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p114RhoGEF Drives RhoA-Dependent Signaling Through the TEAD, SRE, and NFκB Pathways

Allyson Brandon
Biology
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Thomas E. Meigs

Abstract

Gα12 and Gα13 belong to the G12/13 subfamily of heterotrimeric guanine nucleotide-binding proteins. Much work has been done to identify downstream effectors and regulators of Gα12/13 due to their implication in many human cancers. p114RhoGEF is a downstream effector of Gα12 but not Gα13 that regulates the activity of the monomeric GTPase RhoA. Despite the existing evidence of p114RhoGEF interacting with Gα12 and RhoA, the mechanism of these interactions and the cellular functions they facilitate remain unknown. To understand the significance of the Gα12-p114RhoGEF-RhoA signaling cascade, we examined the ability of overexpressed wildtype-p114RhoGEF to drive signaling through four pathways implicated in growth and tumorigenic signaling (SRE, TEAD, NFκB, and AP1) in transcription-based firefly luciferase assays. We used the RhoA inhibitor, *Clostridium botulinum* C3 toxin, to determine which of these processes are RhoA-dependent. p114RhoGEF drove signaling through all pathways except AP1 and required functional RhoA to do so. The most dramatic effect was seen in SRE-luciferase assays, with luminometric readings over twice as high as in NFκB assays. p114RhoGEF also caused a small but reproducible increase in TEAD pathway stimulation as. These results demonstrate a role of p114RhoGEF and RhoA in stimulating three distinct transcriptional responses implicated in oncogenesis. Future work will include resolving the structural determinants of p114RhoGEF that mediate its interaction with Gα12 and RhoA, and examining whether Gα12 increases p114RhoGEF's ability to drive these pathways. Unraveling the mechanism of action in the Gα12-p114RhoGEF-RhoA signaling cascade, as well as its underlying physical structure, could lead to the development of novel cancer therapeutic strategies.

1. Introduction

Guanine nucleotide binding proteins (G proteins) are responsible for driving a multitude of critical cellular events including hormone signaling, vertebrate development, neurologic development and function, muscle contractions, and cellular differentiation and proliferation[1]. In addition to their normal cellular roles, G proteins are also responsible for many human cancers[2]. The G-protein family is composed of several subfamilies, including Gi, G12/13, Gs, and Gq. In the G12/13 subfamily are the alpha subunits Gα12 and Gα13[3], which are the result of a gene duplication early in the evolution of vertebrates[4]. Although both subunits are in the same subfamily, Gα12 and Gα13 perform non-redundant roles in the cell and interact with several unique effector proteins[5]. They exist as heterotrimers with β and γ subunits that associate with various transmembrane G protein coupled receptors (GPCRs) at the cytoplasmic interface of the cell. An assortment of ligands bind the GPCR on its extracellular binding domain, which activate the receptor and initiate a signaling cascade through Gα12/13. In the process of GPCR activation, the α subunit separates from the βγ dimer, exchanging its initially-bound GDPs for one of the many cytoplasmic GTPs[1]. The monomeric GTP-bound α subunit is then free to begin signaling through numerous downstream partners. As a consequence of

inherent GTPase activity, the α subunit can autocatalyze the hydrolysis of its bound GTP, and regulators of G protein signaling (RGS proteins) hasten this process by activating and enhancing the α subunit's GTPase activity[6].

The Meigs lab and others have identified many novel effectors of Gα12 and Gα13[4,5,7,8,9] (Fig. 1). Pinpointing downstream effectors of Gα12 is particularly important, as progress in this area lags behind that of Gα13, mainly due to the lack of a published crystal structure for Ga12 in complex with any of its target proteins[10].

