Differential Interaction of GRK2 with Members of the $G\alpha_q$ Family†

Peter W. Day,‡ Christopher V. Carman,‡,§ Rachel Sterne-Marr,|| Jeffrey L. Benovic,‡ and Philip B. Wedegaertner*,‡

Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Philadelphia, Pennsylvania 19107, and Biology Department, Siena College, 123 Morrell Science Center, 515 Loudon Road, Loudonville, New York 12211

Received March 18, 2003; Revised Manuscript Received June 6, 2003

ABSTRACT: Regulators of G protein signaling (RGS) proteins bind to active Gα subunits and accelerate the rate of GTP hydrolysis and/or block interaction with effector molecules, thereby decreasing signal duration and strength. RGS proteins are defined by the presence of a conserved 120-residue region termed the RGS domain. Recently, it was shown that the G protein-coupled receptor kinase 2 (GRK2) contains an RGS domain that binds to the active form of $G\alpha_q$. Here, the ability of GRK2 to interact with other members of the $G\alpha_q$ family, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$, was tested. The signaling of all members of the $G\alpha_q$ family, with the exception of $G\alpha_{16}$, was inhibited by GRK2. Immunoprecipitation of full-length GRK2 or pull down of GST-GRK2-(45−178) resulted in the detection of $G\alpha_q$, but not $G\alpha_{16}$, in an activation-dependent manner. Moreover, activated $G\alpha_{16}$ failed to promote plasma membrane (PM) recruitment of a GRK2-(45−178)-GFP fusion protein. Assays with chimeric $G\alpha_{q-16}$ subunits indicated that the C-terminus of $G\alpha_q$ mediates binding to GRK2. Despite showing no interaction with GRK2, $G\alpha_{16}$ does interact with RGS2, in both inositol phosphate and PM recruitment assays. Thus, GRK2 is the first identified RGS protein that discriminates between members of the $G\alpha_q$ family, while another RGS protein, RGS2, binds to both $G\alpha_q$ and $G\alpha_{16}$.

The G_q family of heterotrimeric G protein α subunits includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$, all of which link G protein-coupled receptors (GPCRs)[1] to the β isoforms of phospholipase C (PLCβ 1−4) (*1*−*3*). Activated PLCβ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) and produces inositol trisphosphate (IP_3) and diacylglycerol (DAG), leading to activation of downstream effector molecules. The amplification of this signal is limited by return of the Gα subunit to the inactive GDP-bound form by an intrinsic GTPase activity. The rate of GTP hydrolysis by the Gα subunit is increased by GTPase activating proteins (GAPs), including effector molecules, such as PLCβ (*4*), and a family of proteins termed regulators of G protein signaling (RGS) (*5*). The ubiquitously expressed RGS proteins are defined by a conserved 120-residue RGS domain that is responsible for binding to and stabilizing the transition state of Gα-catalyzed GTP hydrolysis (*5*). RGS proteins interact predominantly with $G\alpha_q$ or $G\alpha_i$ families of G proteins. However, in most cases complete characterization of the selectivity among the members of these families does not exist.

A region within the amino terminus of the G protein-coupled receptor kinase (GRK) family of proteins was initially identified by weak sequence identity as a potential RGS domain (*6*), and, subsequently, it was demonstrated that the RGS domain of GRK2 binds to active $G\alpha_{q/11}$, but not active $G\alpha_s$, $G\alpha_{12}$, or $G\alpha_i$ (*7*−*9*). The GRK2-RGS domain binds either GTPγS or AlF_4^-·GDP-bound $G\alpha_q$ (*7*), whereas most RGS proteins exhibit a strong preference for the transition state mimic, AlF_4^-·GDP, form of Gα (*10*−*12*). Binding of the RGS domain of GRK2 to $G\alpha_q$ minimally increases the rate of GTP hydrolysis in the presence of receptor, in contrast to most RGS proteins that have significant GAP activity (*7*). In addition, it has recently been shown that $G\alpha_q$ interacts with a novel surface of the GRK2 RGS domain, termed the "C" site (*13*). This binding site is located on the C-terminus of the α5 helix of the RGS domain distinct from previously identified RGS-Gα interaction sites and thus predicts unique characteristics for the GRK2-$G\alpha_q$ interaction (*13*). These observations in combination with the less conserved RGS domain of the GRKs place them in the category of RGS-like (RGL) proteins with PLCβ, D-AKAP2, and p115RhoGEF (*5*).

To our knowledge, there are no data addressing whether RGS proteins bind selectively to different members of the $G\alpha_q$ family. With this in mind, we decided to investigate whether GRK2 interacts with the remaining $G\alpha_q$ family members, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$, that share 88%, 77%, and 56% sequence identity, respectively, with $G\alpha_q$. This report

† This work was supported by a fellowship from the American Heart Association Pennsylvania−Delaware Affiliate and NRSA Award T32-CA09662 to P.W.D., National Science Foundation Grant MCB9728179 and an American Heart Association Southeastern Pennsylvania Affiliate Beginning Grant-in-Aid to R.S.-M., NIH Grants GM44944 and GM47417 to J.L.B., and NIH Grant GM628884 to P.B.W.

* Corresponding author. Tel: 215-503-3137. Fax: 215-923-2117. E-mail: P_Wedegaertner@mail.jci.tju.edu.

‡ Thomas Jefferson University.

§ Present address: Center for Blood Research and Departments of Pathology and Anesthesia, Harvard Medical School, Boston, MA 02115.

|| Siena College.

[1] Abbreviations: GPCR, G protein-coupled receptor; PLCβ, phospholipase Cβ; RGS, regulator of G protein signaling; PM, plasma membrane; GRK, GPCR kinase; GAP, GTPase activating protein; AlF_4^-, aluminum fluoride; GFP, green fluorescent protein; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphate.

10.1021/bi034442+ CCC: $25.00
Published on Web 07/11/2003

presents the novel finding that the RGS domain of GRK2 binds to $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$ but fails to interact with $G\alpha_{16}$. In contrast, we demonstrate that another RGS protein, RGS2, interacts with both $G\alpha_q$ and $G\alpha_{16}$. Furthermore, using chimeric $G\alpha_q$-$G\alpha_{16}$ proteins, we determined that selectivity for interacting with GRK2 resides within the C-terminal portion of $G\alpha_q$, a region that encompasses the crucial switch regions.

EXPERIMENTAL PROCEDURES

Materials. myo-[^{3}H]Inositol was obtained from Amersham Pharmacia Biotech. Ultima Flo AF and Ultima Gold scintillation cocktails were from Packard Chemical. FuGENE 6 transfection reagent was from Roche Molecular Biochemicals. HEK-293 cells were from American Type Culture Collection (CRL-1573). 9E10 mouse monoclonal antibody was from Covance, and the EE mouse monoclonal antibody was a gift from Henry Bourne. GRK2 mouse monoclonal antibody was from Upstate Biotechnology. HRP-conjugated anti-mouse secondary antibody was from Promega. Super Signal West Pico ECL reagents were from Pierce. Cell culture media were from Cellgro. All other chemicals and reagents were from Sigma Chemical Co. and Fisher Scientific.

Expression Plasmids. $G\alpha_{11}$, $G\alpha_{11}$Q209L, $G\alpha_{14}$, $G\alpha_{16}$, and $G\alpha_{16}$Q212L were gifts from Dr. Mel Simon. EE-tagged $G\alpha_q$ was from Drs. Paul Wilson and Henry Bourne. In $G\alpha_q$ and $G\alpha_{14}$ a glutamine, at position 209 or 205, respectively, was mutated to leucine to inactivate the GTPase activity of the protein. To introduce the internal EE epitope tag into the remaining $G\alpha_q$ family members, $G\alpha_{11}$ residues GYLPTQ (171−176), $G\alpha_{14}$ residues SFVPTQ (167−172), and $G\alpha_{16}$ residues GYVPTA (174−179) were mutated to EYMPTE (*14*). The $G\alpha_{q-16}$ and $G\alpha_{16-q}$ chimeras were created using the sequential PCR method (*15*). All Gα subunits were subcloned into pcDNA3, and mutations were confirmed by sequencing. Kozak-RGS2-myc3-GFP was provided by Drs. Scott Heximer and Ken Blumer. GRK2 constructs have been described previously (*7*, *13*).

