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Gα13 Overexpression Induces Serum Response Element Genes: Membrane Delocalization And Activation State

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Abstract

Heterotrimeric G proteins consist of α, β, and γ subunits, and activating, GTPase-deficient mutations in the α subunit of several classes have been implicated in tumor progression. The G12/13 class of α subunits, comprising Gα12 and Gα13, has the unique ability to stimulate oncogenic transformation of cultured cells though overexpression of the wildtype α subunit. However, the mechanism that allows this signaling property of overexpressed wildtype Gα12 or Gα13 is not understood, as G proteins are not thought to signal outside of the GTP-bound active state. Using transiently transfected HEK293 cells, wildtype forms of Gα13 were examined for ability to stimulate serum response factor (SRF) mediated transcription of genes harboring the serum response element (SRE) promotor, a cell growth signaling mechanism implicated in melanoma and other cancers. Constitutively active Gα13 showed an approximate 100-fold stimulation of SRE, and unexpectedly, wildtype Gα13 showed a response fully half this magnitude. In cell fractionation experiments using an epitope-tagged wildtype Gα13, this transiently expressed protein began appearing in the soluble fraction as its levels increased, whereas endogenous Gα13 remained in the membrane-associated fraction. Under conditions in which Gα13 redistribution was observed, SRE activation showed a sharp increase. This stimulatory effect of wildtype Gα13 was blunted by co-expression of Gβ1 and Gγ2 subunits, suggesting overexpressed Gα13 drives SRE due to stoichiometric imbalance in the heterotrimer. Gβ1/Gγ2 co-expression also changed the subcellular distribution of wildtype Gα13 to a predominantly membrane-associated state. To interrogate Gα activation state within these fractions, the ability of Gα13 to resist trypsin degradation, a feature of GTP-bound Gα subunits, was assessed. Surprisingly, overexpressed wildtype Gα13 was degraded as fully as mutationally inactivated Gα13, suggesting overexpressed Gα13 that is membrane dissociated does not require activation to signal to SRE and promote cell growth.

Keywords: G protein, signal transduction, serum response, subcellular localization, oncogene

1. Introduction

Heterotrimeric guanine nucleotide binding proteins, known as G proteins, and their upstream G protein coupled receptors (known as GPCRs or 7TM receptors) transduce diverse signals in capacities ranging from sensory perception to embryogenesis. These proteins consist of α, β, and γ subunits. The α and γ subunits are peripheral to the membrane by way of a covalent lipid attachment, while the β subunit is membrane-localized by close association with the γ subunit. Upon ligand binding by the GPCR, the α subunit is activated by the exchange of GDP for GTP, whereupon it dissociates from the heterotrimer and GPCR. The α subunit passes the signal along to its effectors until it inactivates itself by its intrinsic GTPase activity, where the GPCR/G protein heterotrimer complex reforms.

Gα13 is one of two members of the G12/13 class of trimeric G proteins, and facilitates numerous cellular responses including transformation, migration, cell-cell and cell-substrate adhesion, and proliferation. For example, knockdown of a miRNA controlling Gα13 promotes the epithelial to mesenchymal transition in colon carcinoma in part due to the role of Gα13 in β-catenin transcriptional activity[1]. These responses are carried out in part by the ability of G12/13 to signal to the Serum Response Factor (SRF), a transcriptional activator that stimulates genes harboring the Serum Response Element (SRE) within their promotor. This pathway is known to promote expression of early response genes involved in cancerous proliferation. Mutations that leave the G protein stuck in a GTP bound, constitutively active state (Q226L in Gα13) are common causes of aberrant G protein signaling. However, comparatively few cancerous tissues from clinical samples contain activating mutations in the G12/13 class [2].

