Atypical activation of the G protein $\text{G}_q$ by the oncogenic mutation Q209P

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The causative role of G-protein–coupled receptor (GPCR) pathway mutations in uveal melanoma (UM) has been well-established. Nearly all UMs bear an activating mutation in a GPCR pathway mediated by G proteins of the $\text{G}_{q/11}$ family, driving tumor initiation and possibly metastatic progression. Thus, targeting this pathway holds therapeutic promise for managing UM. However, direct targeting of oncogenic $\text{G}_{q/11}$ mutants, present in ~90% of UMs, is complicated by the belief that these mutants structurally resemble active $\text{G}_{q/11}$ WT. This notion is solidly founded on previous studies characterizing G proteins in which a conserved catalytic glutamine (Gln-209 in $\text{G}_q$) is replaced by leucine, which leads to GTPase function deficiency and constitutive activation. Whereas Q209L accounts for approximately half of GNAQ mutations in UM, Q209P is as frequent as Q209L and also promotes oncogenesis, but has not been characterized at the molecular level. Here, we characterized the biochemical and signaling properties of $\text{G}_q$, Q209P and found that it is also GTPase-deficient and activates downstream signaling as efficiently as $\text{G}_q$, Q209L. However, $\text{G}_q$, Q209P had distinct molecular and functional features, including in the switch II region of $\text{G}_q$, Q209P, which adopted a conformation different from that of $\text{G}_q$, Q209L or active WT $\text{G}_q$, resulting in altered binding to effectors, $\text{G}_q\gamma$, and regulators of G-protein signaling (RGS) proteins. Our findings reveal that the molecular properties of $\text{G}_q$, Q209P are fundamentally different from those in other active $\text{G}_q$ proteins and could be leveraged as a specific vulnerability for the ~20% of UMs bearing this mutation.

Uveal melanoma (UM) is the second most frequent melanoma (after cutaneous melanoma). Despite progress in monitoring and detection, survival rates have not improved over the past few decades (1, 2). Regardless of tumor classification, UMs are treated by eye enucleation, plaque radiation, or tumor resection, which result in loss of vision (3, 4). For the ~50% of UMs that are metastatic (5, 6), these treatments do not reduce the probability of mortality. Patients with metastatic UM have a grim median survival of less than 1 year (15% 5-year survival) (7–9). UM is largely insensitive to chemotherapeutics or immune checkpoint inhibitors that have shown efficacy in cutaneous melanomas (7, 9). This is easily explained by the fact that the oncogenic drivers of UM are different from those of cutaneous melanoma (i.e., ~90% of UMs are caused by activating mutations in $\text{G}_{q}$ or $\text{G}_{11}$, the genes encoding the G proteins $\text{G}_q$ and $\text{G}_{11}$, and not by mutations in $\text{BRAF}$ (10–14).

Heterotrimeric G proteins, such as $\text{G}_q$ and $\text{G}_{11}$, are composed of a nucleotide-binding $\text{G}_\alpha$ subunit and an obligatory $\text{G}_\beta\gamma$ heterodimer, which form a tight complex in the GDP-bound resting state (15). Upon ligand binding, GPCRs promote their activation by accelerating the exchange of GDP for GTP. In turn, $\text{G}_\alpha$-GTP dissociates from $\text{G}_\beta\gamma$, allowing them both to regulate numerous downstream effectors. Cancer-associated mutations in $\text{G}_q$ and $\text{G}_{11}$ affect residue Gln-209 in ~95% of the cases, whereas mutations in Arg-183 are less frequent (11, 12). These residues are critical for the intrinsic GTPase activity of $\text{G}_q$, and their mutation is known to result in a constitutively active protein (16–18). Active $\text{G}_{q/11}$ can engage with multiple downstream effectors, which trigger various signaling pathways implicated in cell growth and oncogenic behavior. For instance, active $\text{G}_q$ binds to some PLCβ isozymes, which ultimately promotes activation of the MAPK/ERK pathway via Ca$^{2+}$/diacylglycerol/protein kinase C (19–21). Another major $\text{G}_q$-dependent mechanism relies on its direct binding and activation of a subfamily of RhoGEFs (22, 23), such as Trio, which ultimately activate TAZ/YAP to promote oncogenic transformation (24, 25). Recent evidence indicates that nearly all UMs bear one mutation within this GPCR-driven pathway.
Atypical properties of GNAQ Q209P

In addition to mutations in GNAQ or GNA11 found in ~90% of the cases, there are also mutually exclusive mutations in CYSLTR2 (encoding the GPCR cysteinyl leukotriene receptor 2, CysLT2R) (27) and PLCB4 (encoding the G protein effector PLCb4) (28), which operate directly upstream or downstream, respectively, of Gαqq11. Interestingly, a similar pattern of mutually exclusive mutations in GNAQ, GNA11, CYSLTR2, and PLCB4 has been reported to occur in leptomeningeal melanocytic tumors (29–31), another type of noncutaneous melanoma that afflicts the central nervous system.

Given the insensitivity of UM to therapies used for other types of melanoma, targeting the signaling mechanisms triggered by GαqQ209P has been in the limelight for the development of novel therapeutics for this type of cancer (32). Their suitability as targets is supported by many lines of evidence. For example, expression of the active G protein mutants in nontransformed cells is oncogenic (11, 12, 33). Similarly, mouse models in which activated Gαq or Gα11 are expressed in melanocytes develop metastatic UM (34, 35). Importantly from the standpoint of therapeutic targeting, genetic disruption of mutant GαqQ209P or downstream signaling effectors in UM cells impairs proliferation and/or tumor growth in mice (24, 25, 36–38). Unfortunately, attempts to pharmacologically target signaling pathways activated downstream of mutant GαqQ209P have not been successful, even when using multiple drugs in combination (7). A likely explanation for the inefficiency of these approaches in blunting UM is that GαqQ209P activates a complex network of signaling effectors (14), such that targeting individual nodes of this network is insufficient to achieve therapeutic effects. Thus, direct inhibition of mutant GαqQ209P may be required to completely inhibit all the network components required to promote UM and achieve sufficient efficacy and therapeutic benefit.

Direct targeting of oncogenic GαqQ111 mutants is a reasonable therapeutic approach, although it presents challenges, as these mutants are predicted to closely resemble active GαqQ111 WT. If so, strategies to inhibit mutant GαqQ111 would be expected to also cause inhibition of GαqQ111 WT, which could result in undesired side effects related to the major physiological functions of these G proteins (e.g. double GαqQ/Gα11 knockout mice are nonviable (39)). Here, we present evidence that one of the most frequent Gαqq mutants in UM, Q209P, displays properties different from that of active Gαq WT that could be leveraged to achieve specific targeting at the molecular level. Approximately 40–45% of UM have mutations in residue Glu-209 of Gαq, which are split evenly between Q209L and Q209P (11–13). Whereas Gαq Q209L has been extensively characterized and used as a tool to study Gαq signaling for decades, Gαq Q209P has not been adequately studied. Gαq Q209P is not only as frequent as Gαq Q209L in tumors, but it is also the driver mutation of many of the UM cell lines commonly used for cell biological and pharmacological experimentation, such as Mel270, OMM1.3 (also known as OMM2.3), OMM2.2, OMM2.5, and UPMM3 (25, 40). In fact, it has been shown that depletion of Gαq Q209P and/or signaling components downstream of it from UM cells decreases proliferation and/or tumor growth (24, 36, 37). Motivated by the fundamental gap in knowledge about the molecular properties of Gαq Q209P, we characterized this mutant by direct comparison with Gαq Q209L, to discover that while leading to signaling hyperactivation, as expected, it possessed structural features different from other active Gαq species, including active Gαq WT and the Gαq Q209L mutant. From a broader perspective, our findings reveal novel mechanistic insights into how G protein mutants lead to oncogenesis and into their possible suitability as direct targets for pharmacological intervention.

