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N-terminal polybasic motifs are required for plasma membrane localization of Gas AND Gαq

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Abstract

Heterotrimeric G proteins typically localize at the cytoplasmic face of the plasma membrane where they interact with heptahelical receptors. For G protein α subunits, multiple membrane targeting signals, including myristoylation, palmitoylation, and interaction with $\beta\gamma$ subunits, facilitate membrane localization. Here we show that an additional membrane targeting signal, an N-terminal polybasic region, plays a key role in plasma membrane localization of non-myristoylated α subunits. Mutations of N-terminal basic residues in α_s and α_q caused defects in plasma membrane localization, as assessed through immunofluorescence microscopy and biochemical fractionations. In α_s , mutation of four basic residues to glutamine was sufficient to cause a defect, whereas in α_q a defect in membrane localization was not observed unless nine basic residues were mutated to glutamine or if three basic residues were mutated to glutamic acid. $\beta\gamma$ co-expression only partially rescued the membrane localization defects; thus, the polybasic region is also important in the context of the heterotrimer. Introduction of a site for myristoylation into the polybasic mutants of α_s and α_q recovered strong plasma membrane localization, indicating that myristoylation and polybasic motifs may have complementary roles as membrane targeting signals. Loss of plasma membrane localization coincided with defects in palmitoylation. The polybasic mutants of α_s and α_q were still capable of assuming activated conformations and stimulating second messenger production, as demonstrated through GST-RGS4 interaction assays, cAMP assays, and inositol phosphate assays. Electrostatic interactions with membrane lipids have been found to be important in plasma membrane targeting of many proteins, and these results provide evidence that basic residues play a role in localization of G protein α subunits.

Keywords

heterotrimeric G protein; membrane targeting; subcellular localization; palmitoylation

INTRODUCTION

G protein signaling pathways are involved in a wide variety of physiological processes, and aberrant regulation of these pathways results in a multitude of pathological conditions such as cancer, heart disease, hypertension, endocrine disorders, and blindness [1–4]. Therefore

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understanding the mechanism of regulation of these pathways is of utmost importance. The inactive state of the G protein is a heterotrimer of α , β , and γ subunits, with the α subunit ($G\alpha$) bound to GDP. Upon activation by an extracellular agonist the heptahelical G protein-coupled receptor (GPCR) acts as a guanine nucleotide exchange factor (GEF) and catalyzes the exchange of GDP for GTP on $G\alpha$. The binding of GTP causes a conformational change in the α subunit, which decreases the affinity of $G\alpha$ for the β and γ subunits ($\beta\gamma$) and results in at least partial dissociation of the heterotrimer. The separated subunits, $G\alpha$ and $\beta\gamma$, are then able to elicit their own signaling cascades. For inactivation, a family of proteins known as Regulators of G Protein Signaling (RGS) proteins act as GTPase activating proteins (GAPs) for $G\alpha$ and catalyze the hydrolysis of the GTP γ phosphate, thus returning $G\alpha$ to the GDP-bound form. This restores the affinity of $G\alpha$ for $\beta\gamma$ and returns the heterotrimer to the inactive state [5–7].

In order for G proteins to couple the membrane-spanning GPCRs to their effectors, they must be localized at the cytoplasmic face of the plasma membrane (PM) [8–11]. There is a two-signal model for membrane targeting of peripheral membrane proteins, which has been well described for monomeric G proteins and members of the Src family [12–16]. This model states that two signals act synergistically to allow stable membrane binding, and one of these signals may dictate targeting to a specific cellular membrane or compartment. In the case of heterotrimeric G proteins, this second signal would dictate targeting to the PM rather than intracellular membranes [17]. These membrane targeting signals could be covalent lipid modifications, interactions with other membrane-binding proteins, or polybasic stretches of amino acids.

It is well established that lipid modifications are critical in anchoring the G protein heterotrimer to the membrane [9,18]. The γ subunit is prenylated and the $G\alpha$ subunit is fatty acylated. Members of the α_i family are myristoylated and palmitoylated [19–22]. The myristate is attached co-translationally to the N-terminal glycine, after removal of the initiating methionine, through a stable amide bond catalyzed by a highly specific N-myristoyl-transferase (NMT) [23]. Palmitoylation occurs on the cysteine directly next to this glycine through a thioester bond [24]. Members of other $G\alpha$ families, namely α_s , α_q , and α_{12} , are not myristoylated, only palmitoylated [18]. Depending on the family member, palmitoylation could occur at 1, 2, or 3 cysteines in the N-terminus and there does not seem to be any similarities in the location of the cysteines or the surrounding sequences [9,18,25]. Moreover, while a family of palmitoyl protein acyl transferases (PATs) have been recently discovered [26], a PAT specific for $G\alpha$ has yet to be identified, and at which organelle modification of $G\alpha$ occurs has yet to be resolved [27–29]. Also, while there is evidence supporting the idea that the heterotrimer forms at an endomembrane prior to reaching the PM [28,30], it is still unknown at which endomembrane this occurs.

With myristoylation being a co-translational event, it is the earliest step that promotes membrane targeting for members of the α_i family, with palmitoylation as the second signal, as it is a post-translational modification [9,18]. This is supported by mutational analysis of the modified glycine and cysteine. Mutation of the N-terminal glycine to alanine not only inhibits myristoylation, but palmitoylation as well, and causes a shift of the protein from the PM to the cytosol [22,31–33]. On the other hand, mutating the cysteine next to the glycine abolishes palmitoylation, but the protein is still myristoylated and its localization is shifted to intracellular membranes as well as the PM [17,24,34]. Therefore, according to the two-signal model, it seems as though myristoylation can play the role as the initial membrane targeting signal for the α_i family, with palmitoylation being the second signal that dictates localization specifically to the PM.

The situation is less clear for $G\alpha$ families that are only palmitoylated and not myristoylated. It has been demonstrated that palmitoylation is required for PM binding and proper signaling [8,11,25], but according to the two-signal model, another initial membrane targeting signal is needed before this modification can occur. One possibility is interaction with $\beta\gamma$. Crystal structures of α_i and α_t , each in complex with $\beta\gamma$, reveal two surfaces of the α subunit that interact with $\beta\gamma$ [35,36]. The larger of these two contact surfaces involves residues in and adjacent to the switch 1 and 2 regions, where activation-induced conformational changes occur. The second contact surface is in the N-terminal α helix. When residues in the N-termini of α_s and α_q believed to directly contact $\beta\gamma$ were mutated, defects in PM localization, palmitoylation, and signaling were observed [37]. Manipulating α_q so that it could be myristoylated recovered PM binding and palmitoylation of the $\beta\gamma$ -binding mutants [37].