Overexpression of wildtype Gα13 is correlated with negative prognosis in clinically presenting gastric cancers, and acts as a potent driver of tumorigenic growth in culture[3]. Specifically, Gα13 overexpression promoted the G1/S transition via upregulation of cyclin D1 and downregulation of cyclin D1 inhibitors, activation of c-Myc dependent transcription, inhibition of FOXO1 transcription, and activation of AKT and ERK signaling cascades[4]. It is still not understood how overexpressed, wildtype Gα13 drives these pathways in the absence of activating mutations. While assessing the ability of G12/13 mutants to signal to SRE, we observed that overexpression of the alpha subunit without amino acid substitutions drove SRE signaling ~50-fold over background expression levels. As clinical evidence mounts implicating mere overexpression of G12/13 as driving cancer growth and metastasis, the mechanism behind this response remains unclear. Our goal in this study is to better understand how Gα13 mediates signaling by overexpression.

2. Materials and Methods

2.1 Cell Fractionation by Centrifugation

To determine intracellular localization of Gα13, HEK293 Cells were grown to 80% confluence in 10cm dishes using Dulbecco's Modified Eagle's Media (Corning) and were transfected with Gα13 constructs using Lipofectamine 2000, incubated for 36-48 hours. Media was rinsed away with cold phosphate buffered saline and cells were scraped and suspended in 0.75 mL of PBS. Cells were pelleted by centrifugation at 500xg, 5min, 3°C. Cell pellets were suspended in 100µL Negishi Homogenization Buffer (20mM Tris 7.4, 150mM NaCl, mM $MgCl_2$, 1mM DTT, 0.5x MP Biomedicals Protease Inhibitor Cocktail SKU#0215883701, 61 µM TPCK, 58 µM TLCK, 267 µM phenylmethylsulfonyl fluoride). Cell homogenates were lysed by snap-freezing in liquid nitrogen and thawed in icy water. 5µL was removed to act as a pre-fractionation sample. Samples were spun in a Sorvall vacuum ultracentrifuge at 100,000xg for ~20 minutes at 3°C. 40µL of supernatant was removed and set aside as the soluble fraction. Remaining supernatant was removed. Membrane pellet was resuspended in homogenization buffer, centrifuged at 100,000xg for ~20 minutes at 3°C. Supernatant was discarded, leaving a membrane fraction pellet. Cellular fractions were either analyzed by Western immunoblot or taken forward into the following trypsin protection assay.

2.2 Trypsin Protection Assay

To determine the activation state of Gα13 within subcellular fractions the following procedure was carried out. Membrane pellets from the previous fractionation procedure were suspended in 80µL Negishi Homogenization Buffer by agitation with a 25g needle. Soluble fractions were soluble by definition and did not require this step. Trypsin digestion was carried out in 217µL HEDM Buffer (50mM HEPES, 1mM EDTA, 10mM $MgSO_4$, 3mM DTT) 38µL of each fraction sample, and 24µL of 100ng/µL TPCK-Treated trypsin (New England Biolabs, Ipswich, MA) or diH_2O for negative control. Samples were heated for 20 minutes at 30°C, and proteolysis was terminated by addition of 10µL 100 mg/ml lima bean trypsin inhibitor (Worthington, Lakewood, NJ). Proteins were precipitated by 20% trichloroacetic acid and 0.8 mg/ml sodium deoxycholate. Samples were incubated on ice for 30 minutes and centrifuged at 100,000xg for ~20 minutes at 3°C. Supernatant was decanted until ~50µL remained and samples were centrifuged at 100,000xg for 3 minutes at 3°C. All supernatant was removed and pellets were washed with acetone, centrifuged for 15min, 16,000xg, 3°C. Acetone was removed down to the last ~50µL and centrifuged for 5min, 16,000xg, 3°C. Acetone was allowed to evaporate in a fume hood for ~2hrs. Precipitated protein was suspended in 40µL 33% dilute SDS-PAGE 4x loading buffer with 100mM DTT, heated for 10 minutes at 72°C, and vortexed.

Samples were analyzed by SDS-PAGE and immunoblotting using an Anti-Myc antibody (Millipore) and Anti-GNA13 antibody (Thermo-Fisher Scientific).