Cell Culture and Transfection. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 100 units/mL penicillin−streptomycin at 37 °C in 5% CO_2. Cells were transfected with either 1 μg of DNA in a 6-well plate or 3 μg of DNA in a 6 cm plate using FuGENE transfection reagent according to the manufacturer's instructions.

Inositol Phosphate Production Assay. HEK-293 cells were transiently transfected in 6-well plates with the indicated constructs. Twenty-four hours after transfection cells were replated on 24-well plates and labeled for 16 h with 2 μCi/mL [^{3}H]inositol. Inositol phosphate production was determined as previously described (*13*). In assays that measured receptor-mediated stimulation of PLCβ, cells were washed once with 25 mM HEPES-buffered DMEM containing 50 mM LiCl and then treated with 10 μM α_2AR agonist UK 14304 in 25 mM HEPES-buffered DMEM containing 50 mM LiCl for 1 h. Results are the average of three replicates and are represented as [^{3}H]inositol phosphate/([^{3}H]inositol + [^{3}H]inositol phosphate) × 10^3 ± SD cpm. Statistical significance was assessed using a paired *t*-test.

Immunoprecipitation and GST-GRK2 Interaction Assays. HEK-293 cells in 6 cm plates were transfected with the indicated constructs. After transfection (24−36 h) cells were washed with cold PBS and lysed on ice with 0.3 mL of lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 5 mM $MgCl_2$, 0.7% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 5 μg/mL leupeptin and aprotinin). After 1 h of lysis at 4 °C, cells were centrifuged for 3 min at full speed in a microcentrifuge. Two hundred fifty microliters of the supernatant was removed and split into two tubes. Twenty-five microliters of the remaining supernatant was removed from the original tube, and a fraction, indicated by S, was subsequently run alongside the immunoprecipitation or GST pull-down samples. To one of the tubes containing 125 μL of the supernatant were added $AlCl_3$ (25 μM) and NaF (5 mM). Immunoprecipitations were performed with 3 μL of a GRK2-specific polyclonal antibody, as described (*7*), per tube and 20 μL of protein A/G agarose-conjugated beads at 4 °C for 2 h. GST pull-down experiments were performed with 8 μg of GST-GRK2-(45−178) (*13*) immobilized on glutathione−agarose per tube for 1 h at 4 °C. Proteins were then eluted in 50 μL of SDS sample buffer and boiled for 5 min. Samples were then subjected to 12% SDS−PAGE and transferred to PVDF, which was probed with 2 μg/mL EE or 0.5 μg/mL GRK2-specific monoclonal antibodies followed by horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution). Pierce ECL reagents were used to visualize immunoblots.

Confocal Microscopy. HEK-293 cells were transfected in 6-well plates with 0.7 μg of EE-tagged Gα subunits together with 0.02 μg of GRK2-(45−178)-GFP, 0.2 μg of Gβ, and 0.1 μg of Gγ using FuGENE 6 reagent. Alternatively, HEK-293 cells were transfected with 0.25 μg of RGS2-GFP, 0.45 μg of EE-tagged Gα subunits, 0.2 μg of Gβ, and 0.1 μg of Gγ using FuGENE 6 reagent. Twenty-four hours after transfection cells were split onto coverslips and then grown for an additional 24 h before fixation. Cells were fixed with 3.7% formaldehyde for 20 min, then quenched with 50 mM ammonium chloride, and finally washed three additional times with PBS before secondary fixation with methanol at −20 °C for 4 min. This fixation protocol is necessary to reduce the high background associated with the EE monoclonal antibody. Cells were washed with PBS and then incubated in blocking buffer consisting of TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 1% Triton X-100 and 2.5% nonfat milk. Coverslips were then incubated in blocking buffer containing 20 μg/mL EE monoclonal antibody for 1 h. Following washes with blocking buffer, cells were incubated in a 1:100 dilution of Alexa Fluor 594 goat anti-mouse (Molecular Probes) secondary antibody for 30 min. The coverslips were washed and mounted on glass slides with Prolong Antifade reagent (Molecular Probes). Representative images were recorded by confocal microscopy at the Kimmel Cancer Center Bioimaging Facility using a Bio-Rad MRC-600 laser scanning confocal microscope running CoMos 7.0a software and interfaced to a Zeiss Axiovert 100 microscope with Zeiss Plan-Apo 63 × 1.40 NA oil immersion objective. Dual-labeled samples were analyzed using simultaneous excitation at 488 and 568 nm. Images of "*x*−*y*" sections through the middle of a cell were recorded. Images were processed with Adobe Photoshop.

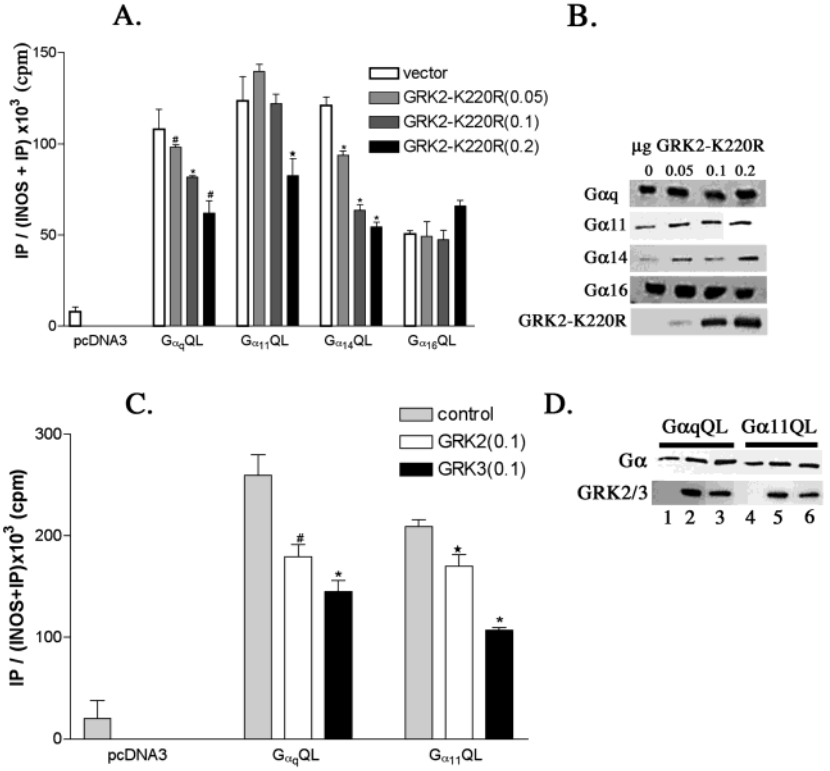

FIGURE 1: Effect of GRK2 expression on $G\alpha_q$ family stimulated inositol phosphate production. (A) HEK-293 cells were cotransfected with EE tagged constitutively active $G\alpha_qQL$, $G\alpha_{11}QL$, $G\alpha_{14}QL$, or $G\alpha_{16}QL$ and increasing amounts of GRK2-K220R, a kinase-deficient mutant. 24 h after transfection the cells were labeled with 2 μCi/mL *myo*-[^{3}H]inositol. The next day total inositol phosphate production was determined, as described under Experimental Procedures. The results shown are from a single experiment representative of at least three experiments each performed in triplicate and displayed as the mean cpm ± SD. In (A), the statistical significance of the difference between the indicated bar and the control bar (Gα in the absence of any GRK2-K220R) is denoted by ★ ($p < 0.05$), # ($p < 0.01$), or * ($p < 0.005$). (B) Western blots of total cellular lysates from inositol phosphate assay shown in (A) probed with the EE monoclonal antibody showing that GRK2-K220R does not affect Gα expression and a western blot of total cellular lysates probed with a GRK2-specific monoclonal antibody showing increased expression of GRK2-K220R as a result of increasing the amount of GRK2-K220R cDNA transfected. (C) HEK-293 cells were cotransfected with 0.05 μg of EE-tagged constitutively active $G\alpha_qQL$ or $G\alpha_{11}QL$ and 0.1 μg of either GRK2 or GRK3 and empty vector to a total of 1.0 μg of DNA, and inositol phosphate assays were performed as in (A). The results shown are from a single experiment representative of at least three experiments each performed in triplicate and displayed as the mean cpm ± SD. In (C), the statistical significance of the difference between the indicated bar and the control bar (Gα in the absence of any exogenously expressed GRK2 or GRK3) is denoted by ★ ($p < 0.05$), # ($p < 0.01$), or * ($p < 0.005$). (D) Western blot of total cellular lysates from the inositol phosphate assay shown in (C) probed with the EE monoclonal antibody showing that GRK2 and GRK3 do not affect $G\alpha_qQL$ (lanes 1−3) or $G\alpha_{11}QL$ (lanes 4−6) expression and a western blot of total cellular lysates probed with a monoclonal antibody that recognizes both GRK2 and GRK3, showing equal expression of GRK2 (lanes 2 and 5) and GRK3 (lanes 3 and 6).