Results and discussion

Binding of signaling effectors to Gαq Q209P is weaker than to Gαq Q209L or GTP-bound Gαq WT

To start characterizing the properties of Gαq Q209P, we compared its ability to bind effectors with that of Gαq Q209L or active Gαq WT. First, we investigated binding to GRK2. GRK2 was the first protein co-crystallized with active Gαq (41). Based on the atomic resolution structure of this complex, it was proposed, and subsequently confirmed, that GRK2 binding to Gαq has effector-like properties. We expressed Gαq WT, Gαq Q209L, and Gαq Q209P in HEK293T cells and carried out pull-downs with the RGS homology (RH) domain of GRK2 (aa 45–178) fused to GST. Cell lysis and pull-downs were carried out rapidly (~1.5 h from lysis to protein complex elution) at 4 °C in a buffer without Mg2+ supplemented with EDTA to minimize GTP hydrolysis during the assay. As a positive control, we also included a condition in which lysates of cells expressing Gαq WT were supplemented with MgAlF4−, which mimics the GTP-bound transition state of the G protein that binds with high affinity to effectors. As expected, Gαq WT loaded with MgAlF4− bound robustly to GRK2, whereas Gαq WT in the absence of MgAlF4−, presumably in its GDP-bound inactive conformation, did not (Fig. 1A). Also, as expected, Gαq Q209L bound to GRK2 as much as Gαq WT supplemented with MgAlF4−, which is consistent with its predicted constitutive GTP-bound status. Surprisingly, Gαq Q209P binding to GRK2 was weaker than Gαq Q209L or active Gαq WT, although consistently stronger than Gαq WT in the absence of MgAlF4− (Fig. 1A).

Prompted by these surprising results, we set out to investigate the binding properties of Gαq Q209P with other Gαq effectors. Gαq works predominantly on two classes of effectors: a subfamily of PLC isoforms (PLCBs), and a subfamily of Rho-GEFs composed of p63RhoGEF (also known as ARHGEF25), Trio (also known as ARHGEF23), and kalirin (also known as ARHGEF24). Both PLCβ3 and p63RhoGEF have been co-crystallized with Gαq (42–44). The atomic resolution structures of these complexes reveal similarities with the GRK2–Gαq complex. More specifically, one of the primary contacts of GRK2, p63RhoGEF, and PLCβ3 with Gαq is mediated by a helix-turn-helix element that docks onto a conserved pocket between the α3 helix and switch II (SwII) region of the G protein (Fig. 1B). Consistent with these structural similarities, we found that the binding pattern of p63RhoGEF to different Gαq species closely resembled that of GRK2 (i.e. Gαq Q209P binding to p63RhoGEF (DH/PHext domain (45), aa 155–493) was weaker than that of Gαq Q209L or MgAlF4−-loaded Gαq WT, although stronger than Gαq WT in the absence of MgAlF4−) (Fig. 1C). Similar results were obtained in pull-downs with the other two
members of the p63RhoGEF subfamily, Trio and kalirin (Fig. 1, D and E), and in co-immunoprecipitations with PLCβ3 (Fig. 1F). The defect in Gαq Q209P binding to effectors was not rescued by the addition of Mg-AlF4 (Fig. S1), suggesting that the diminished binding is not because the G protein is in a GDP-bound state.
Atypical properties of GNAQ Q209P

To rule out that the weaker binding observed for \( \alpha_q \) Q209P was due to the experimental conditions of our biochemical assays in vitro, we carried out bioluminescence resonance energy transfer (BRET) experiments in live cells to monitor the interaction between GRK2 and \( \alpha_q \) (Fig. 1G). Briefly, the same domain of GRK2 used in the pulldowns above (RH domain) was fused to nanoluciferase to generate a BRET donor molecule (GRK2\(^{RH}\)-Nluc). The BRET acceptor consisted of a previously validated \( \alpha_q \) construct internally tagged with Venus (\( \alpha_q^{\text{V}} \)) (46). When co-expressed with GRK2\(^{RH}\)-Nluc in HEK293T cells, \( \alpha_q^{\text{V}} \) Q209L led to a higher BRET signal than \( \alpha_q^{\text{V}} \) WT, whereas \( \alpha_q^{\text{V}} \) Q209P did not (Fig. 1G). This result confirms that \( \alpha_q \) Q209P binding to GRK2 is weaker than Q209L in cells, much like what is observed in the in vitro pulldown experiments. In contrast to the results in pulldown experiments, the BRET assay failed to detect increased binding of \( \alpha_q \) Q209P compared with \( \alpha_q \) WT (Fig. 1G). A likely explanation for this is that nonspecific BRET due to random collision of donor and acceptor molecules restricted to the plane of a lipid membrane masks small signal increases due to weak interactions. Taken together, these findings demonstrate that the effector-binding properties of \( \alpha_q \) Q209P are different from those of other active \( \alpha_q \) proteins, showing diminished binding compared with GTP-bound \( \alpha_q \) WT or the constitutively active mutant Q209L.

\( \alpha_q^{\text{Q209P}} \) activates downstream signaling in cells as efficiently as \( \alpha_q^{\text{Q209L}} \)

The findings above are puzzling because previous evidence indicates that \( \alpha_q \)-dependent signaling in UM cells bearing the Q209P mutation is required to drive cell proliferation and/or tumor growth in mice. More specifically, it has been demonstrated that genetic disruption of \( \alpha_q \) of its signaling effectors like the RhoGEF Trio, or of other downstream components of the signaling cascade, such as YAP or RasGRP3, diminishes the proliferation and/or growth of tumor xenografts of UM cells with Q209P mutation, such as OMM1.3 (also known as OMM2.3) or Mel270 (24, 36, 37). These previous findings argue strongly that the \( \alpha_q^{\text{Q209P}} \) mutant is hyperactive and promotes oncogenic signaling in UM cancer cells.

