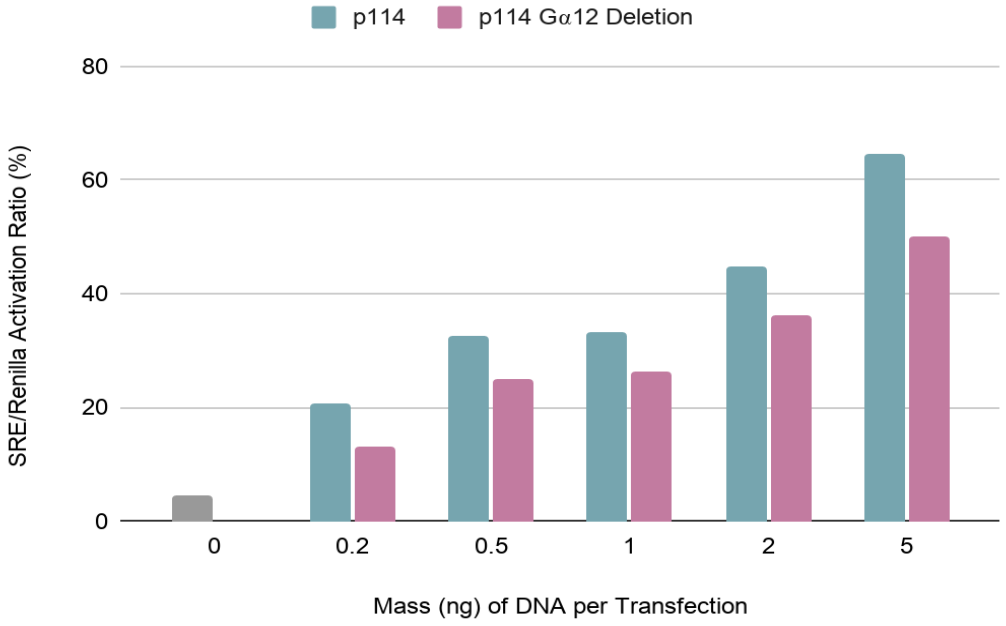

Figure 4. SRE/Renilla Activation Percentage for Gα12-Binding Site Deletion Mutant Versus Unaltered p114RhoGEF along Low-End DNA Concentration Gradient

Figure 4. Luciferase assays were used to measure the SRF-transcriptional activation of p114RhoGEF in an unaltered form versus a mutant missing the Gα12 binding site at low concentrations of DNA per transfection. A control where no p114RhoGEF DNA was transfected into the cells is indicated in grey. This data was to supplement the findings of

the previous SRE experiment that displayed a stepped decline in signalling at the lower range of DNA concentrations used in the transfection of cells. Here we are able to see that at the lower end, Gα12-binding site removal resulted in a loss in signalling strength when compared to untampered p114RhoGEF.

4. Discussion

The intracellular signaling protein p114RhoGEF plays a vital role in the movement and structure of essential junctional positions that govern the cell's ability to function optimally. Through the vast amount of power a small protein such as p114RhoGEF has, it can alter cell morphology, mobility, and structure in ways that, when gone awry, can force oncogenic developments and further metastasis of existing complications [8, 9,12.] Understanding the dynamics of protein interactions is to dissect the regions of said protein for its binding surfaces, unique motifs, and other signaling features and domains. Some domains exist outside traditional models that are far less explored but no less critical. Similar to AKAP-lbc, p114RhoGEF harbors a domain that allows for communication with the α subunit of the G12 subfamily of G proteins. P114RhoGEF appears to have emerged early in vertebrate evolution, being seen in sharks. This bioinformatic approach suggests that a Gα12-interacting surface evolved as a relatively recent domain within p114RhoGEF. This region is completely lacking in invertebrates[9] yet plays an essential role in mammalian cells. Before this region existed, another mechanism would have needed to be used to perform interactions. Such can be seen with Dp114RhoGEF being regulated by the Gβ/ɣ subunit, which is unusual to note. The Gβ/ɣ subunit14 can regulate mammalian p114RhoGEF as it can be in invertebrates, but the region that governs Gα subunit and specifically Gα12 interaction is a relatively new process.

The ability for p114RhoGEF to perform SRE-mediated signaling is less robust when the Gα12 binding site is completely removed from the protein's makeup. Compared to an unaltered version of the protein, this difference in signaling strength is noticeably large, indicating the importance of developing this region on the protein evolutionarily and transductive. The Gα12 binding region development is also relatively new for a similar RhoGEF, AKAP-lbc, which is far more studied than p114RhoGEF. The instance of this region coming into existence for both proteins is different as they are only roughly 47% similar in their amino acid identity. However, two different RhoGEFs similarly manifested the Gα12 binding region is worth dissecting. In future research, it would be interesting to see the impact of splicing the Gα12 binding region of p114RhoGEF into AKAP-lbc and vice versa. Can the two proteins perform optimal signal transduction with the swapped sites, and what are the critical similarities and differences between the binding surfaces? These RhoGEFs notably lack the RH-domain, which other proteins possess in order to mediate Gα12-binding in exchange for their own uniquely different regions with the same general purpose. Why was there a need to have this differentiation, and is it more favorable than the RH-domain model seen in other similar RhoGEFs? If we were able to understand further how the Gα12 subunit and other effectors make contact with p114RhoGEF and similar RhoGEFs, it would be possible to engineer medical solutions for the harm that under/over regulation of these proteins can cause. Thus, further characterization of this binding region would prove insightful to its role in homeostatic regulation for the cell as it moves and interacts with its diverse environment.

5. Acknowledgments

The author of this paper would like to humbly thank the GlaxoSmithKline foundation and the UNC Asheville Undergraduate Research Program for their financial and academic support. Thank you, to UNC Asheville graduate Ally Brandon for their myc-tagged p114RhoGEF construct. I would like to personally thank my fellow lab mates, Sarah Moses, Taylor Durall, Tabor Sykes, and Sarah Young, all of you have been inspiring examples as students and human beings, as well as faces I will never forget once I have graduated from UNCA. It means the world to me to have been able to foster a loving and welcoming environment for which I could pursue my love of biology. I would also like to thank Samantha Nance and Corin O'Shea for being such patient and forthcoming mentors, much of my confidence as a scientist emerged from my time working under your careful and sweet guidance. Lastly, I would like to thank Dr. Ted Meigs for his vast knowledge and passion for this field. It is from his love of the subject that I found my own passion for cell biology, as well as my drive to seek the unknown, and persevere through all manner of challenges. Thank you, everyone.

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