RESULTS

GRK2 Inhibits Signaling by All Members of the $G\alpha_q$ Family with the Exception of $G\alpha_{16}$. All members of the $G\alpha_q$ family when active stimulate the PLCβ isoforms, leading to the production of IP_3 and DAG. Previously, it was shown that GRK2 inhibits $G\alpha_q$-mediated production of inositol phosphate, likely by binding to $G\alpha_q$·GTP and preventing its interaction with PLCβ (*7*). Here, inositol phosphate assays were used to explore the possibility that GRK2 could interfere with the signaling of the remaining members of the $G\alpha_q$ family. HEK-293 cells were transfected with constitutively active forms of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, or $G\alpha_{16}$, in which a conserved glutamine is mutated to leucine (*16*, *17*). HEK-293 cells were also transfected with increasing amounts of GRK2-K220R cDNA, a catalytically inactive mutant (*18*), and production of inositol phosphate was measured. Although it has previously been shown that $G\alpha_q$ is not a substrate for GRK2 phosphorylation, the kinase-deficient mutant of GRK2 was used in all of the inositol phosphate assays shown to avoid any potential kinase-dependent signaling effects (*7*). Expression of GRK2-K220R led to dose-dependent inhibition of $G\alpha_qQL$-, $G\alpha_{11}QL$-, and $G\alpha_{14}QL$-stimulated inositol phosphate production (Figure 1A). Although GRK2-K220R inhibited signaling by $G\alpha_qQL$, $G\alpha_{11}QL$, and $G\alpha_{14}QL$, even the highest amount of GRK2-K220R cDNA transfected had no effect on the $G\alpha_{16}QL$-stimulated signal (Figure 1A). The lower level of $G\alpha_{16}QL$-stimulated inositol phosphate production seen in Figure 1A is consistent and could be due to a reduced ability of $G\alpha_{16}QL$ to stimulate the endogenous PLCβ isoform present in these HEK-293 cells. Western blotting of total cellular lysates suggests that PLCβ1 is the primary isoform expressed in these cells. A previous report indicates that $G\alpha_{16}$ may have a reduced ability to activate PLCβ1, when compared to $G\alpha_q$ and $G\alpha_{11}$ (*19*). Importantly, even this lower level of $G\alpha_{16}QL$-stimulated inositol phosphate production is inhibited by expression of RGS2, as described below (Figure 3A). Figure 1B is a western blot showing that increasing the amount of GRK2-K220R cDNA transfected does not affect the expression level of the Gα subunits. Thus, the decreased production of inositol phosphate, seen in Figure 1A, is most likely due to GRK2-K220R blocking the interaction between the active Gα subunit and PLCβ, not

by decreasing the amount of Gα expressed. This ability to discriminate between $G\alpha_q$ and $G\alpha_{16}$, as measured by inhibition of inositol phosphate signaling, was retained by the core RGS domain (residues 45−178) of GRK2 (data not shown).

GRK2 is able to bind both $G\alpha_q$ and $G\alpha_{11}$ in bovine brain extracts (*7*); however, GRK2 inhibits $G\alpha_q$QL-stimulated inositol phosphate production to 69% of control while signaling stimulated by $G\alpha_{11}$QL is 81% of control (Figure 1C). In contrast, GRK3 inhibits signaling from $G\alpha_q$QL (55% of control) and $G\alpha_{11}$QL (51% of control) equally and is a more potent inhibitor of $G\alpha_{11}$QL signaling compared to GRK2 (Figure 1C). Western blotting suggests that $G\alpha_q$QL and $G\alpha_{11}$QL are overexpressed to similar levels, as are GRK2 and GRK3 (Figure 1D).

GRK2 Blocks Signaling from a Chimeric Gα Subunit Containing the C-Terminus of $G\alpha_q$. Both $G\alpha_q$ and $G\alpha_{16}$ stimulate PLCβ activity (*20*); however, only the $G\alpha_q$-stimulated activity is inhibited by GRK2 (Figure 1). In an attempt to localize the region(s) of $G\alpha_q$ that bind GRK2, $G\alpha_q$-$G\alpha_{16}$ chimeras were constructed and tested for the ability to stimulate IP_3 production and functionally interact with GRK2. Reciprocal chimeras were created. $G\alpha_{q-16}$ consists of the amino-terminal 171 amino acids of $G_{\alpha q}$ and the carboxy-terminal 199 amino acids of $G\alpha_{16}$, and $G\alpha_{16-q}$ is composed of the amino-terminal 174 amino acids of $G\alpha_{16}$ and the carboxy-terminal 187 amino acids of $G\alpha_q$ (Figure 2A). The union of the chimera is located on a loop between α helices αE and αF in the helical domain that is just N-terminal to the switch I region (*21*). This region was chosen as the chimeric union to determine if GRK2 has a binding preference for either the helical or GTPase domain of $G\alpha_q$. This region also coincides with the beginning of an internal EE epitope that has been shown not to affect the ability of $G\alpha_q$ to activate PLCβ (*22*).

The activating QL mutation was introduced into each chimera, and inositol phosphate production was assayed. Both $G\alpha_{q-16}$QL and $G\alpha_{16-q}$QL constitutively stimulated inositol phosphate production in cells (Figure 2B), indicating that the chimeras retained signaling function. Importantly, $G\alpha_{16-q}$QL signaling was inhibited by expression of GRK2, as was signaling by $G\alpha_q$QL (Figure 2B). Although in the experiment presented in Figure 2B $G\alpha_{16-q}$QL displays a substantially higher level of signaling in the absence of GRK2 compared to signaling by $G\alpha_{16}$QL, GRK2 is able to inhibit signaling by $G\alpha_{16-q}$QL even when $G\alpha_{16-q}$QL's basal signaling is lower and similar to $G\alpha_{16}$QL. In contrast, $G\alpha_{q-16}$QL, like $G\alpha_{16}$QL, was refractory to inhibition by GRK2 (Figure 2B).

It is possible that the QL mutation itself somehow affects the ability of $G\alpha_{q-16}$QL and $G\alpha_{16}$QL to interact with GRK2. For this reason we wanted to examine the ability of GRK2 to inhibit receptor-activated signaling from $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{q-16}$, and $G\alpha_{16-q}$. In HEK-293 cells a transfected α_{2A} adrenergic receptor (α_2AR), normally $G\alpha_i$ coupled, is only able to stimulate the production of inositol phosphates when $G\alpha_q$ is also transfected (*23*). $G\alpha_{16}$ is able to couple a wide variety of receptors, including the α_2AR (*24*), to PLCβ activation in cotransfection systems (*2*). The dependence of signal generation on cotransfection of the receptor and G protein allows examination of the signaling properties of mutant or chimeric G proteins in the absence of interference from endogenous $G\alpha_q$ (*23*). $G\alpha_{16}$, $G\alpha_{q-16}$, and $G\alpha_{16-q}$, like

