

An N-Terminal Polybasic Motif of $G\alpha_q$ 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 α_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 α_q in signaling was addressed. For α_q mutants, loss of plasma membrane localization correlated with loss of signaling function, as measured by the ability to couple activated G protein-coupled receptors (GPCRs) to stimulation of inositol phosphate production. However, recovery of plasma membrane localization of α_q polybasic mutants by introduction of a site for myristoylation or by coexpression of $\beta\gamma$ failed to recover signaling, suggesting a role for N-terminal basic amino acids of α_q beyond simple plasma membrane localization. It is

noteworthy that an α_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 α_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. It is noteworthy that α_q 4Q showed a differing localization to plasma membrane nanodomains compared with wild-type α_q . Thus, basic amino acids in the N terminus of α_q can affect its lateral segregation on plasma membranes, and changes in such lateral segregation may be responsible for the observed signaling defects of α_q 4Q.

Introduction

Heterotrimeric ($\alpha\beta\gamma$) 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 $G\alpha$, leading to the binding of GTP to $G\alpha$ and a conformational change in $G\alpha$ (Sprang, 1997; Oldham and Hamm, 2008). 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 (Wedegaertner, 1998; Marrari et al., 2007). $G\gamma$ is prenylated with either a farnesyl or geranylgeranyl group at a C-terminal CaaX motif, whereas the N termini of $G\alpha$ are modified by the fatty acids myristate and/or palmitate. In addition to lipid modifications, other mechanisms contribute to PM targeting

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ABBREVIATIONS: GPCR, G protein-coupled receptor; PM, plasma membrane; HEK, human embryonic kidney; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; RGS, regulator of G protein signaling; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; MOI, multiplicity of infection; FRET, fluorescence resonance energy transfer; PLC- β , phospholipase C β ; mCFP, monomeric cyan fluorescent protein; M3R, M₃ receptor; Sf9, *Spodoptera frugiperda*; α_2 AR, α_2 adrenergic receptor; [³⁵S]GTP- γ S, guanosine 5'-O-(3-[³⁵S]thio)triphosphate; UK14304, 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline; IP, inositol phosphate; U46619, (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid; NTA, nitrilotriacetic acid.

of the heterotrimeric G proteins. For example, it has become clear that individual $G\alpha$ or $G\beta\gamma$ subunits are inefficiently targeted to the PM in the absence of forming a heterotrimer. In other words, interaction of $G\alpha$ with $G\beta\gamma$ is critical for PM targeting (Song et al., 1996; Evanko et al., 2000, 2001; Gotta and Ahringer, 2001; Kosloff et al., 2003; Takida and Wedegaertner, 2003; Marrari et al., 2007; Dupré et al., 2009).

Recent work has demonstrated that clusters of basic amino acid side chains also contribute to PM localization of G proteins (Pedone and Hepler, 2007; Crouthamel et al., 2008). Polybasic motifs are present in the N termini of nonmyristoylated $G\alpha$ family members, namely α_s , α_q , and $\alpha_{12/13}$ (Kosloff et al., 2002). It is noteworthy that these polybasic motifs lie on the opposite face of the N-terminal helix from the $G\beta\gamma$ -contacting residues, and therefore allow $G\alpha$, 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 α_s and α_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 α_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 α_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 α_q family members, α_{14} and α_{16} , had minimal effects on their PM targeting or palmitoylation (Pedone and Hepler, 2007). Defects in PM localization of polybasic mutants of α_s and α_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 $G\alpha$ (Kosloff et al., 2002; Crouthamel et al., 2008).

In this work, we examined whether the polybasic N-terminal region of α_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 α_q also disrupted its ability to couple activated GPCRs to downstream signaling, such as inositol phosphate production (Wedegaertner et al., 1993; Evanko et al., 2000, 2005). Consistent with this, here we demonstrate that polybasic mutants of α_q that were defective in PM localization are also defective in signaling. We were surprised to find that recovery of PM localization of N-terminal polybasic mutants of α_q , through introduction of a site for myristoylation or coexpression of $G\beta\gamma$, did not recover signaling function. Likewise, we identified a polybasic mutant of α_q , termed α_q4Q , 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 seemed to be defective in coupling to GPCRs in cells, although it was able to bind GTP in response to GPCR activation in isolated membranes. It is noteworthy that the nanoscale PM distribution of this mutant differed from that of wild-type α_q . Thus, positively charged residues in α_q seem to contribute to its PM nanodomain localization, and this altered localization of α_q4Q may account for its defect in signaling.

Materials and Methods

Cell Culture and Transfection. HEK293 cells (American Type Culture Collection, Manassas, VA) 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 six-well plates or 6-cm plates 1 day before transfection. Cells in six-well plates were transfected with 1.0 μ g of plasmid DNA, whereas cells in 6-cm plates were transfected with 3.0 μ g of plasmid DNA. Transfections were performed with the FuGENE 6 transfection reagent according to the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN). Experiments were performed approximately 40 h 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, Carlsbad, CA) supplemented with 10% heat-inactivated FBS and gentamicin.

Expression Plasmids. The GST-RGS4 bacterial expression plasmid was provided by Dr. R. Neubig (University of Michigan, Ann Arbor, MI). pcDNA3 encoding the M3 muscarinic receptor was provided by Dr. J. Benovic (Thomas Jefferson University, Philadelphia, PA). α_2AR -pCMV4, MycHis- β_1 pcDNA3, γ_2 -pcDNA3, HA- α_q pcDNA3, and the HA-tagged $\beta\gamma$ -binding mutant α_qIE have been described previously (Levis and Bourne, 1992; Wedegaertner et al., 1993; Snow et al., 1999; Evanko et al., 2000). All α_q constructs described here also contain the internal HA epitope. α_q2Q , α_q3Q , α_q3Qn , α_q4E , α_q2E , α_q3En , α_q4A , α_q4K , and the α_q point mutants were constructed using the sequential polymerase chain reaction method with wild-type α_q pcDNA3 as a template (Ausubel et al., 1992). α_q7Q was made by the sequential polymerase chain reaction method using α_q4Q as a template. Likewise, α_q9Q was constructed using α_q7Q as a template. Myr- α_q9Q and myr- α_q3En were made using QuikChange mutagenesis (Stratagene, La Jolla, CA) with primers that mutate the codon for the initiating methionine along with alanine 8 to glycine (Evanko et al., 2000) using α_q9Q and α_q3En as templates. The Q227L constitutively activating mutation was constructed by digesting pieces of DNA from α_qQL and ligating them into α_q4Q . The G protein anchor constructs of the corresponding heterotrimers were made by ligating the N-terminal 41 amino acids of α_q to the N terminus of a monomeric yellow fluorescent protein (mCit) and the C terminus of γ_2 was ligated to the C terminus of mCit, as described previously for N_qC_γ -mCit (Abankwa and Vogel, 2007).

