An N-terminal polybasic motif of Gαq is required for signaling and influences membrane nanodomain distribution

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ABSTRACT

Regions of basic amino acids in proteins can promote membrane localization through electrostatic interactions with negatively charged membrane lipid head groups. Previous work showed that the heterotrimeric G protein subunit \( \alpha_q \) contains a polybasic region in its N-terminus that contributes to plasma membrane localization. Here, the role of the N-terminal polybasic region of \( \alpha_q \) in signaling was addressed. For \( \alpha_q \) mutants, loss of plasma membrane localization correlated with loss of signaling function, as measured by the ability to couple activated GPCRs to stimulation of inositol phosphate production. However, recovery of plasma membrane localization of \( \alpha_q \) polybasic mutants by introduction of a site for myristoylation or by co-expression of \( \beta \gamma \) failed to recover signaling, suggesting a role for N-terminal basic amino acids of \( \alpha_q \) beyond simple plasma membrane localization. Importantly, a \( \alpha_q^{4Q} \) mutant, containing glutamine substitutions at arginines 27, 30, 31, and 34, was identified that failed to mediate signaling yet retained plasma membrane localization. Although \( \alpha_q^{4Q} \) failed to couple activated receptors to inositol phosphate production, it was able to bind \( \beta \gamma \), bind RGS4 in an activation-dependent manner, stimulate inositol phosphate production in a receptor-independent manner, and productively interact with a GPCR in isolated membranes. Interestingly, \( \alpha_q^{4Q} \) showed a differing localization to plasma membrane nanodomains compared to wild type \( \alpha_q \). Thus, basic amino acids in the N-terminus of \( \alpha_q \) can affect its lateral segregation on plasma membranes, and changes in such lateral segregation may be responsible for the observed signaling defects of \( \alpha_q^{4Q} \).
INTRODUCTION

Heterotrimeric (αβγ) guanine nucleotide-binding proteins (G proteins) transmit signals from extracellular stimuli such as hormones, light, and odorants to intracellular effectors, leading to the production of second messengers and ultimately a physiological response (Neer, 1995). Activation of a heptahelical transmembrane G protein-coupled receptor (GPCR) by an extracellular agonist activates the G protein by catalyzing the release of GDP from Gα, leading to the binding of GTP to Gα and a conformational change in Gα (Oldham and Hamm, 2008; Sprang, 1997). 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).

It is well established that lipid modifications are critical in anchoring the G protein subunits to the membrane (Marrari et al., 2007; Wedegaertner, 1998). Gγ is prenylated with either a farnesyl or geranylgeranyl group at a C-terminal CaaX motif, while the N-termini of Gα are modified by the fatty acids myristate and/or palmitate. In addition to lipid modifications, other mechanisms contribute to PM targeting of the heterotrimeric G proteins. For example, it has become clear that individual Gα or Gβγ subunits are inefficiently targeted to the PM in the absence of forming a heterotrimer. In other words, interaction of Gα with Gβγ is critical for PM targeting (Dupre et al., 2009; Evanko et al., 2001; Evanko et al., 2000; Gotta and Ahringer, 2001; Kosloff et al., 2003; Marrari et al., 2007; Song et al., 1996; Takida and Wedegaertner, 2003).

Recent work has demonstrated that clusters of basic amino acid side chains also contribute to PM localization of G proteins (Crouthamel et al., 2008; Pedone and Hepler, 2007). Polybasic motifs are present in the N-termini of nonmyristoylated Gα family
members, namely \( \alpha_s \), \( \alpha_q \), and \( \alpha_{12/13} \) (Kosloff et al., 2002). Importantly, these polybasic motifs lie on the opposite face of the N-terminal helix as the G\( \beta \gamma \)-contacting residues, and therefore allowing G\( \alpha \), even when bound to G\( \beta \gamma \), to make electrostatic interactions with a negatively charged interface of the PM. Mutation of residues in the polybasic motifs of \( \alpha_s \) and \( \alpha_q \) caused defects in PM localization and palmitoylation (Crouthamel et al., 2008). Substitution of four basic residues with neutral glutamines in the N-terminus of \( \alpha_s \) was sufficient to decrease its PM localization, as observed by immunofluorescence microscopy and cell fractionation. In contrast, a defect in PM localization was not observed for \( \alpha_q \) unless nine basic residues were mutated to glutamine or if three basic residues were mutated to oppositely charged glutamic acids. Thus it seems as though the degree to which the polybasic motif is important for PM targeting varies among G\( \alpha \). Consistent with this variability, mutation of basic residues in other \( \alpha_q \) family members, \( \alpha_{14} \) and \( \alpha_{16} \), had minimal effects on their PM targeting or palmitoylation (Pedone and Hepler, 2007). Defects in PM localization of polybasic mutants of \( \alpha_s \) and \( \alpha_q \) could be overcome by myristoylation of these subunits, supporting the proposal that these targeting signals, myristoylation and polybasic motifs, have overlapping roles in promoting PM localization of G\( \alpha \) (Crouthamel et al., 2008; Kosloff et al., 2002).

In the work described herein, we examined whether the polybasic N-terminal region of \( \alpha_q \) was critical for signaling function, in addition to having a role in PM localization. Previous work had shown that disruption of PM localization of \( \alpha_q \) also disrupted its ability to couple activated GPCRs to downstream signaling, such as inositol phosphate production (Evanko et al., 2005; Evanko et al., 2000; Wedegaertner et al., 1993). Consistent with this, here we demonstrate that polybasic mutants of \( \alpha_q \) that were
defective in PM localization are also defective in signaling. Surprisingly, though, we find that recovery of PM localization of N-terminal polybasic mutants of αq, through introduction of a site for myristoylation or co-expression of Gβγ, does not recover signaling function. Similarly, we identify a polybasic mutant of αq, termed αq4Q that contains substitutions at arginines 27, 30, 31, and 34, which is defective in signaling even though it is properly localized at the PM. This mutant appeared to be defective in coupling to GPCRs in cells, although it was able to bind GTP in response to GPCR activation in isolated membranes. Interestingly, the nanoscale PM distribution of this mutant differed from that of wild type αq. Thus it seems as though positively charged residues in αq contribute to its PM nanodomain localization, and this altered localization of αq4Q may account for its defect in signaling.
MATERIALS AND METHODS

Cell culture and Transfection - HEK293 cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Cells were maintained at 37°C and 5% CO₂. 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 1.0 μg plasmid DNA, while cells in 6-cm plates were transfected with 3.0 μg plasmid DNA. Transfections were performed with the FuGENE 6 transfection reagent according to the manufacturer’s protocol (Roche Applied Sciences). Experiments were performed approximately 40 hours after transfection unless otherwise noted. Spodoptera Frugiperda (SF9) cells (American Type Culture Collection) were maintained in suspension culture at 27°C in SF900 II media (Invitrogen) supplemented with 10% heat-inactivated FBS and gentamycin.