To further substantiate this point, we investigated the ability of \( \alpha_q^{\text{Q209P}} \) to activate signaling in cells by comparing it side by side with the more thoroughly characterized \( \alpha_q^{\text{Q209L}} \) mutant. First, we looked at MAPK/ERK activation, which occurs downstream of \( \text{G}_{\text{q}} \)-mediated activation of PLC. We found that expression of \( \alpha_q^{\text{Q209L}} \) or \( \alpha_q^{\text{Q209P}} \) in HEK293T cells led to similar increases in phospho-ERK1/2 compared with cells expressing \( \alpha_q^{\text{WT}} \) or a vector control (Fig. 2A). Next, we investigated whether \( \alpha_q^{\text{Q209P}} \) was also capable of activating a different \( \text{G}_{\text{q}} \)-mediated pathway triggered by activation of RhoGEFs, as it has been previously shown that UM cells bearing the Q209P mutation also rely on this pathway to maintain their oncogenic properties (24, 37). For this, we used a luminescent reporter based on the serum response element (SRE) that monitors RhoGEF-dependent activity downstream of active \( \text{G}_{\text{q}} \) (42). We found that both \( \alpha_q^{\text{Q209L}} \) and \( \alpha_q^{\text{Q209P}} \) led to a robust and similar increase of SRE reporter activity compared with \( \alpha_q^{\text{WT}} \) or a vector control when expressed in HEK293T cells (Fig. 2B). One possible explanation for this result is that the signaling response is saturated at high levels of active \( \alpha_q \) expression, thereby masking possible differences between the two mutants. However, we found that this is not the case because expression of lower amounts of \( \alpha_q^{\text{Q209L}} \) and \( \alpha_q^{\text{Q209P}} \) led to similar levels of SRE reporter activation. HEK293T cells were co-transfected with HA-\( \alpha_q^{\text{WT}} \) (WT or mutants, as indicated) and a firefly luciferase reporter driven by the SRE-L promoter and cultured overnight in medium with a low concentration of serum (0.5% FBS) before measuring luminescence. Results are mean ± S.E. (error bars) \((n = 4)\).

\( \alpha_q^{\text{Q209P}} \) has impaired GTPase activity in vitro like \( \alpha_q^{\text{Q209L}} \)

To start dissecting the possible causes of the apparent discrepancy between signaling activity (Fig. 2, A and B) and effector binding (Fig. 1) observed when comparing the Q209P and Q209L mutants, we investigated their ability to hydrolyze nucleotides. Gln-209 in \( \alpha_q \) corresponds to a residue with a conserved function in G protein-mediated GTP hydrolysis, and the oncogenicity of mutations at this residue is generally

\( \text{SRE activation} \)

\( \beta/\gamma \)

\( \text{ERK activation} \)

\( \text{IB:} \)

\( \text{pERK1/2} \)

\( \text{total ERK1/2} \)

\( \text{Tubulin} \)

\( \text{WT} \)

\( \text{Q209L} \)

\( \text{Q209P} \)
Atypical properties of GNAQ Q209P

Ascribed to constitutive G protein activity due to GTPase deficiency (16, 17). Whereas prior research indicates that Goq Q209L is GTPase-deficient, the GTPase function of Goq Q209P has not been studied before. To directly investigate GTPase activity in vitro, we purified a novel Goq variant, hereafter named Goq*, that expresses well in Escherichia coli. Goq* is a Goq/Goq chimera in which some residues of Goq not involved in interacting with Gq-specific binding partners or in nucleotide binding/hydrolysis have been replaced by the corresponding residues in Goq WT (Fig. S3A). His-tagged Goq* purified from E. coli binds GTPγS with a level of nucleotide occupancy similar to that previously reported for Goq purified from insect cells (47) and also binds robustly to several known Goq binding partners, such as effectors p63RhoGEF and GRK2. The GEF/chaperone Ric-8A, or the GAP GAIP (also known as RGS19), with the expected activation state dependence (Fig. S3). These results validate that His-Goq* recapitulates many of the properties of Goq WT. Next, we compared the GTPase activity of Goq* Q209P with that of Goq* WT and Goq* Q209L. Because the slow rate of nucleotide exchange of Goq precludes the efficient loading of nucleotide required to carry out GTPase assays under single-turnover conditions (48), we investigated the GTPase activity under multiple-turnover conditions (i.e. at steady state). As expected, Goq* Q209L displayed a lower rate of GTP hydrolysis than Goq* WT (Fig. 3). Moreover, Goq* Q209P also had diminished GTPase activity, which was comparable with that observed in Goq* Q209L (Fig. 3). These results are consistent with Goq* Q209P having a defect in GTPase activity comparable with that of Goq* Q209L.

The switch II region of Goq Q209P adopts a conformation different from other active Goq proteins

The results presented so far indicate that although Goq Q209P is GTPase-deficient and leads to signaling hyperactivation like Goq Q209L, it fails to recapitulate the strong binding to effectors observed for other active Goq proteins. We reasoned that whereas mutation of Glu-209 to any residue (including leucine) might be deleterious for GTPase activity, mutation to proline in particular might have a unique impact on the shape of the effector binding site and thereby account for the observed differences in protein–protein binding. There is a strong rationale for this reasoning based on available structural information. First, Glu-209 is located at the end of the SwII region (Fig. 4A). As its name indicates, the SwII alternates between different conformations, depending on the G protein activation status (49). Upon activation, the SwII region adopts a helical conformation that allows high-affinity binding of effectors to a newly formed pocket between SwII and α3 helix (Fig. 4A). Thus, we hypothesized that mutation of Glu-209 to proline, an amino acid with a cyclical side chain that imposes rotational constraints, might affect the conformation of the SwII region and distort the effector-binding site. To test this hypothesis, we carried out a well-established trypsin proteolysis assay that reports the conformation of the SwII (50). Briefly, when SwII adopts a helical conformation like that observed in active, GTP-bound Goq subunits, it becomes insensitive to trypsin digestion. This results in the formation of a trypsin-resistant species in which only an N-terminal fragment of the protein is cleaved off by trypsin, whereas G proteins in which the SwII region does not adopt such helical conformation, like GDP-bound Goq, are readily digested to low-molecular weight products (Fig. 4B). As expected, when lysates of HEK293T cells expressing Goq WT were incubated with trypsin, the G protein was readily digested, whereas supplementing the reaction with Mg-AlF₄⁻ to mimic the GTP-bound state resulted in the formation of a trypsin-resistant species (Fig. 4C). Trypsination of lysates of cells expressing Goq Q209L also resulted in the formation of the trypsin-resistant species, consistent with the idea that this mutant is constitutively bound to GTP and mimics the canonical active conformation of Goq WT (Fig. 4C). In contrast, the Goq Q209P mutant was not trypsin-resistant under the same conditions (Fig. 4C), indicating that its SwII does not adopt the helical conformation that confers trypsin resistance to active Goq WT. Because SwII is one of the structural elements of Goq that makes direct contact with effectors and shapes the effector binding pocket, these findings help explain the diminished binding of effectors to Goq Q209P mutant compared with Goq WT (Fig. 1).

