Phosphorylation of RGS14 by Protein Kinase A Potentiates Its Activity toward Gαi†

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ABSTRACT: Regulators of G protein signaling (RGS proteins) modulate Gα-directed signals because of the GTPase activating protein (GAP) activity of their conserved RGS domain. RGS14 and RGS12 are unique among RGS proteins in that they also regulate $G\alpha_i$ signals because of the guanine nucleotide dissociation inhibitor (GDI) activity of a GoLoco motif near their carboxy-termini. Little is known about cellular regulation of RGS proteins, although several are phosphorylated in response to G-protein directed signals. Here we show for the first time the phosphorylation of native and recombinant RGS14 in host cells. Direct stimulation of adenylyl cyclase or introduction of dibutyryl-cAMP induces phosphorylation of RGS14 in cells. This phosphorylation occurs through activation of cAMP-dependent protein kinase (PKA) since phosphate incorporation is completely blocked by a selective inhibitor of PKA but only partially or not at all blocked by inhibitors of other G-protein regulated kinases. We show that purified PKA phosphorylates two specific sites on recombinant RGS14, one of which, threonine 494 (Thr494), is immediately adjacent to the GoLoco motif. Because of this proximity, we focused on the possible effects of PKA phosphorylation on the GDI activity of RGS14. We found that mimicking phosphorylation on Thr494 enhanced the GDI activity of RGS14 toward $G\alpha_i$ nearly 3-fold, with no associated effect on the GAP activity toward either $G\alpha_i$ or $G\alpha_o$. These findings implicate cAMP-induced phosphorylation as an important modulator of RGS14 function since phosphorylation could enhance RGS14 binding to $G\alpha_i$−GDP, thereby limiting $G\alpha_i$ interactions with downstream effector(s) and/or enhancing $G\beta\gamma$-dependent signals.

Heterotrimeric G proteins transduce signals from a wide range of hormone and neurotransmitter receptors at the cell surface to the intracellular environment. Following activation, individual members of the four major classes of G proteins (G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$) interact with well-defined effectors such as phopholipase Cβ, adenylyl cyclase, and a number of ion channels. However, because different receptor subtypes linked to the same G protein elicit distinct profiles of cellular responses, emerging models propose that additional proteins and linked signaling pathways contribute to these responses. The most prominent of these protein-binding partners are the regulators of G-protein signaling (RGS)[1].

The family of RGS proteins was first identified as negative regulators of hormone signaling in genetic models of lower eukaryotes. The family now includes more than 30 distinct mammalian proteins, which can modulate a variety of intracellular signals (for review see refs *1*−*3*). The RGS superfamily is defined by a conserved 130 amino acid RGS domain. Biochemical studies show that this domain binds activated Gα subunits to serve as a GTPase activating protein (GAP), thereby accelerating the rate of Gα GTP hydrolysis (*4*). In addition, RGS proteins can also act as effector antagonists, directly interfering with effector binding (*5*, *6*). RGS domains bind the switch regions of the Gα subunit to stabilize the transition state of Gα during GTP hydrolysis, increasing the rate of Gα inactivation to terminate signals from both Gα and G$\beta\gamma$ subunits (*4*, *7*).

Because of the action of their RGS domain, RGS proteins share a capacity to limit the lifetime of Gα signals. However, many family members also contain additional domains that confer a variety of other signaling functions (*2*, *3*, *8*−*10*). For example, RGS proteins can link GPCRs to transmembrane tyrosine kinases (*11*, *12*), monomeric GTPases (*13*, *14*), or ion channels (*15*, *16*) to affect a range of cellular processes such as growth, differentiation, or membrane excitability. The emerging picture of RGS proteins reveals a highly diverse, multifunctional superfamily of signaling proteins that serve as tightly regulated integrators of G-protein signaling pathways.

RGS14, a member of the R12 subfamily of RGS proteins, regulates the activity of Gα subunits through two domains: the RGS domain, which functions as a nonselective GAP for $G\alpha_i$ and $G\alpha_o$ subunits, and the GoLoco or GPR motif, which selectively inhibits nucleotide exchange on $G\alpha_i$ subunits (*14*, *17*−*20*). GoLoco motifs were only recently

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[1] Abbreviations: RGS, regulators of G protein signaling; GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; PKA, cAMP dependent protein kinase; ATP, adenosine trisphosphate; GTPγS, guanosine 5′-*O*-thiotriphosphate; Ni-NTA, nickel-nitrilotriacetic acid; MAPK, mitogen activated protein kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; db-cAMP, dibutyryl adenosine 3′,5′-cyclic monophosphate.

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recognized as protein domains that bind to heterotrimeric G proteins of the $G\alpha_{i/o}$ subfamily and potently inhibit nucleotide exchange (*21*). While a full understanding of proteins containing these motifs awaits further study, some theories as to their functions have been postulated (*21*). Because GoLoco domains compete with $G\beta\gamma$ subunits for Gα binding (*18*), their presence may enhance $G\beta\gamma$ signals independent of activation of Gα subunits. Some proteins that contain GoLoco motifs are also involved in cell division (*21*), although whether this is true for all GoLoco-containing proteins is unclear. The multifunctional nature of RGS14 as a potent regulator of G-protein signals (i.e., by restricting receptor activation of Gα, increasing $G\beta\gamma$ availability, and limiting the lifetime of the Gα) presents the intriguing possibility that these actions could be differentially regulated.

Several posttranslational modifications regulate the signaling capacity of RGS proteins and may be essential for their in vivo function (*3*). Regulated phosphorylation is of particular interest with regard to RGS proteins since a number of kinases are activated following stimulation of G-protein pathways. Phosphorylation has been shown to affect the capacity of RGS proteins to modulate G-protein signals in a variety of ways. Interaction of RGS proteins with non-Gα binding partners such as 14-3-3 (*22*) or the EGF receptor (*23*) is regulated by phosphorylation. Kinase activity can also affect the stability of RGS proteins (*24*), their interaction with Gα subunits (*22*), or their cellular trafficking (*25*). The phosphorylation of several RGS proteins directly interferes with their capacity to act as GAPs for Gα subunits (*26*, *27*), while this modification actually enhances the GAP activity of others (*28*).