Inositol Phosphate Assays. Twenty-four hours after transfection in six-well plates, cells were reseeded onto seven wells of 24-well plates. Six of these wells were labeled with 2 μ Ci/ml [³H]inositol. Three wells would be used to measure basal levels and three would be used to measure agonist-stimulated levels. After labeling for 16 h, IP production was assayed. Calculations were performed using the averages of the three wells. The seventh well was used to assay protein expression. Cells in these wells were lysed in 100 μ l of 2 \times SDS sample buffer and boiled for 5 min. The lysates (25 μ l) were analyzed by SDS-PAGE and Western blotting.

For immunoprecipitation assays, cells were transfected in six-well plates with either 1.0 μ g of pcDNA3, 0.7 μ g of pcDNA3 plus 0.3 μ g of α_2AR pCMV4, or 0.4 μ g of pcDNA3, 0.3 μ g of pcDNA3 encoding an HA- α_q construct, and 0.3 μ g of α_2AR pCMV4. When $\beta_1\gamma_2$ was coexpressed, cells were transfected with 0.3 μ g of α_2AR pCMV4, 0.4 μ g of pcDNA3 encoding the indicated α_q construct, plus 0.2 μ g of mycHis β_1 pcDNA3, and 0.1 μ g of γ_2 pcDNA3. When the signaling of endogenous M₃ muscarinic receptor (M₃R) was examined, 1.0 μ g of pcDNA3 or 0.3 μ g of pcDNA3 containing α_q or α_q4Q was transfected. When M₃R was coexpressed, cells were transfected with the indicated amount of M₃R pcDNA3, either 0.3 μ g of HA- α_q pcDNA3 or 0.7 μ g of HA- α_q4Q pcDNA3, and enough pcDNA3 so that the total amount of transfected DNA is 1.0 μ g. Approximately 40 h after transfection, cells were washed with assay media containing 5 mM lithium chloride after labeling. Cells were then incubated with assay media for 1 h with or without 10 μ M 5-bromo-6-(2-imidazolin-2-

ylamino)quinoxaline (UK14304) or 1 mM carbachol, and the reaction was terminated by adding 0.75 ml of 20 mM formic acid to the cells and incubating them at 4°C for 30 min. Ammonium hydroxide (0.1 ml of 0.7 M) was then added to the samples, which were loaded onto AG1-X8 Dowex columns (Sigma-Aldrich, St. Louis, MO). Ammonium hydroxide (1 ml of 50 mM) was added to the columns and the eluate, which constituted the [³H]inositol fraction, was collected. Columns were then washed with 4 ml of 40 mM ammonium formate and 0.1 M formic acid. The [³H]IP fraction was eluted with 1 ml of 4 M ammonium formate and 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 Pull-Down Assays. HEK293 cells were transfected in 6-cm plates with 1.8 μ g of pcDNA3 encoding the indicated α_q construct, along with either 1.2 μ g of pcDNA3 or 0.9 μ g of MycHis β_1 pcDNA3 plus 0.3 μ g of γ_2 pcDNA3. Forty 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 μ g/ml leupeptin, and 2 μ g/ml aprotinin] for 1 h, after which cellular lysates were centrifuged for 3 min at 13,000 rpm and 4°C to pellet nuclei and debris. A portion (30 μ l) of the postnuclear supernatants (lysate) was saved to analyze total amounts of α_q and MycHis β_1 in lysates. Thirty milliliters of the 2× sample buffer was added to the lysates, which were then boiled for 5 min. Nickel-nitriloacetic acid (nickel-NTA; 10 μ l) magnetic agarose beads (QIAGEN, Valencia, CA) were added to the lysates and incubated for 2 h to precipitate $\beta_1\gamma_2$ and interacting proteins. Beads were subsequently washed three times with lysis buffer C containing 10 mM imidazole. $\beta_1\gamma_2$ and interacting proteins were then eluted from the beads with 50 μ l of lysis buffer C containing 250 mM imidazole. Sample buffer (50 μ l) was added to the eluate, which was subsequently boiled for 5 min. Twenty-five microliters of the pull-downs and 10 μ l 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 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) as described previously (Day et al., 2003, 2004a,b; Sterne-Marr et al., 2003), and then GST-RGS4 pull-downs were performed as described previously (Day et al., 2004a). In brief, HEK293 cells were transfected with wild-type or mutant α_q . Twenty-four hours after transfection, cells were washed with ice-cold phosphate-buffered saline, harvested in 0.3 ml of 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 μ g/ml leupeptin and aprotinin), followed by incubation for 1 h at 4°C for lysis. Cells were then centrifuged at full speed in a microcentrifuge to pellet nuclei and unbroken cells. Fifty microliters of the cleared lysate was saved for analysis of total protein in cellular lysate. Fifty microliters of sample buffer was added to the total cell lysate and boiled for 5 min. The remaining 250 μ l of postnuclear supernatant was equally split into two tubes, and 25 μ M AlCl₃, 5 mM NaF, and 1 mM MgCl₂ were added to one tube. Thereafter, 8 μ g of GST-RGS4-bound beads were added to both sets of tubes, and the lysates were then tumbled for 2 h at 4°C. After this incubation, beads were pelleted by centrifugation at low speed for 3 min at 4°C. Beads were then washed three times with lysis buffer. Protein was eluted from beads with 50 μ l of SDS sample buffer and 5 min of boiling. Twenty microliters of each of total cell lysate and the pull-downs were resolved by SDS-PAGE analysis followed by Western blotting.

Baculovirus Infection. Recombinant baculoviruses expressing the thromboxane A₂ receptor TP α , β_1 , γ_2 , and α_q were described previously (Zhang et al., 2006; Zhang et al., 2009). Production of the recombinant baculovirus expressing α_q4Q was performed using the Bac-to-Bac Baculovirus expression system according to the manufacturer's protocol (Invitrogen). Six million Sf9 cells in 10 ml of SF900 II media (Invitrogen) containing 10% heat-inactivated FBS and gentamicin were seeded onto T-75 flasks. Once the cells had attached, they were infected with four recombinant baculoviruses: one express-

ing the thromboxane A₂ receptor TP α , one expressing β_1 , one expressing γ_2 , and one expressing α_q or α_q4Q . Each virus was infected at a multiplicity of infection (MOI) of 1. Sixteen hours after infection, virus-containing media was removed and replaced with 10 ml of serum-free media. Cells were subsequently incubated for another 24 h at 27°C.

Isolation of Membranes from Sf9 Cells. Approximately 40 h 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 5 min. Cells were then passed through a 27-gauge needle 15 times and centrifuged for 5 min at 2000 rpm and 4°C to pellet nuclei and debris. The postnuclear supernatant was then centrifuged for 35 min at 13,000 rpm and 4°C to pellet membranes. Membranes were resuspended in HE/PI buffer, and the concentration of membrane protein was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Membranes were stored at -80°C until experimental use.

