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Multiple Phosphorylation-Dependent Regulatory Switches in Gα12 and Gα13 Modulate Cell Growth Signaling

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Abstract

The G12/13 subfamily of heterotrimeric guanine nucleotide binding proteins is composed of Gα12 and Gα13, which play important intracellular signaling roles, including activation of serum response factor (SRF). SRF activates transcription of serum response element (SRE) regulated genes involved in cell growth and tumorigenic events. Previous studies have reported serine phosphorylation occurs near the N-terminus of Gα12, and a specific threonine within the Switch I region of Gα13 serves as a phosphorylation site for Protein Kinase A (PKA). To identify and investigate the roles of these residues on SRE signaling, point mutations were engineered in Gα12 and Gα13 to either abolish or mimic phosphorylation. Replacing a Gα12-specific N-terminal serine with the phosphomimic aspartic acid disrupted Gα12 signaling to SRE, as measured by firefly luciferase assays. Signaling by Gα13 was unaffected by phosphomimics of potentially homologous serines, suggesting a Gα12-specific mechanism. Insertion of the Gα12 N-terminus harboring the phosphomimic into Gα13 bestowed negative regulation of SRE signaling upon this chimeric protein. These results point to a kinase-dependent switching mechanism at the N-terminus of Gα12 that governs its growth signaling. The ability of the phosphomimic-containing Gα12 to activate SRF was rescued by inserting a sequence allowing covalent attachment of a myristoyl lipid, suggesting the phosphomimic interferes with palmitoylation of the G protein. To investigate the effects of PKA on SRE signaling, the catalytic subunit of PKA was overexpressed in tandem with phosphomimics and blocks within the Switch I region of Gα12 and Gα13. PKA dramatically increased growth signaling by both G proteins, but this effect was mitigated in the presence of a Thr-to-Ala block suggesting that phosphorylation by PKA within the Switch I region of G12/13 proteins stimulates basal cell growth. Together, these kinase dependent switches may regulate G12/13 activity and serve as targets for anti-cancer therapies.

1. Introduction

For a cell to survive, it must receive extracellular signals and transduce that information intracellularly through a series of complex pathways. G protein coupled receptors, or GPCRs, make up the largest family of cell surface receptors and serve as the first step in G protein mediated functions[1]. G proteins are heterotrimeric in nature, containing a guanosine diphosphate (GDP) bound α subunit, as well as a β and ɣ heterodimer. Following activation of the GPCR, the G protein undergoes a conformational change in which GDP is exchanged for guanosine triphosphate (GTP), leading to the dissociation of the GTP bound α subunit from the βɣ heterodimer. In their active, dissociated forms, these subunits will proceed to interact with various downstream effectors to initiate vital cellular events, such as changes in cytoskeletal arrangement, cell growth, and sensation[1]. For a G protein to return to its inactive state, the third phosphate must be cleaved from GTP, returning it to GDP, at which time the α and βɣ subunits reassociate and bind to a GPCR to await further stimulation[1]. For the α or ɣ subunits to remain functional, they must be tethered to the cell membrane through the attachment of a lipid, an isoprenyl group for ɣ and a palmitoyl or myristoyl fatty acid for an α subunit[2]. There are four subfamilies of G α subunits, Gi, Gq, Gs, and G12/13. Of these, G12/13, which

encompasses Gα12 and Gα13, is of oncogenic interest due to their ability to drive cancerous progression through mere overexpression of the wildtype protein[1].

One of the best studied classes of G12/13 effectors are the Rho guanine nucleotide exchange factors, or RhoGEFs, which stimulate the activity of RhoA. These proteins regulate cytoskeletal actin, the membrane transport pathway, cell growth signaling, and transcription[3], while also playing essential roles in cancer progression and tumorigenesis[4]. Known downstream RhoGEF targets of G12/13 include PDZ-RhoGEF, P115-RhoGEF, and Leukemia associated RhoGEF, or LARG[5]. Gα12/13 directly bind to these RhoGEFs to indirectly regulate RhoA activity. RhoA serves as an important step in the serum response element (SRE) growth signaling pathway. SRE is a regulatory sequence found in several growth-factor mediated promoter regions[6] (Figure 1). Following activation of RhoA by a RhoGEF, myocardin-related transcription factor (MRTF-A) enters the nucleus and forms a complex with serum response factor (SRF). This complex then binds to the SRE promoter region, initiating transcription of the associated gene[7]. SRE-regulated genes are involved in several functions involved in cancer progression, including cell growth, oncogenic transformation, migration, and invasion[8].