2.4 SRE-Luciferase Dual-Reporter Luminometric Assay[5]

In order to quantify the extent that Gα13 overexpression induces expression of genes controlled by SRE elements, HEK293 cells were grown to 80% confluence in 6-well plates and transfected with 0.2 µg SRE-luciferase plasmid, 0.02 µg renilla plasmid pRL-TK, 1 µg Gα13 plasmid, and 1 µg of β_1/γ_2 in corresponding treatments, according to manufacturer's instructions included with the Lipofectamine 2000 transfection kit. Cells were grown for 36-48 hours. Cells were washed in Phosphate-bufered saline and suspended in passive lysis buffer (Promega) on a platform shaker for 25 minutes. Lysates were analyzed with a Dual-luciferase assay system and a GloMax 20/20 luminometer (Promega). Light output from SRE-Luciferase activity was divided by light from the renilla construct to control for constitutive expression.

3. Results

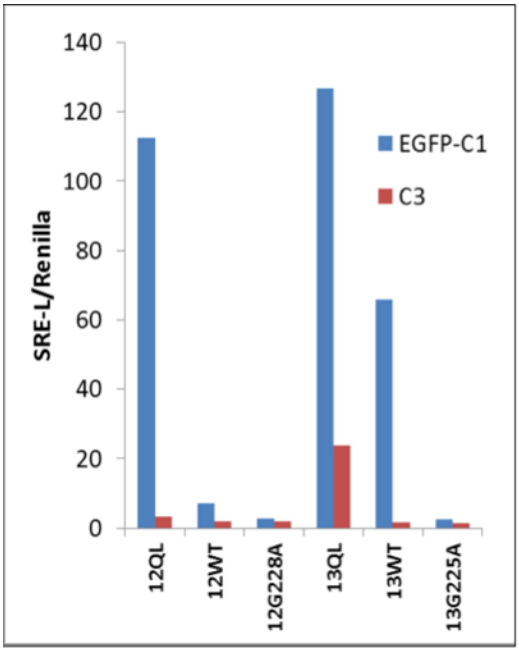

Figure 1. Dual-Glo Luciferase assay for signaling to SRE. Wildtype Gα13 signals to SRE in a Rho dependent manner. Disruption of the Rho signaling axis by the C3 exoenzyme of *Clostridium botulinum* (red) eliminates the ability of overexpressed wildtype Gα13 to drive SRE via SRF.

In luciferase/renilla dual-glo reporter assays for SRE, wildtype Gα13 overexpression drives SRE mediated transcription at fully half the magnitude of constitutively active (Q to L) Gα13. Expression with an enhanced Green Florescent Protein (eGFP) tag established baseline SRE signaling due to overexpression. Constitutively GDP-bound mutants (G to A) were used as a negative control, constitutively GTP-bound mutants (Q to L) were our positive control. In the absence of an activating mutation or an upstream signal, it is surprising that overexpressed wildtype Gα13 is able to signal to SRE at fully half the intensity of overexpressed, constitutively active Gα13. We hypothesized that the overexpressed Gα13 is able to signal during overexpression by outnumbering its corresponding β_1/γ_2 subunits.