Expression Plasmids - The GST-RGS4 bacterial expression plasmid was provided by Dr. R. Neubig (University of Michigan). pcDNA3 encoding the M3 muscarinic receptor was provided by Dr. J. Benovic (Thomas Jefferson University). α₂AR-pCMV4, MycHis-β₁pcDNA3, γ₂-pcDNA3, HA-α₄pcDNA3, and the HA-tagged βγ-binding mutant α₄IE have been described previously (Evan et al., 2000; Levis and Bourne, 1992; Snow et al., 1999; Wedegaertner et al., 1993). All α₄ constructs described here also contain the internal HA epitope. α₄2Q, α₄3Q, α₄3Qn, α₄4E, α₄2E, α₄3En, α₄4A, α₄4K, and the α₄ point mutants were constructed using the sequential PCR method with wild type α₄pcDNA3 as a template (Ausubel et al., 1992). α₄7Q was made by the sequential PCR method using α₄4Q as a template. Similarly, α₄9Q was constructed using α₄7Q as a
template. Myr-$\alpha_q$9Q and myr-$\alpha_q$3En were made using Stratagene QuikChange mutagenesis with primers that mutate the codon for the initiating methionine along with alanine 8 to glycine (Evanko et al., 2000) using $\alpha_q$9Q and $\alpha_q$3En as templates. The Q227L constitutively activating mutation was constructed by digesting pieces of DNA from $\alpha_q$QL and ligating them into $\alpha_q$4Q. The G protein anchor constructs of the corresponding heterotrimers were made by ligating the N-terminal 41 amino acids of $\alpha_q$ to the N-terminus of a monomeric yellow fluorescent protein (mCit) and the C-terminus of $\gamma_2$ was ligated to the C-terminus of mCit, as described previously for N$_q$C$_r$-mCit (Abankwa and Vogel, 2007).

**Inositol phosphate assays** - 24 hours after transfection in 6-well plates, cells were reseeded onto 7 wells of 24-well plates. 6 of these wells were labeled with 2 $\mu$Ci/ml of $[^3H]$ inositol. 3 wells would be used to measure basal levels and three would be used to measure agonist-stimulated levels. After labeling for 16 hours, IP production was assayed. Calculations were performed using the averages of the 3 wells. The seventh well was used to assay protein expression. Cells in these wells were lysed in 100 $\mu$l 2X SDS sample buffer and boiled for 5 minutes. 25 $\mu$l of the lysates were analyzed by SDS PAGE and western blotting.

For IP assays, cells were transfected in 6-well plates with either 1.0 $\mu$g pcDNA3, 0.7 $\mu$g pcDNA3 plus 0.3 $\mu$g $\alpha_{2A}$AR pCMV4, or 0.4 $\mu$g pcDNA3, 0.3 $\mu$g pcDNA3 encoding an HA-$\alpha_q$ construct, and 0.3 $\mu$g $\alpha_{2A}$AR pCMV4. When $\beta_1\gamma_2$ was coexpressed, cells were transfected with 0.3 $\mu$g $\alpha_{2A}$AR pCMV4, 0.4 $\mu$g pcDNA3 encoding the indicated $\alpha_q$ construct, plus 0.2 $\mu$g mycHis$\beta_1$pcDNA3, and 0.1$\mu$g $\gamma_2$pcDNA3. When the signaling of endogenous M$_3$ muscarinic receptor (M$_3$R) was examined, 1.0 $\mu$g pcDNA3 or 0.3 $\mu$g
pcDNA3 containing α_q or α_q4Q were transfected. When M_3R was coexpressed, cells were transfected with the indicated amount of M_3R pcDNA3, either 0.3 μg HA-α_q pcDNA3 or 0.7 μg HA-α_q4Q pcDNA3, and enough pcDNA3 so that the total amount of transfected DNA is 1.0 μg. Approximately 40 hours after transfection cells were washed with assay media containing 5 mM lithium chloride after labeling. Cells were then incubated with assay media for 1 hour with or without 10 μM of UK14304 or 1 mM carbachol, 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.

Nickel-nitriloacetic acid (Ni-NTA) pull-down assays - HEK293 cells were transfected in 6-cm plates with 1.8 μg pcDNA3 encoding the indicated α_q construct, along with either 1.2 μg pcDNA3 or 0.9 μg MycHisβ1 pcDNA3 plus 0.3 μg γ2 pcDNA3. 40 hours after transfection cells were lysed in lysis buffer C (20 mM Hepes, pH 7.5, 100 mM NaCl, 0.7% Triton X-100, 5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml aprotinin) for 1 hour, after which cellular lysates were centrifuged 3 minutes, 13,000 rpm, 4°C, to pellet nuclei and debris. 30 μl of the post-nuclear supernatants (lysate) were saved to analyze total amounts of α_q and
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MycHisβ1 in lysates. 30 μl of 2X sample buffer were added to the lysates, which were then boiled for five minutes. 10 μl Ni-NTA magnetic agarose beads (Qiagen) were added to the lysates and incubated for two hours to precipitate β1γ2 and interacting proteins. Beads were subsequently washed three times with lysis buffer C containing 10 mM imidazole. β1γ2 and interacting proteins were then eluted from the beads with 50 μl lysis buffer C containing 250 mM imidazole. 50 μl of sample buffer was added to the eluate, which was subsequently boiled for 5 minutes. 25 μl of the pull-downs and 10 μl of the lysates were analyzed by SDS PAGE and Western blotting with the 12CA5 and 9E10 antibodies.

**GST-RGS4 pull-down assays** - GST-RGS4 was expressed in BL-21 cells and purified using glutathione-Sepharose 4B beads (Amersham, Biosciences) as previously described (Day et al., 2003; Day et al., 2004a; Day et al., 2004b; Sterne-Marr et al., 2003), and then GST-RGS4 pulldowns were performed as described (Day et al., 2004a). Briefly, HEK293 cells were transfected with wild type or mutant αq. 24 hr after transfection cells were washed with ice cold PBS, harvested in 0.3 ml lysis buffer (20 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 5 mM MgCl2, 0.7% Triton X-100, 1 mM PMSF, 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. 50 μl of the cleared lysate was saved for analysis of total protein in cellular lysate. 50 μl of sample buffer was added to the total cell lysate (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 pull-downs were resolved by SDS PAGE analysis followed by western blotting.