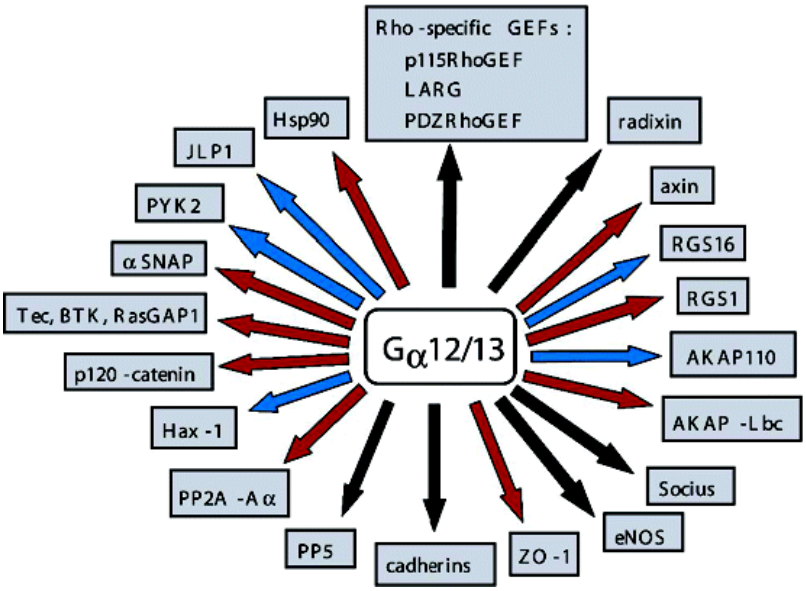

Figure 1. A depiction of identified downstream effectors of the G12/13 subfamily of G proteins. Blue arrows denote a Gα13-specific binding partner, red arrows denote a Gα12-specific binding partner, and black arrows denote proteins that bind both Gα12 and Gα13[5].

A major class of Gα12/13 effectors are the Rho guanine nucleotide exchange factors (RhoGEFs), which are targets of the active (GTP-bound) Gα subunits, and induces the RhoGEF to sequester and activate Rho[11]. These binding partners all contain an RGS-homology (RH) domain, which functions as a protein-binding domain[6], mediating interactions between the RhoGEF and Gα12/13[4]. A unique target of Gα12 is the A kinase anchoring protein (AKAP-Lbc), which does not contain an RH domain[12], but contains other domains characteristic of Rho such as the tandem Dbl homology (DH) and pleckstrin homology (PH) domains[6]. Most RH-RhoGEFs preferentially bind Gα13, but a region of 257 amino acids at the C-terminus of AKAP-Lbc was found by researchers to be necessary for its interaction with Gα12[9]. Moreover, another non-RH RhoGEF, p114RhoGEF[13], shares 47% identity to the Gα12-interaction site in AKAP-Lbc in a region of 106 amino acids located between residues 686-791. In these studies, p114RhoGEF preferentially interacted with Gα12 but not Gα13, suggesting a binding preference for Gα12[9]. Further work involving amino acid substitutions in this region did not reveal any residues necessary for the p114RhoGEF- Gα12 interaction, though two constructs (E759R and E789K) did exhibit slight decrease in binding ability[14].

p114RhoGEF serves as a GEF specific to RhoA, lacking affinity for other monomeric GTPases such as Rac or Cdc42[13]. Several cellular functions utilize p114RhoGEF (presumably in conjunction with Gα12 and RhoA). Normal, physiologic events involving p114RhoGEF include actin stress fiber formation and cytoskeletal rearrangements[13], constructing protective F-actin assemblages at adherens junctions[15], cellular locomotion and normal organ lumen formation[16], and retinal development[17]. However, several detrimental pathologic effects and cancers also implicate p114RhoGEF, including driving metastasis and invasiveness of neuroblastoma cells[18], loss of contact inhibition and induction of epithelial-to-mesenchymal transition in HeLa cells[19], allowing amoeboid tumor cell locomotion[20], and resulting in high lethality and poor prognosis when detected at high levels in some lung cancers[21]. The normal, physiologic functions of p114RhoGEF, particularly its ability to drive cytoskeletal reorganization and cellular migration, are ultimately the same functions that make it effective at driving cancer progression and metastasis[13].

In the present study, we examine p114RhoGEF's ability to drive four oncogenic pathways that lie downstream of Gα12 in human embryonic kidney cells (HEK293 cells) using dual firefly luciferase reporter assays. Previously, p114RhoGEF was shown to signal through the first pathway we chose, the serum response element (SRE) pathway[13], and served as a positive control for the remaining three assays. The remaining reporter assays included three

transcription factor protein families implicated in several human cancers[22, 23, 24]: activating protein-1 (AP1), nuclear factor κ-B (NFκB), and the transcriptional enhanced associate domain (TEAD) reporter assays. The RhoA inhibitor, *Clostridium botulinum* C3 toxin[25], was used to determine if any of these responses were Rho-dependent. The mere overexpression of p114RhoGEF was sufficient in stimulating these pathways, suggesting that like Gα12, mutational activation is not required for p114RhoGEF-mediated signaling. Uncovering the role of p114RhoGEF and its binding partners Gα12 and RhoA in cancer formation and progression could lead to novel cancer therapies, and the discovery of new Gα12 downstream effector proteins.