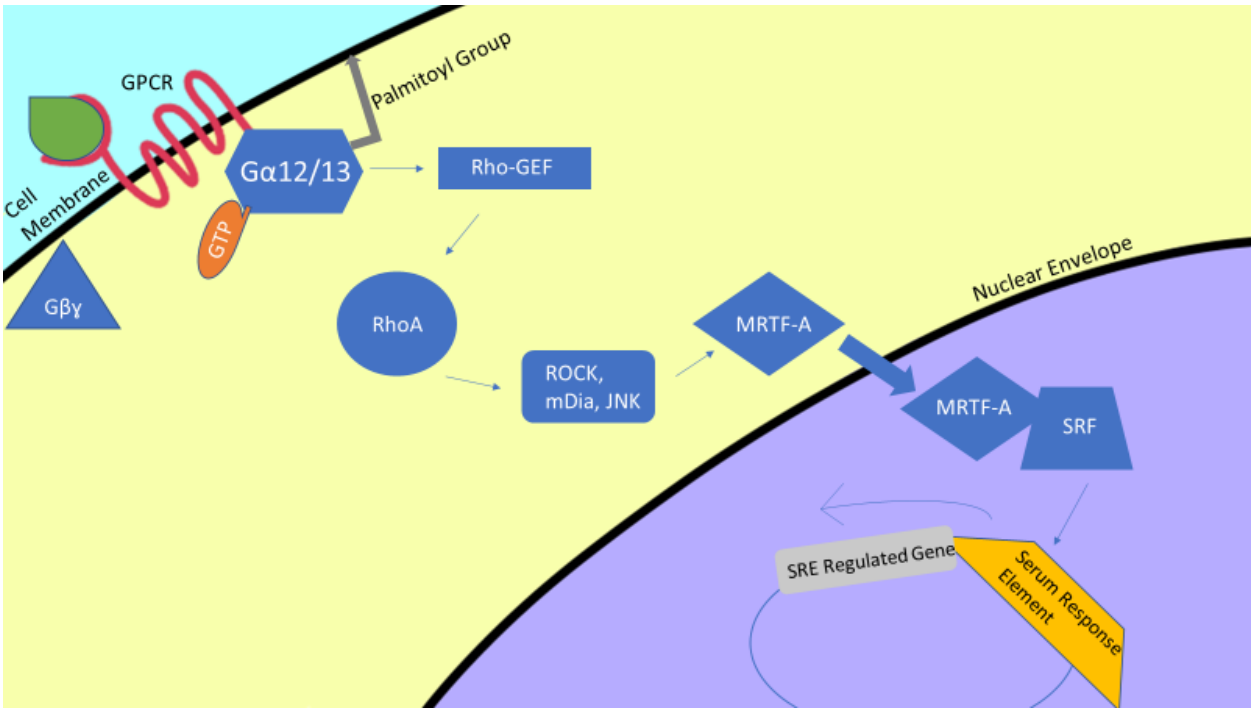

Figure 1. Serum response factor signaling pathway. Following stimulation of the GPCR (red) via an extracellular ligand (green), Gα12 undergoes guanine nucleotide exchange and accepts GTP (orange), resulting in the dissociation of Gα from βγ. Remaining anchored to the cell membrane by a palmitoyl group (dark gray), Gα12/13 stimulate ShoGEFs, which subsequently drive activation of RhoA. RhoA activates a series of downstream effectors, which results in MRTF-A entering the nucleus, binding to SRF, and forming a complex with SRE (yellow). At this time, the SRF regulated gene (light gray) is promoted.

The effects of phosphorylation on the regulation of G are not well understood at a mechanistic level. The roles of kinases as a regulators of G proteins are not well understood. Most of the work on the relationships between G proteins and kinases was performed in the 1990s and focused largely on the role of kinases downstream of Gα activation[9,10,11]. Such kinases include the serine/threonine Protein Kinase Cs (PKC), which are instrumental in the regulation of smooth muscle contraction, perception of opioids, secretion, and memory, as well as the serine/threonine Protein Kinase As (PKA), which serve key roles in carbohydrate and lipid metabolism[9,10,11].

Within the G12/13 subfamily, two key phosphorylation events of the α subunit have been described: phosphorylation by PKC and phosphorylation by PKA[9]. It was discovered that the overexpression of constitutively active Gα12/13 increased Na^+/H^+ exchange within COS-1 cells. However, when exposed to the phorbol ester phorbol 12-myristate 13-acetate (PMA), this increase in activity was blocked in cells transfected with Gα12 but was unaffected in cells expressing Gα13[12]. Phorbol esters, such as PMA, have been shown to trigger activation of several isoforms of PKC and are frequently used to study its effects on a system[13]. These findings suggested that the signaling pathway resulting

in the activation of Na^+/H^+ channels by Gα12 is regulated in part by PKC, while Gα13 utilizes a different pathway and is unaffected by the kinase[12]. It was later revealed using PMA that PKC could directly phosphorylate Gα12 *in vitro*, as well as Gαz[13]. Phosphorylation of Gα12 was also shown to occur *in vivo* following PMA exposure. Consistent with the findings of Dhanasekaran et al., such phosphorylation was not observed in Gα13[12,13]. Protein interaction experiments revealed that following phosphorylation of Gα12, binding affinity between Gα12 and Gβɣ was reduced. This suggested that phosphorylated Gα12 could remain in its active form longer before recoupling with Gβɣ. Using a trypsin protection assay, in which the N-terminus is cleaved from the remainder of a GTP-bound G protein, it was discovered that the PKC-targeted phosphorylation site, or phosphosite, lay at the N-terminus of Gα12[13]. This phosphosite, was not identified by Kozasa and Gilman but was narrowed down to either Ser9 or Ser38. Ser38 appeared the most likely candidate as the surrounding, basic residues, RRRSR, closely resemble the basic residues surrounding the phosphorylated Ser16 in Gαz: RRSRR[13]. Contrary to earlier findings, a separate study found evidence that Gα13 undergoes PKC dependent phosphorylation alongside Gα12 *in vivo* following platelet activation, though *in vitro* phosphorylation of Gα13 by PKC has yet to be observed[14]. A major goal of the present study is to determine whether N-terminal phosphorylation regulates cell growth through a Gα12-specific mechanism.

The second key phosphorylation event reported in the Gα12/13 subfamily is the phosphorylation of Gα13 by PKA. Following the discovery that thromboxane A2 receptor-bound Gα13 was phosphorylated in a PKA dependent process, resulting in inhibition of platelet activation, it remained unclear as to whether PKA was phosphorylating Gα13 directly or if it simply facilitated phosphorylation of Gα13 by a different kinase[15]. It was later discovered that PKA phosphorylates Gα13 directly at Thr203, which lies within the Switch I region[16]. The Switch I region of the α subunit contains key residues essential to the binding of both GTP and the βɣ dimer[17,18]. As a result, the Switch I region plays important roles in regulating both the activation of the α subunit and its recoupling to the βɣ subunits[17]. Phosphorylation by PKA was observed to stabilize the bond between Gα13 and the thromboxane A2 receptor while also reducing the binding affinity between Gα13 and Gβɣ[16]. Additionally, it was revealed that an increase in PKA activity resulted in a significant decrease in RhoA activation via Gα13. This decrease in RhoA activation was not seen following mutation of Thr203, further implicating it as the phosphosite of PKA while also identifying PKA as a regulatory kinase in the Rho signaling pathway[16]. Of interest, there is a homologous threonine in the Switch I region of Gα12, Thr206, that has yet to be investigated.