[³⁵S]GTP γ S Assays. Membranes (20 μ g of protein/assay point) were resuspended in buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 20 mM MgCl₂, and 0.1 μ M GDP). Membranes were stimulated with an appropriate concentration of (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid (U46619; Cayman Chemical, Ann Arbor, MI) for 5 min at 30°C in the presence of 5 nM [³⁵S]GTP γ S (PerkinElmer Life and Analytical Sciences, Waltham, MA). The reaction was terminated with 600 μ l of ice-cold immunoprecipitation 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 min to solubilize protein. The extract was then added to 2 μ l of nonimmune serum preincubated with 100 μ l of a 12% suspension of Pansorbin cells. Nonspecifically bound proteins were removed by incubating for 20 min at 4°C followed by centrifuging for 3 min at 7000 rpm and 4°C. The extract was then incubated for 1 h at 4°C with 10 μ l of an antiserum directed against a peptide corresponding to the C-terminal residues of α_q (Barr et al., 1997). This antiserum (0945) had been preincubated with 100 μ l of a 5% suspension of protein A-Sepharose. Subsequently, immunoprecipitates were collected by centrifugation for 3 min at 7000 rpm and 4°C and washed three times with 1 ml of immunoprecipitation buffer and then once with 1 ml of immunoprecipitation buffer containing no detergent. Immunoprecipitates were subsequently boiled for 30 s in 0.5 ml of 0.5% SDS, after which 5.2 ml of Ecolite + (MP Biomedicals, Solon, OH) was added. The amount of immunoprecipitated [³⁵S]GTP γ S was determined by liquid scintillation spectrometry.

Western Blotting. Samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked with Tris-buffered saline/0.05% Tween/5% milk. Blots were then probed with 0.5 μ g/ml 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 cotransfected with pcDNA3.1 containing Nq-mCit-C γ or Nq4Q-mCit-C γ along with pcDNA3.1 containing a membrane nanodomain marker fused to cyan fluorescent protein using Effectene transfection reagent (QIAGEN). Confocal imaging was performed on HEK293 cells as described previously (Abankwa and Vogel, 2007).

FRET Analysis by Flow Cytometry. FRET analysis was performed on BHK cells as described previously (Abankwa and Vogel, 2007; Abankwa et al., 2008b). An LSRII flow cytometer (BD Biosciences, San Jose, CA) 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 were carried out as described using custom written

algorithms in IgorPro5 (Wavemetrics, Lake Oswego, OR). This procedure performed calibration for normalized acceptor surface concentration and calibrated the FRET-efficiency and donor-acceptor ratio. The FRET-efficiency was calculated per cell by using an adapted sensitized acceptor emission method, and the nanoclustering FRET value, E_{\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 (Crouthamel et al., 2008). Extensive mutagenesis of the 10 basic amino acids, either arginines or lysines, located between amino acids 16 to 34 (Fig. 1) revealed only two mutants for which PM localization was lost, one in which nine basic amino acids were substituted with glutamines (α_q 9Q), and one in which three basic amino acids were substituted with glutamic acids (α_q 3En). Here, our goal was to determine the role of the polybasic motif in the signaling of α_q and to determine whether signaling defects correlated with defects in PM localization. Thus, inositol phosphate assays were performed (Fig. 2). HEK293 cells were transfected with wild-type or mutant α_q constructs along with α_{2A} AR. The α_{2A} AR 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_{2A} AR did not cause an increase in inositol phosphates, either in the absence or presence of the α_{2A} AR agonist UK14304. However, when α_{2A} AR was coexpressed with wild-type α_q , agonist-dependent inositol phosphate stimulation was observed. First, we examined four polybasic mutants that properly localized at the PM (Crouthamel et al., 2008). α_q 3Q (mutations K33Q, R37Q, R38Q), α_q 2Q (mutations R19Q, R20Q), α_q 3Qn (mutations K16Q, R19Q, R20Q), and α_q 2E (mutations R19E, R20E) were able to promote α_{2A} AR agonist-dependent increases in inositol phosphate levels (Fig. 2, A and B), as expected from their retention of PM localization (Crouthamel et al., 2008). α_q 9Q (mutations R19Q, R20Q, R27Q, R30Q, R31Q, K33Q, R34Q, R37Q, R38Q) and α_q 3En (mutations K16E, R19E, R20E), two polybasic mutants that were defective in PM localization (Crouthamel et al., 2008), completely lacked the ability to couple α_{2A} AR to inositol phosphate production (Fig. 2, A and B). This lack of signaling was expected and in agreement with our previous results showing that α_q mutants that lacked PM localization also lacked signaling function (Wedegaertner et al., 1993; Evanko et al., 2000, 2005). We were surprised to identify a third set of α_q N-terminal polybasic mutants (Fig. 1) that lost

signaling function even though they retained PM localization. α_q 4Q (mutations R27Q, R30Q, R31Q, R34Q), α_q 4E (mutations R27E, R30E, R31E, R34E), and α_q 7Q (mutations R27Q, R30Q, R31Q, K33Q, R34Q, R37Q, R38Q) were unable to couple α_{2A} AR to inositol phosphate production (Fig. 2, A and 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 α_q (Crouthamel et al., 2008).

In summary, polybasic mutants of α_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 α_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 α_q Polybasic Mutants. To further examine the relationship between PM localization and signaling function of α_q polybasic mutants, we rescued

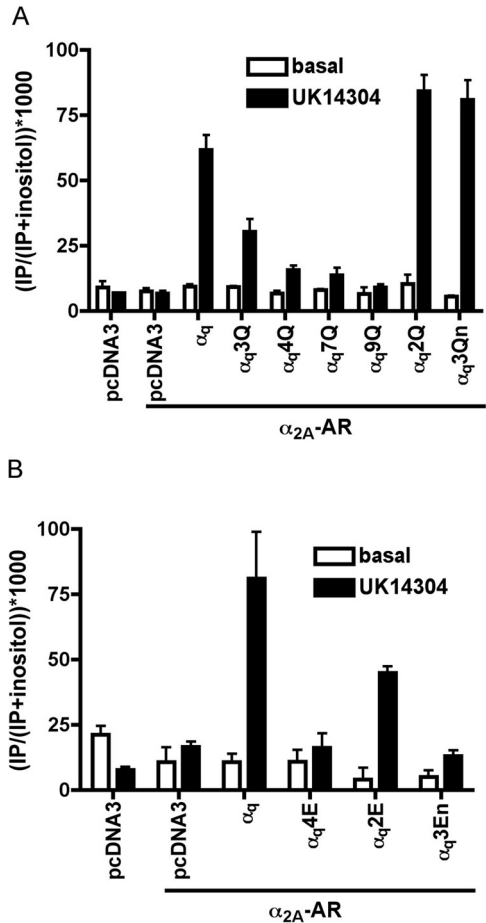


Fig. 2. Signaling by α_q polybasic mutants. HEK293 cells were transfected as indicated with either pcDNA3 alone, pcDNA3 plus α_{2A} AR-pCMV4, or pcDNA3 plus α_{2A} AR-pCMV4 and pcDNA3 encoding either wild-type α_q or the indicated glutamine mutant (A) or the indicated glutamic acid mutant (B). Twenty-four hours after transfection, cells were labeled with [3 H]inositol. Sixteen hours later cells were stimulated with 10 μ M UK14304 for 1 h at 37°C, and inositol phosphate production was assayed as described under *Materials and Methods*. The results shown are the means \pm S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

	1	5	10	15	20	25	30	35	40
α_q	MT	LE	SI	MA	CL	SE	EAK	EARR	REEL
α_q 9Q	-----	-----	-----	-----	Q	Q	-----	Q	-----
α_q 7Q	-----	-----	-----	-----	-----	-----	Q	Q	-----
α_q 4Q	-----	-----	-----	-----	-----	-----	Q	Q	-----
α_q 4E	-----	-----	-----	-----	-----	E	EE	E	-----
α_q 3Q	-----	-----	-----	-----	-----	-----	Q	Q	-----
α_q 3Qn	-----	-----	Q	-----	Q	-----	-----	-----	-----
α_q 3En	-----	-----	-----	E	EE	-----	-----	-----	-----
α_q 2Q	-----	-----	-----	-----	Q	Q	-----	-----	-----
α_q 2E	-----	-----	-----	-----	EE	-----	-----	-----	-----

Fig. 1. N-terminal mutants of α_q . Amino acids 1 to 40 of α_q are shown, and the amino acid substitutions for each α_q mutant are indicated.