While there is strong evidence that interaction with $\beta\gamma$ acts as an initial membrane targeting signal for non-myristoylated $G\alpha$ families, there is also evidence that additional targeting signals may exist in these subunits and the two signal model may be expanded to allow for multiple targeting signals [9]. A potential additional targeting signal is a prominent positively charged patch in the N-termini of non-myristoylated $G\alpha$ subunits, as identified through homology modeling and electrostatic surface maps [38]. Positive charges in a protein can significantly enhance its affinity for the negatively charged interface of the PM via electrostatic interactions [39]. It has been demonstrated for the monomeric GTPase K-Ras that mutations in its C-terminal polybasic residues inhibit PM localization [14]. Also, given that this positively charged motif is much less pronounced in members of the α_i family, it was proposed that the polybasic motif and myristoylation play complementary roles in PM targeting of $G\alpha$ [38]. Moreover, the presence of an N-terminal polybasic region in $G\alpha$ suggests a revised two-signal model, in which the polybasic motif or myristoylation act in conjunction with $\beta\gamma$ -binding as two targeting signals to allow for palmitoylation and consequent PM localization [38].

Here we explore the importance of this polybasic motif in PM targeting of two non-myristoylated $G\alpha$ subunits, α_s and α_q . We present evidence that mutation of residues in this region causes defects in PM localization of these subunits in HEK293 cells, which can be rescued by myristoylation and partially rescued by co-expression of $\beta\gamma$. Mutants defective in PM localization were also defective in palmitoylation. These results provide support for the proposal that N-terminal polybasic regions contribute to the PM localization of α_s and α_q .

EXPERIMENTAL PROCEDURES

Cell culture

HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cells were maintained at 37°C and 5% CO₂.

Transfection

Cells were seeded onto either wells of 6-well plates or 6-cm plates 1 day prior to transfection. Cells in 6-well plates were transfected with 0.6 μ g α_s or α_q expression vector while cells in 6-cm plates were transfected with 1.8 μ g α_s or α_q , along with an appropriate amount of empty vector pcDNA3 to give a final amount of 1 μ g or 3 μ g DNA, respectively. Slightly higher amounts of glutamic acid mutants were transfected for fractionation, palmitoylation, and GST pulldown assays due to expression differences. The α_q 9Q expression vector was transfected in 2 separate plates and the cell populations were combined for fractionation, palmitoylation, and GST pulldown assays. When $\beta\gamma$ was co-expressed, cells were transfected at a ratio of 6:3:1 of α : β : γ expression vectors. Transfections were performed with the Fugene6 transfection

reagent according to the manufacturer's protocol (Roche Applied Sciences). Experiments were performed approximately 40 hours after transfection unless otherwise noted.

Expression Plasmids

The GST-RGS4 bacterial expression plasmid was provided by Dr. R. Neubig (Univ. Michigan). MycHis- β_1 pcDNA3, γ_2 pcDNA3, HA- α_s pcDNA3, HA- α_q pcDNA3, and the HA-tagged $\beta\gamma$ -binding mutant α_q IE have been described previously [8,37,40,41]. All constructs described here also contain the internal HA epitope. α_s 5Q was constructed by Stratagene QuikChange mutagenesis using α_s pcDNA3 as a template. α_s 4QpcDNA3 was prepared by mutating glutamine 34 of α_s 5QpcDNA3 back to lysine using a sequential PCR method [42]. α_q 2Q, α_q 3Q, α_q 3Qn, α_q 4E, α_q 2E, and α_q 3En were constructed in a similar fashion using wild type α_q pcDNA3 as a template. α_q 7Q was made by the sequential PCR method using α_q 4Q as a template. Similarly, α_q 9Q was constructed using α_q 7Q as a template. α_q 7E and α_q 9Q2 were generated using Stratagene QuikChange mutagenesis with α_q 4E and α_q 7Q as templates, respectively. Myr- α_s 5Q was made by Stratagene QuikChange mutagenesis with sense and antisense primers containing the mutations L4T, G5L, and N6S [43] using α_s 5Q as the template. Myr- α_q 9Q and myr- α_q 3En were made using Stratagene QuikChange mutagenesis with primers that mutate the codon for the initiating methionine along with alanine 8 to glycine [37] using α_q 9Q and α_q 3En as templates. The Q227L and Q209L constitutively activating mutations were also introduced by Stratagene QuikChange mutagenesis.

Immunofluorescence Microscopy

24 hours prior to transfection cells were seeded onto 6-well plates containing coverslips. 40 hours after transfection cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature and subsequently permeabilized by incubation with blocking buffer (2.5% nonfat milk and 1% Triton X-100 in Tris-buffered saline (TBS)) for 20 minutes. Coverslips were then incubated with the indicated primary antibodies for a minimum of 1 hour, after which cells were washed with blocking buffer. Subsequently cells were incubated for 30 minutes with a 1:100 dilution of a goat anti-mouse or a goat anti-rabbit secondary antibody conjugated to either Alexa 488 or Alexa 594 (Molecular Probes, Eugene, OR). The coverslips were then washed with 1% Triton X-100 in TBS and subsequently mounted on glass slides with Prolong Gold reagent (Molecular probes, Eugene, OR). Microscopy was performed with an Olympus BX61 microscope and an ORCA-ER (Hamamatsu, Bridgewater, NJ) cooled CCD camera controlled by Slidebook version 4.0 (Intelligent Imaging Innovations, Denver, CO). Images were transferred to Adobe Photoshop for digital processing.

Cell Fractionation Assay

Cells were seeded onto 6-cm dishes for transfection. 24 hours after transfection cells were reseeded onto 10-cm plates. 16 hours later cells were washed and harvested in ice-cold PBS, centrifuged and resuspended in 500 μ l hypotonic lysis buffer (50 mM Tris-HCl, pH 8, 2.5 mM $MgCl_2$, 1mM EDTA, 1mM dithiothreitol) with protease inhibitors (0.5mM phenylmethanysulfonylfluoride, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin). Cells were passed through a 27-gauge needle 10 times to lyse. Lysates were centrifuged at 2,000 rpm for 5 minutes to remove nuclei and debris. The postnuclear supernatant was centrifuged at 60,000 rpm for 20 minutes at 4°C. The supernatant (soluble fraction) was saved and the pellet (particulate fraction) was resuspended in 500 μ l hypotonic lysis buffer. 50 μ l 2X SDS sample buffer was added to 50 μ l each fraction and boiled for 5 minutes. 25 μ l of these fractions were analyzed by SDS-PAGE and western blotting.