Goq Q209P is constitutively dissociated from Gβγ in cells

A possible interpretation for the results obtained in the trypsin protection experiments described above is that Goq Q209P adopts a conformation analogous to that of GDP-bound Goq. This is unlikely because Goq Q209P binds to effectors better than inactive Goq (Fig. 1) and can also trigger signaling in cells (Fig. 2), which is largely incompatible with the properties of GDP-bound Goq. Moreover, Goq Q209P did not become trypsin-resistant upon the addition of Mg-AlF₄⁻ (Fig. S4), suggesting that the trypsin sensitivity is not because the G protein is in a GDP-bound state. Instead, it is more likely that the SwII of Goq adopts a unique conformation that represents neither the active nor the inactive conformation of Goq WT. To further substantiate this idea, we investigated the association of Goq Q209P with Gβγ. Gβγ binds preferentially to Goq subunits in the GDP-bound state by making extensive contacts with the SwII (Fig. 5A). For this, the SwII must adopt an inactive conformation, as formation of the characteristic helical conformation of GTP-bound Goq leads to Gβγ dissociation. Thus, Gβγ bind-
ing is sensitive to the conformational status of the SwII region of Gα. To investigate the association between Gα and Gβγ in cells, we used a previously described BRET-based assay (51, 52). Briefly, a fusion of the C-terminal domain of GRK3 fused to nanoluciferase (GRK3ct-Nluc) is used as the BRET donor, and Gβγ fused to Venus (V-Gβγ) serves as the BRET acceptor. Because GRK3ct and Gα share overlapping binding sites on Gβγ and can bind only in a mutually exclusive manner, BRET due to V-Gβγ binding to GRK3ct-Nluc reflects the dissociation of Gα–Gβγ heterotrimers (Fig. 5B, top).

Consistent with Gα–Gβγ heterotrimer dissociation upon G protein activation and previous observations using this assay system (51–53), agonist-induced activation of the Gαi-coupled GPCR M3 muscarinic acetylcholine receptor (M3R) led to a rapid increase in BRET in HEK293T cells co-expressing the donor/acceptor pair and GαQ WT. The subsequent addition of atropine, an antagonist of the M3 muscarinic receptor, caused the rapid decline of the BRET signal to basal levels, consistent with reassociation of Gαi and V-Gβγ (Fig. 5B). When the same experiment was done in cells expressing GαQ Q209L or GαQ Q209P, the basal levels of BRET before agonist stimulation were as high as that observed for agonist-stimulated BRET in cells expressing GαQ WT (Fig. 5B), indicating that GαQ Q209L and GαQ Q209P are constitutively dissociated from Gβγ. Consistent with constitutive dissociation of Gα and Gβγ, GPCR stimulation of cells expressing GαQ Q209L and GαQ Q209P failed to elicit a BRET response (Fig. 5B). The amount of GαQ WT, GαQ Q209L, and GαQ Q209P in the cells used in these experiments was equal, ruling out the possibility that the BRET differences observed between GαQ WT and the mutants were due to differential expression (Fig. 5B). There are two important points to be drawn from these observations. One is that the SwII of GαQ Q209P adopts a unique conformation different from that of inactive GαQ WT, as it cannot bind to Gβγ (Fig. 5B), and from that of other active Gα proteins, as it does not become trypsin-resistant (Fig. 4). The second point is that GαQ Q209P exists preferentially in a monomeric, Gβγ-free form in cells.

**Binding of RGS proteins to GαQ Q209P is weaker than to GαQ Q209L**

We reasoned that binding of GαQ Q209P to RGS proteins might be impaired because they also utilize the SwII as an important contact point. In fact, crystal structures of RGS8 and RGS2 in complex with GαQ (54, 55) have revealed that, despite adopting different poses, both RGS proteins make contact with Gln-209 and several adjacent residues in the SwII (Fig. 5, C and D). Consistent with our expectation, we found that both GAIP (also known as RGS19) and RGS2, representing the two G protein binding poses of RGS proteins (54), bound to GαQ Q209P less than to GαQ Q209L or GαQ WT activated with Mg-AlF4, but slightly more than inactive GαQ WT (Fig. 5, C and D). Once again, adding Mg-AlF4 did not rescue the defect in RGS binding of GαQ Q209P (Fig. S5), suggesting that the diminished binding is not because the G protein is in a GDP-bound state. These results indicate that GαQ Q209P does not bind to RGS proteins, a family of negative regulators of Gαi signaling.

**Mechanistic model and discussion**

Our findings using in vitro and cell-based reconstitution systems reveal that the oncogenic GαQ Q209P mutant is an active G protein with atypical properties. Although we can only speculate about the mechanism by which this mutant leads to signaling hyperactivation in the context of uveal melanoma, our findings provide a framework to try to explain how two mutants with different biochemical properties, GαQ Q209L and GαQ Q209P, result in similar oncogenicity (Fig. 6). Both GαQ Q209L and GαQ Q209P are GTPase-deficient and trigger constitutive

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**Figure 4. The switch II region of GαQ Q209P adopts a conformation different from other active Gαi proteins.** A. Gln-209 is located at the end of the helix formed by the SwII in active Gαi that is involved in binding effectors. Left panels, surface view of the structure of active Gαi with the SwII in green. The region in the box is shown enlarged on the right as a ribbon representation. Green, SwII; red, Gln-209; blue, GDP-AlF4–. Right panels, surface view of the structure of Gαi with the p63RhoGEF or PLCβ3 binding regions in cyan or purple, respectively, which overlap with the SwII. Red circle, position of Gln-209. B, diagram of limited proteolysis assay used to assess the conformational status of the SwII. The SwII becomes trypsin-resistant when it adopts a helical conformation, such as in active Gαi WT, which results in the formation of a trypsin-resistant fragment of Gαi in which only an N-terminal fragment of the protein has been cleaved off by trypsin. Mock gel depicts potential outcomes, depending on SwII conformation. C. The Sw of Gαi Q209Q, but not of Gαi Q209P adopts a conformation equivalent to that of active Gαi WT (+Mg-AlF4) as determined by limited proteolysis with trypsin. Lysates of HEK293T cells transfected with HA-Gαi WT (WT or mutants, as indicated) were incubated with trypsin in the presence or absence of 10 mM MgCl2, 30 μM AlCl3, and 10 mM NaF (Mg-AlF4) as indicated. The four lanes on the left are controls not treated with trypsin. The arrowhead indicates the position of full-length Gαi and the asterisk indicates the position of the N-terminally cleaved trypsin-resistant fragment of active Gαi. One representative result of at least three independent experiments is shown.
Atypical properties of GNAQ Q209P