Baculovirus infection – Recombinant baculoviruses expressing the thromboxane A₂ receptor TPα, β₁, γ₂, and α₄ were described (Zhang et al., 2009; Zhang et al., 2006). Production of the recombinant baculovirus expressing α₄Q was performed using the Bac-to-Bac Baculovirus expression system according to the manufacturer’s protocol (Invitrogen). Six million SF9 cells in 10 ml SF900 II media (Invitrogen) containing 10% heat-inactivated FBS and gentamycin were seeded onto T-75 flasks. Once the cells had attached, they were infected with 4 recombinant baculoviruses: one expressing the thromboxane A₂ receptor TPα, one expressing β₁, one expressing γ₂, and one expressing α₄ or α₄Q. Each virus was infected at a multiplicity of infection (M.O.I.) of 1. 16 hours after infection virus-containing media was removed and replaced with 10 ml serum free media. Cells were subsequently incubated for another 24 hours at 27°C.

Isolation of membranes from SF9 cells - Approximately 40 hours after infection with recombinant baculoviruses, cells were harvested and washed three times in 0.9% NaCl, then resuspended in HE/PI buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 0.11% aprotinin, 0.02% leupeptin, and 0.1% PMSF) and incubated on ice for five minutes. Cells were then passed through a 27-gauge needle 15 times and centrifuged for 5 minutes, 2,000 rpm, 4°C to pellet nuclei and debris. The post-nuclear supernatant was then centrifuged for 35 minutes, 13,000 rpm, 4°C, to pellet membranes. Membranes
were resuspended in HE/PI buffer and the concentration of membrane protein was determined by Bradford assay (BioRad). Membranes were stored at -80°C until experimental use.

[^35S]GTPγS assays – membranes (20 µg protein/assay point) were resuspended in TME buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 20 mM MgCl₂, and 0.1 µM GDP). Membranes were stimulated with an appropriate concentration of U46619 (Cayman Chemical, Ann Arbor, MI) for 5 minutes at 30°C in the presence of 5 nM[^35S]GTPγS (Perkin Elmer Life and Analytical Sciences). The reaction was terminated with 600 µl ice-cold Immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 150 mM NaCl, 0.5% Nonidet P-40, 0.33% aprotinin, 0.1 mM GDP, and 0.1 mM GTP) and the membranes were incubated for 30 minutes to solubilize protein. The extract was then added to 2 µl of nonimmune serum preincubated with 100 µl of a 12% suspension of Pansorbin cells. Nonspecifically bound proteins were removed by incubating for 20 minutes at 4°C followed by centrifuging for 3 minutes, 7,000 rpm, 4°C. The extract was then incubated for 1 hour at 4°C with 10 µl of an antiserum directed against a peptide corresponding to the C-terminal residues of α₉ (Barr et al., 1997). This antiserum (0945) had been preincubated with 100 µl of a 5% suspension of protein A-Sepharose. Subsequently, immunoprecipitates were collected by centrifugation for 3 minutes, 7,000 rpm, 4°C and washed three times with 1 ml IP buffer, then once with 1 ml IP buffer containing no detergent. Immunoprecipitates were subsequently boiled for 30 seconds in 0.5 ml 0.5% SDS, after which 5.2 ml Ecolite + (MP Biochemicals) was added. The amount of immunoprecipitated[^35S]GTPγS was determined by liquid scintillation spectrometry.
Western Blotting - Samples were subjected to SDS-PAGE, transferred to PVDF membranes, and blocked with Tris-buffered saline (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).

Confocal microscopy – HEK293 cells were co-transfected with pcDNA3.1 containing Nq-mCit-Cγ or Nq4Q-mCit-Cγ along with pcDNA3.1 containing a membrane nanodomain marker fused to cyan fluorescent protein (CFP) using Effectene transfection reagent (Qiagen). Confocal imaging was performed on HEK293 cells as previously described (Abankwa and Vogel, 2007).

FRET analysis by flow cytometry – FRET analysis was performed on BHK cells as previously described (Abankwa et al., 2008b; Abankwa and Vogel, 2007). An LSRII flow cytometer (BD Biosciences) was used to measure fluorescence of cells in donor- (405 nm excitation, 450/50 nm emission), acceptor- (488 nm excitation, 530/30 nm emission) and FRET-channels (405 nm excitation, 560/20 nm emission). Doublet-discrimination was implemented to measure signals of single cells. FRET analysis of flow cytometer data was carried out as described using custom written algorithms in IgorPro5 (Wavemetrics). This procedure performed calibration for normalized acceptor surface concentration cA and calibrated the FRET-efficiency and donor-acceptor ratio. The FRET-efficiency was calculated per cell by using an adapted sensitised acceptor emission method and the nanoclustering FRET-value, $E_{\text{max}}$, was determined as described.
RESULTS

_Mutations in the N-terminal polybasic motif of α_q disrupt signaling_

Our recent work examined the importance of N-terminal basic amino acids of α_q for its PM localization (Croutshamel et al., 2008). Extensive mutagenesis of the 10 basic amino acids, either arginines or lysines, located between amino acids 16-34 (Figure 1) revealed only two mutants for which PM localization was lost, one in which 9 basic amino acids were substituted with glutamines (α_q9Q) and one in which 3 basic amino acids were substituted with glutamic acids (α_q3En). Here, our goal was to determine the role of the polybasic motif in signaling of α_q and to determine whether signaling defects correlated with defects in PM localization. Thus, inositol phosphate assays were performed (Figure 2). HEK293 cells were transfected with wild type or mutant α_q constructs along with α_2AAR. The α_2AAR typically couples to α_i, but when overexpressed together with α_q it couples promiscuously (Conklin et al., 1992; Wedegaertner et al., 1993). This provides a useful assay to examine signaling by transfected α_q constructs without interference from endogenous α_q signals. When expressed alone, α_2AAR did not cause an increase in inositol phosphates, either in the absence or presence of the α_2AAR agonist UK14304. However, when α_2AAR was co-expressed with wild type α_q, agonist-dependent inositol phosphate stimulation was observed. First, we examined four polybasic mutants that properly localized at the PM (Croutshamel et al., 2008). α_q3Q (mutations K33Q, R37Q, R38Q), α_q2Q (mutations R19Q, R20Q), α_q3Qn (mutations K16Q, R19Q, R20Q), and α_q2E (mutations R19E, R20E) were able to promote α_2AAR agonist-dependent increases in inositol phosphate
levels (Figure 2A, B), as expected from their retention of PM localization (Crouthamel et al., 2008). \(\alpha_q9Q\) (mutations R19Q, R20Q, R27Q, R30Q, R31Q, K33Q, R34Q, R37Q, R38Q) and \(\alpha_q3En\) (mutations K16E, R19E, R20E), two polybasic mutants that were defective in PM localization (Crouthamel et al., 2008), completely lacked the ability to couple \(\alpha_{2A}AR\) to inositol phosphate production (Figure 2A, B). This lack of signaling was expected and in agreement with our previous results showing that \(\alpha_q\) mutants that lacked PM localization also lacked signaling function (Evanko et al., 2005; Evanko et al., 2000; Wedegaertner et al., 1993). Surprisingly, we identified a third set of \(\alpha_q\) N-terminal polybasic mutants (Figure 1) that lost signaling function even though they retained PM localization. \(\alpha_q4Q\) (mutations R27Q, R30Q, R31Q, R34Q), \(\alpha_q4E\) (mutations R27E, R30E, R31E, R34E) and \(\alpha_q7Q\) (mutations R27Q, R30Q, R31Q, K33Q, R34Q, R37Q, R38Q) were unable to couple \(\alpha_{2A}AR\) to inositol phosphate production (Figure 2A, B) yet were properly localized at the PM (Crouthamel et al., 2008). The common feature of these mutants is that they all have arginines 27, 30, 31, and 34 mutated; these four amino acids are predicted to form a basic cluster, as demonstrated by helical wheel and helical net diagrams of the N-terminus of \(\alpha_q\) (Crouthamel et al., 2008).