Palmitoylation assays

36 hours after transfection in 6-cm dishes, cells were labeled with 0.5 mCi/ml [^3H] palmitate. After 3 hours of labeling cells were lysed in 500 μl radioimmune precipitation (RIPA) buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 2.5 mM MgCl_2 , 1 mM PMSF, 2 $\mu\text{g/ml}$ leupeptin, and 2 $\mu\text{g/ml}$ aprotinin). After incubation for 1 hour at 4°C lysates were centrifuged at full speed to pellet nuclei and debris. Lysates were then adjusted to 0.1% SDS. For cells transfected with α_q , 10 μl $\alpha_{q/11}$ antibody (Santa Cruz) was added to lysates and 4 μl α_s antibody (Rockland Immunochemicals) was added to lysates of cells transfected with α_s . After 3 hours, 50 μl Protein A/G (Santa Cruz) was added and the lysates were tumbled for 1 hour. Beads were then pelleted by centrifugation for 30 seconds and then washed three times with RIPA buffer. Protein was eluted from the beads with 50 μl 2X SDS PAGE sample buffer and 5 minutes of boiling. 20 μl were resolved by SDS PAGE and transferred to PVDF, which was sprayed with EnHance (Perkin-Elmer) and subjected to fluorography at -80°C for up to 1 week. Another 20 μl were analyzed by SDS and western blotting to confirm equal amounts of protein immunoprecipitated.

cAMP and inositol phosphate assays

24 hours after transfection in 6-well plates, cells were reseeded onto 4 wells of 24-well plates. 3 of these wells were labeled with 2 $\mu\text{Ci/ml}$ of [^3H] adenine for cAMP assays or [^3H] inositol for inositol phosphate (IP) production assays. After labeling for 16 hours cAMP and IP production were assayed. Calculations were performed using the averages of the 3 wells. The fourth well was used to assay protein expression. Cells in these wells were lysed in 100 μl 2X SDS sample buffer and boiled for 5 minutes. 25 μl of the lysates were analyzed by SDS PAGE and western blotting.

For cAMP assays, cells were washed with 0.5 ml assay media (20 mM Hepes-buffered Dulbecco's modified eagle's medium without bicarbonate) containing 1mM isobutylmethylxanthine (IBMX) after labeling. Cells were then incubated with assay media for 1 hour followed by lysing of the cells with 0.5 ml 5% trichloroacetic acid (TCA) containing 1mM cAMP and 1 mM ATP. The samples were loaded onto Dowex AG 50W-X4 columns and [^3H] ATP was eluted from the columns with water. The Dowex columns were placed on top of Alumina columns and washed with water to elute [^3H] cAMP from the Dowex columns onto the Alumina columns. [^3H] cAMP was eluted from the alumina columns with imidazole. The radioactivity in each fraction was determined by liquid scintillation counting. Data are presented as [^3H]cAMP/([^3H]cAMP+[^3H] ATP)*1000.

For IP assays, cells were washed with assay media containing 5 mM lithium chloride after labeling. Cells were then incubated with assay media for 1 hour and the reaction was terminated by adding 0.75 ml 20 mM formic acid to the cells and incubating them at 4°C for 30 minutes. 0.1 ml 0.7 M ammonium hydroxide was then added to the samples, which were loaded onto AG1-X8 Dowex columns. 1 ml of 50 mM ammonium hydroxide was added to the columns and the eluate, which constituted the [^3H] inositol fraction, was collected. Columns were then washed with 4 ml 40 mM ammonium formate, 0.1 M formic acid. The [^3H] IP fraction was eluted with 1ml 4M ammonium formate, 0.2 M formic acid. The radioactivity in each fraction was determined by liquid scintillation counting. Data are presented as [^3H]IP/([^3H]IP+[^3H] inositol)*1000.

GST-RGS4 Interaction Assay

GST-RGS4 was expressed in BL-21 cells and purified using glutathione-Sepharose 4B beads (Amersham, Biosciences) as previously described [44–46], and then GST-RGS4 pulldowns were performed as described [45]. Briefly, HEK293 cells were transfected with wild type or mutant α_q . 24 hr after transfection cells were washed with ice cold PBS, harvested in 0.3 ml

lysis buffer (20mM Tris HCl, pH 7.4, 1mM EDTA, 1 mM dithiothreitol, 100mM NaCl, 5 mM MgCl₂, 0.7% Triton X-100, 1 mM phenylmethanysulfonyl fluoride, and 5 µg/ml leupeptin and aprotinin), followed by incubation for 1 hour at 4°C for lysis. Cells were then centrifuged at full speed in a microcentrifuge to pellet nuclei and unbroken cells. 50µl of the cleared lysate was saved for analysis of total protein in cellular lysate (TCL). 50 µl of sample buffer was added to the TCL and boiled for 5 minutes. The remaining 250 µl of post-nuclear supernatant was equally split into two tubes and 25 µM AlCl₃, 5 mM NaF, and 1 mM MgCl₂ were added to one tube. Subsequently 8 µg GST-RGS4-bound beads were added to both sets of tubes and the lysates were then tumbled for 2 hr at 4°C. After this incubation beads were pelleted by centrifugation at low speed for 3 minutes at 4°C. Beads were then washed three times with lysis buffer. Protein was eluted from beads with 50 µl SDS sample buffer and 5 minutes of boiling. 20 µl each of TCL and the pulldowns were resolved by SDS PAGE analysis followed by western blotting.

Western Blotting

Samples were subjected to SDS-PAGE, transferred to PVDF membranes, and blocked with TBS/0.05% Tween/5% milk. Blots were then probed with 0.5 µg/ml of either anti-HA antibody 12CA5 (Roche Applied Sciences) or anti-myc antibody 9E10 (Roche Applied Sciences), followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody (Promega, Madison, WI). Blots were visualized using SuperSignal West Pico (Pierce Chemical, Rockford, IL).

RESULTS

Mutations in the polybasic motif of α_s and α_q disrupt PM localization

The N-terminal polybasic motif is not evident in the linear sequence of G α (Figure 1A), but was initially identified in the N-termini of non-myristoylated G α using homology modeling and electrostatic surface maps [38]. Indeed, helical wheel diagrams show that this motif maps to one face of the N-terminal helix of G α , more specifically to the opposite side of the helix as residues that contact $\beta\gamma$ (Figure 1B). Consequently these positively charged residues are free to interact with the negatively charged interface of the PM whether or not the α subunit is bound to $\beta\gamma$. Moreover, these basic residues are predicted to be clustered together in helical net diagrams of α_s and α_q (Figure 1C). In these helical net diagrams the N-terminus of α_q contains two discrete basic patches while the basic residues of α_s are more widespread throughout the N-terminus (Figure 1C).

