Selective Regulation of Goq/11 by an RGS Domain in the G Protein-coupled Receptor Kinase, GRK2*

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G protein-coupled receptor kinases (GRKs) are well characterized regulators of G protein-coupled receptors, whereas regulators of G protein signaling (RGS) proteins directly control the activity of G protein α subunits. Interestingly, a recent report (Siderovski, D. P., Hessel, A., Chung, S., Mak, T. W., and Tyers, M. (1996) Curr. Biol. 6, 211–212) identified a region within the N terminus of GRKs that contained homology to RGS domains. Given that RGS domains demonstrate AlF4-dependent binding to G protein α subunits, we tested the ability of G proteins from a crude bovine brain extract to bind to GRK affinity columns in the absence or presence of G proteins from a crude bovine brain extract. Additional studies revealed that bovine brain Goq revealed significant binding of both Goq,GDP/AlF4 and Goq(GTPγS), but not Goq(GDP), to GRK2. Activation-dependent binding was also observed in both COS-1 and HEK293 cells as GRK2 significantly co-immunoprecipitated constitutively activated Goq (R183C) but not wild type Goq. In vitro analysis revealed that GRK2 possesses weak GAP activity toward Goq that is dependent on the presence of a G protein-coupled receptor. However, GRK2 effectively inhibited Goq-mediated activation of phospholipase C-β both in vitro and in cells, possibly through sequestration of activated Goq. These data suggest that a subfamily of the GRKs may be bifunctional regulators of G protein-coupled receptor signaling operating directly on both receptors and G proteins.

G protein-coupled receptors (GPCRs) reside at the plasma membrane where they receive diverse extracellular stimuli, in the form of light, odorants, neurotransmitters, and hormones. This information is translated into intracellular signals when agonist-bound GPCRs activate exchange of GTP for GDP on the α subunit of heterotrimeric G proteins. Activated, GTP-bound Ga (Gs,Gq) then dissociates from Gβγ and each of these G protein components go onto regulate downstream effector molecules. In general the intracellular signal is limited by the presence of the extracellular stimuli and by the intrinsic GTPase activity of Ga. However, in order to selectively modulate the appropriate magnitude and duration of signals in diverse cellular contexts, several ubiquitous mechanisms are utilized to regulate these signaling cascades both at the level of the GPCR and at the level of the G protein.

At the level of the GPCR, agonist-specific loss of receptor responsiveness involves a family of G protein-coupled receptor kinases (GRK1–6). GRKs phosphorylate the agonist-activated form of GPCRs which in turn promotes the high-affinity binding of a second family of proteins termed arrestins (1). These interactions function to uncouple the GPCR from further G protein activation and to promote clathrin-mediated internalization of the receptor (1). Initiation of this process is controlled by GRKs, which are, in turn, regulated by a variety of molecules including the activated GPCRs themselves, Gβγ subunits, PIP2, PKC, calmodulin, and caveolin (1–3). The overall topology of GRKs includes a somewhat conserved catalytic domain of ~270 residues which is flanked by N- and C-terminal regulatory domains. The C terminus is highly variable (~100–230 residues) and has the general function of mediating membrane localization. For example, GRK2 and GRK3 possess a C-terminal pleckstrin homology domain which binds to both PIP2 and free Gβγ promoting membrane recruitment and subsequent receptor phosphorylation (2). Interestingly, the ability of GRK2 and GRK3 to bind to Gβγ has also been implicated as playing a direct role in the regulation of G protein signaling via the sequestration of free Gβγ (4–6). The ~190 residue N terminus of GRKs is modestly conserved and has been suggested to contain receptor binding determinants (7). Recently, calmodulin (8), PIP2 (9), and caveolin (3) have also been shown to interact with the N terminus. However, the overall structure and function of this domain has remained largely uncharacterized.

At the level of the G protein, regulation occurs through

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1 The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PIP2, inositol bisphosphate; GAP, GTPase activating protein; GTPγS, guanosine 5’-O-(thio)triphosphate; PLC, phospholipase C; HA, hemagglutinin; pol II, polymerase II; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; IP3, inositol 1,4,5-trisphosphate; M1AChR, M1 muscarinic cholinergic receptor; TXA2R, thromboxane A2 receptor.
intrinsic GTPase activity possessed by the Gα subunits which hydrolyze bound GTP and promote rebinding of Gβγ. This process has recently been found to be modulated by a ubiquitous family of proteins termed regulators of G protein signaling (RGSs), which serve as GTPase-activating proteins (GAPs) that accelerate the rate of GTP hydrolysis and thereby limit the half-life of the activated species (10, 11). RGS proteins share a ~120-residue region of homology termed an RGS domain which folds into an α-helical module that binds preferentially to the transition state of Gα (12). This preferential binding to the transition state, which can be mimicked in vitro by the addition of GDP/AlF₄⁻ (13), compared with the active state, which can be stably generated in vitro by addition of GTPγS, is thought to serve as the driving force for acceleration of GTPase activity (14, 15).

