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Multiple phosphorylation sites in RGS16 differentially modulate its GAP activity

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Abstract Regulators of G-protein signaling (RGS) are GTPase-activating proteins (GAP) for activated Gα subunits. We found that mouse RGS16, when expressed in HEK293T cells, is phosphorylated constitutively at serine 194 based on in vivo orthophosphate labeling experiments, while serine 53 is phosphorylated in a ligand-dependent manner upon stimulation by epinephrine in cells expressing the α2A adrenergic receptor. Phosphorylation on both sites impairs its GAP activity and subsequent attenuation on heterotrimeric G-protein-stimulated extracellular signal-regulated protein kinase activity. This is the first report of RGS functional downregulation by phosphorylation via a G-protein-coupled receptor.

Key words: Regulators of G-protein signaling; G protein; Phosphorylation; α2A Adrenergic receptor; Epinephrine

1. Introduction

Signal transduction pathways originating from G-protein-coupled receptors (GPCR) are among the most important and widespread signaling cascades in mammalian systems. They mediate crucial body functions ranging from light, odor, and pain perception, blood pressure regulation, inflammatory response, neurotransmission, etc. A family of regulators of G-protein signaling (RGS) have been discovered [1–3] which bind to activated Gα subunits to accelerate their GTP hydrolysis rate, thereby terminating signals initiated from ligand-occupied GPCR, and acting as GTPase-activating proteins (GAP).

There are currently more than 20 mammalian RGS proteins being identified based on a conserved domain of about 120 amino acid residues known as the RGS box [3]. Although these RGS proteins exhibit binding promiscuity for Gα subunits as assayed in vitro, increasing evidence has indicated that individual RGS members have biological specificity. For example, RGS16 preferentially inhibited platelet-activating factor stimulation of the mitogen-activated protein kinase (MAPK) p38 via $G_{q/11}\alpha$ subunits, but not platelet-activating factor initiated extracellular signal-regulated protein kinase (ERK) [4]. The specificity of RGS to G-protein interaction is also shown by a substantial enhancement of GTPase activity of $G_{i2}\alpha$ over $G_{o1}\alpha$ by RGS4 upon α2A adrenergic receptor stimulation [5]. Furthermore, it is likely that the great diversity of sequences beyond the RGS box may confer distinct properties to the various RGS proteins. The G-protein γ subunit-like domain for instance, is found in RGS6, 7, 9, and 11, which enables these RGS proteins to specifically bind to the Gβ5 subunit [6,7]. The pleckstrin homology domain, which binds phosphoinositides, is found in p115-RhoGEF and PDZ-RhoGEF, two RGS proteins that preferentially interact with $G_{12}\alpha$ and $G_{13}\alpha$ subunits [8]. Post-translational modifications of RGS proteins also play roles in modulating the activity of RGS proteins. Palmitoylation of RGSZ1, RGS4, and RGS10 regulated their GAP activity [9], especially if it involved cysteine residues in the RGS box [10]. In addition, palmitoylation of N-terminal cysteine residues may be essential for membrane localization of certain RGS proteins [11].

Recently, there are reports that RGS proteins are phosphoproteins. In the yeast *Saccharomyces cerevisiae*, the RGS protein Sst2p is phosphorylated by MAPK in response to pheromone stimulation [12]. The phosphorylation appears to slow the rate of Sst2p degradation. A mammalian RGS protein, Gα-interacting protein (GAIP), was also observed to be phosphorylated by the MAPK ERK2 [13]. In vitro phosphorylation of GAIP enhances its GTPase activity, and is necessary for stimulating the lysosomal autophagic pathway in human colon cancer HT-29 cells.

In view that post-translational modifications may affect the stability and activity of RGS proteins, we set out to study how such modifications might affect RGS16, which we had previously cloned from a mouse pituitary library [14,15]. RGS16 is known to be palmitoylated at its amino-terminal cysteine residues 2 and 12 based on the observation of Druey et al. [16], and it has an N-terminal amphipathic α-helical domain critical for its plasma membrane localization [17]. However, no study has been conducted on the phosphorylation of RGS16 and its biological effect. We hereby show that the RGS16 protein is phosphorylated both constitutively and in a ligand-dependent manner on different serine residues. Phosphorylation of RGS16 on both sites decreases its GAP activity towards the Gα subunit, and impairs its potency to inhibit ligand-stimulated MAPK activity.

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Abbreviations: RGS, regulators of G-protein signaling; GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; GAP, GTPase-activating protein; GAIP, Gα-interacting protein

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2. Materials and methods

2.1. Construction of plasmids

The plasmids for pCMV5-RGS16 and FLAG-tagged pCMV5-ERK2 were constructed as described previously [4,14]. A FLAG tag was incorporated at the C-terminus of RGS16 in the pCMV5 vector before the stop codon by polymerase chain reaction. Serine to alanine or glutamate mutants of RGS16 were generated using the Transformer™ Site-directed Mutagenesis Kit (Clontech) and correct identities were confirmed by sequencing. The α2A adrenergic receptor was a kind gift from Dr. G. Milligan (University of Glasgow, UK). $(His)_6$-tagged $G_o\alpha$ in the pQE30 plasmid (Qiagen) was generated as described previously [23]. The plasmids for $(His)_6$-tagged wild-type, alanine, and glutamate mutants of RGS16 were constructed by fusing the respective RGS16 cDNAs to the C-terminally $(His)_6$-tagged pQE30 vector.