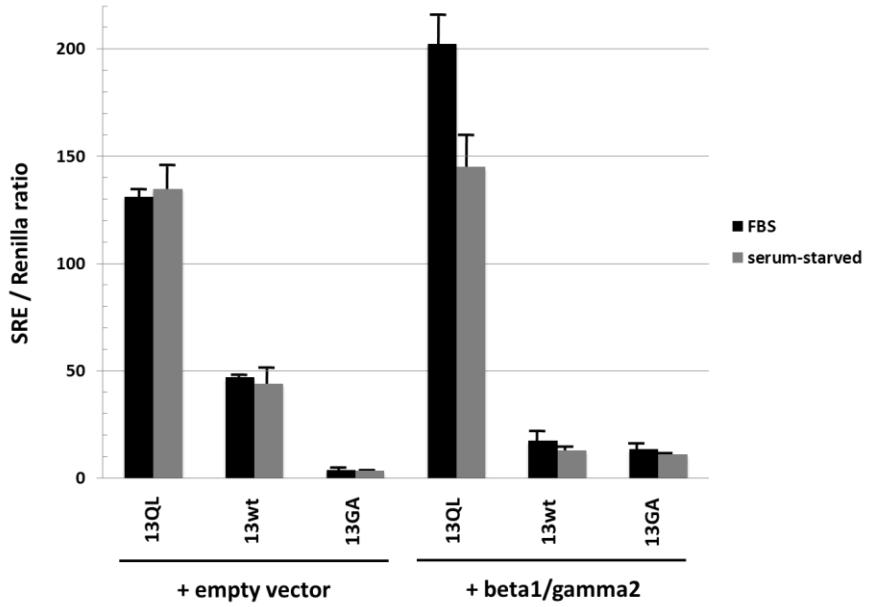

Figure 2. Coexpression with β/γ subunits attenuates SRE response from overexpressed wildtype Gα13. We co-transfected $\beta_1\gamma_2$ subunits alongside α13 to observe any effects with signaling to SRE via SRF. Mean of two independent experiments shown; error bars indicate range.

Co-expression of β/γ attenuated the signal to SRE in wildtype Gα13. Since the β/γ dimer is tethered to the membrane by isoprenylation of γ, we hypothesized that overexpression of the α subunit was causing delocalization of Gα13 from the membrane fraction to the soluble fraction due to stoichiometric imbalance with β/γ.

We turned our focus to the subcellular localization of Gα13 during overexpression. Since the HEK293 cells express a notable level of Gα13 on their own, it became important to differentiate native and recombinant Gα13 (Fig. 3). To further investigate the activation state of overexpressed Gα13 across cellular fractions (Fig. 5), we developed a combined fractionation and trypsin digestion protocol (Fig. 4). Trypsin digestion is an established benchmark in G protein biology to confirm constitutively active Gα protein mutants and chimeras are reaching the activated, GTP bound confirmation which shields critical residues from the activity of trypsin protease. G proteins in the inactive state are completely digested by trypsin.

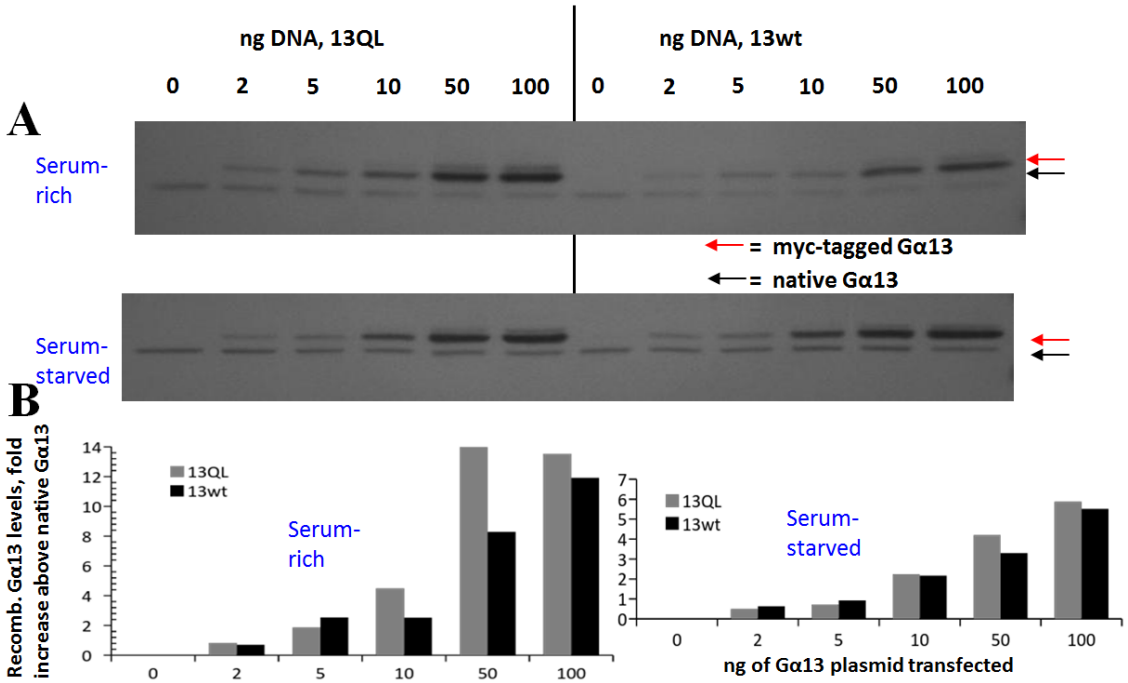

Figure 3. Internal myc-tagging of Gα13 distinguishes the native protein from transfected, overexpressed Gα13. (A). (B) Expression of recombinant Gα13 relative to native protein was quantified by densitometry using a Kodak Gel Logic 100.