2. Materials and Methods

2.1 Cell Culture and Transfection

HEK293 cells incubated in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum albumin until approximately 90% confluence in either 16- or 24-well plates were transfected with 200 ng *Renilla* pRL-TK plasmid, 200 ng SRE-luciferase plasmid (firefly), 200 ng reporter plasmid (SRE, NFκB, TEAD, or AP1) and a combination of either 50 ng p114RhoGEF-*myc* plasmid or 50 ng pcDNA.3.1+ negative control plasmid and either 25 ng RhoA inhibitor *C. botulinum* C3-toxin plasmid or 25 ng enhanced green fluorescent protein (EGFP) negative control plasmid. Cells were incubated an additional 24 hours post-transfection, and serum-starved by removing the original DMEM and replacing it with DMEM with no additions (Corning, Corning, NY).

2.2 Luminometry

After 16 hours, cells were rinsed with phosphate-buffered saline (PBS), and lysed using 1X passive lysis buffer (Promega, Madison, WI). Cell lysates were centrifuged at 16,000 x g for 1 minute, and the top 40 µL of the supernatant was measured for luminescence on a GloMax 20/20 luminometer (Promega, Madison, WI) using a Promega Dual-Glo™ firefly luciferase assay kit.

2.3 Protein Separation and Immunoblotting

To analyze the expression levels of p114RhoGEF-*myc*, 20 µL of the remaining centrifuged cell lysate from the previous step were combined with 1M dithiothreitol (DTT) and 4X Laemmli buffer (Bio-Rad, Hercules, CA) and analyzed using SDS-polyacrylamide gel electrophoresis. Western blots were subsequently performed using 1000-millipore anti-*myc* monoclonal primary antibody (mouse), and a 1:7500 dilution of anti-mouse polyclonal secondary antibody (rabbit). Blots were rinsed thrice in tris-buffered saline with 0.1% Tween20 (TBST) while on a platform shaker. Blots were developed colorimetrically using alkaline phosphatase (AP) buffer combined with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) and stored in sterile 50 mL conical tubes filled with distilled deionized water. Blots were imaged using a Kodak Gel Logic 100 gel documentation instrument with CareStream software.

3. Results

3.1 Overexpressed wildtype p114RhoGEF drives RhoA-dependent signaling through SRE, TEAD, and NFκB

Elevated levels of p114RhoGEF drive signaling through TEAD, SRE, and NFκB, but not AP1 (Fig. 2). Signaling to these pathways is RhoA-dependent, as the presence of the *C. botulinum* C3 toxin greatly reduced signaling (Fig. 3). p114RhoGEF is responsible for the luminescence of these reporter plasmid trials as the empty control plasmid pcDNA3.1+ did not show a significant increase in signaling compared to the experimental samples, except in the case of the AP1 samples (Figs. 2 and 3).