In an effort to determine the importance of the polybasic motif in PM targeting of non-myristoylated G α subunits, we constructed mutants of α_s and α_q in which residues of these basic patches were mutated to glutamine, thus preserving the size of the side chain while removing the positive charge. In α_s , there are five basic residues that map to one face, as shown in the helical wheel diagram of the N-terminus, namely lysines 24, 25, 28, 32, and 34 (Figure 1B). These residues were mutated to glutamine in an HA-tagged α_s (α_s5Q). Wild type α_s and α_s5Q were transiently transfected in HEK293 cells and their localization was assessed through immunofluorescence and cell fractionation assays, using an anti-HA antibody. Wild type α_s exhibited strong PM localization in the majority of the cells when assessed through immunofluorescence microscopy (Figure 2A). In contrast, α_s5Q displayed predominantly cytoplasmic staining (Figure 2B). When localization was assayed through subcellular fractionation, wild type α_s was about evenly distributed between the soluble and particulate fractions, while α_s5Q was mainly in the soluble fraction (Figure 2D). These results suggest that the polybasic motif is involved in targeting α_s to the PM, as removal of positive charges of this motif changes the localization of α_s from the PM to the cytosol. Since lysine 34 lies farther away from the other mutated lysines (Figure 1B), another construct (α_s4Q) was created

with only these four residues mutated. The localization of this mutant was indistinguishable from that of α_s5Q (Figure 2C, D). Therefore, mutation of four basic residues was sufficient to cause a defect in PM localization of α_s .

A mutant similar to α_s4Q was constructed in an HA-tagged α_q (α_q4Q). In this mutant, arginines 27, 30, 31, and 34 were mutated to glutamine (α_q4Q). These residues also map to one face of the α_q N-terminal helical wheel diagram (Figure 1B) and are found clustered together in the helical net diagram of α_q (Figure 1C). α_q4Q and wild type α_q were transiently transfected in HEK293 cells and their localization was assessed through immunofluorescence and cell fractionation assays, again using an anti-HA antibody. Wild type α_q displays predominantly PM localization when assessed through immunofluorescence and is present mainly in the particulate fraction with a small portion in the soluble fraction (Figure 3A, I). As a negative control, the membrane localization of a $\beta\gamma$ -binding mutant, α_qIE , was also examined. As previously shown [37], this mutant displayed cytoplasmic staining and was present predominantly in the soluble fraction (Figure 3B, I). In contrast to α_s4Q , immunofluorescence microscopy and subcellular fractionation studies revealed a pattern of localization of α_q4Q that was similar to wild type α_q (Figure 3D, I). Thus, mutation of these four basic residues was not sufficient to disrupt localization of α_q . We next performed a more extensive mutagenesis of N-terminal basic residues of α_q . A mutant was created in which arginines 37 and 38 along with lysine 33 were mutated (α_q3Q). These residues are part of the large basic cluster, as seen in the helical net diagram, and are in close proximity to the residues that comprise the remainder of the large cluster and that were mutated in α_q4Q (Figure 1C). All of the mutated residues of α_q4Q and α_q3Q were changed to glutamine in another mutant, α_q7Q , where R27, R30, R31, K33, R34, R37, and R38 were all mutated to glutamine. The seven mutated residues of α_q7Q along with arginines 19 and 20 were mutated to glutamine in α_q9Q . A double mutant (α_q2Q) with arginines 19 and 20 mutated to glutamine was also constructed. These arginines, together with lysine 16, form the second, smaller basic patch in the more extreme N-terminus of α_q (Figure 1C). These three residues, lysine 16 and arginines 19 and 20, were mutated in another construct referred to as α_q3Qn to determine whether the basic residues in the more extreme N-terminus were important for PM targeting. Immunofluorescence microscopy revealed that, of these mutants, only α_q9Q displayed cytoplasmic staining (Figure 3H). The rest of the polybasic mutants were properly localized at the PM, similarly to wild type α_q (Figure 3C–G). Consistent with the immunofluorescence images, only α_q9Q displayed a drastic shift to the soluble fraction as assessed through subcellular fractionation assays (Figure 3I). Since α_q2Q was present at the PM and in the particulate fraction (Figure 3F, I) this defect in membrane targeting of α_q9Q cannot be attributed solely to arginines 19 and 20, the only extra residues mutated in α_q9Q compared to α_q7Q . We next investigated whether introducing negative charges into this basic region could cause a defect in localization of α_q . To determine this we constructed mutants α_q4E , α_q2E , and α_q3En , in which the mutated residues of α_q4Q , α_q2Q , and α_q3Qn , respectively, were changed to glutamic acid instead of glutamine. Of these glutamic acid mutants, only α_q3En had an observable defect in PM localization, as demonstrated through immunofluorescence and cell fractionations (Figure 4D, E). α_q4E and α_q2E were both localized at the PM (Figure 4B, C) and were still present to a significant degree in the particulate fraction (Figure 4E). Thus it seems as though mutations in the N-terminal polybasic region of α_q do not have the same effects as they do in α_s . A defect in α_s could be observed upon mutation of only four lysines in the basic patch, whereas a defect in α_q was only observed when 9 residues were mutated to glutamine or when residues 16, 19, and 20 were mutated to glutamic acid. Moreover, it seems as though mutation of the residues in the smaller basic patch in the more extreme N-terminus has more of an effect on the PM localization of α_q , compared to the larger, more downstream basic patch.

To address the possibility that the introduction of multiple glutamines was causing the localization defect independent of the loss of positive charges, we examined two additional

mutants. Although α_q7Q did not display a membrane localization defect (Figure 3), we postulated that mutational replacement of the same residues with glutamic acid, to generate α_q7E , rather than glutamine would decrease membrane localization if indeed charge of the N-terminal region of α_q is critical. In agreement, with this idea α_q7E displayed a pronounced shift to the cytoplasm (Figure 5C) and to the soluble fraction (Figure 5E). Next, we postulated that if indeed the loss of basic charge rather than simply a disrupting effect of multiple glutamines is important in loss of membrane binding of α_q9Q , then introduction of glutamine substitutions at N-terminal sites other than the predicted critical arginines and lysines would not disrupt localization. Thus, we constructed a α_q9Q2 mutant in which glutamic acid 17 and alanine 18 were mutated to glutamine in the context of α_q7Q . These residues are proximal to arginines 19 and 20, the residues mutated in the context of α_q7Q , to generate α_q9Q . α_q9Q2 displayed PM localization (Figure 5D) and an equal distribution between the soluble and particulate fractions (Figure 5E), whereas α_q9Q shows a strong defect in localization at the PM and in the particulate fraction (Figure 3H and 3I, and Figure 5B and 5I), suggesting that it is specifically the loss of charge of arginines 19 and 20 that cause a defect in PM localization and not simply introduction of glutamine residues. Moreover, we compared the predicted helical content of the N-termini of the α_q mutants using a secondary structure prediction algorithm [47]. α_q7E is predicted to have a higher N-terminal helical content than α_q7Q , although α_q7E displays a membrane localization defect (Figure 5). Likewise, α_q9Q2 is predicted to have a substantially decreased N-terminal helical content compared to α_q9Q , but only α_q9Q shows a prominent disruption of membrane localization (Figure 5). Taken together the results with the α_q7E and α_q9Q2 mutants support the conclusion that it is the loss of basic charge rather than simply a disruption of the N-terminal structure that leads to membrane localization defects.