In summary, polybasic mutants of \(\alpha_q\) that were defective in PM localization were also defective in signaling. However, not all mutants that were properly PM-localized were able to signal, suggesting that basic residues may also be involved in the proper functioning of \(\alpha_q\) in a manner additional to simply targeting it to the PM.

**Myristoylation or \(\beta_1\gamma_2\) expression restores PM localization but not signaling of \(\alpha_q\) polybasic mutants**
To further examine the relationship between PM localization and signaling function of αq polybasic mutants, we rescued PM localization of the cytoplasmic αq9Q and αq3En N-terminal polybasic mutants by introducing a myristoylation motif or by co-expressing β1γ2. Normally, αq is palmitoylated at cysteines 9 and 10 (Figure 1) but is not myristoylated due to lack of a required glycine at position 2. However, a mutant form of αq can be generated that undergoes both myristoylation and palmitoylation and, importantly, that retains signaling function (Evanko et al., 2000). Thus, a site for myristoylation was introduced into the αq9Q and αq3En polybasic mutants to create myr-αq9Q and myr-αq3En. Our recent work clearly showed that myr-αq9Q and myr-αq3En were strongly targeted to the PM, demonstrating that myristoylation can rescue the loss of PM binding caused by the N-terminal polybasic mutations of αq (Crouthamel et al., 2008). In contrast, myristoylation did not recover signaling function of αq9Q and αq3En. Myr-αq9Q and myr-αq3En were unable to couple α2AAR to inositol phosphate production (Figure 3A).

Similar to myristoylation, we previously demonstrated that co-expression of β1γ2 can recover PM localization of αq9Q and αq3En (Crouthamel et al., 2008). However, αq9Q and αq3En remained unable to couple α2AAR to inositol phosphate production, even when PM localization was promoted by β1γ2 co-expression (Figure 3B). The signaling defect in αq9Q and αq3En is not simply due to overall structural perturbations of the proteins; previous results demonstrated that αq9Q and αq3En can bind to RGS4 in an activation-dependent manner (Crouthamel et al., 2008). These results showing that recovery of PM localization of αq9Q and αq3En, by myristoylation or co-expression
of $\beta_1\gamma_2$, fails to recover signaling further indicates that the polybasic N-terminal region of $\alpha_q$ plays a critical role beyond membrane targeting.

**$\alpha_q4Q$ is also defective in signaling in response to stimulation of the M$_3$R**

To attempt to understand the defect in signaling of N-terminal polybasic mutants of $\alpha_q$, we further characterized the PM-localized but signaling-defective $\alpha_q4Q$ mutant. First, we wanted to confirm that $\alpha_q4Q$ is also defective in signaling in response to a GPCR that naturally couples to $\alpha_q$, since, as described above, $\alpha_2$AR normally couples to $\alpha_i$. Thus, we examined the ability of $\alpha_q4Q$ to signal through endogenous M$_3$R, a Gq-coupled receptor that is the primary muscarinic acetylcholine receptor subtype in HEK293 cells (Luo et al., 2008). Cells were transfected with either wild type $\alpha_q$ or $\alpha_q4Q$, stimulated with carbachol, and inositol phosphate accumulation was assayed. Both $\alpha_q$ and $\alpha_q4Q$ expressed at similar levels (not shown). In nontransfected cells, there was an increase in inositol phosphate production upon stimulation with carbachol due to activation of endogenous $\alpha_q11$ (Figure 4A). Transfection of $\alpha_q$ led to an even greater increase in inositol production upon carbachol stimulation (Figure 4A). However, when $\alpha_q4Q$ was transfected and the cells were stimulated with carbachol, there was no increase in inositol phosphate production over that which was caused by endogenous $\alpha_q$ (Figure 4A). These results indicate that mutation of these four arginines causes defects in signaling through the M$_3$R in addition to $\alpha_2$AR.

We also examined the ability of overexpressed M$_3$R to signal through $\alpha_q4Q$. We expressed increasing concentrations of M$_3$R cDNA in the presence or absence of co-
expression of wild type $\alpha_q$ or $\alpha_q 4Q$. When $\alpha_q$ is co-expressed with $M_3R$, its expression is much higher compared to when it is expressed alone or with $\alpha_{2AAR}$ (data not shown). This may be due to an increased stabilization of $\alpha_q$ resulting from increased interaction with $M_3R$. On the other hand, when $\alpha_q 4Q$ is co-expressed with $M_3R$, its expression levels remain similar to the levels present when it is expressed alone or with $\alpha_{2AAR}$ (data not shown). This could imply that $\alpha_q 4Q$ may have an impaired interaction with $M_3R$, which could account for its defect in signaling. We therefore had to transfect higher amounts of $\alpha_q 4Q$ cDNA compared to $\alpha_q$ when coexpressing $M_3R$ in order to obtain similar expression levels of $\alpha_q$ and $\alpha_q 4Q$ (data not shown). Transfecting increasing amounts of $M_3R$ cDNA resulted in increasing amounts of inositol phosphate accumulation upon carbachol stimulation (Figure 4B). Co-expressing $\alpha_q$ with increasing amounts of $M_3R$ DNA led to even greater increases in inositol phosphate production upon stimulation with carbachol (Figure 4B). Moreover, basal levels of inositol phosphate increased when $\alpha_q$ was overexpressed with 200 ng $M_3R$ DNA (Figure 4B). On the other hand, co-expressing $\alpha_q 4Q$ had no effect on either basal or carbachol-stimulated levels of inositol phosphate compared to when $M_3R$ was expressed alone (Figure 4B), even when $\alpha_q 4Q$ was expressed at levels comparable to that of $\alpha_q$, as it was in Figure 4B (data not shown). Thus $\alpha_q 4Q$ is defective in signaling in response to both endogenous and overexpressed $M_3R$, indicating that this cluster of basic residues is important for signaling in response to activation of $\alpha_q$-coupled receptors.