At least 18 RGS proteins have been identified. In general, these RGS proteins interact with the α subunits of the Gι and Gq families (10, 11, 16). In addition, a small collection of proteins including GRKs (17), axin (18), D-AKAP (19), and p115 (20), GST-GRK6(1–192), or GST was dialyzed against 3 mM Tris-HCl, pH 8.0, 2 mM MgSO₄, 6 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride, 10 μg/ml apropin, 10 μg/ml leupeptin, 0.2% Triton X-100) per mg of tissue using a Brinkman Polytron (14,000 rpm, 30 s). The homogenate was centrifuged at 45,000 × g for 20 min and the resulting supernatant at 300,000 × g for 60 min. The final supernatant was aliquoted and stored at −70 °C until use. 250-μl aliquots (~125 μg) of GRK2, GST-GRK2, GST, or mock-purified (500 μg) were incubated with 10 μl of the soluble brain extract (~10 mg/ml total protein) and 10 μl of buffer B (20 mM Tris-HCl, pH 8.0, 2 mM MgSO₄, 6 mM β-mercaptoethanol, 100 μM NaCl, 0.05% Lubrol, and 5% glycerol) with 100 μM GDP in the absence or presence of AlF₄⁻ (5 mM sodium fluoride and 30 μM AlCl₃) for ~12 h at 4 °C. The incubation mixture was then centrifuged at 1000 × g for 1 min. The resulting pellet washed four times with buffer B containing 100 μM GDP in the absence or presence of AlF₄⁻. Bound proteins were released from the pelleted resin by addition of 150 μl of SDS sample buffer followed by boiling for 10 min. The eluted proteins were then subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. A specific ~42-kDa protein band was identified by Ponceau-S staining; excised, and subjected to peptide sequence analysis. Alternatively, proteins were transferred to nitrocellulose membrane and subjected to immunoblot analysis.

Identification of GRK-binding Proteins—Fresh bovine calf brain was stripped of connective tissue and minced in ~1 ml of homogenization buffer and homogenized using a Polytron (14,000 rpm, 30 s). This homogenate was centrifuged at 45,000 × g for 20 min and the resulting supernatant at 300,000 × g for 60 min. The final supernatant was aliquoted and stored at −70 °C until use. 250-μl aliquots (~125 μg) of GRK2, GST-GRK2, GST, or mock-purified (500 μg) were incubated with 10 μl of the soluble brain extract (~10 mg/ml total protein) and 10 μl of buffer B (20 mM Tris-HCl, pH 8.0, 2 mM MgSO₄, 6 mM β-mercaptoethanol, 100 μM NaCl, 0.05% Lubrol, and 5% glycerol) with 100 μM GDP in the absence or presence of AlF₄⁻ (5 mM sodium fluoride and 30 μM AlCl₃) for ~12 h at 4 °C. The incubation mixture was then centrifuged at 1000 × g for 1 min. The resulting pellet washed four times with buffer B containing 100 μM GDP in the absence or presence of AlF₄⁻. Bound proteins were released from the pelleted resin by addition of 150 μl of SDS sample buffer followed by boiling for 10 min. The eluted proteins were then subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. A specific ~42-kDa protein band was identified by Ponceau-S staining; excised, and subjected to peptide sequence analysis. Alternatively, proteins were transferred to nitrocellulose membrane and subjected to immunoblot analysis.
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RESULTS AND DISCUSSION

Homology between the N Terminus of GRKs and RGS Domains—Whereas the central catalytic and C-terminal domains of GRKs have been well characterized, the overall structure and function of the ~190 residue N-terminal domain has remained relatively uncharacterized (1, 2). Interestingly, Siderovski et al. (17) identified sequence homology between RGS domains and an ~120 residue region in the N terminus of GRKs through a BLAST search of the NCBI protein data base (17). Indeed, both GRK2 and GRK3 (residues 51–173) are ~20% identical and ~30% similar to various RGS domains (Fig. 1). This compares with an average of 44% identity (~54% similarity) shared among various RGS proteins. Importantly, the majority of the conserved hydrophobic residues shown to make up the hydrophobic core of the RGS domain (12, 15) are shared throughout the GRK family (Fig. 1 and data not shown). This suggests that the N terminus of GRKs may have a three-dimensional topology that is similar to RGS domains. Residues thought to be critical for Ga binding and GAP activity in most RGS proteins are only partially conserved by GRK2 and GRK3 (Fig. 1) (12, 15). However, p115 Rho-GEF, a new member of the RGS family that serves as a GAP for Go12/13, also exhibits only partial conservation of these residues compared with other RGS proteins (Fig. 1) (20, 21).

Finally, it is noteworthy that residues previously conserved as critical caveloin binding determinants in GRKs (residues 60–73 in GRK2 and GRK3 (3)) fall within α-helix 3 of the putative RGS domain (Fig. 1). Interestingly, several RGS proteins including RGS2 and RGS12 possess significant sequence similarity to GRKs within the cavelolin-binding region suggesting that these RGS proteins may possibly interact with cavelolin.