PM localization of the cytoplasmic α_q9Q and α_q3En N-terminal polybasic mutants by introducing a myristoylation motif or by coexpressing $\beta_1\gamma_2$. α_q is palmitoylated at cysteines 9 and 10 (Fig. 1) but is not myristoylated because of the lack of a required glycine at position 2. However, a mutant form of α_q 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 α_q9Q and α_q3En polybasic mutants to create myr- α_q9Q and myr- α_q3En . Our recent work clearly showed that myr- α_q9Q and myr- α_q3En were strongly targeted to the PM, demonstrating that myristoylation can rescue the loss of PM binding caused by the N-terminal polybasic mutations of α_q (Crouthamel et al., 2008). In contrast, myristoylation did not recover signaling function of α_q9Q and α_q3En . Myr- α_q9Q and myr- α_q3En were unable to couple $\alpha_{2A}AR$ to inositol phosphate production (Fig. 3A).

Similar to myristoylation, we demonstrated previously that coexpression of $\beta_1\gamma_2$ can recover PM localization of α_q9Q and α_q3En (Crouthamel et al., 2008). However, α_q9Q and

α_q3En remained unable to couple $\alpha_{2A}AR$ to inositol phosphate production, even when PM localization was promoted by $\beta_1\gamma_2$ coexpression (Fig. 3B). The signaling defect in α_q9Q and α_q3En is not due simply to overall structural perturbations of the proteins; previous results demonstrated that α_q9Q and α_q3En can bind to RGS4 in an activation-dependent manner (Crouthamel et al., 2008). These results showing that recovery of PM localization of α_q9Q and α_q3En , by myristoylation or coexpression of $\beta_1\gamma_2$, fails to recover signaling further indicates that the polybasic N-terminal region of α_q plays a critical role beyond membrane targeting.

α_q4Q Is Also Defective in Signaling in Response to Stimulation of the M_3R . To attempt to understand the defect in signaling of N-terminal polybasic mutants of α_q , we further characterized the PM-localized but signaling-defective α_q4Q mutant. First, we wanted to confirm that α_q4Q is also defective in signaling in response to a GPCR that naturally couples to α_q , because, as described above, α_2AR normally couples to α_i . Thus, we examined the ability of α_q4Q to signal through endogenous M_3R , a G_q -coupled receptor that is the primary muscarinic acetylcholine receptor subtype in HEK293 cells (Luo et al., 2008). Cells were transfected with either wild-type α_q or α_q4Q , stimulated with carbachol, and inositol phosphate accumulation was assayed. Both α_q and α_q4Q expressed at similar levels (data not shown). In non-transfected cells, there was an increase in inositol phosphate production upon stimulation with carbachol as a result of activation of endogenous α_{q11} (Fig. 4A). Transfection of α_q led to an even greater increase in inositol phosphate production upon carbachol stimulation (Fig. 4A). However, when α_q4Q was transfected and the cells were stimulated with carbachol, there was no increase in inositol phosphate production over that caused by endogenous α_q (Fig. 4A). These results indicate that mutation of these four arginines causes defects in signaling through the M_3R in addition to α_2AR .

We also examined the ability of overexpressed M_3R to signal through α_q4Q . We expressed increasing concentrations of M_3R cDNA in the presence or absence of coexpression of wild-type α_q or α_q4Q . When α_q is coexpressed with M_3R , its expression is much higher compared with when it is expressed alone or with $\alpha_{2A}AR$ (data not shown). This may be due to an increased stabilization of α_q resulting from increased interaction with M_3R . On the other hand, when α_q4Q is coexpressed with M_3R , its expression levels remain similar to the levels present when it is expressed alone or with $\alpha_{2A}AR$ (data not shown). This could imply that α_q4Q may have an impaired interaction with M_3R , which could account for its defect in signaling. We therefore had to transfect higher amounts of α_q4Q cDNA compared with α_q when coexpressing M_3R to obtain similar expression levels of α_q and α_q4Q (data not shown). Transfecting increasing amounts of M_3R cDNA resulted in increasing amounts of inositol phosphate accumulation upon carbachol stimulation (Fig. 4B). Coexpressing α_q with increasing amounts of M_3R DNA led to even greater increases in inositol phosphate production upon stimulation with carbachol (Fig. 4B). Moreover, basal levels of inositol phosphate increased when α_q was overexpressed with 200 ng of M_3R DNA (Fig. 4B). On the other hand, coexpressing α_q4Q had no effect on either basal or carbachol-stimulated levels of inositol phosphate compared with when M_3R was expressed alone (Fig. 4B), even when α_q4Q was expressed at levels comparable with that of α_q , as it was in

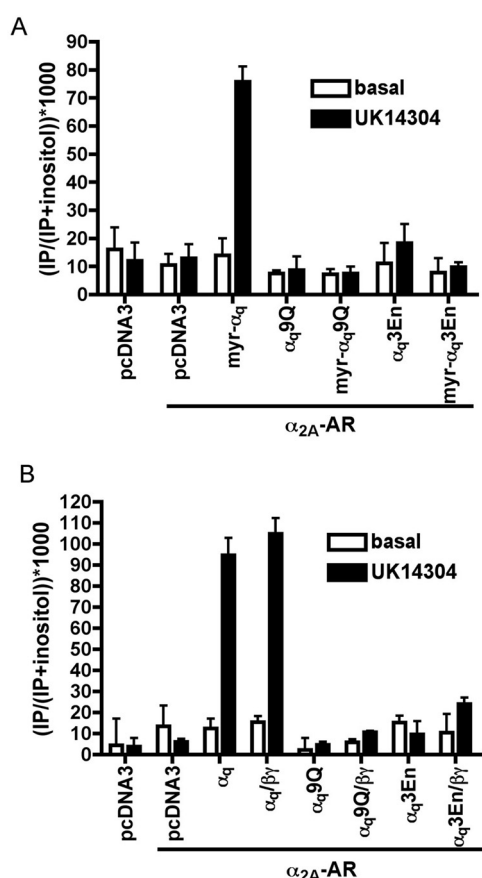


Fig. 3. Recovering PM localization does not recover signaling of α_q polybasic mutants. **A**, HEK293 cells were transfected as indicated with either pcDNA3 alone, pcDNA3 plus $\alpha_{2A}AR$ -pCMV4, or pcDNA3 plus $\alpha_{2A}AR$ -pCMV4 and pcDNA3 encoding the indicated α_q construct. **B**, HEK293 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 α_q construct. In addition, $\beta_1\gamma_2$ was coexpressed, using myc-His- β_1 -pcDNA3 and γ_2 -pcDNA3, as indicated. Twenty-four hours after transfection, cells were labeled with [3H]inositol. Sixteen hours later, cells were stimulated with 10 μM UK14304 for 1 h at 37°C, and inositol phosphate production was assayed as described under *Materials and Methods*. The results shown are the means \pm S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

Fig. 4B (data not shown). Thus α_q4Q 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 α_q -coupled receptors.