Figure 5. Gαq Q209P is constitutively dissociated from Gβγ in cells and binds weakly to RGS proteins. A, Gβγ binds to the SwII region of Gαq. Top, surface view of the structure of Gαq with the SwII in green. Bottom, surface view of the structure of Gαq with the Gβγ binding area in blue. Red circle, position of Gin-209. B, Gαq Q209L and Gαq Q209P are both constitutively dissociated from Gβγ, as observed for GPCR-activated Gαq WT. Top, schematic of the assay used to monitor G protein activity with a BRET reporter of free Gαq. Under resting conditions, Venus-tagged Gαq (V-Gαq, BRET acceptor) associates with inactive Gαq, and BRET signal is low. Upon GPCR stimulation, V-Gαq dissociates from active Gαq and interacts with the C-terminal domain of GRK3 fused to nanoluciferase (GRK3ct-Nluc, BRET donor) inducing a BRET increase. Bottom, Gαq WT and mutants fail to associate efficiently with Gβγ under resting conditions, as indicated by the high BRET before agonist stimulation, and fail to undergo further dissociation upon GPCR stimulation. HEK293T cells transfected with plasmids encoding M3R, Venus(1–155)-Gαq2 (VN-Gαq2) and Venus(155–239)-Gαq1 (VC-Gαq1), HA-Gαq (WT or mutants), and GRK3ct-Nluc were treated with carbachol (100 μM) and atropine (100 μM) as indicated. Twenty-four h after transfection, cells were harvested, and BRET was measured every 0.24 s. Results are mean ± S.E. (error bars) (shown only at 5-s intervals for clarity) of n = 3. Immunoblots of lysates of HEK293T cells, used to confirm the equal expression of Gαq proteins, are shown below the graph. C and D, Gαq Q209P binds to GAIP (C) or RGS2 (D) less than Gαq Q209L or active Gαq WT (+ MgAlF4). Left panels, surface view of the structure of Gαq with the RGS8 (C) or RGS2 (D) binding area in yellow or purple, respectively. Red circle, position of Gin-209. Right panels, lysates of HEK293T cells transfected with HA-Gαq (WT or mutants, as indicated) were incubated with GST-GAIP (C) or GST RGS2 (D) immobilized on GSH-agarose beads in the presence or absence of 10 mM MgCl2, 30 μM AlCl3, and 10 mM NaF (MgAlF4) as indicated. Resin-bound proteins (Pulldown) and aliquots of the lysates were analyzed by Ponceau S staining and immunoblotting (IB) as indicated. One representative result of three independent experiments is shown.
activation of downstream signaling, which explains well their ability to promote cell phenotypic traits that support oncogenic transformation. The fact that the frequency of Q209P mutations in uveal melanoma tumors is similar to that of Q209L mutations supports the idea that both of them provide a similar adaptive advantage during cancer development through abnormal signaling hyperactivation. This is in good agreement with previous observations indicating that both \( \text{G}_{\alpha_q} \) Q209L- and \( \text{G}_{\alpha_q} \) Q209P-dependent signaling in UM-derived cell lines are required for cell proliferation and/or tumor growth in mice (24, 25, 36–38). The main difference between \( \text{G}_{\alpha_q} \) Q209P and other active \( \text{G}_{\alpha_q} \) species (i.e. \( \text{G}_{\alpha_q} \) Q209L and GTP-bound \( \text{G}_{\alpha_q} \) WT) is that it has unique structural properties that impact its ability to bind different interacting partners. More specifically, our results show that the S wil of \( \text{G}_{\alpha_q} \) Q209P adopts a conformation different from that of \( \text{G}_{\alpha_q} \) Q209L or GTP-bound \( \text{G}_{\alpha_q} \), that causes 1) high-affinity binding to effectors (red arrow) and 2) dissociation from \( \text{G}_{\beta\gamma} \) which lead to constitutive signaling activity. Nevertheless, this mutant still binds with high affinity to RGS proteins, which are potent inhibitors of effector binding. In \( \text{G}_{\alpha_q} \) Q209P (bottom), the S wil (dark green) adopts a conformation different from that of \( \text{G}_{\alpha_q} \) Q209L or GTP-bound \( \text{G}_{\alpha_q} \), that causes 1) moderate affinity binding to effectors (orange arrow), 2) dissociation from \( \text{G}_{\beta\gamma} \), and 3) impaired binding to RGS proteins. We speculate that the moderate affinity binding to effectors combined with the loss of \( \text{G}_{\beta\gamma} \)-mediated blockade of the effector-binding site and the lack of RGS-mediated antagonism for effector binding might be sufficient to account for the signaling hyperactivation reported in cells bearing this mutation (11, 24, 25, 37).

Then, how can \( \text{G}_{\alpha_q} \) Q209P lead to hyperactive signaling and oncogenic transformation if its binding to effectors is diminished compared with GTP-bound \( \text{G}_{\alpha_q} \) WT? First, \( \text{G}_{\alpha_q} \) Q209P binding to effectors is stronger than that of inactive \( \text{G}_{\alpha_q} \) WT, so it still retains some ability to engage and activate effectors. Second, \( \text{G}_{\alpha_q} \) Q209P is constitutively dissociated from \( \text{G}_{\beta\gamma} \) and has diminished binding to RGS proteins, both of which are negative regulators of \( \text{G}_{\alpha_q} \) in cells. A frequently overlooked function of \( \text{G}_{\beta\gamma} \) is to prevent the spurious action of \( \text{G}_{\alpha} \) on its effectors by competitive binding (57, 58). The importance of this function is further highlighted by evidence that \( \text{G}_{\beta\gamma} \) can bind to GTP-bound \( \text{G}_{\alpha_q} \) at physiological concentrations and antagonize binding of effectors such as PLC\( \beta \) (48, 59). The role of RGS proteins as negative regulators of G protein signaling is well-documented. This function is not only mediated by their GAP activity, to which GTPase-deficient Gln-209 mutants are presumably insensitive (60), but also by their ability to antagonize the binding of effectors to G proteins. In fact, it has been reported that RGS proteins are potent inhibitors of signaling activation by the \( \text{G}_{\alpha_q} \) Q209L oncogenic mutant (61, 62). Thus, the moderately higher affinity of \( \text{G}_{\alpha_q} \) Q209P for effectors compared with inactive \( \text{G}_{\alpha_q} \) might work concurrently with the relief from \( \text{G}_{\beta\gamma} \)- and RGS-mediated antagonism to attain effective levels of downstream signaling (Fig. 6).

This model shares similarities with the recently proposed mode of action for another \( \text{G}_{\alpha_q} \) oncogenic mutant (i.e. \( \text{G}_{\alpha_q} \) R201C). Hu and Shokat (63) have recently reported that this mutant might exist in a GDP-bound yet active state that accounts for its pro-oncogenic properties in cells. In the presence of \( \text{G}_{\beta\gamma} \), only GDP-bound \( \text{G}_{\alpha_q} \) R201C and not GDP-bound \( \text{G}_{\alpha_q} \) WT can activate a downstream effector (i.e. adenyl cyclase) (63). Thus, although GDP-bound \( \text{G}_{\alpha_q} \) R201C is a weaker activator of adenyl cyclase than GTP-bound \( \text{G}_{\alpha_q} \) in cells. A frequently overlooked function of \( \text{G}_{\alpha_q} \) Q209P–dependent signaling. In summary, our findings reveal a fundamental difference in the molecular properties of \( \text{G}_{\alpha_q} \) Q209P compared with other active \( \text{G}_{\alpha_q} \) proteins, including the other most frequent \( \text{G}_{\alpha_q} \) mutation in uveal melanoma, Q209L. The unique structural features of \( \text{G}_{\alpha_q} \) Q209P could be leveraged as a specific vulnerability of uveal melanomas bearing this mutation, which account for ~20% (11–13), and to overcome current limitations for the treatment of this type of cancer. To date, targeting signaling pathways downstream of \( \text{G}_{\alpha_q} \) in uveal melanoma has not been effective for therapeutic purposes (63). On the other hand, although it is logical to think that targeting mutant \( \text{G}_{\alpha_q} \) directly might be more efficacious than targeting downstream signaling nodes, it has been an area underexplored due to the assumption that it could lead to concurrent blockade of \( \text{G}_{\alpha_q} \) WT function and subsequent undesired side effects. Thus, the safety of cell-permeable peptide-like inhibitors of \( \text{G}_{\alpha_q} \) recently shown to effectively blunt proliferation of uveal melanoma cells bearing activating mutations in \( \text{G}_{\alpha_q} \) remains to be established (64). However, we show that contrary to other mutants like \( \text{G}_{\alpha_q} \) Q209L, \( \text{G}_{\alpha_q} \) Q209P could be specifically inhibited without affecting \( \text{G}_{\alpha_q} \) WT if targeting the unique structural features of the mutant proves to be feasible. Based on our results, it is likely