To differentiate native from transfected Gα13, we inserted a myc-epitope tag into the helical domain of Gα13. This allows for simultaneous detection of native and transfected Gα13 by use of an anti-Gα13 antibody, with the native protein running slightly lower on SDS-PAGE gels. Gα13 expresses in a dose-dependent manner regardless of presense or absence of serum in cell culture.

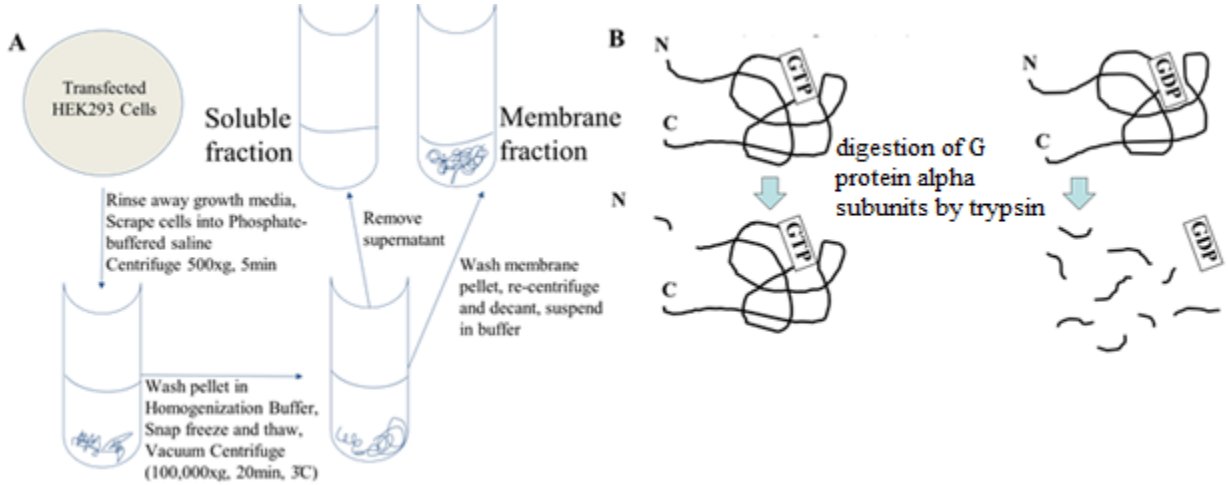

Figure 4. Schematic for (A) Cell Fractionation Procedure and (B) Trypsin Protection Assays.

We developed a combined procedure to overexpress various Gα13 constructs in HEK293 cells, fractionate the membrane-associated and soluble protein populations by centrifugation, and determine the activation state of recombinant Gα13 in these fractions by trypsin digestion. This allows further interrogation of the conformation of

Gα13 GTP-bound Gα-subunits are notable for their resistance to trypsin digestion, losing only a small fragment of the N-terminus, whereas other conformations of Gα proteins are fully degraded by trypsin.