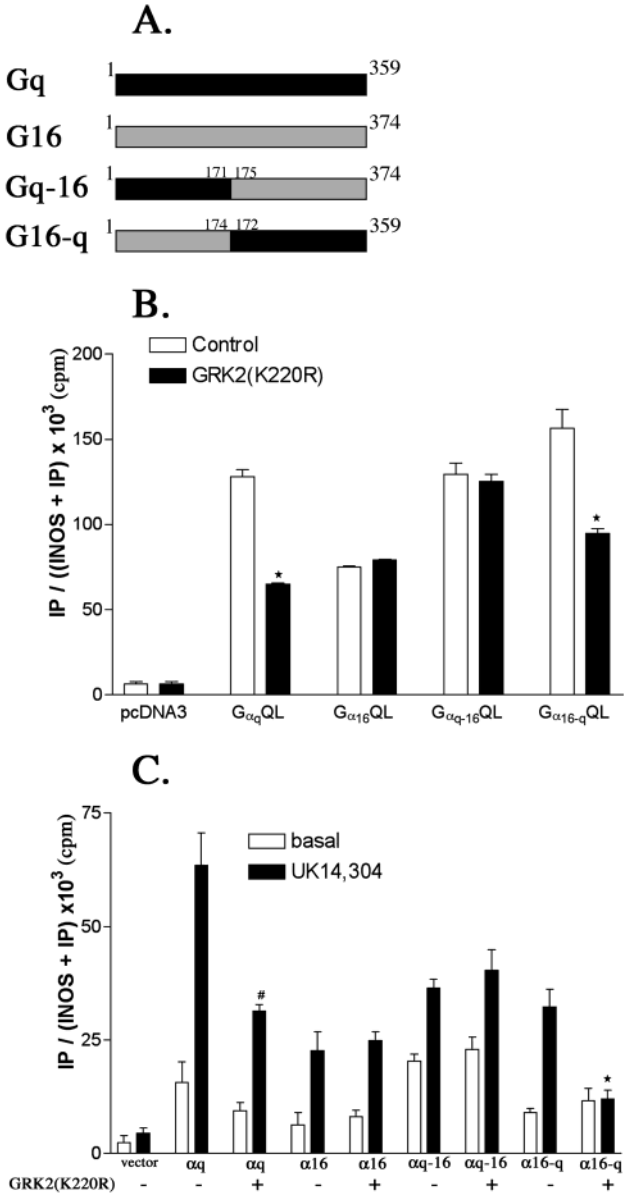

FIGURE 2: Effect of GRK2 expression on the ability of chimeric Gα subunits to stimulate inositol phosphate production. (A) Diagrammatic representations of $G\alpha_q$-$G\alpha_{16}$ chimeras. $G\alpha_q$ and segments of $G\alpha_q$ are represented in black while $G\alpha_{16}$ and segments of $G\alpha_{16}$ are represented in gray. The chimeras were constructed so that the union was between the amino-terminal helical domain and the carboxy-terminal GTPase domain and coincided with the beginning of the internal EE epitope tag. (B) HEK-293 cells were cotransfected with 0.1 μg of the constitutively active $G\alpha_q$QL, $G\alpha_{q-16}$QL, $G\alpha_{16-q}$QL, or $G\alpha_{16}$QL and 0.2 μg of GRK2-K220R and empty vector to a total of 1 μg of DNA. 24 h after transfection the cells were labeled with 2 μCi/mL *myo*-[^{3}H]inositol. The next day total inositol phosphate production was determined, as described under Experimental Procedures. Shown is a single experiment done in triplicate displayed as the mean cpm ± SD that is representative of at least three experiments. The statistical significance of the difference between the indicated bar and the control bar (Gα in the absence of any GRK2-K220R) is denoted by ★ ($p < 0.001$). (C) HEK-293 cells were transfected with 0.4 μg of α_2AR and 0.4 μg of the indicated Gα subunit with or without 0.2 μg of GRK2-K220R. Cells were treated with 10 μM α_2AR agonist, UK 14304, for 1 h, and assays were performed as before. The results from a single experiment are shown and are representative of at least three experiments performed in triplicate and displayed as the mean cpm ± SD. The statistical significance of the difference between the indicated bar and the control bar (Gα in the absence of any GRK2-K220R) is denoted by ★ ($p < 0.005$) or # ($p < 0.001$).

$G\alpha_q$, were all able to stimulate the production of inositol phosphate in response to agonist, UK 14304, when cotransfected with the α_2AR (Figure 2C). When GRK2 is introduced into this system, it only inhibits inositol phosphate production stimulated by $G\alpha_q$ and $G\alpha_{16-q}$ while having no effect on signaling by $G\alpha_{16}$ and $G\alpha_{q-16}$ (Figure 2C). The $G\alpha_{q-16}$ chimera showed an elevated level of activity in the absence of agonist that was increased approximately 2-fold by addition of agonist. Importantly, neither the basal- nor the agonist-induced activities of $G\alpha_{q-16}$ were affected by the expression of GRK2-K220R (Figure 2C). The results of inositol phosphate assays utilizing chimeric $G\alpha_{16-q}$ and $G\alpha_{q-16}$ (Figure 2B,C) suggest that the primary determinants that allow GRK2 to discriminate between $G\alpha_q$ and $G\alpha_{16}$ are found in the C-terminal half of $G\alpha_q$ and/or $G\alpha_{16}$.

RGS2 Interacts with both $G\alpha_q$ and $G\alpha_{16}$. The failure of $G\alpha_{16}$ or the chimera, $G\alpha_{q-16}$, to interact with GRK2 could be due to a general defect in the ability of $G\alpha_{16}$ to interact with RGS proteins. In fact, to our knowledge interaction between $G\alpha_{16}$ and any RGS protein has not been previously reported. RGS2, a $G\alpha_q$-specific RGS protein that has been shown to bind to the GTP-bound form of $G\alpha_q$, was used to examine the capability of $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{q-16}$, and $G\alpha_{16-q}$ to interact with a typical RGS protein (*25*, *26*). HEK-293 cells were transfected with one of the constitutively active forms of $G\alpha_q$, $G\alpha_{16}$, or the chimeras along with increasing amounts of RGS2 cDNA. For all four of the G proteins, increasing the amount of RGS2 transfected led to increased inhibition of inositol phosphate production (Figure 3A). The results with RGS2 are in contrast to the results with GRK2 in which only signals from $G\alpha_qQL$ and $G\alpha_{16-q}QL$ are inhibited. Therefore, either $G\alpha_q$ contains residues that promote binding to the RGS domain of GRK2 or $G\alpha_{16}$ may contain amino acid sequences that specifically inhibit interaction with the RGS domain of GRK2.

Figure 3B is a western blot showing that increasing the amount of RGS2 cDNA transfected does not affect the expression of the Gα subunits. This is an important point because in our hands coexpression of any of several different RGS proteins led to a marked decrease in the expression of the QL forms of the Gα subunits. In fact, in this set of experiments coexpression of the Gβ and Gγ subunits was necessary for the stabilization of Gα expression in the presence of RGS2. Coexpression of G$\beta\gamma$ has been used previously to both stabilize Gα subunits and to increase the amount of $G\alpha_{16}$ at the membrane (*27*, *28*). Expressing the G$\beta\gamma$ heterodimer had no effect on the basal level of inositol phosphate production in the absence of active Gα subunits (Figure 3A). Additionally, coexpressing G$\beta\gamma$ had no effect on the inhibition profiles seen with GRK2. In particular, G$\beta\gamma$ did not induce inhibition of $G\alpha_{16}QL$ or $G\alpha_{q-16}QL$ by GRK2 or decrease the inhibition of $G\alpha_qQL$ or $G\alpha_{16-q}QL$ by GRK2 (data not shown).

GRK2 Binds $G\alpha_q$ but Not $G\alpha_{16}$ in an Activation-Dependent Manner. To extend the inositol phosphate assay results, two different assays were utilized to examine the ability of GRK2 to interact with $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{q-16}$, or $G\alpha_{16-q}$ in cell lysates. In Figure 4A, a GST-GRK2-(45−178) fusion protein coupled to agarose beads was used to investigate the interaction between the RGS domain of GRK2 and $G\alpha_q$, $G\alpha_{q-16}$, $G\alpha_{16-q}$, and $G\alpha_{16}$. Lysates were prepared from HEK-293 cells transfected with the Gα subunit indicated in Figure 4A, and

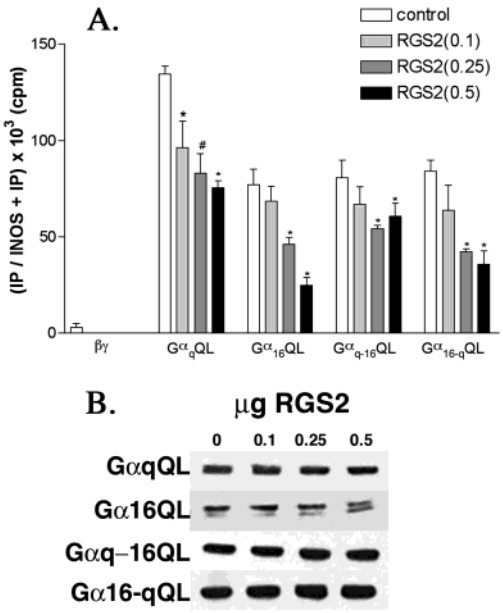

FIGURE 3: Effect of RGS2 expression on Gα-stimulated inositol phosphate production. (A) HEK-293 cells were cotransfected with 0.1 μg of the constitutively active $G\alpha_qQL$, $G\alpha_{q-16}QL$, $G\alpha_{16-q}QL$, or $G\alpha_{16}QL$ and increasing amounts of RGS2 DNA, as indicated. All cells were also transfected with 0.2 μg of the β_1 and 0.1 μg of the γ_2 G protein subunits and empty vector to a total of 1 μg of DNA. 24 h after transfection the cells were labeled with 2 μCi/mL *myo*-[^{3}H]inositol. The next day total inositol phosphate production was determined, as described under Experimental Procedures. The results shown are from a single experiment representative of at least three experiments each performed in triplicate and displayed as the mean cpm ± SD. The statistical significance of the difference between the indicated bar and the control bar (Gα in the absence of any RGS2) is denoted by ★ ($p < 0.05$), # ($p < 0.01$), or * ($p < 0.005$). (B) Western blot of total cellular lysates probed with the EE monoclonal antibody showing that RGS2 does not affect $G\alpha_qQL$, $G\alpha_{q-16}QL$, $G\alpha_{16-q}QL$, or $G\alpha_{16}QL$ expression.