Little is known about the cellular mechanisms that regulate RGS14. As discussed, kinase activation is a major component of G-protein signaling, which can feed back to regulate the interaction of several RGS proteins with their binding partners. The multiple protein interacting domains present in RGS14 could permit fine-tuning of its interactions with Gα subunits at rest or after activation, or with other candidate binding partners such as Rap1/2 (*14*). Our investigations focus on whether phosphorylation by protein kinase A (PKA), which is involved in both $G\alpha_{i/o}$ and Rap1/2 signaling cascades (*29*, *30*), could modulate RGS14 signaling capacity. Here we demonstrate for the first time that both native and recombinant RGS14 are phosphorylated in host cells at rest and following activation of adenylyl cyclase, an enzyme inhibited by $G\alpha_{i/o}$-linked receptors. Phosphorylation of RGS14 in cells is completely blocked by a selective pharmacological inhibitor of PKA but to a much lesser extent or not at all by inhibitors of several other G-protein regulated kinases. In vitro, purified PKA phosphorylates RGS14 at two distinct sites that correspond to consensus PKA phosphorylation sites: Ser258 and Thr494. Thr494 is located immediately adjacent to the GoLoco motif (*19*), which led us to investigate whether modification of this residue affects RGS14 GDI activity. We show that substituting Thr494 with either aspartate or glutamate to mimic phosphorylation enhances RGS14 GDI activity for $G\alpha_i$ almost 3-fold. These mutations do not grossly alter the folding of RGS14, as the GAP activity of the mutant and wild-type proteins toward $G\alpha_i$ and $G\alpha_o$ are nearly identical. These studies provide new insights into the role of kinases in regulating intracellular signals through this class of multifunctional RGS proteins.

MATERIALS AND METHODS

Materials. cDNA encoding H_6-R14-RGS and H_6-R14-R/GL were generated by PCR using primers to the base pairs corresponding to amino acids 1−205 and 299−544, respectively, and cloned into pQE60 as described (*20*). cDNA encoding TxH_6-RGS14 and sera-RGS14 affinity purified polyclonal antisera developed against the full-length RGS14 sequence were a generous gift of D. P. Siderovski (University of North Carolina, Chapel Hill). Affinity purified chicken anti-RGS14 antisera (cRGS14) was raised against full-length purified RGS14 by Aves Labs (Tigard, OR). [^{35}S]GTPγS and [^{32}P]orthophosphate were purchased through Dupont NEN (Boston, MA). B35 neuroblastoma cells were kindly provided by Drs. P. Manness and D. P. Siderovski (University of North Carolina, Chapel Hill).

Immunoblots. Protein samples were denatured, separated by SDS−PAGE, and transferred to nitrocellulose membrane. For sera-RGS14, blots were incubated sequentially with blocking buffer (20 mM Tris, pH 7.6, 140 mM NaCl, 0.1% Tween-20, 5% dry milk), primary antibody in blocking buffer, and horseradish peroxidase (HRP)-conjugated anti-rabbit antibody in TBS 0.1% Tween-20. For cRGS14, blots were incubated sequentially in a 1:10 dilution of Blockhen (Aves Labs) in TBS, primary antibody in TBS 0.1% Tween-20, and HRP conjugated antichicken IgY in 1:10 dilution of Blockhen. Proteins were visualized by chemiluminescence.

Cell Culture. B35 cells were grown in 10% fetal bovine serum containing Dulbecco's Modified Eagle Medium (DMEM) containing 100 μg/mL Gentamycin and Kanamycin. For HA-RGS14 expressing cells, retrovirus was produced as described (*31*). Briefly, Phoenix-ampho producer HEK293 cells were transfected using the $Ca^{2+}PO_4$ method with retroviral plasmid vector pTJ66HA (provided by Dr. T. J. Murphy, Emory University) or the same vector encoding HA-RGS14 in frame. Supernatant was collected every 8 h, and B35 cells were infected by centrifugation. At 48 h post-infection, the media was supplemented with 200 μg/mL Zeocin (Cayla, France). After 10 days, the population was GFP positive, and HA-RGS14 was detected by immunoblot in cell lysates.

Immunoprecipitation. Uninfected B35 cells or HA-RGS14-expressing B35 cells were washed three times with Buffer A (50 mM HEPES, 50 mM NaF, 20 mM Na-pyrophosphate, 20 mM β-mercaptoethanol, and PMSF) and then lysed in Buffer A + 1% Triton-X100 for 30 min at 4 °C. Lysates were centrifuged to remove Triton-X insoluble membranes, and the antibody was added to lysates for 2 h (cRGS14) or overnight (anti-HA). Agarose beads (IgY-agarose for cRGS14 or protein G-agarose for anti-HA, washed three times with Buffer A + 1% Triton-X100) were added to the lysates and rotated at 4 °C for 1 h. The mixture was centrifuged, supernatant removed, and the beads were washed with 30 volumes Buffer A + 1% Triton-X100. Laemmli sample buffer was added, and the samples were heated to remove protein from the beads. Samples were resolved by SDS−PAGE and either coomassie stained and dried, or transferred to nitrocellulose and immunoblotted as described.

In vivo Phosphorylation. Uninfected or HA-RGS14-expressing B35 neuroblastoma cells were grown to confluency in 100-mm cell culture plates. Cells were washed and incubated at 37 °C and 5% CO_2 for 45 min with phosphate-

free DMEM after which 150 μCi [^{32}P]orthophosphate was added. Plates were incubated for 5 h at 37 °C in 5% CO_2 and then treated with H89 (1 μM), bisindolylmaelimide (Bis) (10 μM), PD98059 (10 μM), and KN93 (10 μM) for 30 min and/or forskolin (25 μM) or dibutyryl-cAMP (200 μM) where applicable. After treatment, proteins were immunoprecipitated as described.