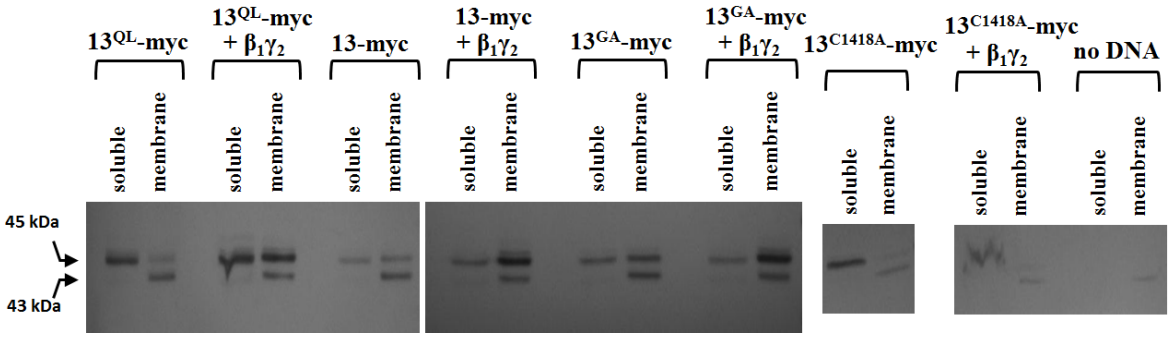

Figure 5. Overexpressed Gα13 cell localization across centrifugal fractions. Using the protocol outlined in Fig. 4, we separated membrane-associated and soluble portions of HEK293 cells and immunoblotted using anti-Gα13 antibody. We were able to visualize and distinguish native (~43kDa) and myc-tagged (~45kDa) proteins.

Native Gα13 remained exclusively membrane associated. Portions of all overexpressed Gα13 constructs were found in the cytoplasm and were recruited to the membrane by co-expression of β/γ. We also tested if soluble localization was the sole requirement for SRE signaling via SRF by wildtype Gα13. A C14/18A mutant of myc tagged Gα13, which lacks the required Cys residues for palmitoylation, remained exclusively in the soluble fraction despite co-expression of β/γ.

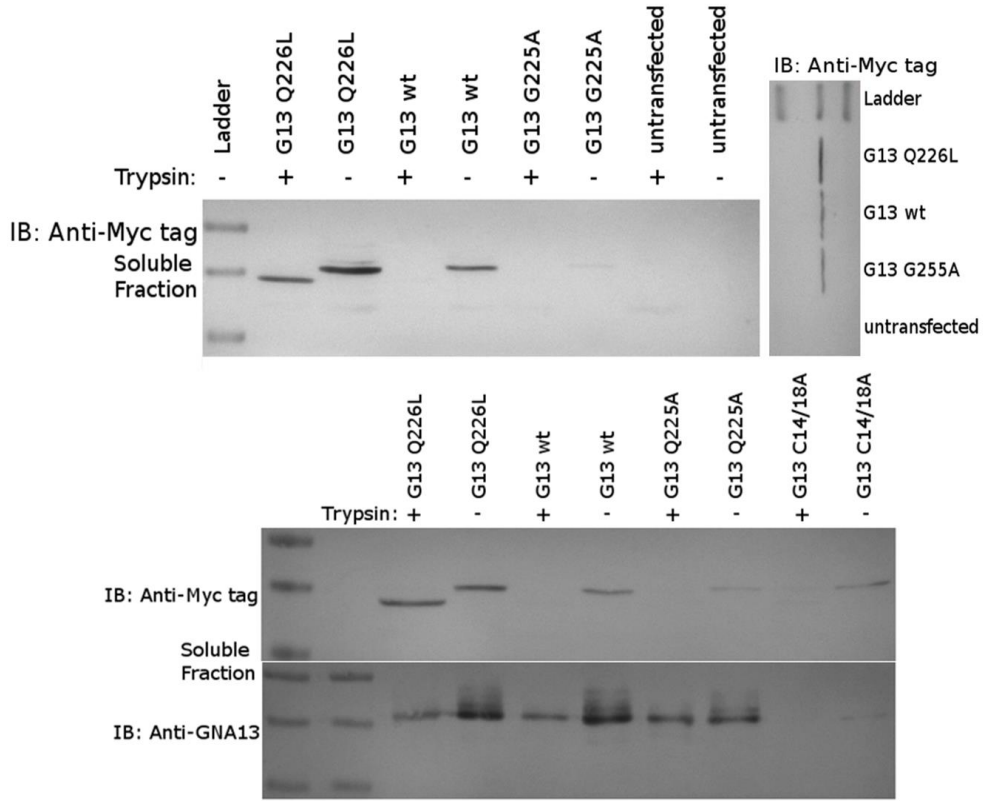

Figure 6. Overexpressed wildtype Gα13 in the soluble fraction is not GTP-bound. (Top Left) Despite being able to drive SRE, overexpressed Gα13 is not protected against trypsin digestion.. Non-transfected protein can be avoided in an immunoblot entirely through use of an anti-myc epitope primary antibody. (Top Right) Immunoblotting of pre-

fractionation cell homogenate confirms similar Gα13 expression across treatments. (Bottom) A representative replicate of the fractionation/protection assay.