Throughout this study, we hoped to explore the effects of N-terminal and Switch I phosphorylation on SRE growth signaling by Gα12/13 within human embryonic kidney cells (HEK293 cells) as measured by dual luciferase assays. A series of N-terminal Gα12 mutations were designed to either mimic and block phosphorylation at the potential phosphosites identified by Kozasa and Gilman[13] in order to identify phosphorylation sites, as well as the effects of phosphorylation on SRE signaling. Due to the surrounding residues, the key N-terminal site in Gα12 was expected to be Ser38. We hypothesized that phosphorylation mimicking mutants would increase SRE-mediated growth signaling because of the observed reduction in binding affinity between the phosphorylated Gα12 subunit and Gβɣ heterodimer. Our findings led us to investigate the N-terminus of Gα13, as well as the effects of N-terminal phosphorylation on post-translational lipidation of the protein. Additionally, we hoped to investigate the effects of phosphorylation of Gα12/13 by PKA on SRE-mediated signaling. Phosphorylation of Gα13 by PKA has been observed to reduce RhoA signaling, leading us to hypothesize a decrease in SRE growth signaling[16]. Our results led us to expand upon earlier work in the Meigs' Lab, in which a series of phosphorylation mimicking and blocking mutations were engineered within the Switch I region of Gα12 and Gα13, to determine their effects on growth signaling.

2. Methods

2.1 PCR-Derived Mutagenesis

All Gα12/Gα13 variants were engineered using polymerase chain reaction (PCR). Initial fragments were created using engineered oligonucleotides containing the desired mutation and DNA containing Gα12QL-myc, Gα13QL-myc, or a previously constructed mutant as a template. Each template plasmid contained both a myc tag, recognizable by the anti-myc antibody (Millipore), and a glutamine to leucine mutation to render the translated G protein constitutively active. The resulting fragments overlapped by 19-20 base pairs and were used as a template for secondary PCR. The final product of the secondary PCR contained the full, desired gene flanked by restriction sites. The PCR product was digested using the site's corresponding restriction enzymes and cloned into the vector plasmid PcDNA 3.1(-). The resulting plasmids were purified and sequenced (Genewiz, NJ).

2.2 Mammalian Cell Culture and Transfection

HEK293 cells were grown in Dulbecco's Modified Eagle's Media with 10% fetal bovine serum and were passaged once they reached confluence. The cells were suspended using a 0.25% trypsin solution, distributed equally throughout 12 well plates, and incubated at 37° C until the cells reached ~80% confluence. At this time, each well was transfected using 200 ng SRE-luciferase plasmid, 10-20 ng Renilla PRL-TK plasmid, and 50 ng of a plasmid encoding a driving protein, be it Gα12QL-myc (with the exception of the N-terminal investigation, in which Gα12QL lacked the myc tag), Gα13QL-myc, their engineered variants, or PcDNA 3.1(-). In all cases, either Gα12QL-myc or Gα13QL-myc served as a positive control. In experiments examining the effects of PKA, cells were also transfected with 50 ng catalytic PKA or PcDNA3.1(-).

2.3 Dual Firefly Luciferase Assays

Forty-eight hours following transfection, cells samples were washed in 1 mL 1x PBS, lysed with 250-400 µL passive lysis buffer, and subjected to light agitation for 25 minutes or until cells were observed to be detached from the plate. Cell lysates were centrifuged at 16,000 x g and the top 40 µL were removed to perform the assay. The dual luciferase assay system was executed using a GloMax 20/20 luminometer (Promega). The luminescence was measured twice per sample. The first measurement was taken after catalyzing a luminescent reaction for the SRE-mediated firefly luciferase. The second measurement was taken immediately following the catalyzation of the Renilla luciferase, partnered with the quenching firefly luciferase reaction to account for transfection efficiency. The strength of the signaling pathway is represented as a ratio of these two measurements and scaled relative to the positive control.

2.4 SDS-Page and Immunoblotting

In order to analyze protein expression, samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. Following execution of the luciferase assay, cell lysates were denatured in 4x Laemmli buffer containing 0.1M dithiothreitol at 72° for approximately ten minutes. The prepared samples were then run on a 12% polyacrylamide gel at 135 volts. Once separated, the samples were transferred from the polyacrylamide gel to nitrocellulose membrane for immunoblotting. Blots were probed using anti-Gα12 (Genetex) or anti-Gα13 (Millipore) polyclonal primary antibodies, followed by secondary anti-mouse or anti- rabbit antibodies (Promega). Western blots were developed using 5-bromo-4- chloro-3-indolyl phosphate, nitroblue tetrazolium, and alkaline phosphatase before being imaged using a Kodak Gel Logic 100 system.

2.5 Analysis

During the investigation of the G protein N-terminus, data was blocked by the date of each experiment where applicable and subjected to statistical analysis via ANOVA with the exclusion of the negative controls for the driving, followed by the Tukey Honest Significant Difference (HSD) *post hoc* test. To determine the effects of Switch I phosphorylation and the overexpression of catalytic PKA on SRE signaling, data was blocked by the date of the experiment and subjected to analysis via two-factor ANOVA with interaction, followed by the Tukey HSD *post hoc* test. In all cases, separate tests were performed for Gα12 and Gα13 derived mutants. This analysis included the negative controls for the driving protein. All analyses were performed using RStudio[19].

3. Results

3.1 N-terminal Phosphomimic Abolishes SRE-mediated Growth Signaling at in Gα12

In order to investigate the effects of serine phosphorylation on the N-terminus of Gα12, Ser9 and Ser38 were subjected to point mutagenesis to produce variants that either, through their negative charge, mimicked constitutive phosphorylation (Ser to Asp) or blocked all phosphorylation (Ser to Ala). The resulting mutants, 12S9D, 12S9A, 12S38D, and 12S38A, were subjected to the luminometry assay to investigate their effects on growth signaling via the SRF pathway, alongside 12QL and 12G228A, a constitutively inactive variant of Gα12 incapable of performing

the guanine nucleotide exchange required to signal. 12S9D failed to drive SRE signaling at 4.05 ± 0.47% of the signaling intensity of Gα12QL, and signaled significantly weaker than any other protein examined ($F_{4,11}$=13.81, p<0.001; Tukey HSD *post hoc* test, p≤0.05) 12S9A, the phosphorylation blocking counterpart of 12S9D, signaled robustly as did 12S38D and 12S38A, with no significant difference between these variants or Gα12QL (Tukey HSD *post hoc* test, p>0.05) (Figure 2).