![Figure 6. Working model for the mechanisms leading to the constitutive signaling activity of \( \text{G}_{\alpha_q} \) Q209L and \( \text{G}_{\alpha_q} \) Q209P in cancer. In the absence of GPCR stimulation, the low signaling activity of \( \text{G}_{\alpha_q} \) WT (top) is ensured by two mechanisms: 1) low affinity for effectors (blue arrow) and 2) the action of negative regulators, such as \( \text{G}_{\beta\gamma} \) and RGS protein, which diminishes coupling to effectors. In \( \text{G}_{\alpha_q} \) Q209L (middle), the SwiI (dark green) adopts an active conformation equivalent to that of GTP-bound \( \text{G}_{\alpha_q} \), that causes 1) high-affinity binding to effectors (red arrow) and 2) dissociation from \( \text{G}_{\beta\gamma} \), which lead to constitutive signaling activity. Nevertheless, this mutant still binds with high affinity to RGS proteins, which are potent inhibitors of effector binding. In \( \text{G}_{\alpha_q} \) Q209P (bottom), the SwiI (dark green) adopts a conformation different from that of \( \text{G}_{\alpha_q} \) Q209L or GTP-bound \( \text{G}_{\alpha_q} \), that causes 1) moderate affinity binding to effectors (orange arrow), 2) dissociation from \( \text{G}_{\beta\gamma} \), and 3) impaired binding to RGS proteins. We speculate that the moderate affinity binding to effectors combined with the loss of \( \text{G}_{\beta\gamma} \)-mediated blockade of the effector-binding site and the lack of RGS-mediated antagonism for effector binding might be sufficient to account for the signaling hyperactivation reported in cells bearing this mutation (11, 24, 25, 37).]
that the α3 helix/SwII groove of Gαq in the Q209P mutant is different than in other active Gαq species. Identifying molecules that specifically bind to this putative pocket would disrupt the binding and activation of effectors downstream of the Q209P mutant. Although disruption of protein–protein interactions is challenging, there are now dozens of different protein–protein interactions that have been targeted by small molecules, and many of them have shown promising therapeutic effects, even entering clinical trials (65–69). An atomic resolution structure of Gαq Q209P might provide additional information on the molecular basis for its unique properties while establishing a framework to explore its druggability.

**Experimental procedures**

**Reagents and antibodies**

Unless otherwise indicated, all chemical reagents were obtained from Sigma or Fisher Scientific. *E. coli* DH5α strain was purchased from New England Biolabs, and the BL21(DE3) strain was purchased from Life Technologies. PfuUltra DNA polymerase was purchased from Agilent. Carbachol (catalog no. AC-10824) was obtained from Acros Organics, and aprotinin (catalog no. AC-10824) was obtained from PerkinElmer Life Sciences. Mouse mAb raised against hemagglutinin (HA) tag (clone 12CA5) was obtained from Roche Applied Science. Mouse monoclonal antibodies raised against α-tubulin (T6074) and FLAG tag (F1804) were from Sigma. Rabbit polyclonal antibody raised against nanoluciferase (Nluc) (catalog no. A10236) was from Alfa Aesar. Leupeptin (catalog no. L-010), pepstatin A (catalog no. P-020), and aprotinin (catalog no. A-655) were from Gold Biotechnology. [γ-32P]GTP was from PerkinElmer Life Sciences. Mouse mAb raised against [γ-32P]GTP was kindly provided by Dr. Lance Encell (Promega). Rabbit polyclonal antibodies raised against Gαq (E-17) were provided by Santa Cruz Biotechnology, Inc. Rabbit antibodies for phospho-ERK1/2 (Thr-202/Tyr-204) (catalog no. 4370) and total ERK1/2 (catalog no. 9102) were obtained from Cell Signaling. Goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800 secondary antibodies were from LI-COR, respectively.

**Plasmids**

pcDNA3.1-Gαq-Venus (Gαq-V, Venus inserted into the b/c loop of Gαq (46)), pcDNA3.1-Venus(1–155)-Gγ2 (VN-Gγ2), and pcDNA3.1-Venus(155–239)-Gβ1 (VC-Gβ1) were kindly provided by N. Lambert (Augusta University, Augusta, GA) (51). pcDNA3.1-masGRK3ct-Nluc (52, 70) was a gift from K. Martemyanov (Scrpps Research Institute, Jupiter, FL). pcDNA3.1-FLAG-PLCβ3 was a kind gift from T. Filtz (Oregon State University). pcDNA3-Gαq-HA (internally tagged) was kindly provided by P. Wedegaertner (Thomas Jefferson University) (71). The plasmid encoding human M3R was a kind gift from T. Filtz (Oregon State University). pcDNA3-GRK3ct-Nluc (clone 12CA5) was obtained from Roche Applied Science. Mouse mAb raised against hemagglutinin (HA) tag (clone 12CA5) was obtained from Roche Applied Science. Mouse monoclonal antibodies raised against α-tubulin (T6074) and FLAG tag (F1804) were from Sigma. Rabbit polyclonal antibody raised against nanoluciferase (Nluc) (catalog no. A10236) was from Alfa Aesar. Leupeptin (catalog no. L-010), pepstatin A (catalog no. P-020), and aprotinin (catalog no. A-655) were from Gold Biotechnology. [γ-32P]GTP was from PerkinElmer Life Sciences. Mouse mAb raised against [γ-32P]GTP was kindly provided by Dr. Lance Encell (Promega). Rabbit polyclonal antibodies raised against Gαq (E-17) were provided by Santa Cruz Biotechnology, Inc. Rabbit antibodies for phospho-ERK1/2 (Thr-202/Tyr-204) (catalog no. 4370) and total ERK1/2 (catalog no. 9102) were obtained from Cell Signaling. Goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800 secondary antibodies were from LI-COR, respectively.

**Protein expression and purification**

GST-Gαq* was purified by adapting a protocol described by Waldo et al. (43). His-Gαq* expression in BL21(DE3) *E. coli* was induced overnight with 1 mM isopropyl-β-D-1-thio-galactopyranoside (IPTG). Bacteria were pelleted from 1 liter of culture and resuspended in 25 ml of buffer (50 mM NaH₂PO₄, pH 7.4, 300 mM NaCl, 10 mM imidazole, 1% (v/v) Triton X-100 supplemented with protease inhibitor mixture (1 μM leupeptin, 2.5 μM pepstatin, 0.2 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride)). After sonication (four 20-s sonication cycles with 1-min intervals), lysates were centrifuged at 12,000 × g for 20 min at 4 °C. The soluble fraction (supernatant) of the lysate was used for affinity purification on GSH-agarose resin (Pierce) and eluted with 50 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM reduced GSH. All purified GST-tagged proteins were dialyzed overnight at 4 °C against PBS. All protein samples were aliquoted and stored at −80 °C.