Binding of Bovine Brain Extracts to GRKs in the Absence or Presence of AlF4−—Based on the identified GRK/RGS sequence homology discussed above we speculated that GRKs may bind to Ga subunits in an AlF4−-dependent fashion. To test this hypothesis an affinity column containing covalently bound GRK2 was generated and 0.2% Triton X-100 solubilized bovine brain extract was passed over it in the presence of either GDP or GDP/AlF4−. After extensive washing, bound proteins were eluted with SDS sample buffer, subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Ponceau-S. This experiment revealed the presence of an ~55 kDa AlF4−-independent band that was identified by immunoblotting as tubulin in agreement with previous studies demonstrating GRK2/tubulin interactions (33, 34). In addition, a ~45-kDa AlF4−-independent band was identified by immunoblotting as actin in agreement with previous studies demonstrating GRK/actin interactions (35). Interestingly, a ~42-kDa protein was identified that bound to the GRK2 column (but not to mock or GST control columns) in a strictly AlF4−-dependent fashion (Fig. 2A). This band was excised and directly microsequenced. This yielded the sequence TLESI(M)MAXXL with the fifth cycle detecting both isoleucine and methionine. A subsequent data base search with this sequence suggests that the 42-kDa band represents a mixture of Goq11 (1MTLESMACCL13) and Goq13 (1MTLESMMACCL13), two G proteins that are highly related in sequence (88% similarity) and function (36).

In order to further analyze the specificity of G protein binding to the GRK2 affinity column, experiments identical to those described above were performed and analyzed by Western blotting. Immunoblotting with Goq11−, Goq2−, Goq3−, and Goq12/13− specific antibodies confirmed the identification of the 42-kDa AlF4−-dependent band as Goq11 and suggested specificity in that Gaq11, Gai, and G12/13 binding was not detected despite
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Their presence in the extract (Fig. 2B). Given the well-characterized sequence (93% similarity) and functional similarity between GRK2 and GRK3 (1, 2), we also generated a GRK3 affinity column and incubated it with the bovine brain extract. This experiment revealed that GRK3, like GRK2, can bind to Goq11, but not Goq7, Goq10, and Go12/13 in an ALF4-dependent fashion (data not shown). In order to establish that GRK/Goq11 binding was dependent on the GRK RGS domain, experiments were also performed using either GRK2 N-terminal (GST-GRK21-178) or C-terminal (GST-GRK2246-689) affinity columns. These studies revealed the specific ALF4-dependent binding of bovine brain Goq11 (Fig. 2C), but not other Ga proteins (data not shown), to GST-GRK21-178, which contains the GRK2 RGS domain. In contrast, Goq11 did not bind to GST-GRK2246-689 which contains the GRK2 pleckstrin homology domain. Interestingly, the N-terminal domains of GRK5 (GST-GRK51-200) (Fig. 2C and data not shown) and GRK6 (GST-GRK61-192) (data not shown) did not bind to Goq11, Goq7, Goq10, or Go12/13. However, this blotting analysis is not exhaustive and it remains possible that the N-terminal domain of other GRKs interact with distinct Go subunits that remain to be identified.

GRK2 and GRK3 Binding to Purified Goq—In order to more thoroughly examine GRK/Goq interactions, we performed experiments with purified Goq. Initially, 100 nm purified Goq was combined with GRK2, GST-GRK21178, GST-GRK22145178, GST-GRK51200, GRK2, GST-GRK51200, GST-GRK61192, GST, and mock affinity columns. These experiments demonstrated binding of nearly 100% of the purified Goq to the GRK2, GST-GRK21178, GST-GRK22145178, and GRK3 affinity columns in a strictly ALF4-dependent manner (data not shown). Goq binding to all other columns was <5% and was unaffected by the presence of ALF4 (data not shown). Identical experiments were then performed using purified Goq, Goq7, and Goq10. Unlike Goq, these other purified Ga proteins did not exhibit significant binding to any of the affinity columns in either the absence or presence of ALF4 (data not shown). Thus, experiments with purified Goq demonstrated the same selectivity with respect to GRKs (i.e. preferential binding to GRK2 and GRK3) that was observed in experiments with bovine brain Goq11 (Fig. 2).

In order to approximate the strength of the GRK2/Goq interaction, fixed amounts of Goq and GRK2 affinity resin were incubated in various volumes of binding buffer in the absence or presence of ALF4. This approach allowed for direct comparison of the amounts of Goq bound to GRK2 at different Goq concentrations. These studies revealed that, even at relatively low concentrations, nearly 100% of the Goq bound to GRK2 in the presence of ALF4 (Fig. 3). In contrast, only ~5% of Goq(GDP) bound to GRK2. These results suggest that Goq binds to GRK2 with high affinity.