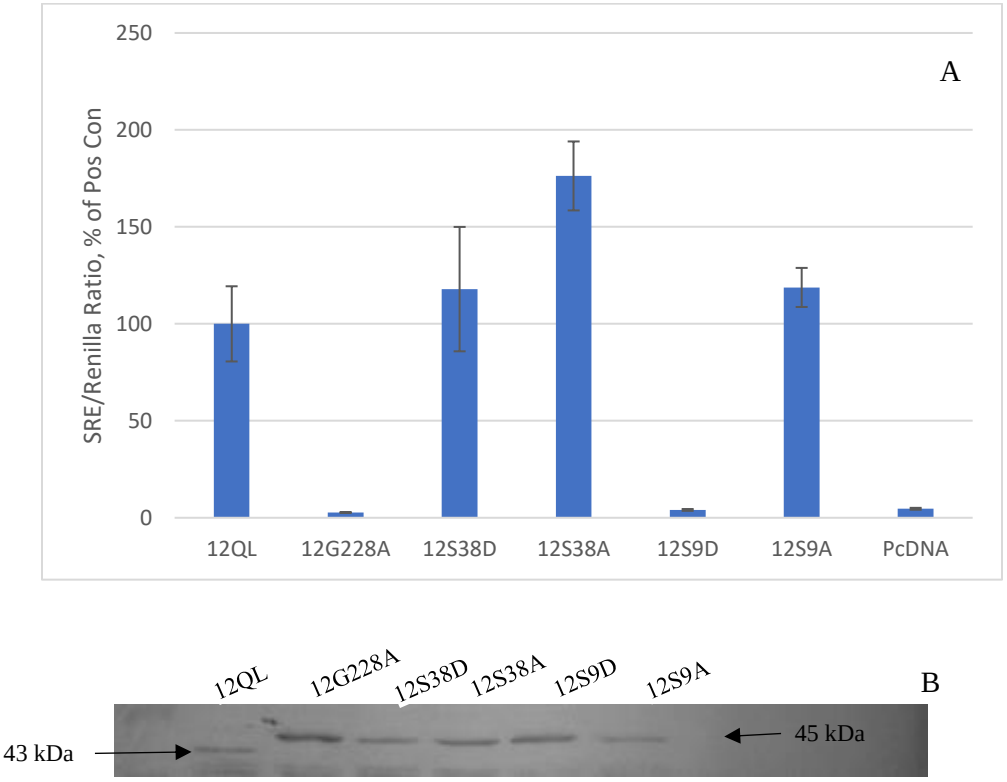

Figure 2. SRE signaling strength relative to Gα12, as measured by luciferase assay (A, *Methods 2.2).* Values are presented as the average percent of the mean positive control value (set at 100% in each experiment) ± standard error of the mean. While 12S9D fails to drive SRE-mediated signaling, all other constructs signal robustly. Equivalent levels of protein expression were verified by immunoblotting (B, *Methods 2.4*). The Western blot qualifies protein expression for a single experiment that is representative of several replicates. While PcDNA was not probed on this Western Blot, no bands were produced at 45 kDa by PcDNA in any replicates.

In order to determine whether this potentially negative regulation of SRE signaling in Gα12 was conserved in Gα13, phosphorylation mimicking mutants were engineered in Gα13 at three potentially homologous serines, Ser7, Ser9, and Ser12 to see if the failure to signal observed in 12S9D was shared with the ortholog of Gα12. Each of the resulting mutants, 13S7D, 13S9D, and 13S12D signaled strongly with no significant difference between any variant and Gα13QL-myc ($F_{3,9}$=3.30, p=0.071) (Figure 3).

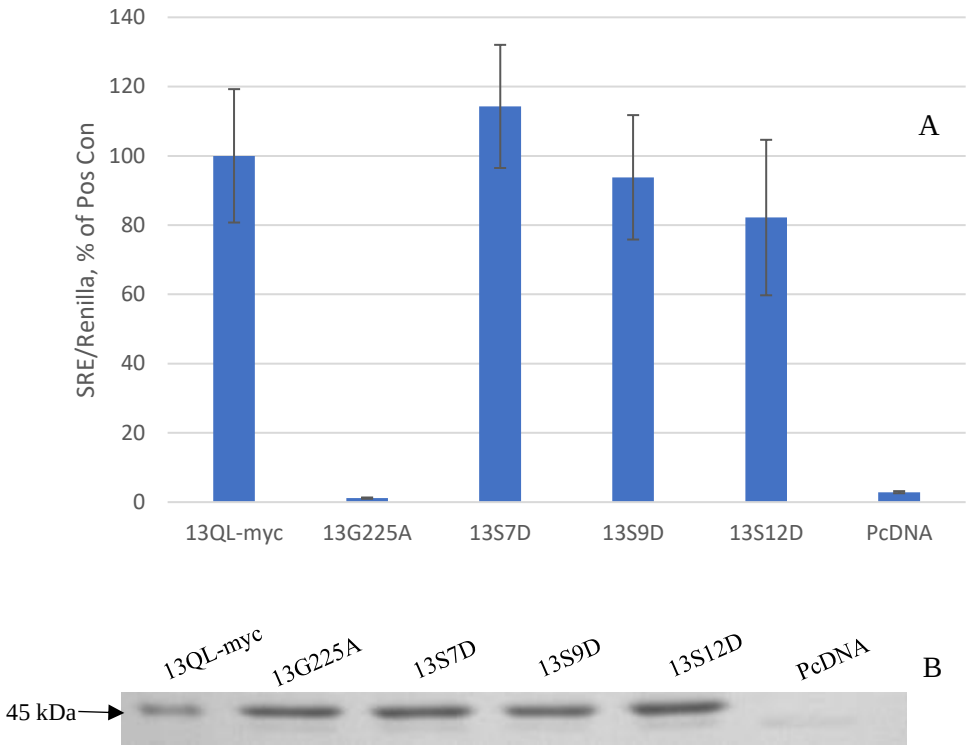

Figure 3. SRE signaling strength relative to Gα13QL-myc, as measured by luciferase assay (A, *Methods 2.2).* Values are presented as the average percent of the mean positive control value (set at 100% in each experiment) ± standard error of the mean. All constructs signal robustly. Equivalent levels of protein expression were verified by immunoblotting (B, *Methods 2.4*). The Western blot qualifies protein expression for a single experiment that is representative of several replicates.