AlF_4^- was added to half of the sample. AlF_4^- binds Gα·GDP and mimics the γ-phosphate of GTP forming GDP·AlF_4^--Gα, a transition state analogue (*29*). The transition state mimicked by GDP·AlF_4^- is thought to be the preferred binding partner of many RGS proteins (*30*), and AlF_4^- is a convenient tool because it allows examination of the same pool of Gα in both the inactive and active state. It has previously been shown that GRK2 binds both GDP·AlF_4^--$G\alpha_q$ and GTPγS-$G\alpha_q$ (*7*). In agreement with this, the GST-GRK2-(45−178) fusion protein bound $G\alpha_q$ only in the presence of AlF_4^- (Figure 4A, $G\alpha_q$ panel, lane 2) or when it contained the activating QL mutation (Figure 4A, $G\alpha_q$ panel, lane 4). In contrast, there was no interaction detected between the GST-GRK2-(45−178) fusion protein and $G\alpha_{16}$, in the absence or presence of AlF_4^- (Figure 4A, $G\alpha_{16}$ panel, lanes 1 and 2) or $G\alpha_{16}QL$ (Figure 4A, $G\alpha_{16}$ panel, lane 4). Wild-type $G\alpha_{q-16}$ (Figure 4A, $G\alpha_{q-16}$ panel, lanes 1 and 2) and $G\alpha_{q-16}QL$ (Figure 4A, $G\alpha_{q-16}$ panel, lane 4), like wild-type $G\alpha_{16}$ or $G\alpha_{16}QL$, did not interact with GST-GRK2-(45−178). In contrast, $G\alpha_{16-q}$ interacted with GST-GRK2-(45−178) in a manner essentially indistinguishable from $G\alpha_q$. Figure 4A shows that $G\alpha_{16-q}$ was only detected in samples to which AlF_4^- was added (Figure 4A, $G\alpha_{16-q}$ panel, lane 2) or when it was in the GTP-bound state, as represented by $G\alpha_{16-q}QL$ (Figure 4A, $G\alpha_{16-q}$ panel, lane 4).

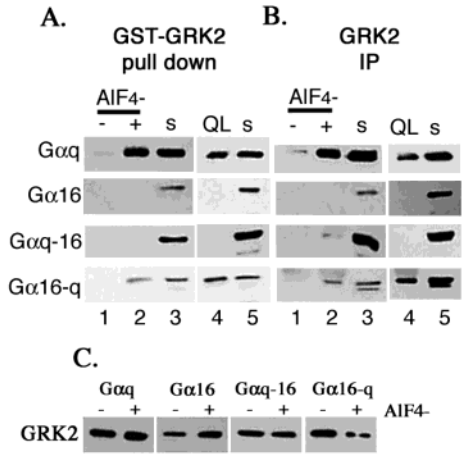

FIGURE 4: Interaction of GRK2 or the RGS domain of GRK2 with Gα subunits. (A) HEK-293 cells were transfected with 3.0 μg of the indicated Gα subunit alone. Cells were lysed, and binding to GST-GRK2-(45−178) in the presence (+) (lane 2) or absence (−) (lanes 1 and 4) of AlF_4^- was determined as described in Experimental Procedures. (B) HEK-293 cells were cotransfected with 0.3 μg of GRK2 and 2.7 μg of $G\alpha_q$, $G\alpha_q$QL, $G\alpha_{16}$, $G\alpha_{16}$QL, $G\alpha_{q-16}$, $G\alpha_{q-16}$QL, $G\alpha_{16-q}$, or $G\alpha_{16-q}$QL. 24−36 h after transfection the cells were lysed, and GRK2 was immunoprecipitated in the presence (+) (lane 2) or absence (−) (lanes 1 and 4) of AlF_4^- with a GRK2-specific polyclonal antibody. Samples were subjected to an EE immunoblot as described in Experimental Procedures. The lower band observed in some of the cell lysates, particularly in panel $G\alpha_{16-q}$ (panel B, lanes 3 and 5) and to a lesser extent in panel $G\alpha_{q-16}$ (panel A, lane 5, and panel B, lane 3), represents a background band variably detected by the EE antibody, as described previously (*47*). (C) Immunoblot with a GRK2-specific monoclonal antibody showing equal immunoprecipitation of GRK2 under the conditions described in (B). Lanes marked S in (A) and (B) were loaded with the cell lysate supernatant and represent 4% of the total amount of protein available for IP or pull down.

As an additional assay for the interaction between $G\alpha_q$, $G\alpha_{q-16}$, $G\alpha_{16-q}$, or $G\alpha_{16}$ and GRK2, cellular lysates from cells coexpressing full-length GRK2 and the indicated Gα subunit (Figure 4B) were incubated with a GRK2-specific polyclonal antibody in the absence or presence of AlF_4^-. Consistent with previous results (*7*−*9*), $G\alpha_q$ was detected only in lysates that contain AlF_4^- (Figure 4B, $G\alpha_q$ panel, lane 2), whereas the constitutively active mutant $G\alpha_q$QL co-immunoprecipitated with GRK2 in the absence of AlF_4^- (Figure 4B, $G\alpha_q$ panel, lane 4). Similarly, GRK2 was only detected in samples incubated with a $G\alpha_q$ C-terminal-specific antibody when AlF_4^- was present (data not shown). When lysates from cells cotransfected with GRK2 and $G\alpha_{16}$ were subjected to the same conditions, $G\alpha_{16}$ was not detected in the absence or presence of AlF_4^- (Figure 4B, $G\alpha_{16}$ panel, lanes 1 and 2), and $G\alpha_{16}$QL (Figure 4B, $G\alpha_{16}$ panel, lane 4) could not be detected in any GRK2 immunoprecipitation. Similarly, $G\alpha_{q-16}$ did not co-immunoprecipitate with GRK2 in an AlF_4^--dependent (compare Figure 4B, $G\alpha_{q-16}$ panel, lanes 1 and 2) or activating QL mutation-dependent manner (Figure 4B, $G\alpha_{q-16}$ panel, lanes 4). The detection of $G\alpha_{16-q}$ in GRK2 immunoprecipitations was AlF_4^- or $G\alpha_{16-q}$QL dependent (Figure 4B, $G\alpha_{16-q}$ panel, lanes 2 and 4) and consistent with the GST-GRK2-(45−178) pull-down assay (Figure 4A). In both the GST-GRK2-(45−178) pull-down and GRK2 co-immunoprecipitation experiments, a comparison to the amount of expressed Gα in the lysates (Figure 4A,B, lanes 3 and 5) indicated that 10−20% of available $G\alpha_q$ (+AlF_4^-), $G\alpha_{16-q}$ (+AlF_4^-), $G\alpha_q$QL, and $G\alpha_{16-q}$QL