The Positive Charges of the Arginines Are Important for Signaling. α_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 (α_q4K) so that the positive charge of the side chains is preserved, and we also mutated these arginines to alanines (α_q4A), which removes not only the charge but also the long side chain. Inositol phosphate assays display that α_q4K was capable of eliciting responses from α_2AR upon stimulation, whereas α_q4A is identical with α_q4Q in that it is defective in signaling (Fig. 5A). These results suggest that it is the positive charges of these arginines that are somehow required for proper signaling, because 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 α_q4Q , we performed a more extensive mutagenesis. We created four point mutants in which each of the arginines mutated in α_q4Q were individually mutated to glutamine, namely α_qR27Q , α_qR30Q , α_qR31Q , and α_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 α_q4Q cannot be attributed to one specific arginine (Fig. 5B). Rather, the defect is due to the combined loss of all four arginines.

The Signaling Defect of α_q4Q Is Not Due to an Impaired Interaction with $G\beta\gamma$. The four arginines that are mutated in α_q4Q lie within the N-terminal $G\beta\gamma$ contact site. Thus, we asked whether the observed signaling defects could be caused by a defective interaction with $G\beta\gamma$. First, we tested its ability to interact with $G\beta\gamma$ through a nickel-NTA pull-down assay. HEK293 cells were transfected with wild-type α_q or α_q4Q with or without transient coexpression of $\beta_1\gamma_2$ in which the β_1 subunit contains a hexahistidine tag. When $\beta_1\gamma_2$ was isolated from cell lysates using nickel-NTA beads, both α_q and α_q4Q were also found in the pull down (Fig. 6A), suggesting that mutation of these basic residues does not perturb an interaction with $G\beta\gamma$. As a control, the previously characterized $G\beta\gamma$ binding-deficient mutant α_qIE did not bind $G\beta\gamma$ (Evanko et al., 2000, 2001, 2005).

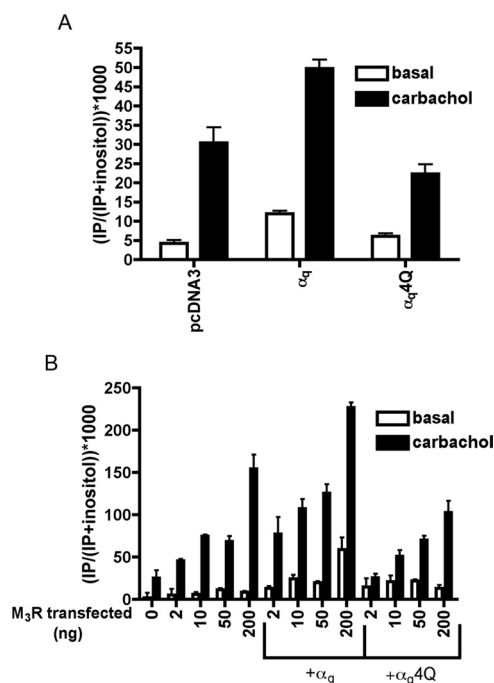


Fig. 4. α_q4Q is defective in signaling through M_3R . A, HEK293 cells were transfected with pcDNA3 or pcDNA3 plus pcDNA3 encoding the indicated α_q construct. B, HEK293 cells were transfected as indicated with either pcDNA3 or the indicated amount of pcDNA3 encoding M_3R , either alone or along with α_q -pcDNA3 or α_q4Q -pcDNA3. Higher amounts of α_q4Q were transfected to make up for expression differences between α_q and α_q4Q that occur when M_3R is coexpressed. Twenty-four hours after transfection, cells were labeled with [3H]inositol. Sixteen hours later, cells were stimulated with 1 mM carbachol for 1 h at 37°C, and inositol phosphate production was assayed as described under *Materials and Methods*. The results shown are the means \pm S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

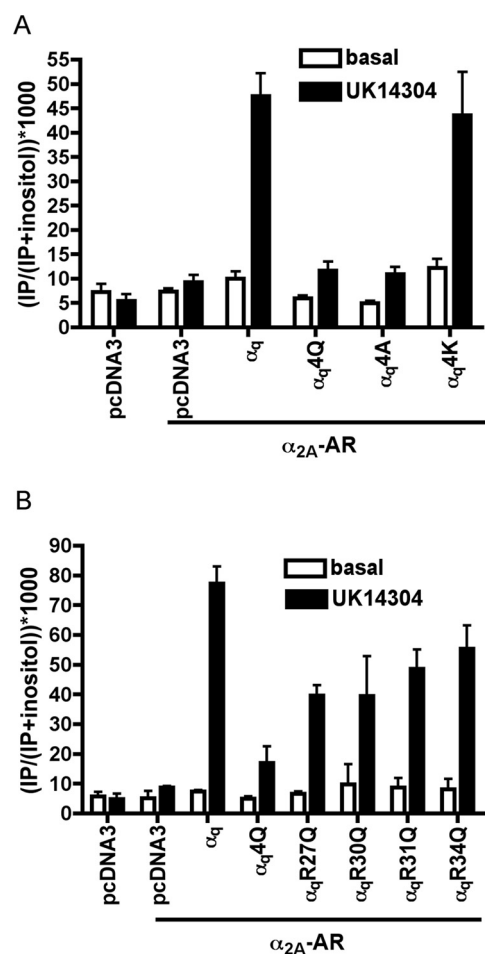


Fig. 5. The positive charge of the N terminus of α_q is important for signaling. HEK293 cells were transfected as indicated with either pcDNA3 alone, pcDNA3 plus α_2AR -pCMV4, or pcDNA3 plus α_2AR -pCMV4 and pcDNA3 encoding either wild-type α_q , α_q4Q , α_q4A , or α_q4K (A) or the indicated single-point mutant (B). Twenty-four hours after transfection, cells were labeled with [3H]inositol. Sixteen hours later, cells were stimulated with 10 μ M UK14304 for 1 h at 37°C, and inositol phosphate production was assayed as described under *Materials and Methods*. The results shown are the means \pm S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

Next, we tested whether overexpression of $\beta_1\gamma_2$ could promote signaling by α_q4Q . However, coexpression of $\beta_1\gamma_2$ could not recover signaling of α_q4Q (Fig. 6B). These results further strengthen the notion that the signaling defect of α_q4Q is not due to a defect in $G\beta\gamma$ -binding.