In order to determine the significance of the effect of the phosphorylation mimicking mutation on the rest of the overall functionality of the protein, two chimeras were engineered in which the first 46 residues of Gα13QL-myc were replaced with the N-terminus of Gα12QL-myc. The splice point was positioned in a region of extreme homology. One mutant, 12QL-Nterm, contained no further mutations, while 12S9D-Nterm contained the phosphomimic at Ser9. The SRE-luciferase assay revealed that 12QL-Nterm did not significantly affect signaling relative to Gα13QL-myc ($F_{2,5}$=62.14, p≤0.001; Tukey HSD *post hoc* test, p>0.05). Alternatively, 12S9D-Nterm experienced a significant reduction in SRE signaling at 7.55 ± 2.24% of the signaling strength of 13QL-myc (Tukey HSD *post hoc* test, p≤0.05) (Figure 4).

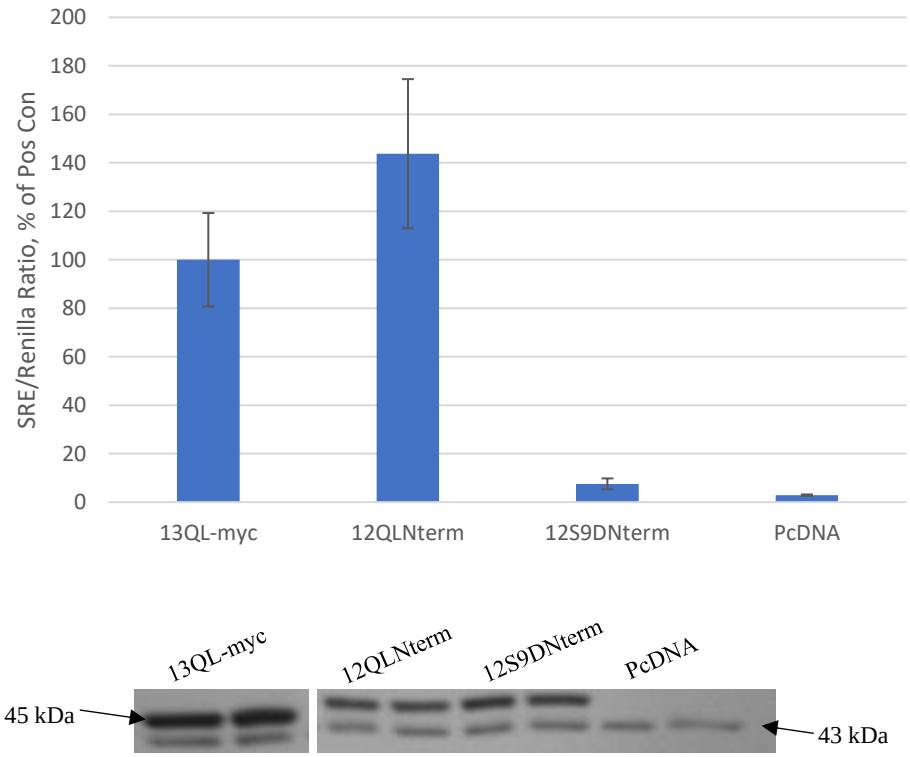

Figure 4. SRE signaling strength relative to Gα13QL-myc, as measured by luciferase assay (A, *Methods 2.2).* Values are presented as the average percent of the mean positive control value (set at 100% in each experiment) ± standard error of the mean. While 12S9DNterm fails to drive SRE-mediated signaling, 12QLNterm signals strongly. Equivalent levels of protein expression were verified by immunoblotting (B, *Methods 2.4*). The Western blot qualifies protein expression for a single experiment that is representative of several replicates. Bands present at 43 kDa represent native Gα13, which is chromosomally encoded and lacks the QL mutation and the myc tag.

3.2 Insertion of a Myristoylation Sequence Rescues Ability of 12S9D to Drive SRE-mediated Growth Signaling

Like all G proteins, Gα12/13 must be lipidated in order to localize to the cell membrane[2]. Proteins within the G12/13 subfamily undergo palmitoylation, the post translational attachment of a palmitoyl group to an N-terminal cysteine. While Gα13 is palmitoylated at two locations, Cys14 and Cys18, Gα12 is palmitoylated only once at Cys11. Failure to palmitoylate results in a loss of signaling capability[2]. However, previous work has shown that signaling via the SRF pathway in depalmitoylated Gα12 and Gα13 can be rescued by inserting a sequence allowing for the covalent attachment of a myristoyl group at the N-terminus via PCR mutagenesis[20]. This myristoyl group does not affect SRE signaling in successfully palmitoylated Gα12 or Gα13 but provides the necessary lipid anchor in variants where palmitoylation cannot occur. To determine if the phosphorylation mimic at Ser9 in Gα12 affected palmitoylation, a series of four mutants were engineered containing the myristoylation consensus sequence. These variants were myristoylated forms of Gα12QL-myc (12QL-myc-myrst), 12QL-NTerm (12QLNterm-myrst), 12S9D-NTerm (12S9DNterm-myrst), and 12S9D (12S9D-myrst). Following analysis of the chimeric variants relative to Gα13QL-myc, it was revealed that while 12S9D-NTerm remained incapable of driving SRE signaling, there was no significant difference between to Gα13QL-myc, 12QL-NTerm, 12QLNterm-myrst, or 12S9DNterm-myrst ($F_{4,5}$=11.83, p=0.009; Tukey HSD *post hoc* test, p>0.05) (Figure 5A). Insertion of a myristoylation group resulted in a significant increase in SRE-signaling ability between 12S9D-NTerm and 12S9DNterm-myrst (Tukey HSD *post hoc* test, p0.05). Similarly, while 12S9D-myrst also experienced significantly stronger SRE signaling than 12S9D ($F_{3,4}$=43.32, p=0.002; Tukey HSD *post hoc* test, p≤0.05) there was no significant difference in SRE-mediated growth signaling between Gα12QL-myc, 12QL-myc-myrst, or 12S9D-myrst (Tukey HSD *post hoc* test, p>0.05) (Figure 5B). These findings suggest a relationship between palmitoylation of Gα12 and phosphorylation of Ser9.