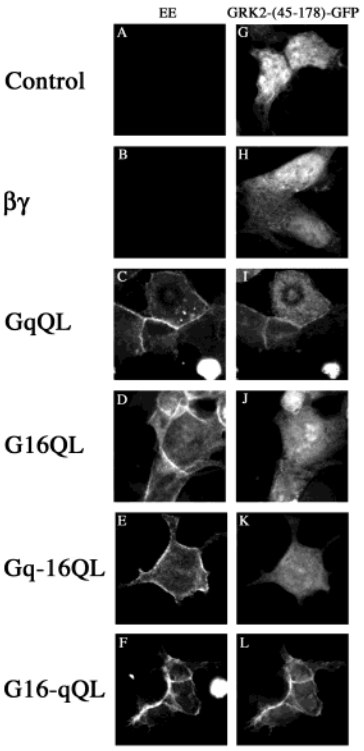

FIGURE 5: Confocal microscopy comparing the subcellular localization of GRK2-(45−178)-GFP in cells cotransfected with activated Gα subunits. HEK-293 cells were transiently transfected with 0.7 μg of the active QL mutants of $G\alpha_q$ (C and I), $G\alpha_{16}$ (D and J), $G\alpha_{q-16}$ (E and K), or $G\alpha_{16-q}$ (F and L) and 0.02 μg of GRK2-(45−178)-GFP. All cells were also transfected with 0.2 μg of the β_1 and 0.1 μg of the γ_2 G protein subunits. Panel G represents the localization of GRK2-(45−178)-GFP alone, while panel H represents the localization of GRK2-(45−178)-GFP in the presence of $\beta_1\gamma_2$. The panels (A−F) in the column labeled EE represent the localization of the Gα subunits as visualized by immunostaining described in Experimental Procedures. The panels (G−L) in the column labeled GRK2-(45−178)-GFP depict the localization of GRK2-(45−178)-GFP. The cells were fixed and stained 48 h after transfection as described in Experimental Procedures. More than 100 cells were examined in at least three independent experiments.

was successfully precipitated. Taken together, the results of the GST-GRK2-(45−178) pull down and the GRK2 co-immunoprecipitation confirm the ability of GRK2 to discriminate between $G\alpha_q$ and $G\alpha_{16}$ and indicate that the determinants for the specific activation-dependent binding of $G\alpha_q$ to GRK2 are located in the C-terminal GTPase domain.

GRK2-(45−178)-GFP Is Recruited to the Plasma Membrane by $G\alpha_q$QL and $G\alpha_{16-q}$QL but Not $G\alpha_{16}$ QL or $G\alpha_{q-16}$QL. Confocal microscopy studies were performed to examine the ability of $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{q-16}$, and $G\alpha_{16-q}$ to recruit GRK2 or RGS2 to the plasma membrane (PM). Recently, we have demonstrated that $G\alpha_q$QL can recruit GRK2-(45−178)-GFP to the plasma membrane (*13*). Panels C−F of Figure 5 show the PM localization of all four G protein α subunits, $G\alpha_q$QL, $G\alpha_{16}$QL, $G\alpha_{q-16}$QL, and $G\alpha_{16-q}$QL. In HEK-293 cells that expressed GRK2-(45−178)-GFP alone (Figure 5G) (*13*) or together with the $G\beta\gamma$ heterodimer (Figure 5H) or the inactive, GDP-bound, G protein α subunits (not shown), GRK2-(45−178)-GFP was distributed throughout the cytoplasm and nucleus. In contrast, when the constitutively active mutants $G\alpha_q$QL and $G\alpha_{16-q}$QL were expressed, GRK2-(45−178)-GFP was partially re-

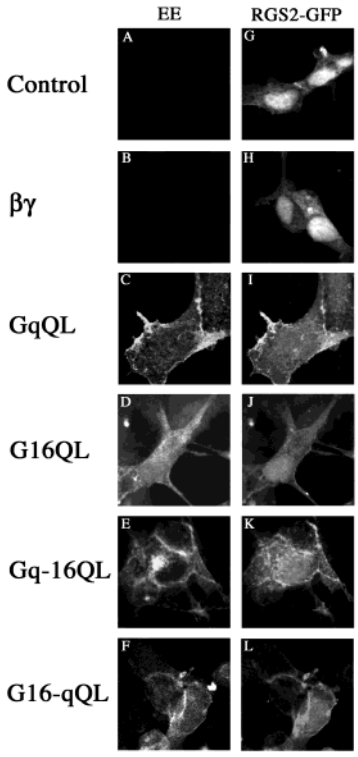

FIGURE 6: Confocal microscopy comparing the subcellular localization of RGS2-GFP in cells cotransfected with activated Gα subunits. Under the column heading RGS2-GFP, HEK-293 cells were transiently transfected with 0.25 μg of RGS2-GFP and 0.45 μg of the active QL mutants of $G\alpha_q$ (H), $G\alpha_{16}$ (I), $G\alpha_{q-16}$ (J), or $G\alpha_{16-q}$ (K). All cells were also transfected with 0.2 μg of the β_1 and 0.1 μg of the γ_2 G protein subunits. Panel G represents the localization of RGS2-GFP alone, while panel H represents the localization of RGS2-GFP in the presence of $\beta_1\gamma_2$. The panels (A−F) in the column labeled EE represent the localization of the Gα subunits as visualized by immunostaining described in Experimental Procedures. The panels (G−L) in the column labeled RGS2-GFP depict the localization of RGS2-GFP. The cells were fixed and stained 48 h after transfection as described in Experimental Procedures. More than 100 cells were examined in at least three independent experiments.

cruited to the PM, as observed by concentration at the cell periphery and at cell−cell contacts (Figure 5I,L). $G\alpha_{16}$QL and the chimera, $G\alpha_{q-16}$QL, that contains the C-terminus of $G\alpha_{16}$ did not interact with GRK2-(45−178)-GFP, as indicated by the retention of cytoplasmic and nuclear distribution of fluorescence and no observable PM localization in cells expressing these constructs (Figure 5J,K).

RGS2-GFP Was Recruited to the PM by $G\alpha_q$QL, $G\alpha_{16}$QL, $G\alpha_{q-16}$QL, and $G\alpha_{16-q}$QL. Localization of RGS2-GFP in the cytoplasm and nucleus of cells cotransfected with wild-type $G\alpha_q$ and at the plasma membrane in the presence of $G\alpha_q$QL has been previously reported (*31*). It is important to show that the Gα constructs that are not able to recruit GRK2-(45−178)-GFP to the PM are able to recruit a typical RGS protein to the plasma membrane. Panels C−F of Figure 6 show the PM localization of all four G protein α subunits, $G\alpha_q$QL, $G\alpha_{16}$QL, $G\alpha_{q-16}$QL, and $G\alpha_{16-q}$QL. The RGS2-GFP fluorescence was seen throughout the cytoplasm and strongly in the nucleus when it was transfected alone (Figure 6G) with $G\beta\gamma$ (Figure 6H) or with $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{q-16}$, or $G\alpha_{16-q}$ (data not shown). The GTPase-deficient, QL, forms of $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{q-16}$ and $G\alpha_{16-q}$ (Panels I−L of Figure 6, respectively) were able to recruit RGS2-GFP out of the nucleus and partially to the PM, in agreement with what has been previously reported for $G\alpha_q$QL (*31*). These observations agree with the inositol phosphate assay results and suggest the level of selectivity of the binding of GRK2 to $G\alpha_q$ is distinct among RGS proteins.

DISCUSSION

In this study we present the novel finding that the RGS domain of GRK2 can discriminate between the $G\alpha_q$ family members $G\alpha_q$ and $G\alpha_{16}$. In assays using either constitutively active or receptor-activated G protein α subunits, coexpression of GRK2 had no effect on the ability of $G\alpha_{16}$ to stimulate inositol phosphate production (Figures 1 and 2), although GRK2 did inhibit signaling by $G\alpha_q$ (Figures 1 and 2) (*7*). In addition, we demonstrated that GRK2 can decrease inositol phosphate signaling by the other two $G\alpha_q$ family members, $G\alpha_{11}$ and $G\alpha_{14}$ (Figure 1). The ability of GRK2 to differentiate between $G\alpha_q$ and $G\alpha_{16}$ was confirmed by several additional assays. Activated forms of $G\alpha_q$ but not $G\alpha_{16}$ interacted with GST-GRK2-(45−178) (Figure 4A), immunoprecipitated in a complex with full-length GRK2 (Figure 4B), and were able to promote plasma membrane recruitment of a GFP-tagged GRK2-(45−178) (Figure 5). Moreover, we constructed $G\alpha_q$-$G\alpha_{16}$ chimeras and, using the above cell-based assays, showed that the $G\alpha_q$ versus $G\alpha_{16}$ selectivity of interacting with GRK2 is mediated primarily by the C-terminal half of the G protein α subunit. Last, our results suggest that the $G\alpha_q$ versus $G\alpha_{16}$ selectivity may be unique to GRK2 among RGS domain-containing proteins, since we observed that RGS2, unlike GRK2, can functionally interact with both $G\alpha_q$ and $G\alpha_{16}$.