α_q4Q Is Capable of Undergoing Activation-Induced Conformational Changes and of Activating Effectors.

We demonstrated previously that the more extensive N-terminal polybasic mutant α_q9Q was able to interact with RGS4 in an activation-dependent manner (Crouthamel et al., 2008), and thus, we expected that α_q4Q would also interact with RGS4 and thereby demonstrate the ability of α_q4Q to undergo the typical activation-dependent conformational change that characterizes G protein α -subunits. Lysates of cells transfected with wild-type α_q or α_q4Q were incubated with GST-RGS4-conjugated beads in the presence or absence of aluminum fluoride (AlF_4^-), followed by centrifugation to isolate interacting proteins. In general, RGS proteins preferentially bind active GDP \cdot 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 α_q nor α_q4Q is pulled down with GST-RGS4 in the absence of AlF_4^- . On the other hand, both wild-type α_q and α_q4Q were efficiently pulled down with GST-RGS4 in the presence of AlF_4^- (Fig. 7A), indicating that mutation of these four basic residues does not affect the ability of α_q to undergo activation-induced conformational changes.

The fact that α_q4Q is capable of undergoing activation-induced conformational changes (Fig. 7A) combined with a lack of evidence for $G\alpha$ N termini as sites of effector interaction suggests that α_q4Q would be capable of effector activation. To test this, we generated a constitutively active form of α_q4Q by introducing the glutamine 209 to leucine (QL) mutation into α_q4Q (α_q4Q QL). This mutation inhibits the GTPase activity of α_q and thus renders it capable of stimulating the effector PLC- β in the absence of receptor activation. When α_q QL was transfected alone into HEK293 cells,

there was a large increase in inositol phosphate levels compared with those of cells transfected with an empty vector or with wild-type α_q (Fig. 7B). The expression of α_q4Q QL caused a similarly large increase in constitutive inositol phosphate production. Thus, the above results indicate that the signaling defect of α_q4Q cannot be ascribed to an inability to assume an active conformation or to an inability to interact with PLC- β .

α_q4Q /GPCR Coupling in Sf9 Membranes. So far, the results suggest that α_q4Q localizes to the PM, can bind $G\beta\gamma$, can undergo activation-dependent conformational changes, and can interact with the effector PLC- β ; thus, none of these factors seem to explain the defect in α_q4Q signaling. On the other hand, studies with the $\alpha_{2A}AR$ and M_3R are consistent with the idea that α_q4Q is defective in coupling to GPCRs (Figs. 2, 4, and 5). To examine this proposal more directly, we tested GPCR coupling in isolated membranes using a well characterized [^{35}S]GTP γ S binding assay with Sf9 insect cell membranes (Windh and Manning, 2002). Sf9 cells were infected with baculoviruses to express the G_q -coupled thromboxane A_2 receptor TP α , the $G\beta_1$ subunit, the $G\gamma_2$ subunit, and either wild-type α_q or α_q4Q . Membranes were isolated from infected Sf9 cells, and [^{35}S]GTP γ S binding to α_q or α_q4Q was determined in the absence or presence of the TP α agonist U46619. A 3- to 4-fold increase in [^{35}S]GTP γ S binding to α_q was observed upon U46619 activation of TP α (Fig. 8A). When TP α was expressed alone, only a small increase in [^{35}S]GTP γ S binding was observed, consistent with Sf9 cells having relatively low levels of endogenous G proteins. Surprisingly, α_q4Q also displayed a 3- to 4-fold increase in TP α -stimulated [^{35}S]GTP γ S binding (Fig. 8A), similar to wild-type α_q . When various concentrations of the agonist were used to stimulate membranes, and the amounts of bound [^{35}S]GTP γ S were fitted to dose-response curves, the EC_{50} values for α_q and for that of α_q4Q were similar (Fig. 8B). These results indicate that α_q4Q is able to couple to GPCRs; mutation of these four arginines does not affect the ability of α_q to couple to its cognate GPCRs. This is in apparent contrast to the inability of α_q4Q to mediate GPCR signaling in

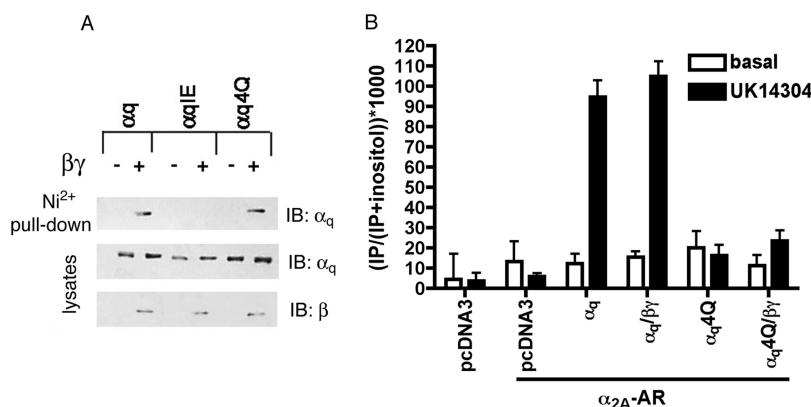


Fig. 6. The signaling defect of α_q4Q is not due to impaired interaction with $G\beta\gamma$. **A**, HEK293 cells were transiently transfected in 6-cm plates with pcDNA3 encoding the indicated α_q construct alone or with pcDNA3 encoding the indicated α_q construct plus MycHis- β_1 -pcDNA3 and γ_2 -pcDNA3. Approximately 40 h after transfection, cell lysates were prepared and incubated with nickel-NTA magnetic agarose beads to precipitate MycHis $\beta_1\gamma_2$. Beads were washed, and MycHis $\beta_1\gamma_2$ was eluted as described under *Materials and Methods*. 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_q or α_q mutants (top) or the anti-myc antibody 9E10 to detect expressed β_1 (bottom). **B**, HEK293 cells were transfected with either pcDNA3 alone, pcDNA3 plus $\alpha_{2A}AR$ -pCMV4, or pcDNA3 plus $\alpha_{2A}AR$ -pCMV4 and α_q -pcDNA3 or α_q4Q -pcDNA3. In addition, $\beta_1\gamma_2$ was coexpressed, using mycHis- β_1 -pcDNA3 and γ_2 -pcDNA3, as indicated. Twenty-four hours after transfection, cells were labeled with [3H]inositol. Sixteen hours later, cells were stimulated with 10 μ M UK14304 for 1 h at 37°C, and inositol phosphate production was assayed as described under *Materials and Methods*. The results shown are the means \pm S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

transfected cells; however, [35 S]GTP γ S binding using membranes directly measures α_q4Q activation by a GPCR, whereas cell-based inositol phosphate signaling is an indirect measure of productive GPCR-G α -effector function.