Myristoylation recovers PM localization of polybasic mutants of α_s and α_q

Since it has been proposed that myristoylation and this polybasic motif have complementary roles as an initial membrane targeting signal in Ga [38], we sought to determine whether myristoylation could rescue PM localization of polybasic mutants. Replacing residues 4 through 6 of α_s with residues 4 through 6 of α_i results in myristoylation of α_s [43]. This mutant (myr- α_s) also exhibits increased palmitoylation and tighter membrane association compared to wild type α_s [43]. When these mutations were introduced into α_s5Q , strong PM localization was observed through immunofluorescence (Figure 6A) and a drastic shift to the particulate fraction was observed through cell fractionations (figure 6D). Myristoylation can be introduced into α_q by mutating the codon for the initiating methionine, causing methionine 7 to become the initiating methionine. This mutation in conjunction with mutation of alanine 8 to glycine results in myristoylation, in addition to palmitoylation, of α_q [37]. It was previously shown that introduction of these mutations into α_q $\beta\gamma$ -binding mutants rescued their PM localization and palmitoylation [37]. We thus introduced these mutations into the α_q polybasic mutants that were deficient in PM localization, namely α_q9Q (myr- α_q9Q) and α_q3En (myr- α_q3En). Immunofluorescence microscopy revealed strong PM localization of these mutants (Figure 6B, C), and myr- α_q9Q and myr- α_q3En were also present exclusively in the particulate fraction (Figure 6E). These studies suggest that for α_s and α_q , the polybasic motif serves as the initial membrane targeting signal while for members of the α_i family, myristoylation plays this role. These results are in accordance with the speculation that myristoylation and the polybasic motif have complementary roles as an initial membrane targeting signal [38].

Expression of $\beta\gamma$ rescues PM localization of α_s and α_q polybasic mutants

We next examined the effects of transient overexpression of $\beta_1\gamma_2$ on PM localization of the polybasic mutants. HEK293 cells were transfected with the α subunit constructs with or without co-transfection of MycHis-tagged β_1 and γ_2 subunits ($\beta_1\gamma_2$). Transient co-expression of $\beta_1\gamma_2$ restored PM localization of α_s5Q , as determined through immunofluorescence microscopy with an anti-HA antibody (Figure 7A–C). Co-staining of the cells with an anti- γ_2 antibody

indicated that $\beta_1\gamma_2$ co-localized with α_s5Q at the PM (Figure 7C). Similar results were observed for α_s4Q (data not shown), α_q9Q (Figure 7D–F), and α_q3En (Figure 7G–I). The effects of $\beta_1\gamma_2$ co-expression were also assessed through cell fractionations. Co-expression of $\beta_1\gamma_2$ with wild type α_s and α_q resulted in an increased amount of each G α in the particulate fraction relative to the soluble fraction (Figure 7J,K), such that 90% or more of wild type α_s and α_q were found in the particulate fraction when $\beta_1\gamma_2$ were also overexpressed. There was also a shift of the polybasic mutants to the particulate fraction when $\beta_1\gamma_2$ was co-expressed (Figure 7J, K). Interestingly, α_s4Q and α_q9Q were still not present in the particulate fraction to the same degree as wild type α_s and α_q . The polybasic mutants were distributed about 50:50 soluble:particulate when $\beta_1\gamma_2$ was co-expressed, while wild type α_s and α_q were present almost exclusively in the particulate fraction. Thus the effects of mutations in the polybasic motif can be observed even in the context of the heterotrimer.

Polybasic mutants defective in PM localization are also defective in palmitoylation

Next, we investigated whether defects in PM localization are linked with defects in palmitoylation. G α was immunoprecipitated from lysates of cells labeled with [3H] palmitate, and immunoprecipitates were analyzed by SDS PAGE and fluorography. The $\beta\gamma$ -binding mutant α_sIEK+ displayed a slightly weaker signal than wild type α_s , as has been demonstrated previously [37]. α_s5Q displayed a significantly weaker signal compared to wild type α_s (Figure 8A). In accordance with these results, wild type α_q displayed a strong signal, while α_q9Q and α_q3En displayed markedly reduced amounts of incorporated radiolabel (Figure 8B). These results imply that defects in PM localization of polybasic mutants are also indicative of defects in palmitoylation.

Polybasic mutants can assume an activated conformation and elicit signaling responses

We next wanted to examine the effects of mutations in the polybasic motif on signaling abilities of α_s and α_q . For α_s , we performed cAMP assays with constitutively active forms of the polybasic mutants. We introduced the glutamine 227 to leucine (QL) mutation into α_s5Q (α_s5Q QL). This mutation inhibits GTPase activity, rendering the G α trapped in an active conformation and able to stimulate effectors independently of receptor activation [48]. HEK293 cells were transfected with wild type or mutant α_s . Both α_sQL and α_s5Q QL were capable of stimulating cAMP (Figure 9A). This indicates that α_s5Q is capable of effector activation, even though it is not present at the PM. This is not surprising, as a constitutively active palmitoylation mutant of α_s , also not at the PM, was able to signal [8]. These results also indicate that the switch regions have not been perturbed, strongly suggesting that the mutant is structurally intact.

A similar constitutively activating mutation was also introduced at position 209 into the polybasic mutants of α_q [49]. α_q9Q QL and α_q3En QL were capable of stimulating inositol phosphate production over basal levels, albeit to a much lesser extent than α_qQL (Figure 9B), even though expression levels were similar (not shown). This could be due to the fact these mutants are presumably not present at the PM, and therefore less available to the effector, PLC β ; similarly, a constitutively active palmitoylation mutant of α_q was strongly defective in its ability to stimulate inositol phosphate production in cells [8]. To rule out the possibility that this lowered activity results from altered structure of the mutants, we further confirmed the structural integrity of α_q9Q and α_q3En through examination of the ability of the mutants to interact with GST-RGS4 in the presence and absence of aluminum fluoride (AlF_4^-). AlF_4^- binds to G α -GDP in the position that would be filled by the γ phosphate of GTP, thereby promoting a G α conformation that mimics the transition state of GTP-hydrolysis [50]. Typically, RGS proteins preferentially bind active GDP· AlF_4^- -bound rather than the inactive GDP-bound G α [5]; thus AlF_4^- -dependent binding of an RGS protein and a G α demonstrates the ability of the G α to undergo activation-induced conformational changes. Wild type α_q ,

α_q^{9Q} , and α_q^{3En} were expressed in HEK293 cells and cell lysates were prepared. The lysates were incubated with GST-RGS4-conjugated beads in the presence or absence of AlF_4^- , followed by centrifugation to isolate interacting proteins. Western blot analysis of the pulldowns with an anti-HA antibody indicates that no wild type or mutant α_q construct was pulled down in the absence of AlF_4^- . On the other hand, the presence of AlF_4^- caused similar amounts of wild type and mutant α_q to be pulled down with GST-RGS4 (Figure 9C), indicating that the global structure and ability to undergo activation-induced conformational changes is not perturbed by N-terminal polybasic mutations in α_q .