*The positive charges of the arginines are important for signaling*
\(\alpha_q4Q\) contains mutations of four arginines at positions 27, 30, 31, and 34. To determine whether the positive charge of the arginines or the arginine side chains themselves are required for signaling, we further extended our mutagenesis studies. We mutated the four arginines to lysines (\(\alpha_q4K\)) so that the positive charge of the side chains is preserved, and we also mutated these arginines to alanines (\(\alpha_q4A\)) which removes not only the charge, but also the long side chain. Inositol phosphate assays display that \(\alpha_q4K\) was capable of eliciting responses from \(\alpha_2\)AR upon stimulation, while \(\alpha_q4A\) is identical to \(\alpha_q4Q\) in that it is defective in signaling (Figure 5A). These results suggest that it is the positive charges of these arginines that are somehow required for proper signaling, as mutating them to other positively charged residues results in a mutant that is still able to signal upon receptor stimulation.

To further characterize the defect in signaling of \(\alpha_q4Q\), we performed a more extensive mutagenesis. We created four point mutants in which each of the arginines mutated in \(\alpha_q4Q\) were individually mutated to glutamine, namely \(\alpha_qR27Q\), \(\alpha_qR30Q\), \(\alpha_qR31Q\), and \(\alpha_qR34Q\). When inositol phosphate assays were performed it was observed that all four of these mutants were capable of stimulating inositol phosphate production, indicating that the defect in signaling observed in \(\alpha_q4Q\) cannot be attributed to one specific arginine (Figure 5B). Rather, the defect is due to the combined loss of all four arginines.

**The signaling defect of \(\alpha_q4Q\) is not due to an impaired interaction with G\(\beta\)\(\gamma\)**

The four arginines that are mutated in \(\alpha_q4Q\) lie within the N-terminal G\(\beta\)\(\gamma\) contact site. Thus, we asked whether the observed signaling defects could be caused by
defective interaction with Gβγ. First, we tested its ability to interact with Gβγ through a Ni-NTA pull-down assay. HEK293 cells were transfected with wild type αq or αq4Q with or without transient coexpression of β1γ2 in which the β1 subunit contains a hexahistidine tag. When β1γ2 was isolated from cell lysates using Ni-NTA beads, both αq and αq4Q were also found in the pull down (Figure 6A), suggesting that mutation of these basic residues does not perturb interaction with Gβγ. As a control, the previously characterized Gβγ binding-deficient mutant αqIE did not bind Gβγ (Evanko et al., 2001; Evanko et al., 2005; Evanko et al., 2000).

Next, we tested whether overexpression of β1γ2 could promote signaling by αq4Q. However, coexpression of β1γ2 could not recover signaling of αq4Q (Figure 6B). These results further strengthen the notion that the signaling defect of αq4Q is not due to a defect in Gβγ-binding.

αq4Q is capable of undergoing activation-induced conformational changes and of activating effectors

We previously demonstrated that the more extensive N-terminal polybasic mutant αq9Q was able to interact with RGS4 in an activation-dependent manner (Crouthamel et al., 2008), and thus we expected that αq4Q would also interact with RGS4 and thereby demonstrate the ability of αq4Q to undergo the typical activation-dependent conformational change that characterizes G protein α subunits. Lysates of cells transfected with wild type αq or αq4Q were incubated with GST-RGS4-conjugated beads in the presence or absence of aluminum fluoride (AlF4−), followed by
centrifugation to isolate interacting proteins. Typically, RGS proteins preferentially bind active GDP-AlF$_4^-$-bound rather than the inactive GDP-bound G$_\alpha$ (Watson et al., 1996); thus AlF$_4^-$-dependent binding of an RGS protein and G$_\alpha$ demonstrates the ability of the G$_\alpha$ to undergo activation-induced conformational changes. Neither wild type $\alpha_q$ nor $\alpha_q4Q$ is pulled down with GST-RGS4 in the absence of AlF$_4^-$. On the other hand, both wild type $\alpha_q$ and $\alpha_q4Q$ were efficiently pulled down with GST-RGS4 in the presence of AlF$_4^-$ (Figure 7A), indicating that mutation of these four basic residues does not affect the ability of $\alpha_q$ to undergo activation-induced conformational changes.

The fact that $\alpha_q4Q$ is capable of undergoing activation-induced conformational changes (Figure 7A) combined with a lack of evidence for G$_\alpha$ N-termini as sites of effector interaction suggests that $\alpha_q4Q$ would be capable of effector activation. To test this, we generated a constitutively active form of $\alpha_q4Q$ by introducing the glutamine 209 to leucine (QL) mutation into $\alpha_q4Q$ ($\alpha_q4Q$ QL). This mutation inhibits the GTPase activity of $\alpha_q$ and thus renders it capable of stimulating the effector PLC-$\beta$ in the absence of receptor activation. When $\alpha_q$QL was transfected alone into HEK293 cells, there was a large increase in inositol phosphate levels compared to those of cells transfected with an empty vector or with wild type $\alpha_q$ (Figure 7B). Expression of $\alpha_q4Q$ QL caused a similarly large increase in constitutive inositol phosphate production. Thus, the above results indicate that the signaling defect of $\alpha_q4Q$ cannot be ascribed to an inability to assume an active conformation or to an inability to interact with PLC-$\beta$.

$\alpha_q4Q$/GPCR coupling in Sf9 membranes
So far, the results suggest that $\alpha_q4Q$ localizes to the PM, can bind $G\beta\gamma$, can undergo activation-dependent conformational changes, and can interact with the effector PLC-\(\beta\); thus, none of these factors appear to explain the defect in $\alpha_q4Q$ signaling. On the other hand, studies with the $\alpha_{2A}AR$ and M3R are consistent with the idea that $\alpha_q4Q$ is defective in coupling to GPCRs (Figures 2, 4 and 5). To examine this proposal more directly, we tested GPCR coupling in isolated membranes using a well characterized $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay with Sf9 insect cell membranes (Windh and Manning, 2002). Sf9 cells were infected with baculoviruses to express the Gq-coupled thromboxane A\(_2\) receptor TP\(\alpha\), the G\(\beta_1\) subunit, the G\(\gamma_2\) subunit, and either wild type $\alpha_q$ or $\alpha_q4Q$. Membranes were isolated from infected Sf9 cells, and $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to $\alpha_q$ or $\alpha_q4Q$ was determined in the absence or presence of the TP\(\alpha\) agonist U46619. A 3 to 4-fold increase in $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to $\alpha_q$ was observed upon U46619 activation of TP\(\alpha\) (Figure 8A). When TP\(\alpha\) was expressed alone, only a small increase in $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding was observed, consisted with Sf9 cells having relatively low levels of endogenous G proteins. Surprisingly, $\alpha_q4Q$ also displayed a 3 to 4-fold increase in TP\(\alpha\)-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (Figure 8A), similar to wild type $\alpha_q$. When various concentrations of the agonist were used to stimulate membranes, and the amounts of bound $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ were fitted to dose response curves, the EC\(_{50}\) for $\alpha_q$ and for that of $\alpha_q4Q$ were similar (Figure 8B). These results indicate that $\alpha_q4Q$ is able to couple to GPCRs; mutation of these four arginines does not affect the ability of $\alpha_q$ to couple to its cognate GPCRs. This is in apparent contrast to the inability of $\alpha_q4Q$ to mediate GPCR signaling in transfected cells; however, $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding using membranes directly
measures α₉4Q activation by a GPCR, whereas cell-based inositol phosphate signaling is an indirect measure of productive GPCR-Gα-effector function.