Binding of Transition and Active States of Goq to GRK2—Addition of ALF4 to inactive Goq(GDP) produces a stable conformation that is thought to represent the transition state produced during hydrolysis of Goq(GTP) to Goq(GDP) (13). Many molecules, such as the effector adenyl cyclase, do not appear to discriminate between the active state (Goq(GTP)) or Goq(GTPS) and the transition state (Goq(GDP)/ALF4) of the Ga subunit. However, RGS proteins are unique in that they exhibit preferential binding to Goq(GDP)/ALF4 (12, 14, 15). This preference for the transition state is thought to enable RGS proteins to stabilize this conformation and thus promote GTP hydrolysis (12, 14, 15). To determine if GRK2 binding to Goq is also selective for the Goq transition state we compared GRK2 binding to 3 nm Goq(GDP), Goq(GDP/ALF4), and Goq(GTPS). Interestingly, significant GRK2 binding was observed to both the Goq(GDP/ALF4) (~95% binding) and Goq(GTPS) (~45% binding), whereas Goq(GDP) failed to demonstrate significant binding (Fig. 4). When a similar analysis was performed using Goq(R183C), a GTPase-deficient mutant, both GTP and GTPS forms bound to GRK2 to a similar extent (35~40% binding), whereas the GDP/ALF4 form again bound more extensively

FIG. 1. Alignment of GRK2 and GRK3 N termini with RGS domains. Top, overall topology of GRK2 and GRK3 is shown as a hatched bar (GRK2/3). The N-terminal RGS domain of ~120 residues is shaded. The central catalytic domain of ~270 residues is shown in light gray, whereas the C-terminal pleckstrin homology domain of ~100 residues is shown in dark gray. Black bars above and below GRK2/3 indicate regions shown previously to contain critical binding determinants for Gβγ (1, 2) and caveolin (3). Bottom, GRK2 and GRK3 (residues 51–173) were aligned with the RGS domains of RGS12 (residues 712–830), RGS14 (64–182), RGS2 (80–197), RGS4 (59–176), GAP (87–204), and p115 Rho-GEF (p115) (45–170). The predicted secondary structure is represented by black bars labeled α1-α9 for each of the α-helices in this structure (12). Hydrophobic residues thought to be largely involved in forming the hydrophobic core of this structure (12, 15) are shown in gray. Residues in RGS4 shown to contact Goq (12, 15) are designated α. Residues in GRK2 shown to be critical caveolin binding determinants (3) are designated β.
and were immunoblotted with Gαs, similar to those shown above (Fig. 3). Bound Gαs was eluted from the affinity column with SDS, subjected to SDS-PAGE, and immunoblotting with a Gαs-specific antibody. A representative immunoblot of Gαs (0.1–30 nM) binding to GRK2 or GST columns is shown along with standards representing 25, 50, and 100% of the Gαs loaded into the binding experiments (% Load).

Fig. 3. GRK2/Gαs binding in the absence or presence of AlF4. 1.5 pmol of purified Gαs (GDP) was incubated with 6.25 pmol of GRK2 (covalently coupled to CNBr-activated Sepharose) in a total volume from 50 μl to 15 ml in the absence or presence of AlF4 as described under “Experimental Procedures.” Bound Gαs was eluted from the affinity column with SDS, subjected to SDS-PAGE, and immunoblotting with a Gαs-specific antibody. Representative GRK2/Gαs binding in the absence or presence of AlF4 is shown.

Fig. 2. Binding of soluble bovine brain extract to GRK affinity columns in the absence or presence of AlF4. Covalently bound GRK, GST-GRK, GST, and mock affinity columns were prepared and combined with a soluble bovine brain extract in the absence or presence of AlF4 as described under “Experimental Procedures.” After washing the columns extensively, bound proteins were eluted by boiling with SDS sample buffer and then subjected to SDS-PAGE. A, brain proteins were eluted from GRK2, GST, and mock-coupled affinity resins from experiments performed in the absence (–) or presence of AlF4 were visualized by Ponceau-S staining. Total brain extract (Ext) and molecular weight standards (Std) are shown on the right. Identity of specific bands including GRK2, tubulin, actin, and Gαq, as well as an unidentified protein of ~70 kDa are shown on the right. B, experiments identical to those shown above were subjected to immunoblotting with Gαq, Gαs, Gαt, and Gα12/13-specific antibodies (indicated on right). C, experiments similar to those shown above (A) were performed using GST-GRK5(1–200), GST-GRK2(1–178), and GST-GRK2(469–689) affinity columns and were immunoblotted with Gαq11-specific antibodies.

(-90% binding) (Fig. 4). Because, the loading of GTP or GTPγS onto Gαs proteins in the absence of an activated GPCR is significantly less efficient than that of AlF4 (29, 30), the observed binding of GRK2 to the GTP- and GTPγS-bound forms of Gαs is likely underestimated. Nevertheless, GRK2 appears to bind extensively to both the “active” and “transition” states of Gαs. This binding profile is in contrast to other RGS proteins which, when analyzed in a similar fashion, bound preferentially to the transition state of Gαi/o or Gαq (14, 15, 37–39).