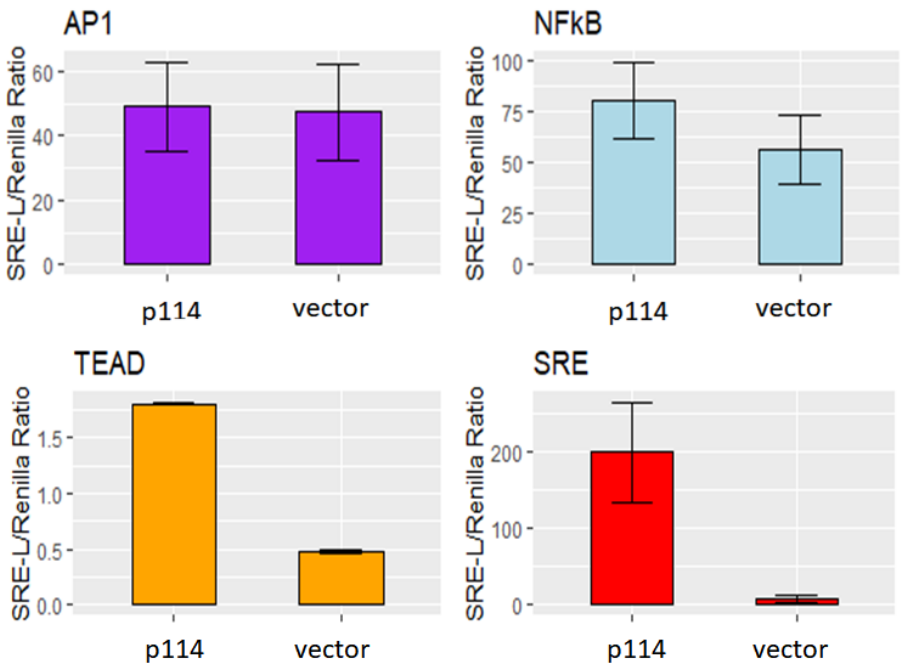

Figure 2. Firefly SRE-luciferase assays showing overexpressed wildtype p114RhoGEF's ability to drive signaling through different oncogenic pathways compared against the empty vector plasmid pcDNA3.1+ without the presence of the RhoA inhibitor, *Clostridium botulinum* C3 toxin. This figure represents a single experiment that is representative of several replicate experiments.

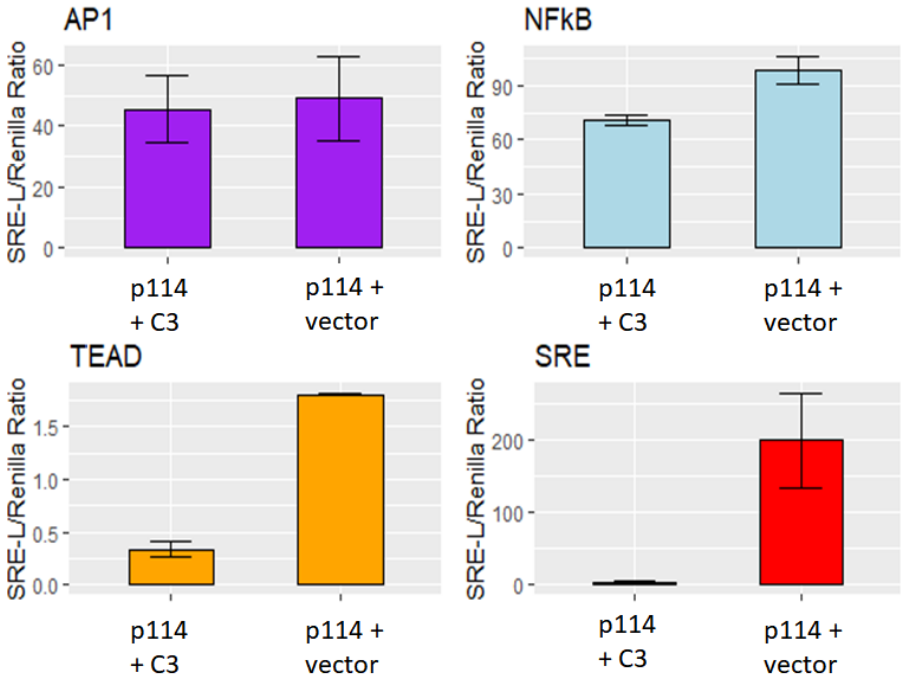

Figure 3. Firefly SRE-luciferase assays showing the effect of the *Clostridium botulinum* C3 toxin on overexpressed wildtype p114RhoGEF signaling through four cancer-causing pathways (TEAD, AP1, NFκB, and SRE). This figure represents a single experiment that is representative of several replicate experiments.