In vitro Phosphorylation. For each reaction, 2 μg of protein was incubated at 30 °C in Buffer A (50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, 100 μM ATP + 2000 cpm/pmol [^{32}P]-ATP) with or without five units of the catalytic subunit of PKA (PKA_{cat}) (Calbiochem). Sample buffer was added, and proteins were subjected to SDS−PAGE, stained with coomassie, dried, and exposed to film. To quantify the stoichiometry of phosphorylation, 2 μg of RGS14 was incubated in Buffer A in the presence of PKA_{cat}. In separate assays, buffer pH and PKA_{cat} concentration were varied to optimize stoichiometric incorporation of phosphate. Reactions were incubated for varying times (0 min to 4 h), terminated, and samples were subjected to SDS−PAGE. The gels were stained with coomassie, dried, and exposed to film, after which the bands corresponding to RGS14 were excised, solubilized in scintillation fluid, and incorporated [^{32}P] was counted in a scintillation counter. After the results were obtained, the pmol of [^{32}P] recovered was compared with the pmol RGS14 protein in the assay to calculate the stoichiometry of phosphorylation (mol [^{32}P]ATP incorporated/mol protein). This method accounts for only the [^{32}P] incorporated into full-length RGS14, in contrast to precipitation procedures in which all protein-bound [^{32}P] will be counted.

Protein Expression. Thioredoxin and hexahistidine tagged RGS14 (TxH_6-R14), hexahistidine tagged H_6-R14-RGS (amino acids 1−205), and hexahistidine tagged H_6-R14-R/GL (amino acids 299−544) were constructed and expressed in BL21DE3 cells as described (*20*). The cells were grown to mid-log phase, and protein production was induced with 1 mM IPTG for 2 h. Cells were lysed using the French Press method, and the supernatant was recovered, loaded to a Ni^{2+} HiTrap affinity column (Amersham Pharmacia, NJ), and purified by FPLC. Proteins were eluted with an imidazole gradient from 20−200 mM imidazole in 50 mM HEPES, pH 7.4, and 150 mM NaCl. For TxH_6-R14, cell supernatant was loaded to Ni-NTA agarose beads, washed, and eluted using 200 mM imidazole and further purified by FPLC using a superdex-200 column (Pharmacia-Biotech).

GTPase Assays. GTPase assays were performed as described (*32*). Gα subunits were loaded with [^{32}P]GTP at room temperature for either 20 min ($G\alpha_o$) or for 1 h ($G\alpha_{i1}$). Proteins were cooled to 4 °C and then added to a reaction tube containing an excess of GTP (100 μM final) and $MgCl_2$ (5 mM final), as well as either buffer or buffer containing given concentrations of RGS protein. At 60 s, the reaction was quenched by the addition of activated charcoal. The [^{32}P]i released in the supernatant was measured, and the [^{32}P]i present at $t = 0$ was subtracted. The difference was defined as GTP hydrolyzed and was expressed as % maximal GTP hydrolysis (defined as [^{32}P]i accumulation in the presence of 30 μM RGS4).

GTPγS Binding Assays. GTPγS binding assays were performed as described (*20*, *33*). Briefly, 2.5 pmol of $G\alpha_{i1}$ or $G\alpha_o$ (diluted in 50 mM HEPES, 5 mM EDTA, 1 mM DTT, and 0.1% lubrol) were incubated with [^{35}S]GTPγS (2000 cpm/pmol) with or without indicated amounts of TxH_6-RGS14, H_6-R14-R/GL, or H_6-R14-RGS at 30 °C in reaction buffer (50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM DTT, and 10 mM $MgSO_4$) to a total volume of 50 μL. Reactions were terminated with ice cold reaction buffer, rapidly filtered over nitrocellulose, and washed with 12 mL of ice cold reaction buffer.

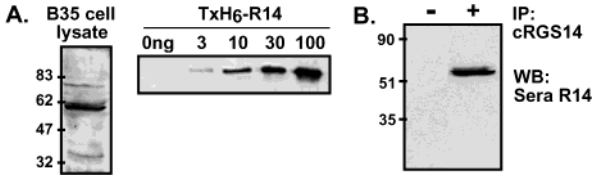

FIGURE 1: Native RGS14 is detected in B35 neuroblastoma cells and immunoprecipitated by antibody cRGS14. (A) Immunoblot using cRGS14 antibody (1:1000). Left panel, B35 cell lysates; right panel, purified TxH_6-R14 at 3, 10, 100, and 300 ng. (B) Immunoprecipitation of native RGS14 from B35 cell lysates by cRGS14 was carried out as described in Materials and Methods. Cell lysates were incubated with or without cRGS14 for 2 h at 4 °C, then IgY conjugated agarose was added, and incubation continued for 1 h at 4 °C. The mixture was centrifuged, beads were washed three times, and proteins eluted in sample buffer. Proteins were separated by SDS−PAGE and immunoblotted with rabbit polyclonal sera-RGS14 (1:2000) [*20*].

RESULTS

Native and Recombinant RGS14 Are Phosphorylated in Cells. Our initial investigations focused on the regulated phosphorylation of RGS14 in its native environment, which has not been examined previously. To identify cell lines that express native RGS14 for further study, we developed an affinity purified chicken antibody (cRGS14) using purified full-length recombinant RGS14 as an antigen. cRGS14 readily recognizes as little as 3 ng of purified RGS14 (Figure 1a). We identified native RGS14 in the rat neuroblastoma B35 cell line using both cRGS14 (Figure 1a) and the previously described rabbit polyclonal sera-R14 (data not shown) (*20*). Native full-length RGS14 can also be immunoprecipitated from these cells using cRGS14 and subsequently recognized using sera-R14 (Figure 1b). After incubation with [^{32}P]orthophosphate, a [^{32}P]-labeled protein was immunoprecipitated from B35 cells corresponding to the RGS14 immunoreactive band (Figure 2a). Treating the cells with forskolin to activate adenylyl cyclase and raise cellular cAMP levels enhanced the phosphorylation of this band (Figure 2a). Because native RGS14 is expressed at low levels, recovery of sufficient protein for biochemical analysis proved limiting. We therefore performed further experiments using amino-terminal HA-epitope tagged, recombinant RGS14 expressed in B35 cells. For these studies, we generated a homogeneous population of B35 neuroblastoma cells stably expressing HA-RGS14 derived from cells infected with a retrovirus encoding N-terminally HA-tagged RGS14. After [^{32}P]orthophosphate labeling of these cells, HA-RGS14 was immunoprecipitated using anti-HA antibody and detected by immunoblot using a previously described affinity purified rabbit antibody that recognizes the extreme C-terminus of RGS14 (see Materials and Methods) (Figure 2b). Consistent with our observations with native RGS14, phosphorylation of HA-RGS14 was also enhanced by treatment with forskolin, reaching maximal levels by 30 min of treatment (Figure