Function of GRK2/Gαs Interaction—Given the ability of RGS proteins to serve as GAPs for Gα proteins, we next investigated whether GRK2 may serve as a GAP for Gαs. To test this possibility we initially utilized a single turnover assay, which involves pre-loading of [γ-32P]GTP onto Gα in the absence of MgSO4 to slow hydrolysis. Unfortunately, GDP dissociation, and therefore GTP loading, in the absence of activated GPCR is particularly inefficient compared with the kcat for GTP hydrolysis for Gαs, precluding use of the single turnover assay (30).

However, the kcat for GTP hydrolysis of Gαs(R183C) is significantly reduced allowing GTP loading to occur more efficiently (29). Moreover, it was recently shown that the GTPase activity of Gαs(R183C) can be promoted by RGS4 in a single turnover assay (29). Thus, we utilized this assay to monitor the GTPase activity of Gαs(R183C) in the absence or presence of purified RGS4, GRK2, GST-GRK2(1–178), or Gαi/o alone. While 100 nM RGS4 promoted rapid GTP hydrolysis releasing up to 5 pmol of Pγ (GRK2 and GST-GRK2(1–178)) at concentrations up to 500 nM failed to enhance GTP hydrolysis (Fig. 5A). We also performed single turnover GTPase assays on wild type Gαs, Gαi/o, Gαq, Gαt, and Gα12 in the absence or presence of RGS4 (100 nM) and GRK2 (100 nM). As previously shown, RGS4 significantly promoted the GTPase activity of Gαi/o and Gαq while having no effect on either Gαs or Gα12 (Ref. 26, data not shown).

It was previously demonstrated that while RGS2 does not serve as a GAP for Gαs in single turnover GTPase assays, it is...
an effective GAP in the presence of purified heterotrimeric Gi and the Gb-coupled M3AChR (29). Thus, we reconstituted heterotrimeric Gq and Gb-coupled M3AChR into phospholipid vesicles allowing measurement of agonist-promoted steady-state GTPase activity of Gαq. Addition of carbachol produced a steady-state rate of Gαq-mediated [γ-32P]GTP hydrolysis of ~5 fmol/min (basal activity). The basal activity was not significantly altered by addition of buffer control (Fig. 5B) or GST (500 nM) (~7 fmol/min). Addition of RGS4 (50 nM), however, produced an ~33-fold increase in the rate of GTP hydrolysis (~166 fmol/min) (Fig. 5B). Addition of GRK2 (300 nM) or GST-GRK2(1–178) (500 nM) produced more modest enhancements of GTPase activity of ~7-fold (~34 fmol/min) and ~9-fold (~46 fmol/min), respectively. Thus, it appears that GRK2 may have a weak ability to function as a GAP for Gαq. The fact that the GRK2-dependent GAP activity was only ~25% that of RGS4 and required concentrations up to 10-fold greater than that of RGS4 raises the question of whether this activity is important under physiological conditions. However, the fact that this GRK2 GAP activity is apparent only in the presence of an activated GPCR suggests the possibility that receptors could have a critical role in potentiating GRK2-dependent GAP activity in cells. Perhaps in the presence of other GPCRs, GRK2 may serve as a more efficient GAP for Gαq. Indeed it has been demonstrated that the ability of RGS2 to inhibit Gαq-mediated signals in cells is highly dependent on the nature of the receptors that are being stimulated (40). The authors of this study suggested that regulatory selectivity may be conferred by specific receptor-RGS complexes. Moreover, it has been well established that the kinase activity of GRK2 can be stimulated by binding to an activated GPCR (41). Thus, further investigation of the role of receptors in modulating GRK/Gαq interactions seem warranted.

The primary role of activated Gαq in cells is stimulation of PLC-β which hydrolyses PIP2 (and other phosphoinositides) to the second messengers IP3 (and other inositol phosphates) and diacylglycerol (36). Given the high affinity binding that GRK2 possesses for activated Gαq (Figs. 2–4), we speculated that even in the absence of significant GAP activity, GRK2 may be able to regulate PLC-β activity simply by competing for binding to activated Gαq. To test this, purified PLC-β1 was combined with phospholipid vesicles containing [3H]PIP2 and Gαq in the absence or presence of AlF4− in the presence of 0–3 μM RGS4, GST-GRK2(1–178), or GST as described under “Experimental Procedures.” Reactions were quenched by addition of 10% trichloroacetic acid and bovine serum albumin and [3H]IP3 production was quantified by liquid scintillation counting and plotted against the concentration of added proteins. All values are averages of duplicate reactions from a representative experiment.

The authors of this study suggested that regulatory selectivity may be conferred by specific receptor-RGS complexes. Moreover, it has been well established that the kinase activity of GRK2 can be stimulated by binding to an activated GPCR (41). Thus, further investigation of the role of receptors in modulating GRK/Gαq interactions seem warranted.