As predicted, the constitutively active QL mutant is digested at the N-terminus, but a ~40kDa polypeptide is protected. Strikingly, overexpressed Gα13-myc in the soluble fraction is completely degraded by trypsin, in a manner similar to the inactive GA mutant. A weak GA band in the negative trypsin lane is expected, as GA mutants localized poorly to the soluble fraction (Fig. 5) Anti-GNA13 blot was included to demonstrate the advantage of use of the anti-myc antibody to stain only for transiently expressed protein. The unpalmitoylatable C1418A mutant does not appear to have signaling activity, performed very poorly with the Anti-GNA13 antibody, and does not drive SRE by overexpression (Fig.7).

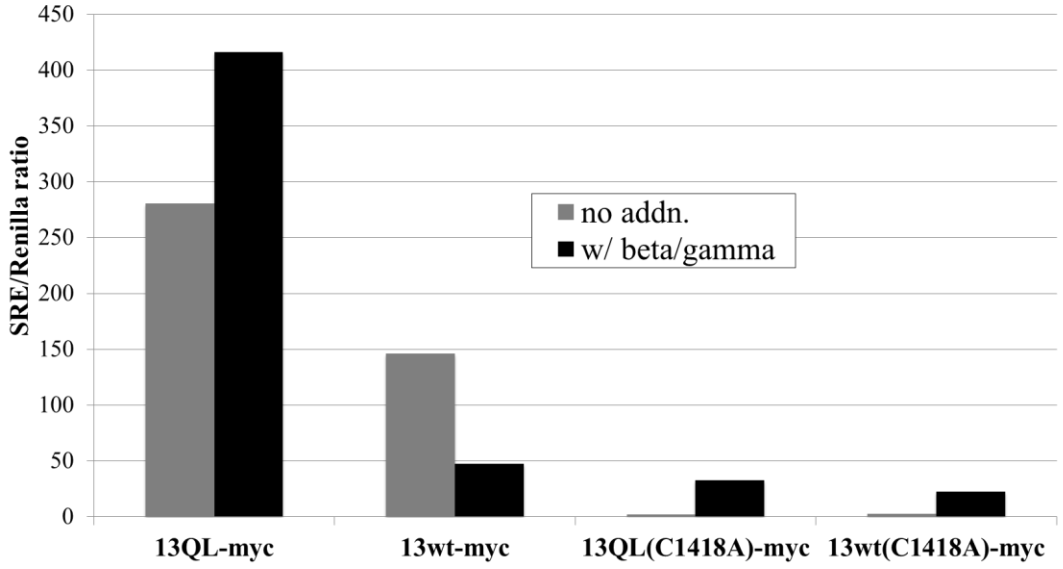

Figure 7. Palmitoylation of overexpressed Gα13 is necessary to drive SRE.

We observed that co-expression of β/γ to complete the heterotrimer attenuated SRE signaling by Gα13 overexpression (Fig. 7, Fig. 1). β/γ is membrane associated suggesting that the SRE is driven through Gα13 residing in soluble fraction. We hypothesize that Gα13 overexpression saturates the membrane and β/γ dimers within the membrane with α subunits to the point that newly translated Gα13 fails to localize to the membrane. Indeed, Gα13 was observed in the soluble fraction of cell lysates during recombinant expression, irrelevant of binding state caused by constitutive activity or inactivity (Fig. 5). The only overexpressed Gα13 that did not associate with the membrane lacked the Cys residues needed to do so (C1418A), a mutant that we have found to be impaired in SRE signaling (Fig. 7). The unpalmitoylated mutant does not drive SRE at all, and in pulldown assays for known Gα13 effectors, was severely impaired in binding to non-RhoGEF effectors. This suggests palmitoylation is a critical feature of Gα13, and overexpressed wildtype Gα13 is likely palmitoylated, even when delocalized from the membrane fraction. Palmitoylation of Gα13 is a known requirement for signaling through RhoGEFs[6], confirming our original finding (Fig. 1) that overexpression effects of Gα13 are RhoGEF mediated. Palmitoylation also appears to be important for binding to β/γ (Fig. 5).