There are over 20 members of the RGS family of proteins that, with only a few exceptions, bind to members of the $G\alpha_i$ and $G\alpha_q$ families of G-proteins (*5*). Investigations into the selectivity of RGS protein interactions with the $G\alpha_i$ and $G\alpha_q$ families have found varying levels of specificity (*5*). Studies in cells and with purified proteins indicate a degree of promiscuity in the interactions between RGS proteins and the G protein targets (*30*, *32*, *33*). For example, RGS4 has been shown to bind and act as a GAP for both $G\alpha_q$ and $G\alpha_i$ (*30*, *34*). However, there are also studies that show a preference by an RGS protein for $G\alpha_i$ over $G\alpha_q$ family members and vice versa. RGS2 is an example of a mammalian RGS protein that preferentially acts as a GAP on members of the $G\alpha_q$ subfamily due to unique sequence characteristics of both RGS2 and $G\alpha_q$ (*26*). In addition, there are reports of RGS proteins, such as RGS4 and GAIP, that are able to differentiate between members of the $G\alpha_i$ family (*35*−*37*). In *Caenorhabditis elegans*, EGL-10 specifically inhibits GOA-1 ($G\alpha_o$), and EAT-16 specifically inhibits EGL-30 ($G\alpha_q$); proper regulation of these two opposing pathways is essential for appropriate egg-laying and locomotion behavior (*38*). GRK2 provides the first example of an RGS protein able to discriminate between $G\alpha_q$ family members. In Figure 1C, GRK3 inhibits signaling stimulated by $G\alpha_{11}$ more effectively than does GRK2. It is possible that in selected tissues or cell types this increased sensitivity of $G\alpha_{11}$ to GRK3 provides a mechanism for targeted inhibition of specific receptors.

The $G\alpha_q$-$G\alpha_{16}$ chimeras used in this study suggest that the selectivity of binding to GRK2 is largely mediated by

the C-terminal switch regions. RGS proteins act as GAPs for G proteins by binding to the switch regions of the Gα subunit and stabilizing the transition state of the molecule (*39*). RGS proteins may also block G protein activation of effector molecules by competitive binding to regions in the Gα necessary for effector activation (*34*). GRK2 displays only very weak GAP activity toward $G\alpha_q$ (*7*), but the ability of GRK2 to bind specifically to AlF_4^-·GDP and GTP-bound forms of $G\alpha_q$ and to inhibit $G\alpha_q$ stimulation of PLCβ suggests the importance of the $G\alpha_q$ switch regions. Consistent with this, only the $G\alpha_{16-q}$ chimera, but not the reciprocal $G\alpha_{q-16}$ chimera, interacted with GRK2, as measured in our studies by inositol phosphate assays, GST pull-down experiments, co-immunoprecipitations, and PM recruitment. A number of amino acid differences exist in switch regions of $G\alpha_q$ and $G\alpha_{16}$, and present work is directed toward further defining the $G\alpha_q$ versus $G\alpha_{16}$ selectivity.

The overall structural similarity found in the Gα family has allowed several groups to use chimeric Gα subunits as tools to identify regions that interact with either receptors, effectors, or RGS proteins (*21*, *40*, *41*). In contrast to results with the $G\alpha_q$-$G\alpha_{16}$ chimeras, a recent study used chimeric $G\alpha_i$-$G\alpha_t$ subunits to demonstrate that the helical domain of $G\alpha_t$, rather than the C-terminal switch regions, mediates the selectivity of interacting with RGS9 (*42*). Our experiments do not rule out the possibility that the $G\alpha_q$ helical domain also contains important determinants for mediating interaction with GRK2.

Although $G\alpha_{16}$ failed to interact with GRK2, our results demonstrate that this specificity does not extend to another RGS protein, RGS2. In contrast to the results with the RGS domain of GRK2, RGS2 coexpression inhibits inositol phosphate production stimulated by both constitutively active Gα subunits, $G\alpha_q$QL and $G\alpha_{16}$QL, as well as the chimeras $G\alpha_{q-16}$QL and $G\alpha_{16-q}$QL (Figure 3). Moreover, all four activated subunits were able to recruit RGS2-GFP to the plasma membrane (Figure 6). We cannot rule out that the in vivo interaction between RGS2-GFP and $G\alpha_{16}$QL in these experiments is a result of overexpression; however, overexpression of any of the GRK2 constructs did not induce a detectable interaction with $G\alpha_{16}$. We have only been able to detect relatively weak pull downs of $G\alpha_{16}$QL and the two chimeras with GST-RGS2 (data not shown). Thus, interaction of RGS2 and $G\alpha_{16}$ may be weaker compared to RGS2 and $G\alpha_q$. The in vivo assays of inositol phosphate accumulation and subcellular redistribution are likely more sensitive than the GST pull down for detecting functional interaction of RGS2 with Gα subunits.

Nonetheless, the ability of RGS2 to inhibit both $G\alpha_q$QL and $G\alpha_{16}$QL and to translocate to the PM in response to both $G\alpha_q$QL and $G\alpha_{16}$QL accentuates the unique specificity of the RGS domain of GRK2 for $G\alpha_q$. In addition, these results provide the first evidence for a functional interaction between $G\alpha_{16}$ and any RGS protein. Thus, RGS proteins that are expressed in cells of hematopoietic lineage could compensate for the lack of regulation of $G\alpha_{16}$ signaling by GRK2. The RGS2 interaction with $G\alpha_{16}$ demonstrates that the inability of the GRK2-RGS domain to bind $G\alpha_{16}$ is in fact due to a selectivity of GRK2 for $G\alpha_q$, as opposed to a structural feature of $G\alpha_{16}$ that hinders interaction with RGS proteins in general.

However, several features of $G\alpha_{16}$ set it apart from other Gα subunits. $G\alpha_{16}$ has been labeled a promiscuous G protein due to its ability to couple to a wide variety of receptors, including the β_2 adrenergic and muscarinic M2 receptors, that do not normally couple to members of the $G\alpha_q$ family (*2*, *43*). Perhaps as a way to avoid aberrant signaling from receptors inappropriately coupled to $G\alpha_{16}$, evolution has strictly confined the expression of $G\alpha_{16}$ to pluripotent cells of hematopoietic lineage (*44*). Interestingly, a previous study indicated that $G\alpha_{16}$ may not bind AlF_4^- and assume the conformation of the transition state mimic that is typical of Gα subunits; GTPγS, but not AlF_4^-, allowed $G\alpha_{16}$ to stimulate PLCβ (*28*). In agreement with this, we were unable to pull down $G\alpha_{16}$ with GST-RGS4 in an AlF_4^--dependent manner, although GST-RGS4 effectively precipitated AlF_4^--activated $G\alpha_q$ (data not shown). It has been hypothesized that the inability of $G\alpha_{16}$ to bind AlF_4^- is due to the substitution of a conserved alanine with a proline in an amino-terminal portion of the guanine nucleotide binding domain in $G\alpha_{16}$ (*28*). However, we demonstrated that addition of AlF_4^- to lysates of cells expressing $G\alpha_{16-q}$ allowed this chimera to interact with both GST-GRK2-(45−178) and full-length GRK2 (Figure 4). The $G\alpha_{16-q}$ chimera contains the proline suggested to interfere with AlF_4^- binding, yet binds to GRK2 in an AlF_4^--dependent manner. If $G\alpha_{16}$ does not bind AlF_4^-, then there must be other $G\alpha_{16}$ sequence characteristics that alone or in combination with proline 51 preclude binding of AlF_4^-. Although $G\alpha_{16}$ may simply not bind to AlF_4^- in pull-down and co-immunoprecipitation experiments (Figure 4), $G\alpha_{16}$QL is constitutively active (Figures 1−3) and able to functionally interact with RGS2, yet unable to interact with GRK2. In contrast, $G\alpha_q$QL binds to GRK2 (Figure 4), allowing us to clearly demonstrate a defect in GRK2 binding to the constitutively active $G\alpha_{16}$QL.