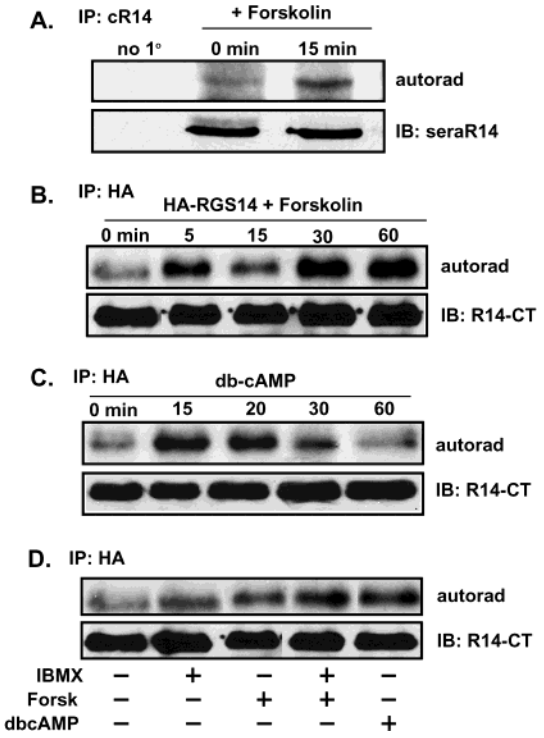

FIGURE 2: RGS14 is phosphorylated following forskolin treatment of B35 cells. (A) [^{32}P]-labeled B35 cells were treated with 25 μM forskolin for indicated times, cells were lysed, and then native RGS14 was immunoprecipitated as described in Materials and Methods using cRGS14. Radiolabeled protein was separated by SDS−PAGE, transferred to nitrocellulose, and then exposed to film (top panel) or immunoblotted using sera-RGS14 (1:2000) (bottom panel). (B) B35 cells were infected with retrovirus coding for HA-RGS14 (see Materials and Methods), and cells were selected using Zeocin (200 μg/mL). HA-RGS14 expressing cells were then [^{32}P]-labeled and treated with 25 μM forskolin for indicated times. Immunoprecipitation was carried out using anti-HA antibody (1:400 dilution). Proteins were separated by SDS−PAGE, transferred to nitrocellulose, and either exposed to film (top panel) or immunoblotted using rabbit polyclonal R14-CT [*20*] (bottom panel). (C) HA-RGS14 expressing B35 cells were [^{32}P]-labeled and treated with 200 μM db-cAMP for indicated times. Immunoprecipitation was carried out using anti-HA antibody. Proteins were separated by SDS−PAGE, transferred to nitrocellulose, and either exposed to film (top panel) or immunoblotted using R14-CT (bottom panel). (D) HA-RGS14 expressing B35 cells were [^{32}P]-labeled and treated with 200 μM IBMX, 25 μM forskolin, 200 μM db-cAMP, or a combination of the above as indicated for 15 min. Immunoprecipitation was carried out using anti-HA antibody. Proteins were separated by SDS−PAGE, transferred to nitrocellulose, and either exposed to film (top panel) or immunoblotted using R14-CT (bottom panel).