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These data suggest that, even in the absence of significant GAP activity, GRK/Gαq binding may be able to regulate G protein signaling simply by sequestration of activated Gαq. This is particularly interesting in light of extensive previous studies that the C terminus of GRK2 and GRK3 can sequester free Gβγ subunits and thereby inhibit their signaling in cells (4–6). Thus, the possibility exists that these GRKs may be able to concomitantly sequester both components of the bifurcating Gαq signal (i.e. Gαq(GTP) and free Gβγ). Alternatively, if Gαq/11
and Gβγ binding to GRKs are mutually exclusive, this would provide a mechanism for complex regulation of GRK2 and GRK3 activity.

Another possible consequence of GRK2/Gao and GRK3/Gao interactions would be phosphorylation of Gao. Indeed, members of the Gao family have previously been shown to be regulated by tyrosine phosphorylation and by PKC-mediated serine phosphorylation (42, 43). In order to test whether GRK2 or GRK3 phosphorylate Gao, these GRKs were combined with [γ-32P]ATP and Gao(GDP) in the absence or presence of AIF4 for 0 to 60 min at 37 °C. These experiments failed to produce any detectable phosphorylation of Gao by these GRKs (data not shown). Additional experiments were performed in the presence of Gβγ and/or light-activated rhodopsin in order to test the possibility that these GRK activators might be required for Gao phosphorylation. These experiments also failed to produce detectable GRK-mediated Gao phosphorylation (data not shown).

Thus, Gao does not appear to be a substrate for GRK2 or GRK3. GRK2/Gao Interaction in Intact Cells—In order to determine if the GRK/Gao interaction occurs in intact cells we co-expressed GRK2 and either Gao or Gao(R183C) in COS-1 cells. This particular mutation (R183C) in Gao nearly abolishes GTPase activity trapping the GTP-bound active state of Gao but does not directly confer the active state. Thus, a portion of the expressed Gao will accumulate in the active state over time due to basal stimulation of GPCRs during cell culture. Presumably, acute GPCR stimulation should drive further accumulation of Gao(R183C) trapped in this active, GTP-bound state. Therefore, COS-1 cells were transfected with GRK2 and either HA-Gao or HA-Gao(R183C) cDNAs. Immunoblotting of lysates with an HA-specific antibody revealed that the total expression of HA-Gao and HA-Gao(R183C) was similar (Fig. 7). Moreover, GRK2 expression levels were also similar in cells co-expressing HA-Gao and those co-expressing HA-Gao(R183C) (data not shown). For immunoprecipitation, lysates were incubated with either GRK2- or pol II (control)-specific polyclonal antibodies. Subsequent blotting of these immunoprecipitation reactions with a GRK2-specific monoclonal antibody revealed that the GRK2 monoclonal antibody effectively immunoprecipitated GRK2 in both cells co-expressing HA-Gao and those expressing HA-Gao(R183C), while the pol II antibody did not precipitate GRK2 (data not shown). Immunoblotting the immunoprecipitates with an HA-specific monoclonal antibody to detect Gao revealed that only a small amount (~1%) of wild type Gao co-precipitated with GRK2 whereas a significant amount (~20%) of Gao(R183C) co-immunoprecipitated with GRK2 (Fig. 7). Similar results were obtained using HEK293 cells (data not shown). Importantly, this experiment is in agreement with our in vitro data (Fig. 4) and underscores the ability of GRK2 to bind tightly to the active state of Gao, as opposed to other RGS proteins which appear to require the transition state for significant binding (12, 14, 15, 37–39).

In order to examine the effect of GPCR stimulation on GRK2/Gao interaction, COS-1 cells were co-transfected with the M2AChR, GRK2, and either HA-Gao or HA-Gao(R183C). Incubation of these cells with 100 μM carbachol for 20 min enhanced the amount of Gao(R183C) co-immunoprecipitated by nearly 2-fold, while stimulation for longer periods (up to 60 min) did not produce further enhancement (data not shown). In contrast, wild type HA-Gao co-immunoprecipitation was not significantly enhanced by carbachol although this may be a consequence of GTP hydrolysis over the duration of the immunoprecipitation (~3–4 h). These studies support the idea that under physiological conditions GPCR stimulation may promote GRK2/Gao interaction. We next examined the selectivity of these interactions by co-expressing GRK2 with HA-Gao, HA-Gao(R201C) (a GTPase-deficient mutant), EE-Gao, or EE-Gao(R179C) (a GTPase-deficient mutant), in HEK293 cells and performed immunoprecipitation experiments analogous to those described above. Here, neither the wild type nor GTPase-deficient mutants of Gao or Gao were co-immunoprecipitated with GRK2 (data not shown) demonstrating that the selectivity that we observed in vitro occurs in intact cells.