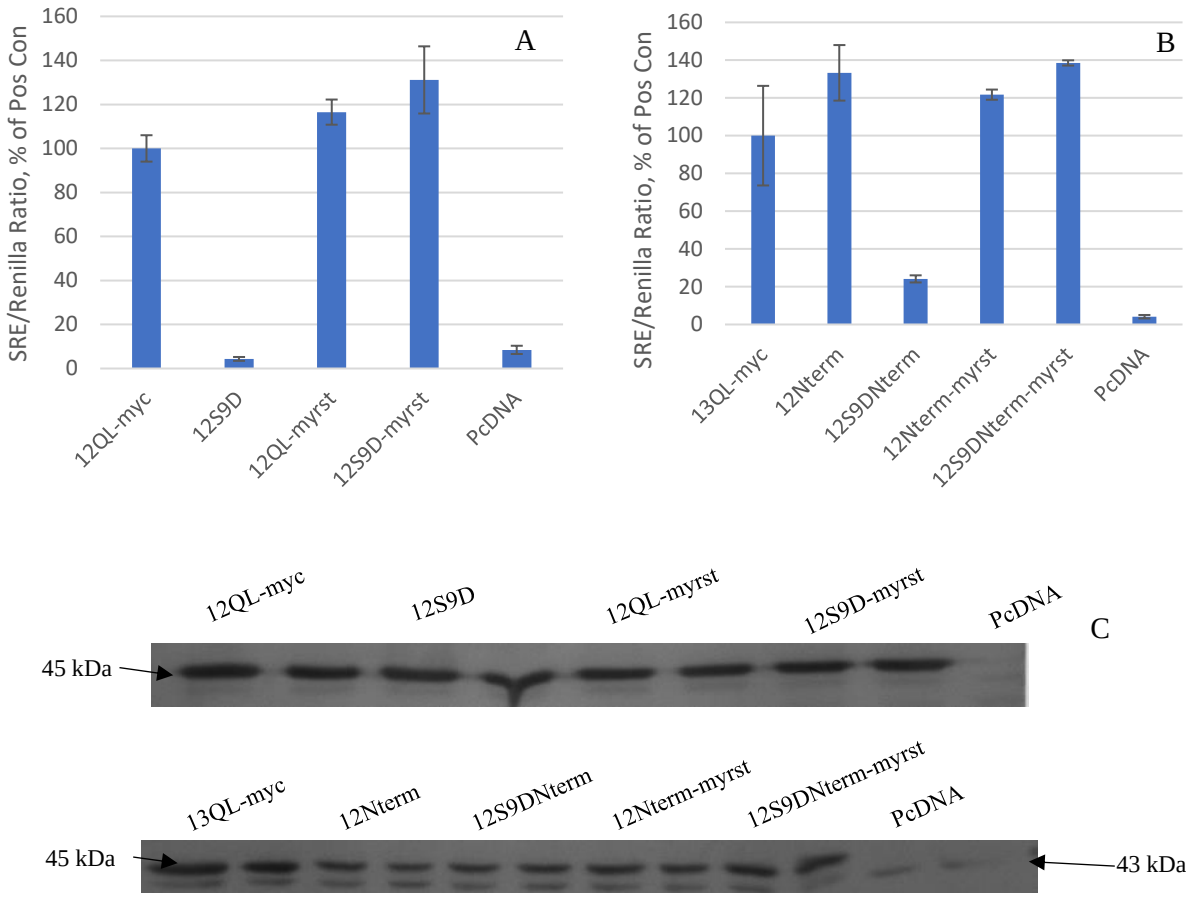

Figure 5. SRE signaling strength relative to Gα12QL-myc (A) or Gα13QL-myc (B), as measured by luciferase assay (*Methods 2.2*). Values are presented as the average percent of the mean positive control value (set at 100% in each experiment) ± standard error of the mean. Myristoylation of 12S9D and 12S9DNterm rescues SRE signaling. Equivalent levels of protein expression were verified by immunoblotting (C, *Methods 2.4*). The Western blot qualifies protein expression for a single experiment that is representative of several replicates. Bands present at 43 kDa represent native Gα13, which lack the QL mutation and the myc tag.

3.3 Catalytic PKA Stimulates SRE-mediated Growth Through Phosphorylation of the Switch I Region in Both Gα12 and Gα13

Following the research of Manganello et al., 2003[16] and previous work in the Meigs' lab, the Switch I region of the G12/13 subfamily was investigated to determine the effects of phosphorylation of this region on SRE signaling. The luciferase assay revealed a significant interactive effect between the driving and modifying protein on SRE signaling ability in both Gα12 ($F_{3,43}$=16.71, $p<0.001$) and Gα13-derived variants ($F_{3,32}$=16.82, $p<0.001$). When co-expressed with an overabundant catalytic subunit of PKA, both Gα12QL-myc and Gα13QL-myc experienced highly significant increase in SRE-mediated growth signaling (Tukey HSD *post hoc* test, $p\leq0.05$). Overexpression of catalytic PKA also increased basal SRE signaling in cells transfected with an empty vector, though this difference was not significant when analyzed alongside either Gα12 or Gα13-derived variants (Tukey HSD *post hoc* test, $p>0.05$) (Figure 7). Previous work in the Meigs' lab resulted in the creation of phosphorylation blocks and phosphorylation mimics using both aspartic and glutamic acid at Gα13 Thr203 (13T203A, 13T203D, and 13T203E) and the homologous Gα12 Thr206 (12T206A, 12T206D, and 12T206E). In all cases, the mutation resulted in a decreased ability to drive growth signaling, though the decrease was much more substantial in the phosphorylation blocking variants, 13T203A,and

12T206A. Additionally, all mutants were shown to block the dramatic increase of signaling ability associated with the overexpression of catalytic PKA, with no significant difference between mutants transfected with an empty vector with those transfected with the overexpressed catalytic subunit of PKA (Tukey HSD *post hoc* test, $p>0.05$) (Figure 7). These findings further implicating the Switch I Thr as the site of phosphorylation by PKA.