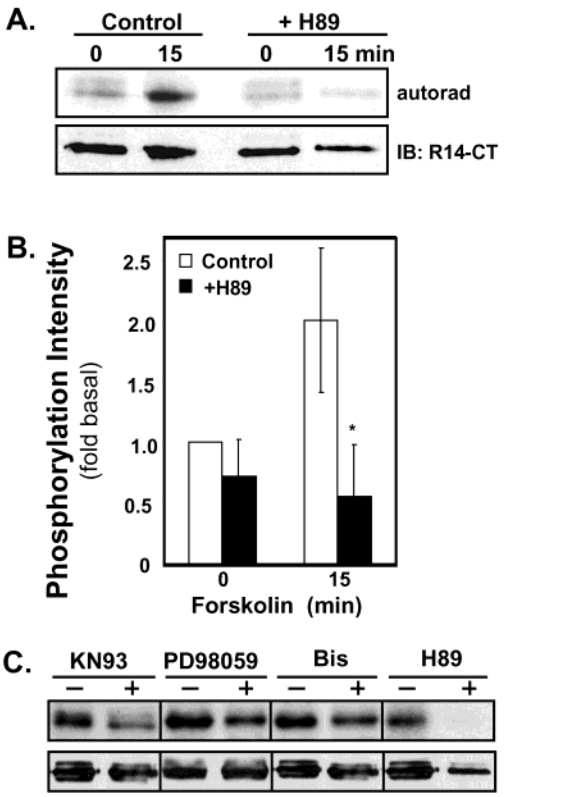

FIGURE 3: Cellular phosphorylation of RGS14 by forskolin is mediated by protein kinase A (PKA). B35 cells were infected with retrovirus coding for HA-RGS14 (see Materials and Methods), and cells were selected using zeocin (200 μg/mL). (A) The HA-RGS14 expressing cells were [^{32}P]-labeled for 5 h, incubated with 1 μM kinase inhibitor H89 for 30 min, and then treated with 25 mM forskolin for 0 or 15 min. Immunoprecipitation was carried out using anti-HA antibody (see Materials and Methods). The proteins were separated by SDS−PAGE, transferred to nitrocellulose, exposed to film, and then immunoblotted using rabbit polyclonal R14-CT. Representative autoradiogram (top) and immunoblot (bottom). (B) Densitometry was performed, and net intensity of autoradiograms from five experiments was plotted, normalized to basal phosphorylation. *$p < 0.05$ (Student's two-sample t-test). (C) HA-RGS14 expressing B35 cells were [^{32}P]-labeled for 5 h, incubated with kinase inhibitors for 30 min, and then treated with 200 μM db-cAMP for 15 min. Immunoprecipitation was carried out using anti-HA antibody (see Materials and Methods). The proteins were separated by SDS−PAGE, transferred to nitrocellulose, exposed to film, and then immunoblotted using R14-CT. Representative autoradiogram (top) and immunoblot (bottom). Kinase inhibitors: 10 μM KN93 (CaMKII/IV); 10 μM PD98059 (MEK); 10 μM Bis (PKC); and 1 μM H89 (PKA).

2b). Additional experiments were performed to maximize cellular cAMP levels, including treatment of the cells with the stable cAMP analogue db-cAMP. Treatment of cells with db-cAMP markedly stimulated phosphorylation of RGS14 (Figure 2c,d), reaching maximal levels by approximately 15 min. Further experiments investigated additional mechanisms to maximize cAMP levels, including supplementing the media with a phosphodiesterase inhibitor (IBMX) in the presence or absence of forskolin. Treatment of cells with IBMX increased RGS14 phosphorylation; however, only marginally enhanced forskolin-stimulated phosphorylation (Figure 2d).

Inhibiting PKA Activity Blocks Forskolin-Induced Phosphorylation of RGS14 in Cells. We next considered which kinase was responsible for the phosphorylation of RGS14 by forskolin or db-cAMP. Forskolin stimulates adenylyl cyclase, increasing the cellular pool of cAMP, which can initiate several kinase cascades either directly or indirectly. The most direct of these is the activation of protein kinase A (PKA), the catalytic subunit of which is released when cAMP binds to the PKA regulatory subunit. Because RGS14 contains several consensus recognition motifs for PKA (see Figure 4a), we investigated whether inhibition of PKA could limit the forskolin-induced phosphorylation of RGS14. We incubated B35 cells expressing HA-tagged RGS14 with the kinase inhibitor H89 at 1 μM for 30 min. Under these conditions, H89 is specific for PKA and RhoKinase II (*34*); however, RGS14 does not contain a recognition motif for RhoKinase II. After incubation with H89, we stimulated the

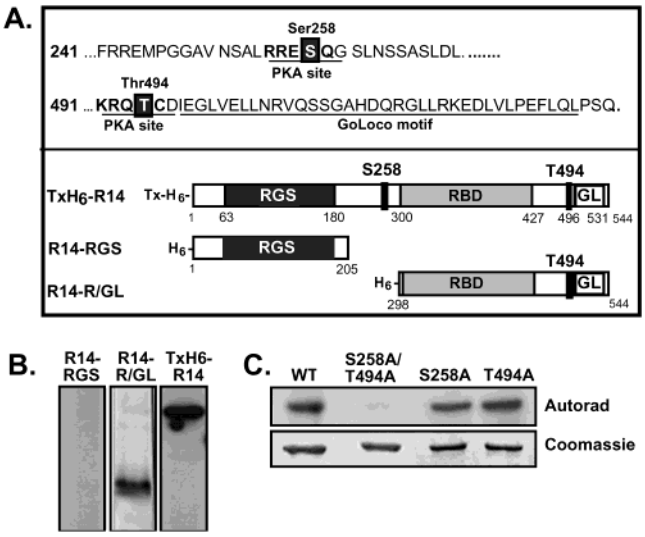

FIGURE 4: RGS14 is phosphorylated in vitro at Ser258 and Thr494 by PKA. (A) Rat RGS14 amino acid sequence and diagram of full length and truncated forms of RGS14, indicating location of PKA recognition motifs. (B) TxH_6-R14, R14-R/GL, and R14-RGS were purified as described [*20*]. A total of 2 μg of each protein was incubated with [^{32}P]ATP and PKA_{cat} for 30 min at 30 °C (see Materials and Methods), separated by SDS−PAGE, stained with coomassie blue, dried, and exposed to film. (C) TxH_6-R14, R14-(S258/T494A), R14(S258A), and R14(T494A) were purified as described in Materials and Methods. A total of 2 μg of each protein was incubated with [^{32}P]ATP and PKA_{cat} (see Materials and Methods) for 15 min at 30 °C, separated by SDS−PAGE, stained with coomassie blue (bottom), dried, and then exposed to film (top).

cells with forskolin for 15 min and compared the level of phosphorylation with and without treatment (Figure 3a,b). We found that preincubation with H89 did not affect basal phosphorylation of RGS14 (Figure 3b, $t = 0$) but completely blocked forskolin-induced phosphorylation ($p < 0.05$), leading us to conclude that activation of PKA underlies forskolin-induced cellular phosphorylation of RGS14 (Figure 3a,b). In most cases, H89 treatment reduced the level of phosphorylation of RGS14 after forskolin treatment to below basal without directly affecting basal levels (Figure 3a,b). This leads us to hypothesize that RGS14 phosphorylation by PKA either encourages phosphorylation by an additional kinase or reduces interaction with a phosphatase.

To investigate the influence of other kinases on cAMP-mediated phosphorylation of RGS14, we examined the effects of an array of well-described inhibitors on phosphorylation by db-cAMP. For this, we compared the relative effects of H89 with those of Bis, a selective inhibitor of protein kinase C isoforms (*35*); PD98059, a selective inhibitor of MEK and Erk phosphorylation (*36*); and KN93, a selective inhibitor of Ca^{2+}/calmodulin-dependent kinases (CaMK) II and IV (*37*). We show that Bis and PD98059 do not significantly alter db-cAMP-induced phosphorylation of RGS14 (Figure 3c), whereas the CaMK inhibitor KN93 reduces RGS14 phosphorylation by approximately 50% in separate experiments (Figure 3c and data not shown). However, the selective PKA inhibitor H89 completely blocked db-cAMP induced phosphorylation of RGS14 (Figure 3c), an even more pronounced inhibition than observed for forskolin-induced phosphorylation (Figure 3a).