α_q and α_q4Q Localize to Different PM Nanodomains. Although both α_q and α_q4Q 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 α_q and α_q4Q 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 and Vogel, 2007; Abankwa et al., 2008a,b) to examine the ability of the N termini of α_q and α_q4Q to elicit FRET signals with different membrane nanodomain markers (Fig. 9A). Constructs were made in which the N-terminal 41 amino acids of either α_q or α_q4Q were fused to the N terminus of a monomeric yellow fluorescent protein (mCit) with the C-terminal 11 amino acids of γ_2 fused to the C terminus of mCit (N_qC_{γ} -mCit). N_qC_{γ} -mCit was recently constructed and used in a study comparing nanodomain localization of PM targeting motifs of α_q and α_i (Abankwa and Vogel, 2007). Here, we used the nanodomain markers described previously (Fig. 9C) (Abankwa and Vogel, 2007). In addition, FRET signals between N_qC_{γ} -mCit and a similar construct containing the N

terminus of growth-associated protein 43 and the C terminus of $G\gamma_2$ fused to mCFP ($N_{GAP43}C_{\gamma}$ -mCFP) were examined, as well as FRET signals between mCFP fused only to the N terminus of α_{i2} (N_{i2} -mCFP) or the N terminus of GAP43 (N_{GAP43} -mCFP). The mutations in α_q4Q were introduced into N_qC_{γ} -mCit ($N_{q4Q}C_{\gamma}$ -mCit), and its nanodomain localization was compared with that of N_qC_{γ} -mCit. $N_{q4Q}C_{\gamma}$ -mCit was found to be properly localized at the PM through confocal microscopy, similar to full-length α_q4Q (Fig. 9B). It is noteworthy that $N_{q4Q}C_{\gamma}$ -mCit displayed significantly lower FRET values with all of the nanodomain markers compared with α_q with the exception of the tR marker, indicating that there is an altered nanoscale distribution of $N_{q4Q}C_{\gamma}$ -mCit in different membrane nanodomains (Fig. 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 and Vogel, 2007; Abankwa et al., 2008b). These results indicate that these basic residues in the N terminus of α_q are involved in properly localizing the protein to PM

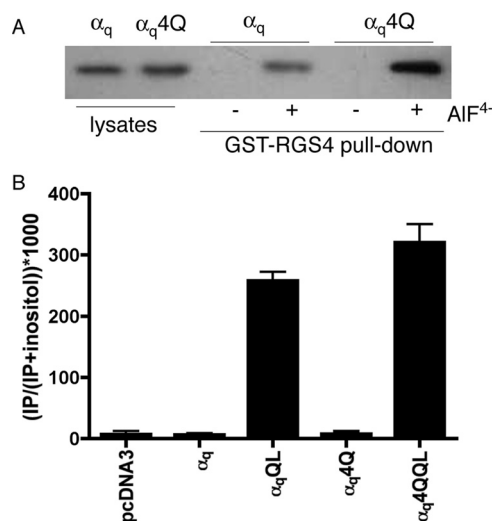


Fig. 7. α_q4Q displays activation-dependent interaction with RGS4 and constitutively activating mutation-dependent stimulation of inositol phosphates. A, HEK293 cells were transfected in 6-cm plates with the indicated α_q construct. Twenty-four hours after transfection, cell lysates were prepared and incubated in the presence or absence of AIF $_4^-$ for 2 h at 4°C with GST-RGS4, purified as described under *Materials and Methods*. 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 α_q and α_q4Q were visualized by immunoblotting with the 12CA5 antibody. B, HEK293 cells were transfected in six-well plates with either pcDNA3 alone or pcDNA3 plus pcDNA3 encoding the indicated α_q mutant. Twenty-four hours after transfection, cells were labeled with [3 H]inositol. Sixteen hours later, inositol phosphate production was assayed in the absence of any agonist stimulation as described under *Materials and Methods*. The results shown are the means \pm S.D. from a representative experiment assayed in triplicate. Similar results were obtained in at least two additional experiments.

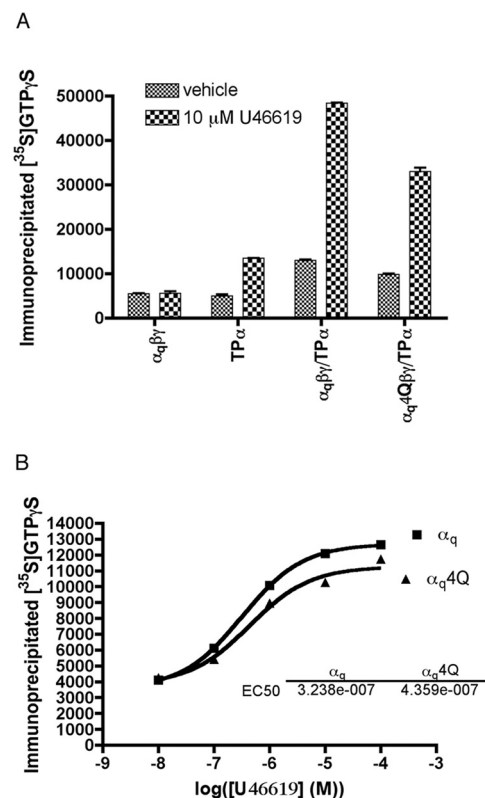


Fig. 8. α_q4Q is capable of coupling to receptors. A, Sf9 cells were infected with baculoviruses expressing TP α , β_1 , γ_2 , and with or without a baculovirus expressing α_q or α_q4Q at an MOI of 1. Approximately 40 h after transfection, membranes from infected cells were isolated as described under *Materials and Methods*. Membranes were then stimulated with the indicated concentration of U46619 for 5 min at 30°C in the presence of [35 S]GTP γ S. α_q or α_q4Q was immunoprecipitated as described under *Materials and Methods*, and the amount of bound [35 S]GTP γ S was measured by scintillation spectrometry. B, Sf9 cells were infected with baculoviruses expressing TP α , β_1 , γ_2 , and either α_q or α_q4Q at an MOI of 1. Membranes isolated from these infected cells were stimulated with the indicated concentration of U46619 for 5 min at 30°C in the presence of [35 S]GTP γ S. α_q was immunoprecipitated as described under *Materials and Methods*, and the amount of bound [35 S]GTP γ S was measured by scintillation spectrometry. Dose-response curves were plotted using Prism software (GraphPad Software Inc., San Diego, CA).