**α₉ and α₉₄Q localize to different PM nanodomains**

Although both α₉ and α₉₄Q contain the N-terminal sites of palmitoylation at cysteines 9 and 10 and localize at the PM, it is clear that amino acids near sites of lipid modification can influence a protein’s partitioning into PM nanodomains. Moreover, localization of G proteins to PM nanodomains, or lipid rafts, can influence the ability to couple GPCR activation to signaling responses (Marrari et al., 2007). Thus, we tested the possibility that the N-termini of α₉ and α₉₄Q when in the heterotrimeric, receptor-coupled state promoted localization to different PM nanodomains. To accomplish this, we used a well-characterized FRET-based assay (Abankwa et al., 2008a; Abankwa et al., 2008b; Abankwa and Vogel, 2007) to examine the ability of the N-termini of α₉ and α₉₄Q to elicit FRET signals with different membrane nanodomain markers (Figure 9A). Constructs were made in which the N-terminal 41 amino acids of either α₉ or α₉₄Q were fused to the N-terminus of a monomeric yellow fluorescent protein (mCIT) with the C-terminal 11 amino acids of γ₂ fused to the C-terminus of mCIT (N₉Cγ₉-mCIT). N₉Cγ₉-mCIT was recently constructed and used in a study comparing nanodomain localization of PM targeting motifs of α₉ and α₄ (Abankwa and Vogel, 2007). Here, we utilized the previously described nanodomain markers (Abankwa and Vogel, 2007): tH, the C-terminal tail of H-Ras fused to monomeric cyan fluorescent protein (mCFP); tK, the C-terminal tail of K-Ras fused to mCFP; and tR, the C-terminal tail of Rac1 fused to mCFP. Also, FRET signals between N₉Cγ₉-mCIT and a similar construct containing the N-
terminus of GAP-43 and the C-terminus of Gγ2 fused to mCFP (N\textsubscript{GAP43}C\textsubscript{γ}-mCFP) were examined, as well as FRET signals between mCFP fused only to the N-terminus of α\textsubscript{i2} (N\textsubscript{i2}-mCFP) or the N-terminus of GAP43 (N\textsubscript{GAP43}-mCFP). The mutations in α\textsubscript{q}4Q were introduced into N\textsubscript{q}C\textsubscript{γ}-mCit (N\textsubscript{q4Q}C\textsubscript{γ}-mCit) and its nanodomain localization was compared to that of N\textsubscript{q}C\textsubscript{γ}-mCit. N\textsubscript{q4Q}C\textsubscript{γ}-mCit was found to be properly localized at the PM through confocal microscopy, similar to full length α\textsubscript{q}4Q (Figure 9B). Interestingly, N\textsubscript{q4Q}C\textsubscript{γ}-mCit displayed significantly lower FRET values with all of the nanodomain markers compared to α\textsubscript{q} with the exception of the tR marker, indicating that there is an altered nanoscale distribution of N\textsubscript{q4Q}C\textsubscript{γ}-mCit in different membrane nanodomains (Figure 9C). The magnitude of the FRET changes observed here are similar to that observed in recent reports analyzing the lateral segregation of different G protein subunits and orientation changes of H-ras (Abankwa et al., 2008b; Abankwa and Vogel, 2007). These results indicate that these basic residues in the N-terminus of α\textsubscript{q} are involved in properly localizing the protein to PM nanodomains. This mislocalization may contribute to the defect in signaling of α\textsubscript{q}4Q.
DISCUSSION

In summary, the work presented here shows the novel finding that the polybasic region in the N-terminus of \( \alpha_q \) is required for proper signaling. Previous work indicated that basic residues in the N-terminus of \( \alpha_q \) play a role in localization at the PM (Crouthamel et al., 2008), and now we extend this analysis by showing that mutations in the basic residues can disrupt signaling even when PM localization is not affected. Several key findings support a novel role for the \( \alpha_q \) N-terminal polybasic region. First, two PM localization-defective polybasic mutants, \( \alpha_q9Q \) and \( \alpha_q3En \), were unable to couple an activated GPCR to PLC\( \beta \)-stimulated production of inositol phosphates. This lack of signaling function was true even when \( \alpha_q9Q \) and \( \alpha_q3En \) were directed to the PM through the introduction of myristoylation or by co-expression of G\( \beta\gamma \), indicating a role for the N-terminal polybasic region beyond simple localization to the PM. Second, we identified \( \alpha_q \) N-terminal polybasic mutants, such as \( \alpha_q4Q \), which were not defective in PM localization, yet were still unable to couple a GPCR to inositol phosphate production. This provided further support for the importance of N-terminal basic residues in \( \alpha_q \) signaling function independent of a general role in PM targeting. Third, the positive charge of the N-terminus is crucial. Mutation of the key arginines in the \( \alpha_q4Q \) mutant (positions 27, 30, 31, and 34) to lysines (\( \alpha_q4K \)) did not disrupt signaling while mutation to glutamines (\( \alpha_q4Q \)) or alanines (\( \alpha_q4A \)) did. Lastly, we show that the \( \alpha_q \) basic residues are critical for plasma membrane nanodomain localization; \( \alpha_q \) and \( \alpha_q4Q \) show differing localization to nanodomains, suggesting one possible mechanism whereby N-terminal basic residues of \( \alpha_q \) could affect signaling function.
Previous work has implicated the N-terminus of various Gα subunits in interacting with GPCRs. Mutagenesis, cross-linking studies, and peptide competition studies are all consistent with the proposal that the Gα N-terminus is an important point of contact between a heterotrimeric G protein and a GPCR (Hamm et al., 1988; Itoh et al., 2001; Kostenis et al., 1997; Kostenis et al., 1998; Onrust et al., 1997; Taylor et al., 1994), although in some of the previous studies interpretations regarding the importance of the Gα N-terminus for functional GPCR coupling are complicated by the knowledge that the Gα N-terminus is essential for direct binding to Gβ. Here, however, the mutations in \( \alpha_{q4Q} \) do not disrupt binding to Gβγ. The four basic residues at positions 27, 30, 31, and 34 are predicted to be on the opposite face of the N-terminal helix from the key residues that interact with Gβ (Crouthamel et al., 2008), and \( \alpha_{q4Q} \) interacts with Gβγ in pull-down experiments (Figure 6A). Thus, we hypothesized that \( \alpha_{q4Q} \) was defective in GPCR interaction based on its inability to promote receptor-dependent inositol phosphate production in transfected cells (Figures 2-4). Surprisingly, however, \( \alpha_{q4Q} \) displayed no defect compared to wild type \( \alpha_{q} \) in receptor-dependent GTPγS binding, as measured in isolated membranes. These latter GTPγS-binding studies indicate that \( \alpha_{q4Q} \) does not have a general defect in GPCR interaction. The mechanistic reason for the inability of \( \alpha_{q4Q} \) to couple activated GPCRs to stimulation of PLCβ remains unclear. Although our results imply that \( \alpha_{q4Q} \) is generally defective in coupling activated GPCRs to productive signaling in cells, it will be important to test additional Gq-coupled receptors to test for specificity.