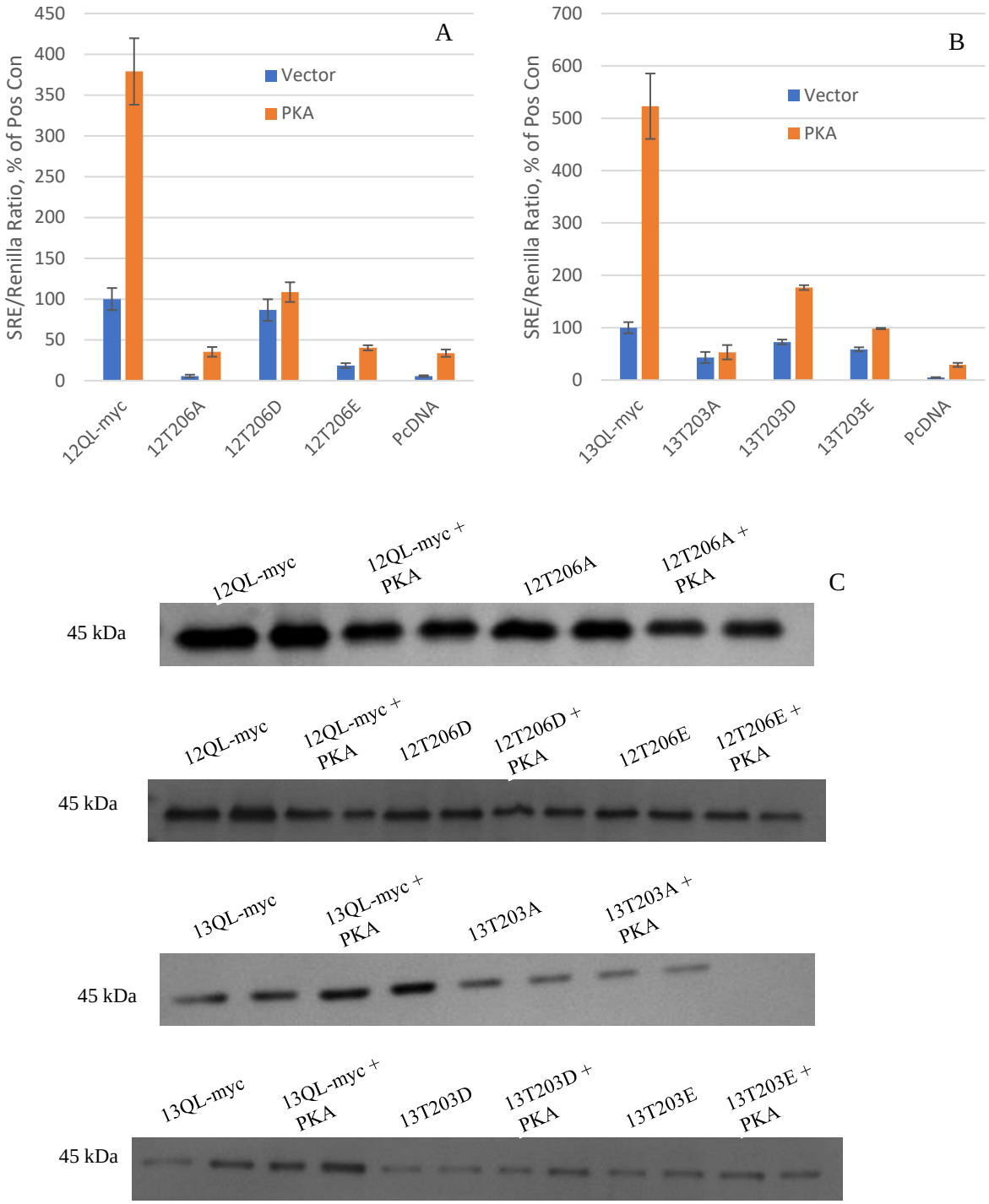

Figure 7. SRE signaling strength relative to Gα12QL-myc (A) or Gα13QL-myc (B), as measured by luciferase assay (A, *Methods 2.3)*. Values are presented as the average percent of the mean positive control value (set at 100% in each experiment) ± standard error of the mean. Samples were either transfected alongside an empty vector (blue) or

the catalytic subunit of PKA (orange, *Methods 2.*2). Expression of the catalytic subunit of PKA led to a sharp increase in SRE-mediated signaling. This increase was mitigated through mutation of the Switch I Thr. Equivalent levels of protein expression were verified by immunoblotting (C, *Methods 2.4*). The Western blot qualifies protein expression for a single experiment that is representative of several replicates.