DISCUSSION

Overall, we have presented the first evidence that α_s and α_q contain N-terminal clusters of basic amino acids that contribute to PM localization. Mutational replacement of critical lysines and arginines with neutral glutamines strongly inhibited PM localization and palmitoylation of α_s and α_q . In addition, PM localization of N-terminal polybasic mutants of α_s and α_q could be rescued by another targeting signals, such as myristoylation. Importantly, the polybasic clusters of α_s and α_q also play a role in membrane localization in the context of the G protein heterotrimer; the N-terminal polybasic mutants of α_s and α_q showed a defect in membrane localization even when $\beta\gamma$ was co-expressed. Thus, these results highlight a new membrane targeting signal for non-myristoylated $G\alpha$ and are consistent with a revised model for membrane targeting of G proteins, in which multiple signals, including multiple lipid modifications, positively-charged patches of amino acids, and heterotrimer interactions, provide a complex combination of signals to direct G proteins to cellular membranes [9].

Although PM localization of both α_s and α_q could be disrupted by mutation of N-terminal basic amino acids, the studies reported here also indicate interesting differences in the N-terminal basic regions of α_s and α_q . Mutation of as few as four basic residues in the N-terminus of α_s , as in α_s^{4Q} , was sufficient to cause a defect in PM localization, as observed by immunofluorescence microscopy and cell fractionation (Figure 2). These four critical amino acids in α_s , lysines 24, 25, 28, and 32, are predicted to form a central basic patch in the N-terminus of α_s (Figure 1). In contrast, a defect in PM localization was not observed for α_q unless nine basic residues were mutated to glutamine (α_q^{9Q}) or three residues were mutated to glutamic acid (α_q^{3En}) (Figure 3–Figure 4). The α_q^{9Q} mutant contained substitutions in two out of the three basic residues in the first basic patch and all seven basic residues in the second, more extensive, basic patch (Figure 1). Surprisingly, mutational substitution of all seven basic residues of the second patch (α_q^{7Q}) failed to disrupt PM localization (Figure 3). The first basic patch in the N-terminus of α_q thus plays an important role in PM localization, although it only consists of three basic amino acids (Figure 1). Accordingly, mutational replacement of the three basic residues of the first patch with glutamic acids (α_q^{3En}), to switch the charge of the side chains, strongly disrupted PM localization. Our extensive α_q mutagenesis results (Figure 3–Figure 4) suggest that numerous basic residues in the N-terminus of α_q contribute to membrane binding. Thus, PM localization of α_s is more readily perturbed by N-terminal mutation of basic residues compared to α_q . Interestingly, the opposite trend was observed in mutational analysis of $\beta\gamma$ -contacting residues. A strong defect in $\beta\gamma$ -binding, PM localization, and palmitoylation was not observed unless five $\beta\gamma$ -contacting-residues in the N-terminus of α_s were mutated [37]. On the other hand, mutation of only one $\beta\gamma$ -interacting residue, isoleucine 25, was sufficient to cause a complete defect in $\beta\gamma$ -binding, PM localization, and palmitoylation of α_q [51]. These results could imply that the polybasic motif is more important for PM targeting of α_s , whereas interaction with $\beta\gamma$ is the more crucial PM-targeting signal for α_q .

Not only did we observe differences in the importance of the N-terminal polybasic motifs in α_s compared to α_q , it appears that differences also exist within members of the α_q subfamily. A recent report addressed mechanisms of PM localization of the α_q subfamily members α_{14}

and α_{16} , and the data was consistent with little or no role for N-terminal basic clusters in PM localization of those two G α [25]. The N-terminus of α_{14} contains nine basic residues at identical positions as nine of the ten basic residues in α_q . Fusion of the N-terminal 34 amino acids of α_{14} to GFP was sufficient to target GFP to the PM. When all nine basic residues of the N-terminus of α_{14} were changed to glutamines, PM localization of the fusion protein was not disrupted, suggesting that the basic residues were not required for PM localization of α_{14} . These mutations, however, were not examined in the context of full length α_{14} . α_{16} contains eight N-terminal basic residues, although they are spread along the entire N-terminus and not predicted to comprise strong basic clusters, as in α_q and α_{14} . Mutation of five of the basic residues in the N-terminus of α_{16} did not cause a defect in PM localization of a GFP fusion construct and resulted in little or no change in the particulate/soluble partitioning of full-length α_{16} [25]. Those results combined with our results presented here suggest that the role of polybasic motifs can differ among members of the same G α subfamily. These differences may be due to the fact that α_{14} and α_{16} have three cysteines that can serve as sites of palmitoylation [25], while α_q has two [9]. This could render the polybasic motif more important for α_q than it is for α_{14} and α_{16} . Likewise α_s , for which PM localization is disrupted by mutation of only four basic residues, has only one cysteine site of palmitoylation, consistent with the possibility that the presence of multiple palmitoylated cysteines decreases the relative importance of polybasic motifs. It will be important to determine the role of polybasic motifs in other non-myristoylated G α subunits, such as α_{12} and α_{13} .

In view of the fact that this N-terminal polybasic motif is only present in the non-myristoylated G α families, Kosloff, et al. hypothesized that this motif and myristoylation have complementary roles as a targeting signal. This group also proposed a revised two signal model in which for α_i , myristoylation and interaction with $\beta\gamma$ act in conjunction to allow for palmitoylation and PM localization, whereas for the other G α subunit families, the polybasic motif and interaction with $\beta\gamma$ act synergistically to result in palmitoylation and PM localization [38]. Our results demonstrating that the α_s and α_q polybasic mutants defective in PM localization are also defective in palmitoylation (Figure 8) support this model, as well as our results demonstrating that myristoylation of α_s and α_q can restore localization of PM-defective polybasic mutants (Figure 6). We were able to introduce N-terminal myristoylation signals into α_s5Q , α_q9Q and α_q3En , and introduction of such myristoylation motifs into the background of these cytoplasmic α_s and α_q polybasic mutants resulted in strong PM localization and drastic shifts to the particulate fractions (Figure 6).