PKA Phosphorylates Recombinant RGS14 at Two Distinct Sites. We next assessed whether PKA could phosphorylate recombinant RGS14 in vitro. Analysis of the RGS14 sequence reveals two consensus sites (i.e., [R/K][R/K]X[S/T]) for PKA phosphorylation, Ser258, and Thr494 (Figure 4a)). We expressed and purified recombinant RGS14 (TxH_6-R14), the N-terminal portion of RGS14 containing the RGS domain (R14-RGS), and the C-terminal portion containing the Rap binding domain and GoLoco domains (R14-R/GL) as previously described (*20*). In phosphorylation assays using the purified catalytic subunit of PKA (PKA_{cat}) (see Materials and Methods), we show that both full-length TxH_6-R14 and R14-R/GL are phosphorylated in vitro, while the R14-RGS domain is not (Figure 4b). Because R14-RGS is truncated at Lys201 and R14-R/GL begins at Gly298, neither protein contains Ser258. Phosphorylation of R14-R/GL is therefore most likely at Thr494. To confirm that PKA phosphorylates RGS14 at these two sites, we mutated Ser258 and Thr494 by means of site-directed mutagenesis. We substituted each residue with alanine either individually (R14(S258A) and R14(T494A)) or together (R14(S258/T494A)). We assessed the capacity of PKA to phosphorylate these mutants in vitro. Wild-type RGS14 and each point mutant are phosphorylated by the purified catalytic subunit of PKA (PKA_{cat}) (Figure 4c), while the full-length double mutant, R14(S258/T494A), is not. Importantly, the activity of R14(S258/T494A), as measured by both GAP and GDI assays, was not affected (data not shown), indicating that the lack of phosphorylation was not due to gross conformational changes in the protein. These data indicate that Ser258 and Thr494 are preferred sites for PKA phosphorylation.

Altering Thr494 Does Not Affect GAP Activity. Because of the close proximity of Thr494 to the recognized GoLoco motif (*38*) (Figure 4a), we investigated the potential effects of phosphorylating Thr494 on RGS14 interactions with Gα. For these studies, we initially attempted to use wild-type RGS14 phosphorylated with PKA_{cat}. However, initial experiments revealed that recombinant RGS14 is phosphorylated in vitro to a maximal stoichiometry (mol phosphate/mol protein) of only 20% (data not shown). In an effort to increase the efficiency of phosphate incorporation, we varied the protocol by increasing the concentration of PKA, altering the pH of the reaction solution, and introducing fresh enzyme at successive time points. However, none of these modifications to our protocol significantly increased the incorporation of phosphate. Additional efforts to separate phosphorylated from nonphosphorylated RGS14 also were not feasible because recombinant RGS14 is highly prone to degradation, resulting in low yields of purified protein. Attempts to concentrate RGS14 were unsuccessful because the purified protein readily precipitates out of solution during the concentration steps. Therefore, investigating the effects of direct phosphorylation of RGS14 on its defined functions has thus far proven technically unapproachable.

Because of these technical limitations, we instead investigated the effect of mutating Thr494 to either glutamate (R14(T494E)) or aspartate (R14(T494D)), which mimic phosphorylation, or to alanine (R14(T494A)), which serves as a control. We performed initial tests to determine if any of these mutations have any effect on RGS14 Gα GAP activity. Because Thr494 is located distal to the RGS domain, we did not expect to see an effect unless our mutation caused a conformational change affecting the overall folding of the protein. A comparison of the wild type and mutated forms

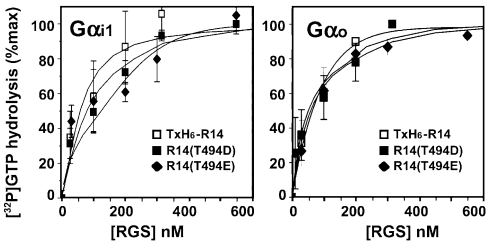

FIGURE 5: RGS14 GAP activity toward $G\alpha_i$ and $G\alpha_o$ is not affected by mimicking phosphorylation of Thr494. TxH_6-R14, R14(T494E), and R14(T494D) were purified by affinity chromatography (see Materials and Methods), and GTPase assays were performed as described (see Materials and Methods). Increasing concentrations of TxH_6-R14 (open squares); R14(T494E) (black diamonds); and R14(T494D) (black squares) were incubated with 0.5 μM preactivated $G\alpha_{i1}$ (left panel) or $G\alpha_o$ (right panel) for 60 s. Basal [^{32}P]i released was subtracted, and numbers were normalized to % maximal as defined by [^{32}P]i released by Gα incubation with 30 μM RGS4 for 60 s.

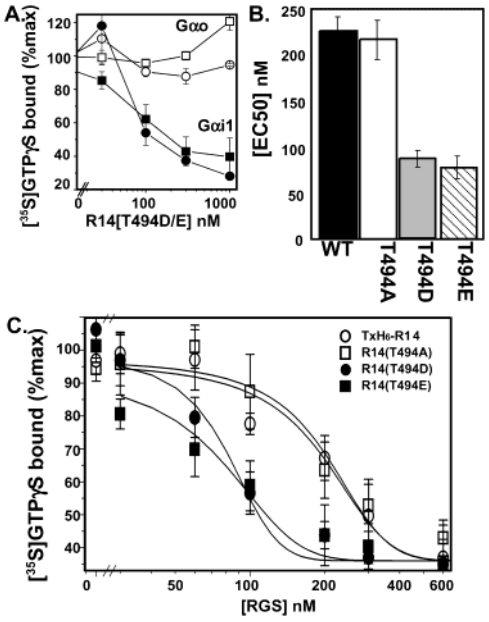

FIGURE 6: RGS14 GDI activity toward $G\alpha_i$ is potentiated by mimicking phosphorylation of Thr494. Proteins were purified by affinity chromatography (see Materials and Methods), and GTPγS binding assays were performed as described. (A) Comparison of R14(T494D) (circles) and R14(T494E) (squares) GDI activity toward $G\alpha_{i1}$ (black) versus $G\alpha_o$ (open). (B) EC50 values in nM of wild-type TxH_6-R14 (black), R14(T494A) (white), R14(T494D) (gray), and R14(T494E) (hatched) GDI activity toward $G\alpha_{i1}$. (C) Inhibition of GTPγS binding to $G\alpha_{i1}$ by increasing concentrations of RGS14. TxH_6-R14 (open circles), R14(T494A) (open squares), R14(T494D) (black circles), and R14(T494E) (black squares) (see Materials and Methods).