His-Gαq* was purified by adapting a protocol described by Waldo et al. (43). His-Gαq* expression in BL21(DE3) *E. coli* was induced overnight with 1 mM isopropyl-β-D-1-thio-galactopyranoside at 23 °C. Bacteria were pelleted, lysed by sonication, and cleared from insoluble material as described above but using a different lysis buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 15 mM imidazole, 10% glycerol, 50 μM GDP, 30 μM AlCl₃, and 10 mM NaF supplemented with protease inhibitor mixture (1 μM leupeptin, 2.5 μM pepstatin, 0.2 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride)). The soluble fraction of the lysate was incubated with HisPur cobalt resins (Pierce, catalog no. 89964) for 90–120 min at 4 °C with rotation, followed by four cycles of washes with ~20 resin volumes of lysis buffer and centrifugation (2,000 × g, 2 min), and eluted with lysis buffer supplemented with imidazole to obtain a final concentration of 500 mM. The eluate was supplemented with 100 mM EDTA and incubated on ice for 20
min before passing it over a Superdex 200 HR10/30 gel filtration column using a running buffer consisting of 20 mM Hepes, pH 7.0, 100 mM NaCl, 1 mM MgCl₂, 2 mM DTT, 2% glycerol, and 10 μM GDP. In some cases, proteins were concentrated using an Amicon centrifugal filter with a molecular mass cut-off of 10 kDa. The typical yield was 0.5 mg of protein/liter of culture. Purified His-Gα₉* was aliquoted and stored at −80 °C.

**In vitro protein-binding assays with GST-fused proteins**

For pulldowns of proteins expressed in mammalian cells, ∼2 × 10⁶ HEK293T cells were seeded on 100-mm dishes and transfected the following day using the calcium phosphate method with plasmids encoding the following constructs (DNA amounts in parenthesis): Gα₉-HA (WT or mutants, 3 μg) or Gα₉-V (WT or mutants, 3 μg). Cell medium was changed 6 h after transfection. Twenty-four h after transfection, cells were lysed on ice with lysis buffer (20 mM Hepes, pH 7.2, 125 mM K(CH₃COO), 0.4% (v/v) Triton X-100, 10 μM β-glycerophosphate, 30 μM GDP, and 0.5 mM Na₃VO₄ supplemented with a protease inhibitor (SigmaFAST, catalog no. S8830)). For some conditions, the lysis buffer was supplemented with 30 μM AlCl₃, 10 mM NaF, and 10 mM MgCl₂ (Mg-AlF₄ condition). The cell lysates were cleared (14,000 × g, 10 min) and used as the source of soluble protein ligands for immobilized GST-fused proteins in pulldown assays. The following GST-fused proteins were immobilized on GSH-agarose beads for 90 min at room temperature in PBS (amounts per condition indicated in parenthesis): GST-GRK2R²H (5 μg), GST-p63RhoGEF²²H³⁵²⁵ext (7.5 μg), GST-Trio²²H³⁵²⁵ext (5 μg), GST-kalirin²²H³⁵²⁵ext (5 μg), GST-GAI² (7.5 μg), and GST-RGS² (7.5 μg). Beads were washed twice with PBS and resuspended in 400 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% (v/v) Nonidet P-40, 5 mM EDTA, 30 μM GDP, 2 mM DTT), which in some cases was supplemented with 30 μM AlCl₃, 10 mM NaF, and 10 mM MgCl₂ (Mg-AlF₄ condition). After the addition of cleared HEK293T cell lysates (∼400 μg of total protein), tubes were incubated for 90 min at 4 °C with constant rotation. Beads were rapidly washed four times with 1 ml of wash buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 30 μM GDP, 5 mM EDTA, 1 mM DTT), which in some cases was supplemented with 30 μM AlCl₃, 10 mM NaF, and 10 mM MgCl₂ (Mg-AlF₄ condition). Resin-bound proteins were eluted with Laemmli sample buffer by incubation at 37 °C for 10 min. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, stained with Ponceau S, and immunoblotted with the corresponding antibodies.

**For pulldowns of purified His-Gα₉* (Fig. S1), the following GST-fused proteins were immobilized on GSH-agarose beads by incubation at room temperature for 90 min in PBS (amounts indicated in parenthesis): GST-GRK2R²H (10 μg), GST-GAI² (10 μg), GST-p63RhoGEF²²H³⁵²⁵ext (10 μg), and GST-Ric-8A (10 μg). Beads were washed twice with PBS and resuspended in 300 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% (v/v) Nonidet P-40, 5 mM EDTA, 10 mM MgCl₂, 1 mM DTT) supplemented with 30 μM GDP or 30 μM GDP plus 30 μM AlCl₃ and 10 mM NaF (GDP-AlF₄⁻ condition) as indicated. After the addition of 1.7 μg of purified His-Gα₉*, tubes were incubated 4 h at 4 °C with constant rotation. Beads were washed four times with 1 ml of wash buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 10 mM MgCl₂, 5 mM EDTA, 1 mM DTT) supplemented with 30 μM GDP or 30 μM GDP plus 30 μM AlCl₃ and 10 mM NaF (GDP-AlF₄⁻ condition) as indicated. Resin-bound proteins were eluted with Laemmli sample buffer by incubation at 37 °C for 10 min. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, stained with Ponceau S, and immunoblotted with the corresponding antibodies.

**Immunoprecipitation**

Approximately 2 × 10⁶ HEK293T cells were seeded on 100-mm dishes and transfected the day after using the calcium phosphate method with plasmids encoding Gα₉-HA (WT or mutants, 3 μg) and FLAG-PLCβ3 (6 μg). Cell medium was changed 6 h after transfection. Twenty-four h after transfection, cells were lysed on ice with 500 μl of lysis buffer (20 mM Hepes, pH 7.2, 125 mM K(CH₃COO), 0.4% (v/v) Triton X-100, 10 mM EDTA, 10 μM β-glycerophosphate, 30 μM GDP, and 0.5 mM Na₃VO₄ supplemented with a protease inhibitor (SigmaFAST, catalog no. S8830)). For some conditions, the lysis buffer was supplemented with 30 μM AlCl₃, 10 mM NaF, and 10 mM MgCl₂ (Mg-AlF₄⁻ condition). Cell lysates were cleared (14,000 × g, 10 min) and incubated with 2 μg of anti-FLAG antibodies (Sigma, F1804) immobilized on Protein G–agarose beads (Thermo Scientific, ∼35 μl of beads preblocked with 5% BSA) for 90 min at 4 °C with rotation. Beads were washed three times (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 30 μM GDP, 5 mM EDTA, 1 mM DTT), and proteins were eluted by adding Laemmli sample buffer and boiling for 5 min. For some conditions, the wash buffer was supplemented with 30 μM AlCl₃, 10 mM NaF, and 10 mM MgCl₂ (Mg-AlF₄⁻ condition). Eluted proteins were separated by SDS-PAGE and analyzed by immunoblotting.