Finally, given the ability of GRK2 to bind to activated Gao in intact cells (Fig. 7) along with the regulatory function of GRK2 toward PLC-β shown in vitro (Fig. 6), we examined whether GRKs may regulate PLC-β activity in intact cells. Initially, we took advantage of the functional properties of Gao(R183C). We found that expression of HA-Gao(R183C) in HEK293 cells, in the absence of specific GPCR stimulation, generated a significantly elevated production (~10-fold) of inositol phosphates as compared with cells transfected with vector control or wild type HA-Gao (data not shown). To examine the effect of GRKs on inositol phosphate production, cells were co-transfected with HA-Gao(R183C) and either vector (100% control), GRK2, GRK2(K220R), HA-GRK2(45–178), GRK3, GRK3(K220R), GRK5, GRK6, RGS4, or GAIP. Importantly, levels of HA-Gao(R183C) expression, as assessed by Western analysis, were similar regardless of the nature of the co-transfected DNA (data not shown). These experiments reveal, as predicted, that RG54 and GAIP expression both lead to a significant (~40%) inhibition of PLC-β activity (Fig. 8A). Expression of GRK2 or GRK3 lead to a similar inhibition of PLC-β activity (~40%), while the catalytically inactive versions of these kinases ((GRK2(K220R) and GRK3(K220R)) were equally effective at blunting PLC-β activity (~45% inhibition) (Fig. 8A). This demonstrates that this inhibition does not require phosphorylation activity. Alternatively, expression of full-length GRK5 had no effect on PLC-β activity, while GRK6 exhibited a small (~15%) inhibition (Fig. 8A) in general agreement with the in vitro GRK selectivity demonstrated above. Since a GRK2 RGS domain construct (G5R-K2(R45–178)) can bind Gao in vitro (data not shown), we also generated an HA-tagged pcDNA3 minigene construct containing this domain (residues 45–178). Co-expression of the GRK2 RGS construct produced a dramatic (~65%) inhibition of PLC-β activity (Fig. 8A). Given that PLC-β activity is dependent on overexpressed HA-Gao(R183C) in this system, the level of competing GRK constructs would seem to be critical. Thus, we believe that the enhanced effectiveness of the GRK2 RGS domain construct is likely a consequence of a higher molar expression of this relatively small construct compared with the full-length GRK2 and GRK3 constructs. Taken together, these data demonstrate that the RGS domains of GRK2 and GRK3 can effectively inhibit Gao(R183C)-stimulated PLC-β activity.
Regulation of Goq/11 by GRK2

A

B

FIG. 8. Inhibition of Goq, signaling by RGS and GRK constructs in HEK293 cells. A, HEK293 cells expressing HA-Goq(R183C) along with vector (control) or the indicated GRK or RGS constructs were metabolically labeled with myo-[3H]inositol and the total [3H]inositol phosphates produced were isolated as described under “Experimental Procedures.” Total [3H]inositol phosphates were quantitated by liquid scintillation counting, expressed as a percent of control and plotted against the indicated experimental conditions (i.e. co-expressed constructs). B, HEK293 cells expressing TXA2Rα along with vector (control) or the indicated GRK or RGS constructs were metabolically labeled with myo-[3H]inositol and then stimulated for 10 min with 100 nM U46619. Total [3H]inositol phosphate production was measured as described under “Experimental Procedures” and plotted as above. All values are mean ± S.E. from three to eight separate experiments.

Given that the approach used above provides a direct stimulation of PLC-β activity via Goq(R183C), we can state with relative certainty that the inhibition observed above is mediated at the level of the Goq, as opposed to, for example, the GPCR. Having established this, we were next interested in examining a more physiologically relevant system involving receptor-stimulated activation of PLC-β. To accomplish this, HEK293 cells were co-transfected with cDNA for TXA2Rα, a Gq-coupled GPCR, along with all of the GRK and RGS constructs described above. Stimulation of TXA2Rα-containing cells with the agonist U46619 (100 nM) for 10 min led to a significant enhancement of total inositol phosphate production (7-fold) which was taken as the 100% control. This U46619-dependent enhancement of PLC-β activity was completely dependent on the expression of TXA2Rα (data not shown). In cells co-expressing TXA2Rα and the various GRK or RGS constructs, basal inositol phosphate production (i.e. without U46619) was similar to that of cells co-transfected with TXA2Rα and vector (data not shown). However, the U46619-stimulated inositol phosphate production was inhibited by ~40 and ~25% by RGS4 and GAIP co-expression, respectively, in agreement with previous studies (Fig. 8B) (31). Expression of GRK2, GRK3, and, importantly their kinase-deficient counterparts also led to a substantial inhibition (~40%) of inositol phosphate production, whereas expression of the GRK2 RGS domain minigene gave ~45% inhibition (Fig. 8B). GRK5 and GRK6 co-expression, in agreement with other experiments (Fig. 8A), was without effect (Fig. 8B). In addition, expression of the GRK2 C-terminus (GRK2R(468–689)) was also without effect (data not shown), demonstrating that the U46619-stimulated inositol phosphate production was independent of Gβγ subunits. An analogous set of experiments was also performed with the M2ACHR, another Gq-coupled GPCR. In these studies, carbachol-stimulated inositol phosphate production was inhibited by the above constructs in a manner that was qualitatively identical to the observed effects on TXA2Rα (Fig. 8B), although the maximal extent of RGS-mediated (~15%) and GRK2/GRK3-mediated (~35%) inhibition was somewhat reduced (data not shown). These experiments are generally in agreement with those shown above (Fig. 8A) with respect to the relative function of various GRK and RGS constructs. The reason for the apparent increase in effectiveness of the GRK2 RGS construct toward HA-Goq(R183C)-mediated PLC-β activity relative to that of the receptor-stimulated system is not yet clear. Perhaps, in these receptor systems, where the endogenous Goq, is more limiting, the relative differences in molar expression of the GRK constructs is diminished. Overall, however, the experiments with TXA2Rα and M2ACHR support the hypothesis that GRK2 and GRK3 can dynamically interact with activated Goq, in response to GPCR agonists and thereby regulate downstream signaling.