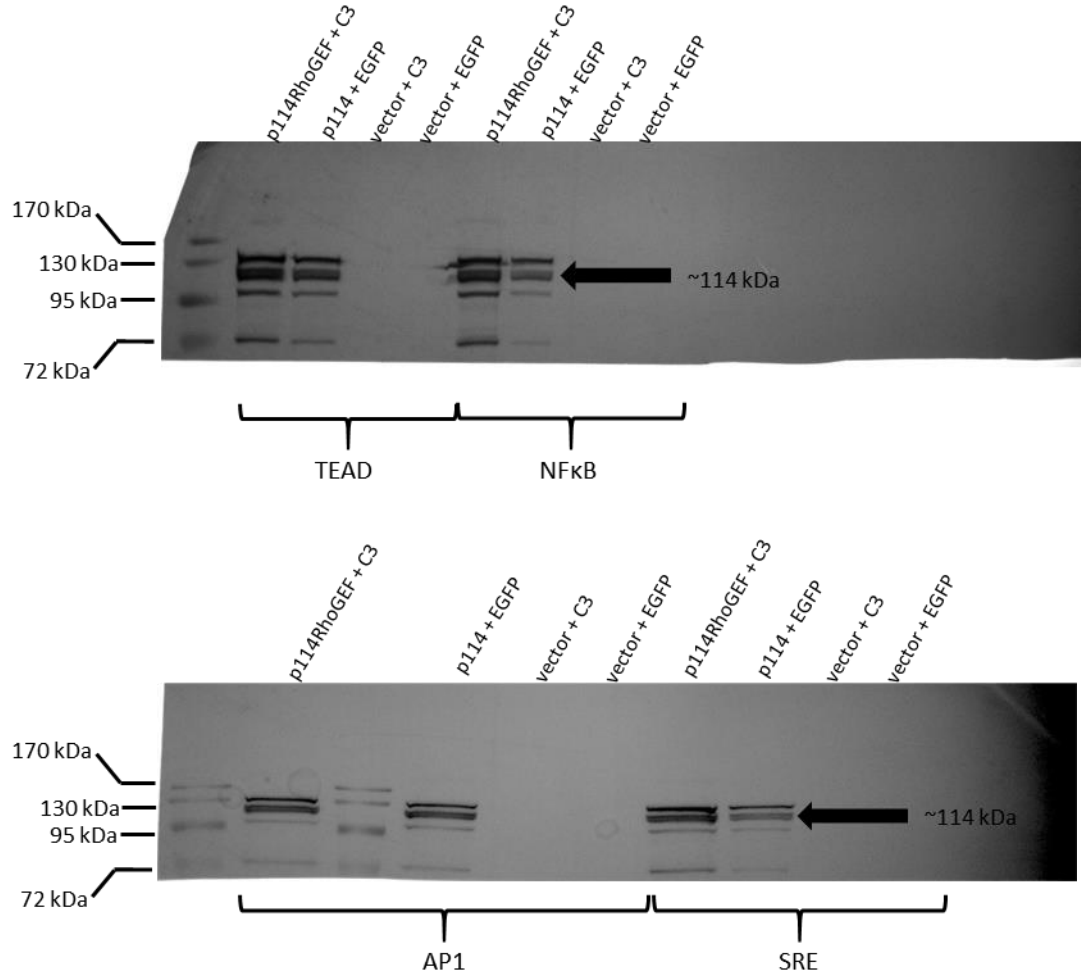

Figure 4. Western blots showing the expression levels of the p114RhoGEF-*myc* plasmid from the assays in figures 2 and 3. PageRuler (ThermoFisher Scientific, Waltham, MA) ladder was used in lane 1 for the top blot and in lanes 1 and 3 on the bottom blot. The four experimental treatments describe the combinations of plasmids in that sample (e.g., the p114+C3 label means that the p114RhoGEF-*myc* plasmid was combined with the *C. botulinum* C3 toxin for that sample, etc.). The reporter plasmid used for the corresponding samples is denoted with brackets (e.g., TEAD means that for all four of the corresponding samples, the TEAD reporter plasmid was added in those reactions). The bands present in the p114 lanes occur at the expected location, with some breakdown product present just above and below the bands at ~114 kDa.

4. Discussion and Conclusion

G proteins and their receptors are the target for many widely-used cancer drugs, so identifying their downstream effectors and the role they play in driving tumorigenesis is vital in developing effective cancer therapies[2]. The serum response element pathway was most responsive to p114RhoGEF, while the TEAD pathway showed a small but reproducible response. The AP1 pathway was not stimulated by p114RhoGEF or RhoA, suggesting Gα12 uses other intermediary proteins besides p114RhoGEF to signal to this pathway. Our results demonstrate for the first time that p114RhoGEF drives signaling through the TEAD and NFκB pathways and support the findings of previous studies showing that p114RhoGEF drives signaling through the SRE pathway[13]. It must be emphasized that the p114RhoGEF plasmid used in this study was not modified other than containing a *myc* tag for easy detection on a western blot; these pathways were stimulated by the mere overexpression of wildtype p114RhoGEF. Also, there was no additional Gα12 or RhoA added or modified in these assays. Other studies have shown that overexpression of Gα12 and Gα13 is

enough to promote oncogenesis, and that mutational activation is not required[26]. Our findings suggest that, like Gα12/13, overexpressed p114RhoGEF is enough to drive signaling through known cancer-implicated pathways in cells. Taken together, these experiments reinforce p114RhoGEF's place in the signaling cascades of Gα12 and RhoA. Future work should focus on creating a constitutively active form of p114RhoGEF, and targeted mutagenesis of p114RhoGEF to determine the mechanism behind its association with Gα12 and RhoA. Further research could reveal p114RhoGEF as an attractive cancer drug target because of its specificity for Gα12, and unravel the Gα12-p114RhoGEF-RhoA signaling axis.

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