The defect in \( \alpha_{q4Q} \) signaling does not appear to lie in an inherent inability to undergo an activating conformation or to productively interact with PLCβ. \( \alpha_{q4Q} \) binds
RGS4 in the presence of AlF$_4^-$ indicating that it is capable of activation-induced conformational changes (Figure 7A), and therefore likely capable of effector interaction. Moreover, $\alpha_q4Q$ containing the constitutively activating Q209L mutation ($\alpha_q4Q$ QL) stimulated inositol production when expressed in cells (Figure 7B), supporting the notion that $\alpha_q4Q$ is able to interact with PLC-$\beta$. Thus, our results suggest that the signaling defect of $\alpha_q4Q$ is not due to a loss of key amino side chains involved in interaction with important protein partners, such as GPCRs, G$\beta\gamma$ or the effector PLC-$\beta$.

On the other hand, a FRET analysis of PM nanodomain localization of $\alpha_q4Q$-heterotrimer (N$\alpha4Q$C$\gamma$mCit) indicated differences in nanodomain localization compared to wild type $\alpha_q$-heterotrimer (N$q$C$\gamma$mCit) (Figure 9). These nanoscale localization changes could reduce the probability of $\alpha_q4Q$ encountering certain GPCRs or other proteins, such as effectors or RGS proteins, and thus changes in nanodomain localization could impair the ability of $\alpha_q4Q$ to form productive signaling complexes inside cells. The results in Figure 9 imply that residues in this basic cluster in the N-terminus of $\alpha_q$ serve to direct localization to specific nanodomains. The importance of basic residues in differential nanodomain localization of proteins is exemplified by Ras proteins. K-Ras, which contains a prenyl group and a basic cluster in its C-terminus, localizes to different nanodomains than H-Ras, which contains prenyl and palmitoylation sites in its C-terminus (Abankwa et al., 2008b; Abankwa and Vogel, 2007; Prior et al., 2003). Indeed, FRET analysis demonstrates differing lateral segregation of different Ras isoforms (Abankwa et al., 2008b; Abankwa and Vogel, 2007). Moreover, it has been shown that peptides of the C-terminal tails of monomeric G proteins with basic clusters, such as Rit and K-Ras, bind to PIP$_2$ and PIP$_3$, which have been shown to be
concentrated in lipid rafts in some cell types (Heo et al., 2006). Being that PIP$_2$ is the substrate for the $\alpha_q$ effector PLC-$\beta$, targeting of $\alpha_q$ to certain nanodomains may be necessary for proper activation of the signaling pathway. In a recent analysis of lateral segregation of heterotrimer G protein constructs using the FRET technique as used here, it was proposed that N-terminal polybasic sequences of G$\alpha$ proteins affect their nanodomain localization (Abankwa and Vogel, 2007). This has now been confirmed with the $\alpha_q 4Q$ mutant (Figure 9). Thus, it is tempting to speculate that differences in nanodomain localization of $\alpha_q$ versus $\alpha_q 4Q$-heterotrimers may contribute to the observed signaling differences. Nonetheless, we cannot completely rule out alternative mechanisms, such as reduced affinity of $\alpha_q 4Q$ for one or more key members of a signaling complex. Further studies will be required to fully understand the mechanism of how this polybasic region of $\alpha_q$ regulates nanolocalization and signaling function.
REFERENCES


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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. N-terminal mutants of $\alpha_q$. Amino acids 1-40 of $\alpha_q$ are shown, and the amino acid substitutions for each $\alpha_q$ mutant are indicated.

Figure 2. Signaling by $\alpha_q$ polybasic mutants. HEK293 cells were transfected as indicated with either pcDNA3 alone, pcDNA3 plus $\alpha_{2A}$AR-pCMV4, or pcDNA3 plus $\alpha_{2A}$AR-pCMV4 and pcDNA3 encoding either wild type $\alpha_q$ or the indicated glutamine mutant (A) or the indicated glutamic acid mutant (B). 24 hours after transfection cells were labeled with $[^3]$H inositol. 16 hours later cells were stimulated with 10 $\mu$M UK14304 for 1 hour at 37°C, and inositol phosphate production was assayed as described under “Experimental Procedures.” The results shown are the means ± S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

Figure 3. Recovering PM localization does not recover signaling of $\alpha_q$ polybasic mutants. A, HEK293 cells were transfected as indicated with either pcDNA3 alone, pcDNA3 plus $\alpha_{2A}$AR-pCMV4, or pcDNA3 plus $\alpha_{2A}$AR-pCMV4 and pcDNA3 encoding the indicated $\alpha_q$ construct. B, HEK293 cells were transfected with either pcDNA3 alone, pcDNA3 plus $\alpha_{2A}$AR-pCMV4, or pcDNA3 plus $\alpha_{2A}$AR-pCMV4 and pcDNA3 encoding the indicated $\alpha_q$ construct. In addition, $\beta_1\gamma_2$ was co-expressed, using mycHis-$\beta_1$-pcDNA3 and $\gamma_2$-pcDNA3, as indicated. 24 hours after transfection cells were labeled with $[^3]$H inositol. 16 hours later cells were stimulated with 10 $\mu$M UK14304 for 1 hour at 37°C,
and inositol phosphate production was assayed as described under “Experimental Procedures.” The results shown are the means ± S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

**Figure 4. α₄⁴Q is defective in signaling through M₃R.** 
**A,** HEK293 cells were transfected with pcDNA3 or pcDNA3 plus pcDNA3 encoding the indicated α₄ construct. 
**B,** HEK293 cells were transfected as indicated with either pcDNA3 or the indicated amount of pcDNA3 encoding M₃R, either alone or along with α₄-pcDNA3 or α₄⁴Q-pcDNA3. Higher amounts of α₄⁴Q were transfected to make up for expression differences between α₄ and α₄⁴Q that occur when M₃R is coexpressed. 24 hours after transfection cells were labeled with [³H] inositol. 16 hours later cells were stimulated with 1 mM carbachol for 1 hour at 37°C, and inositol phosphate production was assayed as described under “Experimental Procedures.” The results shown are the means ± S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