of RGS14 in GTPase assays indicated that mimicking phosphorylation on Thr494 has little or no effect on the capacity of RGS14 to act as a GTPase activating protein for either $G\alpha_{i1}$ or $G\alpha_o$ (Figure 5). These data indicate that the mutated RGS14 is properly folded and that altering the C-terminus does not affect the activity of the RGS domain.

Altering Thr494 to Mimic Phosphorylation Enhances GDI Activity. We next assessed the effects of mutating Thr494 on the capacity of RGS14 to inhibit nucleotide exchange on Gα subunits (GDI activity) as assessed by GTPγS binding. In these assays, nonhydrolyzable [^{35}S]GTPγS was incubated with Gα in the presence or absence of RGS14, and the amount of [^{35}S] radiolabel bound to the Gα subunit was measured; less radiolabel bound indicates GDI activity. We first examined whether phosphorylation by PKA changes the preference of the RGS14 GoLoco motif for Gα subunits. Previous studies have shown that RGS14 is a selective GDI for $G\alpha_i$ over $G\alpha_o$ subunits (*19*, *20*). Substituting either glutamate or aspartate for Thr494 caused no changes in the selectivity of RGS14 for $G\alpha_i$ over $G\alpha_o$ in GDI assays (Figure 6a). We next investigated whether phosphorylation by PKA could change the affinity of RGS14 for $G\alpha_i$-GDP. We found that both R14(T494E) and R14(T494D), but not R14-(T494A), exhibit an increased capacity to inhibit nucleotide exchange on $G\alpha_i$ over the wild-type RGS14 (Figure 6b,c). Mutating Thr494 to mimic phosphorylation renders RGS14 a nearly 3-fold more potent GDI than either the wild-type RGS14 or the alanine mutant of the same residue (EC50: TxH_6-R14 = 223 $\pm$ 21 nM, R14(T494A) = 216 $\pm$ 18 nM, R14(T494D) = 87 $\pm$ 8 nM, and R14(T494E) = 78 $\pm$ 12 nM) (Figure 6b).

DISCUSSION

Although investigations into the cellular roles of RGS proteins are still in the earliest stages, the importance of this protein family in cell and organ physiology is already clear (*1*−*3*). Because signals within the cell can modify the properties of these proteins, studies of RGS regulation are vital for a complete understanding of how these proteins function in vivo. In this report, we have begun to investigate the cellular mechanisms regulating the activity of one of the most complex RGS family members, RGS14. With its multiple Gα regulatory domains and potential rap-binding activity (*14*, *17*, *19*, *20*), cellular functions of this protein have thus far proven difficult to model and are likely to be highly regulated. Here we show that phosphorylation of both native and recombinant RGS14 is measurable in a cell line that expresses native RGS14 and that phosphorylation is enhanced through the activation of adenylyl cyclase by either forskolin or a stable cAMP analogue. The induced phosphorylation is completely blocked by a selective PKA inhibitor, indicating that phosphorylation is due, at least in part, to the actions of PKA. A possible role for Ca^{2+}/calmodulin kinases (CaMK) as a contributor to this phosphorylation cannot be ruled out since cAMP-induced phosphorylation is also partially blocked by a selective CaMK inhibitor but not by inhibitors of PKC or MAPKinase. These observations led us to investigate the functional consequences of PKA phosphorylation on RGS14 function. We have shown that two PKA consensus recognition sites present in recombinant RGS14 are phosphorylated in vitro (Ser258, Thr494) and that mimicking the phosphorylation of Thr494 adjacent to the GoLoco motif selectively potentiates the capacity of RGS14 to act as a GDI for $G\alpha_{i1}$ without affecting the GAP activity of RGS14 for either $G\alpha_{i1}$ or $G\alpha_o$.

Kinases are involved at a number of levels in the regulation of GPCR signaling. Some kinases such as the GRKs limit G-protein signals by binding to and phosphorylating activated receptors. Other kinases, such as PKA, PKC, and CaMK, are activated by G-protein regulated signals and can feedback to regulate their own activation through phosphorylation of signaling molecules. Given their importance in G-protein signaling, RGS proteins are obvious

targets for feedback phosphorylation and regulation by G-protein activated kinases. In the growing list of studies investigating regulated phosphorylation of RGS proteins, most have explored the effects on GAP activity toward Gα subunits (*22*−*24*, *28*, *39*−*41*). Depending on the RGS and kinase involved, phosphorylation can either enhance or diminish GAP activity. For instance, PKC phosphorylates RGS2 and reduces its Gα GAP activity, thereby enhancing Gq/11 signals (*27*). Another report shows a similar effect of phosphorylation on RGS16, which is phosphorylated following activation of $G\alpha_{i/o}$-linked α_{2A}-adrenergic receptors in cells (*26*). This modification reduces RGS16 GAP activity toward $G\alpha_{i/o}$, resulting in increased α_{2A}-adrenergic receptor signals. A separate study (*23*) showed that activation of muscarinic cholinergic receptors elicited RGS16 phosphorylation at conserved tyrosine residues in the RGS domain. In this case, phosphorylation is mediated by transactivation of the EGF receptor, which binds directly to RGS16 (*23*). In contrast to the inhibitory effects on RGS16 GAP activity of phosphorylation by α_{2A}-adrenergic receptors, phosphorylation of RGS16 by the EGFR enhances its GAP activity toward $G\alpha_i$. Similarly, MAPKinase phosphorylation of the RGS domain of RGS-GAIP enhances its GAP activity (*28*), which in this case could constitute a feedback mechanism to reduce the Gβγ available to initiate MAPKinase cascades. Together, these studies highlight the complexity of cellular regulation of RGS proteins.