4. Discussion

This project investigated phosphorylation sites at the N-terminus of Gα12/13, the effects of N-terminal phosphorylation on lipidation, and the effects of PKA phosphorylation in the Switch I region on SRE growth signaling. We investigated potentially kinase-targeted serines at the N-terminus of Gα12 using a series of phosphomimics and blocks and discovered a possible phosphosite at Ser9, which, when replaced with a phosphorylation-mimicking aspartic acid, halted SRE-mediated growth signaling. This appears to be a Gα12 specific behavior, due to the failure of potentially homologous serines in Gα13 to replicate this inhibition of signaling. Additionally, when we inserted the N-terminus containing the phosphomimicking mutation into Gα13, the negative regulation of SRE signaling was transferred as well. Together, our findings suggest a kinase-dependent switching mechanism at the N-terminus of Gα12 that regulates its growth signaling. Such a mechanism could serve as a therapeutic site and be utilized to slow the progression of oncogenic processes[8]. However, these findings do not necessarily implicate Ser9 as the PKC observed by Kozasa and Gilman, nor do they dismiss the hypothesized Ser38 phosphosite[13]. It is possible that Ser9 is the target of a different kinase altogether. To determine which serine is phosphorylated by PKC, the true effects of PKC on Gα12-driven SRE signaling would need to be observed, then blocked by a non-phosphorylatable variant. PKC activity could be increased by overexpressing it in an activated form, or through exposure to phorbol esters. While this method would provide a better understanding of the true effects of PKC on Gα12, it must be remembered that the kinase will not interact with Gα12 exclusively, but may bind with other targets. These confounding variables may result in a change in growth signaling that cannot be attributed to phosphorylated Gα12.

Gα12/13, like all trimeric G protein α subunits, must undergo post-translational lipidation to allow signaling function. The N-terminal addition of palmitic acid to a specific cysteine localizes Gα12/13 to the cell membrane. Without proper localization, Gα12/13 cannot signal properly[2]. It has been shown that Gα12 is palmitoylated at Cys11. Should this palmitoylation be mutationally blocked, Gα12 will fail to signal[2]. Additionally, Gα13 is palmitoylated dually at Cys14 and Cys18, and will also fail to localize to the membrane should lipidation at either point be interrupted[21]. However, previous work in the Meigs' lab has shown that growth signaling can be rescued in a nonpalmitoylated protein through myristoylation. By mutationally inserting a N-terminal sequence allowing for the covalent attachment of a myristic acid, the lost palmitic acid is replaced, and localization is restored[20]. The proximity of the possible phosphosite Ser9 to the palmitoylation site Cys11 in Gα12 led us to investigate whether phosphorylation could interfere with lipidation of the protein. While regulation of lipidation by phosphorylation has not been reported for G proteins, such a mechanism has been observed in phosphodiesterase 10A (PDE10A), for which phosphorylation of an N-terminal threonine blocks the palmitoylation of a nearby cysteine[22]. This allows for regulation of trafficking and localization of PDE10A within the cell[22].

In order to pursue this possibility, consensus sequences allowing for myristoylation were inserted into the phosphomimicking variants 12S9D and 12S9DNterm. Through the addition of a myristoyl group, SRE-mediated growth signaling was restored. These findings suggest that phosphorylation of Ser9 in Gα12 could negatively regulate palmitoylation, and by extension, the ability of the protein to localize.

To better understand the effects of phosphorylation on the localization of Gα12, it would be necessary to perform cellular fractionation experiments to determine the location of the 12S9D in the cell as compared to a non-palmitoylated, non-phosphorylated counterpart, 12C11A. Our findings appear to support the hypothesis that the proposed kinase-dependent switch is a Gα12 specific mechanism. We replaced three homologous Gα13 serines with phosphorylation blocking alanines and found that they were capable of driving rubust SRE signaling. These serines also lie closely to a palmitoylation site of Gα13. Should the proposed regulatory mechanism be shared within the G12/13 subfamily, we expect that these phosphomimcis would fail to localize to the cellular membrane and therefore fail to drive SRE-mediated signaling. However, it is worth noting that a Thr21 lies near the second palmitoylation site Cys18 in Gα13. Future work may involve replacing this residue with a phosphomimic, alone or alongside any of the three engineered mimics, observing whether this mutation results in a loss of signaling ability, and if such a loss could be rescued through the insertion of a myristoylation consensus sequence.

PKA has been shown to phosphorylate Gα13 at Thr203 within the Switch I region, a region key in the activation of Gα13 and its reassociation with Gβγ[16]. This project elaborated on previous research in the Meigs' lab, in which mutations blocking and mimicking phosphorylation at this location and its Gα12 homolog, Thr206, were engineered.

To investigate the effects of PKA on SRE signaling, we overexpressed the catalytic subunit of PKA alongside a driving G protein, including the previously engineered variants. We found that the presence of the catalytic subunit of PKA sharply increased growth signaling in both Gα12 and Gα13. This increase was blocked when Thr206/203 underwent mutation, further implicating it as the target of PKA and suggesting that PKA provides a strong up-regulation of Gα12/13 to SRE. However, it must be mentioned that phosphomimics of Thr206/203 failed to recreate the sharp increase in signaling we observed. This emphasizes the risk of utilizing phosphomimics. While the negatively charged aspartic or glutamic acid residues can resemble the negative charge bestowed upon phosphorylation, it is not always possible to replicate kinase-mediated modification perfectly through a genetic introduction of a phosphomimic. In the case of the current study, it appears that aspartic acid serves as a better mimic than glutamic acid. In all cases, the phosphomimics were able to signal growth stronger than their blocking counterparts, suggesting some conformational similarity between the variants and their phosphorylated counterparts. Additionally, due to the sharp decrease in ability to signal growth seen in the phosphorylation blocking Thr-to-Ala mutations, it is possible that phosphorylation of the Switch I region of Gα12/13 is necessary to drive SRE-mediated growth signaling.

These findings contradicted our earlier hypothesis that PKA would result in a reduction of SRE-mediated growth signaling as a result of a decrease in RhoA activation[16]. It is possible that phosphorylation of Gα12/13 could stimulate binding with RhoGEFs, which are key in the regulation of RhoA. Future work could investigate *in vitro* binding with RhoGEFs through the usage of protein interaction assays. Due to the ability of PKA to stimulate such strong growth signaling by Gα12 and Gα13, the Switch I phosphosite is an attractive target for anti-tumor therapies.

Due to the influence of Gα12/13 on cancer progression, any regulatory mechanism for SRF mediated processes is of medical importance. Increasing understanding of the role of phosphorylation on the regulation of Gα12/13 is worth further pursuit. The kinase dependent switch mechanisms discovered during this project have potential as targets for cancer fighting drugs, and it is crucial their structural features be investigated further.

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