Basic motifs are present in a multitude of PM-targeted proteins. For example, while H-Ras and N-Ras are farnesylated and palmitoylated, K-Ras is farnesylated and has a cluster of basic residues in its C-terminal hypervariable region as its additional PM targeting signal [52]. When the localization of 125 CFP-tagged constitutively active small GTPases was examined, 37 out of 48 PM-localized small GTPases had C-terminal polybasic clusters consisting of four or more lysine or arginine residues at positions 5 to 20 from the C-terminus [53,54]. Basic residues have also been implicated in the PM-localization of growth-associated protein 43 (GAP43), Src, myristoylated alanine-rich C kinase substrate (MARCKS), G-protein-coupled receptor kinases (GRKs), and other signaling proteins [16,39,55,56]. In some cases, extensive polybasic domains are sufficient for PM targeting, while in many cases polybasic motifs function together with other membrane targeting signals, such as lipid modifications, to mediate membrane localization. Here we provide evidence that N-terminal basic residues are required for proper PM localization of heterotrimeric G α subunits, thus adding to the growing list of proteins that utilize basic patches on their surfaces to bind membranes.

Regions of positive charges on the surface of a protein function as membrane binding signals via electrostatic interactions with negatively charged headgroups of membrane lipids [16,39,56]. Potential lipid-binding partners for basic clusters could be the monovalent acidic

phosphatidylserine (PS) or the more negatively charged phosphatidylinositol 4,5-bisphosphate (PIP₂) [57]. Moreover, these acidic lipids are concentrated at the PM compared to other intracellular membranes [56,58], thus providing a simple mechanism for basic clusters directing a protein specifically to the inner surface of the PM. MARCKS has been shown to interact strongly with membranes containing physiological fractions of PS [59]. However, it was demonstrated recently that combined depletion of PIP₂ and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) at the PM affects PM localization of several peptides containing basic clusters, such as the C-terminal tails of Rit, Rin, K-Ras, as well as the effector domain of MARCKS [54], suggesting that the phosphoinositides may be more important than PS for binding basic surfaces of proteins. It will be important in future work to define which membrane lipids are interacting with the polybasic motifs of G α .

Polybasic-mediated protein binding to acidic lipids is not merely a static event but can be dynamic and subject to regulation through changes in the membrane lipids and changes in the electrostatics of the polybasic region. For example, decreases in PM content of phosphatidylinositol phosphates can be affected by phospholipases and lipid phosphatases, and this would cause a decrease in the acidic nature of the PM and a consequent decrease in the ability of polybasics to interact with the PM [54]. The polybasic regions themselves can be regulated through binding of other proteins to the polybasics, which would serve to sequester the polybasic region and prevent it from interacting with the acidic lipids. Ca²⁺/calmodulin (Ca²⁺/CaM) is an abundant cellular protein that often binds with high affinity to basic regions of proteins. Indeed, changes in cellular Ca²⁺ can promote Ca²⁺/CaM binding to MARCKS and induce the translocation of MARCKS from the PM [56]. Another way to disrupt the positive charge nature of a polybasic surface is through phosphorylation within the polybasic region [60]. As an example, a recent report showed that phosphorylation of K-Ras within its C-terminal polybasic region by PKC causes K-Ras to translocate from the PM to the mitochondria, where it induces apoptosis [61]. For G α , there is some evidence for N-terminal phosphorylation [62]; it will be interesting to determine whether N-terminal phosphorylation plays a physiological role in regulating G α interaction with the PM. In addition, it is tempting to speculate that differences in polybasic-mediated PM binding may somehow account for why α_s translocates to the cytosol upon activation while other G α subunits do not [9].

CONCLUSION

In conclusion, N-terminal basic residues can promote PM targeting of α_s and α_q . These studies showed similarities between the N-terminal polybasic regions of α_s and α_q , namely the ability of other targeting signals such as myristoylation or $\beta\gamma$ co-expression or to restore or partially restore, respectively, PM localization of polybasic mutants. There were also some critical differences between α_s and α_q , such as the difference in sensitivity to these mutations of N-terminal basic residues and the ability of polybasic mutants to stimulate second messenger production. Thus, the role of basic residues in PM targeting may vary amongst G α subunits, as has been shown through studies of the polybasic motifs of α_{14} and α_{16} [25]. Our results support the hypothesis that myristoylation and interaction with $\beta\gamma$ lead to palmitoylation and PM localization of α_i family members, whereas for the non-myristoylated G α families, the polybasic motif compensates for myristoylation and acts in conjunction with $\beta\gamma$ and palmitoylation to target the heterotrimer to the PM [38]. These studies shed light on mechanisms of trafficking of the G protein heterotrimer, and future studies to further discern where the heterotrimer forms and where in the cell palmitoylation occurs will provide additional insight into how the polybasic motif is targeting the heterotrimer to the PM.

The abbreviations used are

G protein, guanine nucleotide-binding protein; PM, plasma membrane; HEK293 cells, human embryonic kidney cells; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; RGS, regulator of G protein signaling.

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Figure 1. N-termini of α_s and α_q

A, Linear sequences of the N-termini of α_s (amino acids 1–41) and α_q (amino acids 1–40). Asterisks indicate amino acid side chains that contact $\beta\gamma$. Plus signs indicate basic residues. **B**, Helical wheel diagrams of the N-termini of α_s (amino acids 20–37) and α_q (amino acids 19–36). Asterisks indicate amino acid side chains that contact $\beta\gamma$. Basic residues are indicated in gray. **C**, Helical net diagrams of the N-termini of α_s (amino acids 13–41) and α_q (amino acids 15–40). Basic residues are indicated by the dark circles, and predicted clusters of basic residues are shaded in gray.

Figure 2. Localization of α_s polybasic mutants

HEK293 cells were transfected in 6-well plates containing coverslips with 0.4 μ g pcDNA3 along with 0.6 μ g α_s pcDNA3 (**A**), α_s5Q pcDNA3 (**B**), or α_s4Q pcDNA3 (**C**). 40 hours after transfection cells were fixed with 3.7% formaldehyde and visualized as described under “Experimental Procedures.” **D**, HEK293 cells were transfected in 6-cm plates with 1.2 μ g pcDNA3 along with 1.8 μ g pcDNA3 containing the indicated α subunit. Approximately 40 hours after transfection cells were lysed in hypotonic lysis buffer and soluble and particulate cell fractions were separated as described under “Experimental Procedures.” 2.5% of each fraction was resolved on 12% SDS-PAGE and the α subunits were visualized by immunoblotting with the 12CA5 antibody.

Figure 3. Localization of α_q glutamine mutants

HEK293 cells were transfected in 6-well plates containing coverslips with 0.4 μ g pcDNA3 along with 0.6 μ g α_q pcDNA3 (A), α_q IEpcDNA3 (B), α_q 3QpcDNA3 (C), α_q 4QpcDNA3 (D), α_q 7QpcDNA3 (E), α_q 2QpcDNA3 (F), α_q 3QnpcDNA3 (G), or α_q 9QpcDNA3 (H). 40 hours after transfection cells were fixed with 3.7% formaldehyde and visualized as described under "Experimental Procedures." I, HEK293 cells were transfected in 6-cm plates with 1.2 μ g pcDNA3 along with 1.8 μ g pcDNA3 containing the indicated α subunit. Approximately 40 hours after transfection cells were lysed in hypotonic lysis buffer and soluble and particulate cell fractions were separated as described under "Experimental Procedures." 2.5% of each fraction was resolved on 12% SDS-PAGE and the α subunits were visualized by immunoblotting with the 12CA5 antibody.