Taken together, the demonstrated selectivity for GRK2 and GRK3 for Goq/11 binding and the apparent ability of these GRKs to inhibit Goq/11-mediated signaling leads to the question of what the overall role of these GRKs may be with respect to regulation of Goq/11-coupled signaling events. Signaling through many of the Goq/11-coupledGPCRs including the α1-AR, M2ACHR, M3AChR, TXA2Rα, endothelin-1A, endothelin-1B, bradykinin-B2, thrombin, substance-P, bombesin, parathyroid hormone, and angiotensin II-1A receptors have previously been shown to be effectively regulated by GRK2 and/or GRK3 (1, 32, 44–48). Characterization of such regulation has been performed using diverse experimental methods including measurement of inositol phosphate production (1, 44–48). While regulatory effects of GRK2 and GRK3 on signaling have been presumed to be a consequence of GPCR phosphorylation, our results suggest that GRK2- or GRK3-dependent decreases in PLC-β activity may reflect combined effects of these GRKs on (i) the initiation of GPCR desensitization (through phosphorylation of GPCRs) and (ii) sequestration of activated Goq, away from PLC-β. This suggestion is supported by a previous study of α1-AR signaling which demonstrated that GRK2- and GRK3-mediated regulation can be divided into both α1-AR phosphorylation-dependent and phosphorylation-independent events (45). Indeed, the authors speculated that this might reflect the interaction of GRK2 with other molecules downstream of the receptor (45) which we now believe to be Goq/11. Another study has demonstrated that endothelin-1A/B receptor stimulation of PLC-β activity was significantly blunted (~85% reduction) by overexpression of either wild type GRK2 or GRK2(K220R), while GRK5 had only modest effects (~15% reduction) (46). Moreover, this study demonstrated that while the C terminus of GRK2 had no effect, a N-terminal const of GRK2 alone was sufficient to mediate substantial (30%) inhibition of endothelin-stimulated PLC-β activity (46). Similar observations were also made with the angiotensin II-1A (47) and parathy-
roid hormone (48) receptors in which angiotensin- or parathyroid hormone-mediated PLC-β activity was diminished ~90 or ~70%, respectively, by overexpression of GRK2/K220R. The authors of these studies concluded that such inhibitory effects are likely a result of direct GPCR/GRK interaction (46–48). However, it now seems likely that these observations can also be explained, at least in part, by direct interaction of GRK2 and Gaq/11. Gaq-coupled receptors are known for their ability to stimulate PLC-β which causes hydrolysis of PIP2 leading to elevation of intracellular Ca²⁺ and activation of PKC. Interestingly, each of these molecules, PIP2, Ca²⁺/calmodulin, and PKC, have been previously shown to regulate GRK2 activity (2). These observations, together with the present study and previous studies of GRK regulation of Gaq-coupled receptors (44–48) suggest that GRK2 and GRK3 may indeed have a specialized role in the regulation of Gaq-coupled signaling. However, achieving a precise understanding of such a role in cells may be impeded by the complexity of all of the possible GRK2 and GRK3 interactions that may take place during activation of Gaq-coupled GPCRs. Another important aspect to consider is what effect, if any, Gaq/11 binding may have on GRK2 and GRK3 activity and/or cellular localization. Binding of Gaq13 to p115 Rh-GEF has been shown to enhance guanine nucleotide exchange factor activity toward RhoA, demonstrating the ability of Gaq to regulate RGS domain-containing proteins as effectors (21). The high affinity binding of GRK2 and GRK3 to activated Gaq may be explained, at least in part, by direct interaction of GRK2 and GRK3 and co-workers (51) have demonstrated that a pool of GRK2 minus of the GRK family in general may have a three-dimensional topology that is related to RGS domains with particular respect to hydrophobic core residues (Ref. 17, data not shown). Thus, we speculate that the N terminus of GRKs we identified and characterized a selective, high affinity and activation-dependent binding interaction between GRK2 and Gaq/11. Our data suggest that, in addition to their well characterized role in desensitization of GPCRs, GRKs may also regulate signaling at the level of the Gaq protein directly. As further investigation of GRK/Gaq interactions proceed it will be important to determine whether Gaq binding exerts any regulatory effects on GRKs possibly directing previously unidentified functions for these kinases, an area which we are currently investigating. As well, the possibility that other members of the GRK family may also exhibit selective binding to Gaq proteins remains to be explored.

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