Last, the results presented here demonstrating a binding preference for $G\alpha_q$ over $G\alpha_{16}$ by GRK2 provide further evidence of the uniqueness of the GRK2-$G\alpha_q$ interaction compared to other studied RGS-Gα pairs. The RGS domain of GRK2 displays low amino acid identity to other RGS proteins, but a number of the residues in the hydrophobic core are conserved, suggesting that the N-terminus of GRK2 assumes the typical RGS domain conformation (*7*). However, recent mutagenesis studies have defined the $G\alpha_q$ binding site on the GRK2-RGS domain, and these studies predict a unique interaction site on an RGS domain, termed the C site (*13*) that differs from the region used by other RGS proteins to bind Gα subunits. Moreover, mutation of glycine 188 to serine in $G\alpha_q$, a mutation that disrupts interaction with other RGS proteins (*45*, *46*), has no effect on the interaction between $G\alpha_q$ and the GRK2-RGS domain (*13*). The RGS domain of GRK2 also has the ability to bind to two different constitutively active mutants, R183C and Q209L, that are presumably GTP bound (*7*); this is in contrast to RGS4, which specifically binds to the transition state of the Gα subunit, mimicked by GDP·AlF_4^--$G\alpha_q$ (*39*). Identification of specific residues in $G\alpha_q$ responsible for the interaction with GRK2 should help to further elucidate the unique specificity of the interaction.

In conclusion, we have identified the unique selectivity of GRK2 for $G\alpha_q$ over a member of the $G\alpha_q$ family, $G\alpha_{16}$. This selectivity is determined in part by the C-terminal

GTPase domain of $G\alpha_q$. Studies are underway to identify the specific residues that impart the specificity of the GRK2-$G\alpha_q$ interaction. In addition, we have for the first time identified an interaction between an RGS protein, RGS2, and $G\alpha_{16}$. It will be of interest to investigate the possibility that the conserved N-terminal RGS domain of one of the remaining GRK family members interacts with $G\alpha_{16}$ in a manner similar to the GRK2-$G\alpha_q$ interaction.

ACKNOWLEDGMENT

We thank Chris Fischer for excellent technical assistance and Drs. Ray Penn, Scott Heximer, and Mel Simon for providing plasmids.

REFERENCES

1. Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., and Gilman, A. G. (1993) *J. Biol. Chem. 268*, 14367−14375.
2. Offermanns, S., and Simon, M. I. (1995) *J. Biol. Chem. 270*, 15175−15180.
3. Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton, J. H. (1991) *Nature 350*, 516−518.
4. Berstein, G., Blank, J. L., Jhon, D. Y., Exton, J. H., Rhee, S. G., and Ross, E. M. (1992) *Cell 70*, 411−418.
5. Ross, E. M., and Wilkie, T. M. (2000) *Annu. Rev. Biochem. 69*, 795−827.
6. Siderovski, D. P., Hessel, A., Chung, S., Mak, T. W., and Tyers, M. (1996) *Curr. Biol. 6*, 211−212.
7. Carman, C. V., Parent, J. L., Day, P. W., Pronin, A. N., Sternweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) *J. Biol. Chem. 274*, 34483−34492.
8. Sallese, M., Mariggio, S., D'Urbano, E., Iacovelli, L., and De Blasi, A. (2000) *Mol. Pharmacol. 57*, 826−831.
9. Usui, H., Nishiyama, M., Moroi, K., Shibasaki, T., Zhou, J., Ishida, J., Fukamizu, A., Haga, T., Sekiya, S., and Kimura, S. (2000) *Int. J. Mol. Med. 5*, 335−340.
10. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) *J. Biol. Chem. 271*, 27209−27212.
11. Chen, C. K., Wieland, T., and Simon, M. I. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12885−12889.
12. Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) *Nature 383*, 172−175.
13. Sterne-Marr, R., Tesmer, J. J., Day, P. W., Stracquatanio, R. P., Cilente, J. A., O'Connor, K. E., Pronin, A. N., Benovic, J. L., and Wedegaertner, P. B. (2003) *J. Biol. Chem. 278*, 6050−6058.
14. Grussenmeyer, T., Scheidtmann, K. H., Hutchinson, M. A., Eckhart, W., and Walter, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7952−7954.
15. Ausubel, F. M., Brent, R. E., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992) *Short Protocols in Molecular Biology*, 2nd ed., John Wiley & Sons, New York.
16. Graziano, M. P., and Gilman, A. G. (1989) *J. Biol. Chem. 264*, 15475−15482.
17. Qian, N.-X., Winitz, S., and Johnson, G. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4077−4081.
18. Carman, C. V., and Benovic, J. L. (1998) *Curr. Opin. Neurobiol. 8*, 335−344.
19. Lee, C. H., Park, D., Wu, D., Rhee, S. G., and Simon, M. I. (1992) *J. Biol. Chem. 267*, 16044−16047.
20. Rhee, S. G. (2001) *Annu. Rev. Biochem. 70*, 281−312.
21. Medina, R., Grishina, G., Meloni, E. G., Muth, T. R., and Berlot, C. H. (1996) *J. Biol. Chem. 271*, 24720−24727.
22. Wilson, P. T., and Bourne, H. R. (1995) *J. Biol. Chem. 270*, 9667−9675.
23. Conklin, B. R., Chabre, O., Wong, Y. H., Federman, A. D., and Bourne, H. R. (1992) *J. Biol. Chem. 267*, 31−34.
24. Mody, S. M., Ho, M. K., Joshi, S. A., and Wong, Y. H. (2000) *Mol. Pharmacol. 57*, 13−23.
25. Heximer, S. P., Watson, N., Linder, M. E., Blumer, K. J., and Hepler, J. R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14389−14393.
26. Heximer, S. P., Srinivasa, S. P., Bernstein, L. S., Bernard, J. L., Linder, M. E., Hepler, J. R., and Blumer, K. J. (1999) *J. Biol. Chem. 274*, 34253−34259.
27. Posner, B. A., Mixon, M. B., Wall, M. A., Sprang, S. R., and Gilman, A. G. (1998) *J. Biol. Chem. 273*, 21752−21758.
28. Kozasa, T., Hepler, J. R., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., and Gilman, A. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9176−9180.
29. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) *Science 265*, 1405−1412.
30. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) *Cell 86*, 445−452.
31. Heximer, S. P., Lim, H., Bernard, J. L., and Blumer, K. J. (2001) *J. Biol. Chem. 276*, 14195−14203.
32. Diverse Pierluissi, M. A., Fischer, T., Jordan, J. D., Schiff, M., Ortiz, D. F., Farquhar, M. G., and De Vries, L. (1999) *J. Biol. Chem. 274*, 14490−14494.
33. Huang, C., Hepler, J. R., Gilman, A. G., and Mumby, S. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6159−6163.
34. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 428−432.
35. De Vries, L., Mousli, M., Wurmser, A., and Farquhar, M. G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11916−11920.
36. Woulfe, D. S., and Stadel, J. M. (1999) *J. Biol. Chem. 274*, 17718−17724.
37. Cavalli, A., Druey, K. M., and Milligan, G. (2000) *J. Biol. Chem. 275*, 23693−23699.
38. Hajdu Cronin, Y. M., Chen, W. J., Patikoglou, G., Koelle, M. R., and Sternberg, P. W. (1999) *Genes Dev. 13*, 1780−1793.
39. Tesmer, J. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) *Cell 89*, 251−261.
40. Blahos, J., Fischer, T., Brabet, I., Stauffer, D., Rovelli, G., Bockaert, J., and Pin, J. P. (2001) *J. Biol. Chem. 276*, 3262−3269.
41. Venkatakrishnan, G., and Exton, J. H. (1996) *J. Biol. Chem. 271*, 5066−5072.
42. Skiba, N. P., Yang, C. S., Huang, T., Bae, H., and Hamm, H. E. (1999) *J. Biol. Chem. 274*, 8770−8778.
43. Milligan, G., Marshall, F., and Rees, S. (1996) *Trends Pharmacol. Sci. 17*, 235−237.
44. Amatruda, T. T., III, Steele, D. A., Slepak, V. Z., and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5587−5591.
45. DiBello, P. R., Garrison, T. R., Apanovitch, D. M., Hoffman, G., Shuey, D. J., Mason, K., Cockett, M. I., and Dohlman, H. G. (1998) *J. Biol. Chem. 273*, 5780−5784.
46. Lan, K. L., Sarvazyan, N. A., Taussig, R., Mackenzie, R. G., DiBello, P. R., Dohlman, H. G., and Neubig, R. R. (1998) *J. Biol. Chem. 273*, 12794−12797.
47. Wedegaertner, P. B. (2002) *BMC Cell Biol. 3*, 12.

BI034442+