**BRET**

HEK293T cells were grown at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% l-glutamine. HEK293T cells were seeded on 6-well plates (∼400,000 cells/well) coated with gelatin and after 1 day transfected using the calcium phosphate method. For the detection of Gα₉-V binding to GRK2²H⁻Nluc, the following constructs were used (DNA amounts per well in parenthesis): Gα₉-V (0.5 μg, WT or mutants) and mas-GRK2²²H⁻Nluc (0.05 μg). For the detection of Gα₉-V association with GRK3ct-Nluc, we followed previously described protocols (53, 78, 79). For these transfections, the following constructs were used (DNA amounts per well in parenthesis): Gα₉-V (0.5 μg), VC-GBβ (0.2 μg), VN-Gγ₂ (0.2 μg), mas-GRK3ct-Nluc (0.2 μg), and M3R (0.2 μg). Cell medium was changed 6 h after transfection. Approximately 16–24 h after transfection, cells were washed and gently scraped in warm PBS, centrifuged (5 min at 550 × g), and resuspended in Tyrode’s solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.37 mM NaH₂PO₄, 24 mM NaHCO₃, 10 mM HEPES, pH 7.4, 0.1% glucose) at a concentration of ∼10⁶ cells/ml. 25,000 cells were added to a white opaque 96-well plate (Opti-Plate,
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PerkinElmer Life Sciences) and mixed with the nanoluciferase substrate Nano-Glo (Promega, final dilution 1:200) for 2 min before measuring. A POLARStar OMEGA plate reader (BMG Labtech) was used to measure luminescence signals at 460 ± 20 and 528 ± 10 nm at 28 °C, and BRET signals were calculated as the ratio between the emission intensity at 528 ± 10 nm over the emission intensity at 460 ± 20 nm. For the kinetic experiments, BRET measurements were performed every 0.24 s. An aliquot of cells from each experiment was processed for subsequent immunoblot analysis as follows. Cells were centrifuged (1 min at 14,000 × g) and resuspended on ice with lysis buffer (20 mM Hepes, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% (v/v) Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, and 0.5 mM Na₂VO₄ supplemented with a protease inhibitor mixture (SigmaFAST, catalog no. S8830)). Lysates were cleared by centrifugation (14,000 × g, 10 min, 4 °C) and boiled for 5 min in Laemmli sample buffer prior to protein separation by SDS-PAGE and immunoblotting.

ERK1/2 phosphorylation

HEK293T cells were seeded on 6-well plates (~200,000 cells/well) coated with gelatin and after 1 day were transfected using the calcium phosphate method with plasmids encoding Gαq-β-HA WT or mutants (0.5 μg/well) or with an empty vector. Cell medium was changed 6 h after transfection. Approximately 16–24 h after transfection, cells were scraped in PBS, centrifuged (30 s at 10,000 × g), and resuspended on ice with lysis buffer (20 mM Hepes, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% (v/v) Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, and 0.5 mM Na₂VO₄ supplemented with a protease inhibitor mixture (SigmaFAST, catalog no. S8830)). Lysates were cleared by centrifugation (14,000 × g, 10 min, 4 °C) and boiled for 5 min in Laemmli sample buffer prior to protein separation by SDS-PAGE and immunoblotting.

SRE reporter activation

These experiments were performed using a luciferase reporter assay as described by Lutz et al. (42) with minor modifications. HEK293T cells were seeded on 6-well plates (~350,000 cells/well) coated with gelatin and after 1 day were transfected using the calcium phosphate method with plasmids encoding Gαq-β-HA WT or mutants, 1 μg/well) or with an empty vector, along with the luciferase reporter plasmids pGL3-SRE.L and pRL-TK (0.5 μg of each per well). Cell medium was changed 6 h after transfection by medium containing a reduced concentration of FBS (0.5% (v/v) final). Approximately 16–24 h after transfection, cells were harvested for measurement of firefly and Renilla luciferase activity using the Dual-Glo® Luciferase Assay System (Promega, catalog no. E2920). Approximately one-sixth of the transfected cells were used for each 96-well condition in this assay using the Passive Lysis Buffer (Promega, catalog no. E1941). SRE activity–related counts (firefly) were normalized by the counts obtained for the Renilla luciferase under the control of a constitutive promoter, and then results were expressed as -fold activation compared with control cells transfected with an empty plasmid instead of Gαq.

GTPase activity

This assay was performed as described previously (47). Briefly, reactions were started by mixing equal volumes of 200 mM His-Gαq* (WT or mutants) and 1 μM [γ-32P]GTP (~50 cpm/fmol) at 20 °C in assay buffer (50 mM Na-HEPES, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 0.3 mM MgCl₂, 1 mM DTT, 0.2 mM (NH₄)_2SO₄, 0.05% (w/v) C₁₂E₁₀). Duplicate aliquots (25 μl) were removed at 0, 5, 10, 15, 20, 30, and 45 min, and reactions were stopped by mixing with 975 μl of ice-cold 5% (w/v) activated charcoal in 20 mM H₃PO₄, pH 3. Samples were then centrifuged for 10 min at 10,000 × g, and 500 μl of the resultant supernatant were scintillation-counted to quantify released [32P]P, which was subsequently converted to moles.

Limited proteolysis

HEK293T cells were seeded on 100-mm dishes (~2 × 10⁶ cells/dish) coated with gelatin and after 1 day were transfected using the calcium phosphate method with plasmids encoding Gαq-β-HA WT or mutants (3 μg/dish). Cell medium was changed 6 h after transfection. Approximately 16–24 h after transfection, cells were scraped in PBS, centrifuged (5 min at 550 × g) and resuspended on ice with 2 ml of lysis buffer (20 mM Hepes, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% (v/v) Triton X-100, 30 μM GDP, 1 mM DTT, 10 mM β-glycerophosphate, and 0.5 mM Na₂VO₄ supplemented with a protease inhibitor mixture (SigmaFAST, catalog no. S8830)). For some conditions, the lysis buffer was supplemented with 30 μM AlCl₃, 10 mM NaF, and 10 mM MgCl₂ (MgAlF₄⁻ condition). Ten μl of the cleared lysate (14,000 × g, 10 min) were used for each condition. Digestions were started by the addition of 100 ng of trypsin (or an equivalent volume of PBS) to each tube and incubated for 20 min at 30 °C. Reactions were stopped by the addition of Laemmli sample buffer and boiling followed by protein separation by SDS-PAGE and immunoblotting.

Immunoblotting

Proteins separated by SDS-PAGE were transferred to PVDF membranes. Membranes were blocked for 30 min with 5% (w/v) nonfat milk (or 5% (w/v) BSA for antibodies for phosphoproteins) and then incubated overnight at 4 °C with primary antibodies. The primary antibodies were used at the following dilutions: Gαq, 1:1,000; tubulin, 1:2,500; FLAG, 1:1,000; HA, 1:250; nanoluciferase, 1:1,000; phospho-ERK1/2, 1:1,000, total ERK1/2, 1:1,000. Following primary antibody incubation, membranes were washed with PBS + 0.1% Tween 20 and incubated in secondary antibodies (goat anti-rabbit Alexa Fluor 680 and goat anti-mouse or IRDye 800) at 1:10,000 dilution. IR imaging of Western blots was performed according to the manufacturer’s protocols using an Odyssey IR Imaging System (LI-COR Biosciences). Images were processed using ImageJ software (National Institutes of Health) and assembled for presentation using Photoshop and Illustrator software (Adobe).

Statistical analyses

Each experiment was performed at least three times unless otherwise indicated. The data shown are presented as means with error bars representing the S.E. or as one representative...
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