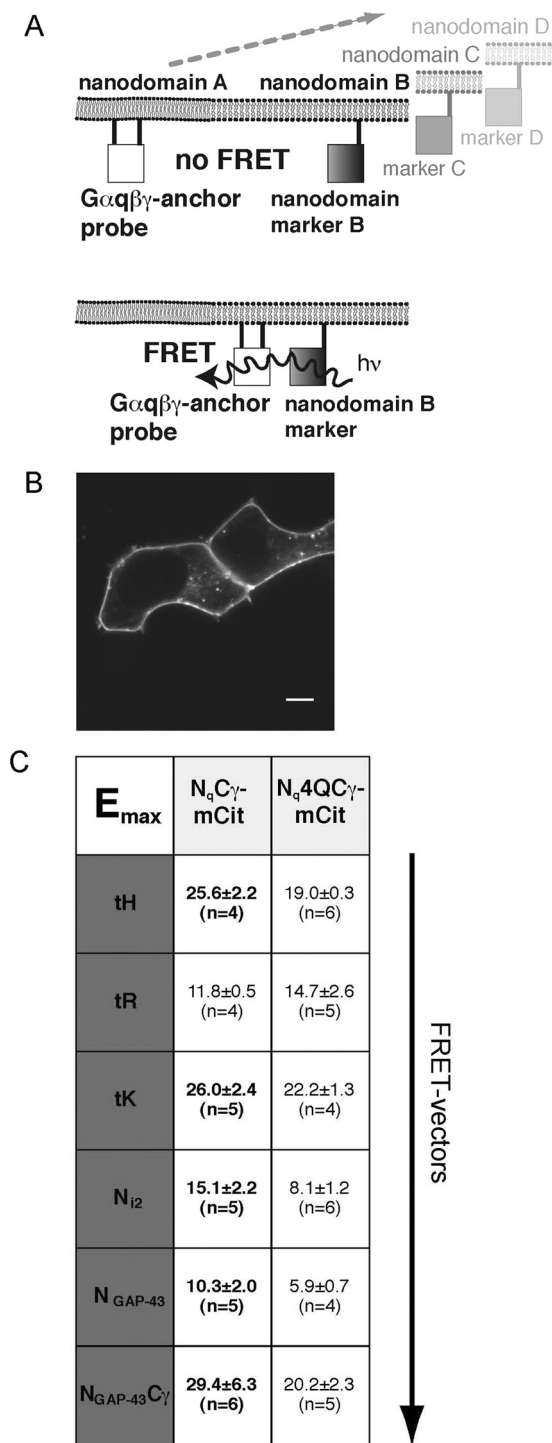


Fig. 9. Localization of α_q in PM nanodomains. **A**, FRET vectors are determined by evaluating the coclustering of a heterotrimeric α_q -anchor probe with a given nanodomain marker. **B**, $N_q4QC\gamma$ -mCit localized predominantly at the plasma membrane of BHK cells, as determined by confocal microscopy. **C**, lateral segregation FRET vectors of heterotrimer mimicking constructs $N_qC\gamma$ -mCit and the mutant $N_q4QC\gamma$ -mCit are significantly different. Lateral segregation FRET vectors are a set of E_{\max} values, which reflect coclustering of a probing membrane-anchored construct and a nanodomain marker. Probes and nanodomain markers were pair-wise coexpressed in BHK cells. E_{\max} values are shown \pm S.E.M. from a number of independent experiments, n . Differences between boldface and lightface E_{\max} values in one column (i.e., for each nanodomain marker) are significantly different (Student's t test). 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.

nanodomains. This mislocalization may contribute to the defect in signaling of α_q4Q .

Discussion

In summary, the work presented here shows the novel finding that the polybasic region in the N terminus of α_q is required for proper signaling. Previous work indicated that basic residues in the N terminus of α_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 α_q N-terminal polybasic region. First, two PM localization-defective polybasic mutants, α_q9Q and α_q3En , were unable to couple an activated GPCR to PLC- β -stimulated production of inositol phosphates. This lack of signaling function was true even when α_q9Q and α_q3En were directed to the PM through the introduction of myristoylation or by coexpression of $G\beta\gamma$, indicating a role for the N-terminal polybasic region beyond simple localization to the PM. Second, we identified α_q N-terminal polybasic mutants, such as α_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 α_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 α_q4Q mutant (positions 27, 30, 31, and 34) to lysines (α_q4K) did not disrupt signaling, whereas mutation to glutamines (α_q4Q) or alanines (α_q4A) did. Finally, we show that the α_q basic residues are critical for plasma membrane nanodomain localization; α_q and α_q4Q show differing localization to nanodomains, suggesting one possible mechanism whereby N-terminal basic residues of α_q could affect signaling function.

Previous work has implicated the N terminus of various $G\alpha$ subunits in interacting with GPCRs. Mutagenesis, cross-linking studies, and peptide competition studies are all consistent with the proposal that the $G\alpha$ N terminus is an important point of contact between a heterotrimeric G protein and a GPCR (Hamm et al., 1988; Taylor et al., 1994; Kostenis et al., 1997, 1998; Onrust et al., 1997; Itoh et al., 2001), although in some of the previous studies, interpretations regarding the importance of the $G\alpha$ N terminus for functional GPCR coupling are complicated by the knowledge that the $G\alpha$ N terminus is essential for direct binding to $G\beta$. Here, however, the mutations in α_q4Q do not disrupt binding to $G\beta\gamma$. 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 $G\beta$ (Crouthamel et al., 2008), and α_q4Q interacts with $G\beta\gamma$ in pull-down experiments (Fig. 6A). Thus, we hypothesized that α_q4Q was defective in GPCR interaction based on its inability to promote receptor-dependent inositol phosphate production in transfected cells (Figs. 2–4). Surprisingly, however, α_q4Q displayed no defect compared with wild-type α_q in receptor-dependent GTP γ S binding, as measured in isolated membranes. These latter GTP γ S binding studies indicate that α_q4Q does not have a general defect in GPCR interaction. The mechanistic reason for the inability of α_q4Q to couple activated GPCRs to the stimulation of PLC- β remains unclear. Although our results imply that α_q4Q is generally

defective in coupling activated GPCRs to productive signaling in cells, it will be important to test additional G_q -coupled receptors to test for specificity.

The defect in α_q4Q signaling does not seem to lie in an inherent inability to undergo an activating conformation or to productively interact with PLC- β . α_q4Q binds RGS4 in the presence of AlF_4^- , indicating that it is capable of activation-induced conformational changes (Fig. 7A) and therefore probably capable of effector interaction. Moreover, α_q4Q containing the constitutively activating Q209L mutation (α_q4Q QL) stimulated inositol production when expressed in cells (Fig. 7B), supporting the notion that α_q4Q is able to interact with PLC- β . Thus, our results suggest that the signaling defect of α_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- β .

On the other hand, a FRET analysis of PM nanodomain localization of α_q4Q -heterotrimer ($N_{q4Q}C_{\gamma}$ -mCit) indicated differences in nanodomain localization compared with wild-type α_q -heterotrimer (N_qC_{γ} -mCit) (Fig. 9). These nanoscale localization changes could reduce the probability of α_q4Q encountering certain GPCRs or other proteins, such as effectors or RGS proteins, and thus changes in nanodomain localization could impair the ability of α_q4Q to form productive signaling complexes inside cells. The results in Fig. 9 imply that residues in this basic cluster in the N terminus of α_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 (Prior et al., 2003; Abankwa and Vogel, 2007; Abankwa et al., 2008b). Indeed, FRET analysis demonstrates differing lateral segregation of different Ras isoforms (Abankwa and Vogel, 2007; Abankwa et al., 2008b). 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 phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate, which have been shown to be concentrated in lipid rafts in some cell types (Heo et al., 2006). Because phosphatidylinositol 4,5-bisphosphate is the substrate for the α_q effector PLC- β , targeting of α_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 α_q4Q mutant (Fig. 9). Thus, it is tempting to speculate that differences in nanodomain localization of α_q - versus α_q4Q -heterotrimers may contribute to the observed signaling differences. Nonetheless, we cannot completely rule out alternative mechanisms, such as reduced affinity of α_q4Q 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 α_q regulates nanolocalization and signaling function.

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