5. Discussion

Our investigation began with the insight that overexpression of Gα13 is able to drive SRE without any modifications to GDP-GTP cycling activity. This cycling activity has been thought of as a prerequisite for G protein signaling, with non-GTP bound Gα subunits being more or less invisible with regards to cell signaling. Early on, we observed distribution of Gα13 into the soluble fraction coincided with increased signaling to SRE. To further elucidate what

was occurring with the activation state of Gα13 in the soluble fraction, we designed a combined fractionation/trypsin protection protocol. Wildtype, overexpressed Gα13 does not appear to be GTP-bound in the soluble fraction. Mere overexpression of Gα13 does not include an obvious mechanism of signaling, which is a "limitation" in explaining its potency in oncogenesis[4]. We cannot decisively conclude that overexpressed, wildtype Gα13 is inactive within the membrane fraction. Another explanation for inactivity of Gα13 within the soluble fraction is that the soluble Gα13 we isolated was GTP bound at one point, but intrinsic GTPase activity of Gα13 left it GDP bound by the time trypsin was introduced.

Optimization of the fractionation and trypsin protection assay is still needed to examine the activation state of Gα13 in the membrane fraction. Protein precipitation using trichloroacetic acid and sodium deoxycholate causes the membrane fraction to form into a hardened pellet that is not soluble despite heating and agitation at 72°C or sonication. In order to better address the question of the mechanism of SRE signaling by Gα13 overexpression, we would need to make the precipitated membrane fraction more accessible to trypsin digestion. This is a challenge, as the substances capable of dissolving this pellet are likely not compatible with SDS-PAGE. We have been advised (Dr. Heidi Hamm, Vanderbilt University School of Medicine) to lower our percentage of trichloroacetic acid to 5%-10% w/v during protein precipitation.

G protein overexpression is known to increase signaling through cognate GPCRs by stabilizing active confirmations of the receptor in an allosteric manner. This can increase signaling activity of the G protein coupled receptor in the presence of the same concentration of ligands[7]. This signaling paradigm by overexpression has been well investigated in β-adrenoreceptor signaling through G protein Gαsβ1γ2, but less so in Gα12/13 and its cognate receptors. This phenomenon could explain signaling to SRE over baseline despite retrieval of soluble Gα13 to the membrane fraction by coexpression of β1/γ2. However, this model raises the question regarding why Gα alone would mediate a larger signal to SRE than the complete G protein. G protein overexpression strikes at the root of signal transduction, and G protein signaling outside of the GTP bound state is a growing branch among the many roles of G proteins.

It is possible that the presence of more Gα13 allows an existing signal from outside the cell to be more quickly re-transduced by more rapid formation of the GPCR:G protein heterotrimer complex, as the previous α subunit leaves the complex following activation. Gα competition for βγ dimers has been observed in other classes (αi/αs) of G proteins[8]. This would result in the same concentration of GPCR ligands outside the cell causing a larger response through Gα13 than under baseline conditions. However, our evidence that β/γ co-expression attenuates SRE response by re-localizing Gα13 to the membrane would seem to argue against this model. In addition, overexpression of all three subunits of the heterotrimer, which under this model would amplify the signal to SRE from outside the cell, does not occur.

Future directions include co-expression with known Gα13-interacting RhoGEFs, as signaling by Gα13 expression seems to be Rho dependent, and the signal to SRE could be carried through a specific RhoGEF. Acquisition of more diverse luciferase reporter plasmids will reveal if overexpression acts in an SRE specific manner, or through additional elements such as AP-1 and c-Fos. Finally, efforts are currently underway to engineer a myristoylated Gα13 to see if this alternate lipid modification can restore palmitoyl-dependent signaling functions, an experiment previously carried out with Gα12[9]. When studying heterotrimeric G protein signaling, it is important to consider activation independent G protein signaling. The majority of Gα12/13 literature examines constituatively activated (QL) subunits, which provide unmistakable results with high affinity binding and obvious phenotypic effects, but may gloss over more nuanced roles of physiological Gα12/13 signaling.

6. Acknowledgements

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7. Resources

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