Recent reports provide evidence that phosphorylation can dictate RGS interaction with non-G-protein binding partners. For example, several studies show that certain RGS proteins can interact with the intracellular scaffolding protein 14-3-3 by way of a conserved binding motif (*22*, *42*, *43*). In the case of RGS7, PKC phosphorylation allows association with cytosolic 14-3-3 in lieu of $G\alpha_{i/o}$, thereby reducing RGS7-mediated inhibition of $G\alpha_{i/o}$ signals (*22*). Tumor necrosis factor-α transiently inhibits this phosphorylation, limiting RGS7 interaction with 14-3-3 and thereby releasing RGS7 to inhibit $G\alpha_{i/o}$ linked signals (*43*).

Phosphorylation may serve an important role in regulating G-protein regulation of visual signaling, in particular RGS9 modulation of transducin (*40*, *41*, *44*). Kinases in the photoreceptor phosphorylate RGS9-1 at several sites, and in one case, phosphorylation is mediated by PKA (*41*, *44*). The kinase seems to be localized to plasma membrane microdomains, and phosphorylation of RGS9-1 is dependent upon recruitment to these domains by activated Gα. However, mimicking the putative phosphorylation sites reduces GAP activity (*41*), possibly constituting a feedback mechanism to potentiate photoreceptor signals under conditions where phosphorylation is enhanced, for example, in dark-adapted cells.

Other studies also indicate that phosphorylation may depend on RGS subcellular localization and, in turn, that phosphorylation might regulate RGS protein localization and function. For example, RGS-GAIP is tightly regulated in cells (*45*−*47*) and has been isolated from cell lysates as a phosphoprotein (*39*). The phosphorylation state of RGS-GAIP appears to be related to its cellular localization, as the protein is found in a subpopulation of intracellular vesicles that have the capacity to phosphorylate RGS-GAIP in vitro. Kinases may also dynamically regulate the subcellular localization and function of certain RGS proteins. For example, phosphorylation by PKA causes RGS10 to shuttle to the nucleus (*25*), thereby making it unavailable to limit plasma membrane delimited G-protein signals but fully available to regulate possible nontraditional G-protein signaling events or G-protein-independent signals in the nucleus (see ref *21*). Related to this, certain forms of $G\alpha_i$ are enriched in golgi-derived vesicles and in the nucleus (*48*, *49*), organelles far removed from GPCR signaling at the plasma membrane. RGS12 and other GoLoco-motif containing proteins also are localized to the nucleus (*50*, *51*). New ideas postulate that the RGS and GoLoco containing proteins may link the unusually localized G-proteins to undefined signaling pathways involved with cell division (*21*). In these cases, phosphorylation may contribute to short latency, signal-induced regulation of RGS and GoLoco domain functions that are vital for these cellular processes.

Defined roles for RGS14 phosphorylation in a cellular context, particularly with regard to GoLoco function and Gi-linked signals, remain uncertain. RGS14 and RGS12 are unique among signaling proteins in that they contain domains that confer opposing GAP and GDI activities for $G\alpha_i$. As discussed, GoLoco-motif containing proteins and certain $G\alpha_i$ subunits are reported to be present in the nucleus where they may contribute to cell division by as yet undefined mechanisms. Thus, any working model of phosphorylation-induced modulation of RGS14 function must consider this complexity. We favor a mechanistic model (*20*, *21*) in which the RGS domain of RGS14 is recruited to activated $G\alpha_i$ and $G\alpha_o$ subunits to serve as a GAP. Because RGS domains do not bind Gα-GDP, the RGS domain dissociates from Gα, which permits Gα reassociation with Gβγ. However, in the case of RGS14, the GoLoco motif is available to complex with the Gα-GDP monomer, thereby delaying heterotrimer reformation and enhancing $G\alpha_i$-derived Gβγ signaling (including activation of PI_3Kinase, phospholipase C, MAP-Kinases, and regulation of ion channels). Roles for RGS14 phosphorylation by PKA in this cellular model remain undefined at this point. However, it is plausible that enhancing the GDI activity of RGS14 through PKA phosphorylation may augment Gβγ signaling by favoring RGS14/Gα-GDP complex formation and prolonging the lifetime of free Gβγ in the cell. Because many of the known signals initiated by Gβγ can cause variable consequences depending on the level of activation, one testable idea is that increasing the duration of these signals could amplify the downstream outcome. Alternatively, some models propose that GoLoco motifs can serve to initiate G-protein βγ signals independent of receptor activation (*52*). In much the same way that a ligand activates a GPCR, PKA phosphorylation of Thr494 in RGS14 might activate the RGS14 GoLoco interaction with $G\alpha_i$. Further studies that focus on the roles of phosphorylated RGS14 in a cellular context will be needed to test these ideas.

In conclusion, we have shown that phosphorylation of both native and recombinant RGS14 is measurable in cells and enhanced through the activation of adenylyl cyclase. A selective inhibitor of PKA completely blocks the induced phosphorylation under conditions where inhibitors of other kinases either do not block (PKC or Erk) or only partially limit (CaMK) phosphorylation, indicating that phosphorylation in cells is due at least in part to PKA. We have identified two sites that are phosphorylated by PKA in vitro and showed that altering the PKA recognition site near the

GoLoco motif to mimic phosphorylation modulates the capacity of RGS14 to act as a guanine nucleotide dissociation inhibitor for $G\alpha_{i1}$. Ongoing studies are aimed at understanding the role of phosphorylation in modulating RGS14 function in cells.

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