**Figure 5. The positive charge of the N-terminus of α₄ is important for signaling.** 
HEK293 cells were transfected as indicated with either pcDNA3 alone, pcDNA3 plus α₂AAR-pCMV4, or pcDNA3 plus α₂AAR-pCMV4 and pcDNA3 encoding either wild type α₄, α₄⁴Q, α₄⁴A, or α₄⁴K (A), or the indicated single point mutant (B). 24 hours after transfection cells were labeled with [³H] inositol. 16 hours later cells were stimulated with 10 μM UK14304 for 1 hour at 37°C, and inositol phosphate production was
assayed as described under “Experimental Procedures.” The results shown are the means ± S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

**Figure 6. The signaling defect of α<sub>q</sub>4Q is not due to impaired interaction with Gβγ.**

**A,** HEK293 cells were transiently transfected in 6-cm plates with pcDNA3 encoding the indicated α<sub>q</sub> construct alone or with pcDNA3 encoding the indicated α<sub>q</sub> construct plus MycHis-β<sub>1</sub>-pcDNA3 and γ<sub>2</sub>-pcDNA3. Approximately 40 hours after transfection cell lysates were prepared and incubated with Ni-NTA magnetic agarose beads to precipitate MycHisβ<sub>1</sub>γ<sub>2</sub>. Beads were washed and MycHisβ<sub>1</sub>γ<sub>2</sub> was eluted as described under “Experimental Procedures.” Samples (16% of lysates and 25% of pull-downs) were resolved by SDS PAGE and immunoblotted with either the anti-HA antibody 12CA5 to detect the expressed α<sub>q</sub> or α<sub>q</sub> mutants (upper panels) or the anti-myc antibody 9E10 to detect expressed β<sub>1</sub> (lower panel).

**B,** HEK293 cells were transfected with either pcDNA3 alone, pcDNA3 plus α<sub>2</sub>AAR-pCMV4, or pcDNA3 plus α<sub>2</sub>AAR-pCMV4 and α<sub>q</sub>-pcDNA3 or α<sub>q</sub>4Q-pcDNA3. In addition, β<sub>1</sub>γ<sub>2</sub> was co-expressed, using mycHis-β<sub>1</sub>-pcDNA3 and γ<sub>2</sub>-pcDNA3, as indicated. 24 hours after transfection cells were labeled with [³H] inositol. 16 hours later cells were stimulated with 10 μM UK14304 for 1 hour at 37°C, and inositol phosphate production was assayed as described under “Experimental Procedures.” The results shown are the means ± S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.
Figure 7. $\alpha_q4Q$ displays activation-dependent interaction with RGS4 and constitutively activating mutation-dependent stimulation of inositol phosphates. **A,** HEK293 cells were transfected in 6-cm plates with the indicated $\alpha_q$ construct. 24 hours after transfection cell lysates were prepared and incubated in the presence or absence of AlF$_4^-$ for 2 hours at 4°C with GST-RGS4, purified as described under “Experimental Procedures.” GST-RGS4 along with bound proteins was isolated, and proteins were eluted from the beads with SDS-PAGE sample buffer. 3.3% of the lysate and 40% of the pull downs were resolved by SDS-PAGE, and $\alpha_q$ and $\alpha_q4Q$ were visualized by immunoblotting with the 12CA5 antibody. **B,** HEK293 cells were transfected in 6-well plates with either pcDNA3 alone or pcDNA3 plus pcDNA3 encoding the indicated $\alpha_q$ mutant. 24 hours after transfection cells were labeled with [$^3$H] inositol. 16 hours later inositol phosphate production was assayed in the absence of any agonist stimulation as described under “Experimental Procedures.” The results shown are the means ± S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

Figure 8. $\alpha_q4Q$ is capable of coupling to receptors. **A,** SF9 cells were infected with baculoviruses expressing TP$\alpha$, $\beta_1$, $\gamma_2$, and with or without a baculovirus expressing $\alpha_q$ or $\alpha_q4Q$ at an M.O.I. of 1. Approximately 40 hours after transfection, membranes from infected cells were isolated as described under “Experimental Procedures.” Membranes were then stimulated with the indicated concentration of U46619 for 5 minutes at 30°C in the presence of [$^{35}$S]GTP$\gamma$S. $\alpha_q$ or $\alpha_q4Q$ was immunoprecipitated as described under “Experimental Procedures” and the amount of bound-[[$^{35}$S]GTP$\gamma$S was measured by
scintillation spectrometry. **B**, SF9 cells were infected with baculoviruses expressing TPα, β1, γ2, and either αq or αq4Q at an M.O.I. of 1. Membranes isolated from these infected cells were stimulated with the indicated concentration of U46619 for five minutes at 30°C in the presence of [35S]GTPγS. αq was immunoprecipitated as described under “Experimental Procedures” and the amount of bound-[35S]GTPγS was measured by scintillation spectrometry. Dose response curves were plotted using Prism.

**Figure 9. Localization of αq in PM nanodomains.** **A**, FRET-vectors are determined by evaluating the co-clustering of a heterotrimeric αq-anchor probe with a given nanodomain marker. **B**, Nq4QCγ-mCit localized predominantly at the plasma membrane of BHK cells, as determined by confocal microscopy. **C**, Lateral segregation FRET-vectors of heterotrimer mimicking constructs NqCγ-mCit and the mutant Nq4QCγ-mCit are significantly different. Lateral segregation FRET-vectors are a set of Emax values, which reflect co-clustering of a probing membrane-anchored construct and a nanodomain marker. Probes and nanodomain markers were pairwise co-expressed in BHK cells. Emax values are shown ± s.e.m. and number of independent experiments, n. Differences between bold and regular Emax values in one column (i.e. for each nanodomain marker) are significantly different (Student’s t-test).
Figure 1
Figure 2
Figure 3
Figure 4

(A) Graph showing the effect of carbachol on the ratio of IP/IPinositol for different transfected constructs.

(B) Graph showing the effect of M₃R transfected (ng) on the ratio of IP/IPinositol for different conditions (+α_q and +α_q⁴Q).

Figure 4
Figure 6
Figure 7

(A) Western blot showing the expression levels of α_q and α_q4Q in lysates with or without GST-RGS4 pull-down and AIF^4-.

(B) Bar graph showing the ratio of IP/IP+inositol for different treatments: pcDNA3, α_β, QL, α_q4Q, and α_βQL. The error bars represent the standard error of the mean.