Figure 4. Localization of α_q glutamic acid mutants

HEK293 cells were transfected in 6-well plates containing coverslips with 0.4 μ g pcDNA3 along with 0.6 μ g α_q pcDNA3 (A), $\alpha_q 4E$ pcDNA3 (B), $\alpha_q 2E$ pcDNA3 (C), or $\alpha_q 3En$ pcDNA3 (D). 40 hours after transfection cells were fixed with 3.7% formaldehyde and visualized as described under “Experimental Procedures.” E, HEK293 cells were transfected in 6-cm plates with 0.6 μ g pcDNA3 along with 2.4 μ g pcDNA3 containing a glutamic acid mutant. Approximately 40 hours after transfection cells were lysed in hypotonic lysis buffer and soluble and particulate cell fractions were separated as described under “Experimental Procedures.” 2.5% of each fraction was resolved on 12% SDS-PAGE and the α subunits were visualized by immunoblotting with the 12CA5 antibody.

Figure 5. Localization of α_q7E and α_q9Q2

HEK293 cells were transfected in 6-well plate containing coverslips with 0.4 μ g pcDNA3 along with 0.6 μ g pcDNA3 containing α_q7Q (A), α_q9Q (B), α_q7E (C), or α_q9Q2 (D). 40 hours after transfection cells were fixed and stained with 12CA5 antibody as described under “Experimental Procedures.” E, HEK293 cells were transfected with 0.6 μ g pcDNA3 along with 2.4 μ g pcDNA3 containing an α_q mutant. 40 hours after transfection cells were lysed in hypotonic lysis buffer and soluble and particulate cell fractions were separated as described under “Experimental Procedures.”

Figure 6. Localization of myristoylated polybasic mutants

HEK293 cells were transfected in 6-well plates containing coverslips with 0.4 μ g pcDNA3 along with 0.6 μ g pcDNA3 containing myr- α_s5Q pcDNA3 (**A**), myr- α_q9Q pcDNA3 (**B**), or myr- α_q3En pcDNA3 (**C**). 40 hours after transfection cells were fixed with 3.7% formaldehyde and visualized as described under “Experimental Procedures.” For fractionations, HEK293 cells were transfected in 6-cm plates with 1.2 μ g pcDNA3 along with 1.8 μ g pcDNA3 containing the indicated α_s construct (**D**) or α_q construct (**E**). 40 hours after transfection cells were lysed and fractionated as described under “Experimental Procedures.” Note that myr- α_q9Q and myr- α_q3En migrate slightly faster than α_q9Q and α_q3En , respectively. This is likely due to deletion of amino acids 1–6 in the myristoylated forms [37].

Figure 7. Effects of coexpressing $\beta_1\gamma_2$ on the localization of polybasic mutants

HEK293 cells were transfected in 6-well plates containing coverslips with 0.6 μ g of either α_s5Q pcDNA3 (A–C), α_q9Q pcDNA3 (D–F) or α_q3En pcDNA3 (G–I). α subunits were transfected along with either 0.4 μ g pcDNA3 (A, D, G) or 0.3 μ g mycHis- β_1 pcDNA3 and 0.1 μ g γ_2 pcDNA3 (B, C, E, F, H, I). 40 hours after transfection cells were fixed and stained with 12CA5 antibody (A, B, D, E, G, H) and anti- γ_2 antibody (C, F, I) as described under “Experimental Procedures.” J, K, HEK293 cells were transfected with 1.8 μ g pcDNA3 containing the indicated α subunit along with either 1.2 μ g pcDNA3 or 0.9 μ g mycHis- β_1 pcDNA3 plus 0.3 μ g γ_2 pcDNA3. 40 hours after transfection cells were lysed and fractionated as described under “Experimental Procedures.” A portion of each fraction was resolved by

SDS-PAGE and immunoblotted with either the 12CA5 antibody (upper panels) or 9E10 anti-myc antibody (lower panels).

Figure 8. Palmitoylation of α_s and α_q polybasic mutants

HEK293 cells were transfected in 6-cm plates with pcDNA3 containing either wild type or the indicated mutant α_s (**A**) or wild type or the indicated mutant α_q (**B**) in the amounts indicated under “Experimental Procedures.” α_s IEK+ and α_q IE are $\beta\gamma$ -binding mutants described previously [37]. 40 hours after transfection, cells were labeled with 0.5 mCi/ml [3 H] palmitate for 3 hours, after which cells were lysed and G α was immunoprecipitated as described under “Experimental Procedures.” A portion of each immunoprecipitation was resolved by SDS-PAGE and [3 H] palmitate incorporated into G α was visualized by fluorography (upper panels) after a one-week exposure for α_s and a two-day exposure for α_q . A separate portion of each immunoprecipitation was analyzed by SDS-PAGE and immunoblotted with the 12CA5

antibody to confirm that similar amounts of wild type and mutant G α were immunoprecipitated (lower panels). In each panel, samples were run together on the same gel. The samples in lower panel of *A* are separated to indicate that intervening lanes were removed.

Figure 9. Signaling by α_s and α_q polybasic mutants

A, HEK293 cells were transfected in 6-well plates with either 1 μ g pcDNA3, 1 μ g α_s pcDNA3 or 0.8 μ g pcDNA3 plus 0.2 μ g pcDNA3 containing the indicated mutant α_s . 24 hours after transfection cells were labeled with [³H] adenine. 16 hours later cAMP accumulation was measured as described under "Experimental Procedures." **B**, HEK293 cells were transfected in 6-well plates with either 1 μ g pcDNA3 or 0.7 μ g pcDNA3 plus 0.3 μ g pcDNA3 containing the indicated α_q construct. 24 hours after transfection cells were labeled with [³H] inositol. 16 hours later inositol phosphate was assayed as described under "Experimental Procedures." **C**, HEK293 cells were transfected in 6-cm plates with the indicated α_q construct. 24 hours after transfection cells were lysed and incubated in the presence or absence of AIF₄⁻ for 2 hours at

4°C with GST-RGS4, purified as described under “Experimental Procedures.” Beads were then washed 3 times with lysis buffer. Protein was eluted from the beads by boiling in sample buffer for 5 minutes. 3.3% of the lysate and 40% of the pull downs were resolved by SDS-PAGE and visualized by immunoblotting with the 12CA5 antibody.