2.2. Cell culture, transfection, and immunoprecipitation

Human embryonic kidney 293T (HEK293T) cells were maintained in RPMI 1640 medium as previously described [4] and transfected using DOSPER (Boehringer Mannheim) according to the manufacturer's instructions. Empty vector pCMV5 was used to normalize the amount of DNA in each transfection. Cell lysis and immunoprecipitation of FLAG-tagged proteins using M2 (anti-FLAG) beads (Sigma) were performed essentially as described previously [16].

2.3. In vivo orthophosphate labeling

In vivo orthophosphate labeling was carried out as described by Ploegh [18]. Briefly, HEK293T cells were plated onto 35 mm dishes for transfection with the desired plasmids. At 36 h post-transfection, the cells were rinsed with, and incubated for 3 h in, phosphate-free MEM medium (Sigma) containing 1% dialyzed fetal bovine serum (FBS, Gibco BRL, Life Technologies). This was followed by another rinse and an additional 1 h incubation in the same medium before changing to 0.5 ml of phosphate-free medium containing 5% dialyzed FBS for 30 min. [^{32}P]-labeled orthophosphate [^{32}Pi] was then added to each dish to give a final concentration of 1.2 mCi/ml, for a duration of 4–6 h. Cells were stimulated with or without epinephrine (100 μM) for 30 min and then washed once with ice-cold PBS before lysing in 600 μl of lysate buffer. Following immunoprecipitation of the [^{32}Pi]-labeled FLAG-tagged RGS16 protein, the immunoprecipitates were boiled in 60 μl of Laemmli sample buffer and separated on a 12% SDS–PAGE gel. Resolved proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane for phosphoamino acid mapping or a nitrocellulose membrane for cyanogen bromide cleavage analysis.

2.4. Phosphoamino acid mapping

Phosphoamino acid analysis was performed using two-dimensional thin-layer chromatography (2D-TLC). Briefly, the bands on the PVDF membrane which corresponded to the [^{32}Pi]-labeled RGS16 protein were excised and cut into fine pieces before incubating in 200 μl of 5.7 N HCl at 110°C for 1 h. The supernatant containing the acid-hydrolyzed protein was transferred to a new tube and dried for 2 h in a speed-vacuum dryer. The resultant pellet was resuspended in 9 μl of 1D buffer (pH 1.9) containing 2.25% formic acid and 7.8% glacial acetic acid, before mixing with 2 μl of phosphoamino acid standards (Sigma). The sample was spotted onto a cellulose-coated glass plate for separation in a TLC electrophoresis system. The first dimension electrophoresis was performed at 1.5 kV, 20 mA current for 22 min in 1D buffer; electrophoresis of the second dimension was performed at 1.4 kV for 18 min in 2D buffer (pH 3.9) containing 5% glacial acetic acid and 0.5% pyridine. The sample plate was air-dried for 30 min and sprayed with 0.25% ninhydrin, followed by incubation in an oven set at 65°C for 10–15 min until the standard amino acid appeared. Autoradiography of the plate revealed phosphorylated amino acids.

2.5. Cyanogen bromide cleavage

[^{32}Pi]-labeled proteins transferred onto the nitrocellulose membrane were digested in 200 μl of cyanogen bromide (100 mg/ml in 70% formic acid) in the dark at room temperature for 90 min. The digested proteins in the supernatant were dried in a speed-vac for 1 h, and resuspended in 500 μl of H_2O, followed by evaporation for another 2 h. The final sample was dissolved in 30 μl of sample buffer, boiled for 5 min, and then separated on a small-pore 16.5% T, 6% C tricine–SDS–PAGE gel with 6 M urea as described by Schägger et al. [19].

2.6. GAP activity assays

The GAP activity of individual RGS16 proteins was carried out based on a single-step GTP hydrolysis according to previous description [23]. Specifically, the $G_o\alpha$ protein and individual RGS16 proteins were generated and purified from bacteria as N-terminal $(His)_6$ fusion proteins. 0.25 μM of $G_o\alpha$ protein was incubated with 1 μM [γ-^{32}P]GTP in the absence of Mg^{2+} at 30°C for 30 min, and then transferred to an ice bath for 5 min. The hydrolysis of GTP to GDP was initiated by incubating the GTP-preloaded $G_o\alpha$ mixture with 2 μM RGS16 protein and 10 mM Mg^{2+} at 8°C. The GAP activity was determined by subtracting remaining [^{32}P] counts at the indicated time-point from counts on filters at zero time-point.

2.7. Phospho-ERK blotting

RGS16 wild-type, alanine, and glutamate mutants of S53 and S194 (0.7 μg each) were expressed in HEK293T cells plated on 60 mm dishes, together with the α2A adrenergic receptor (0.5 μg) and FLAG-tagged ERK2 (0.5 μg). At 30 h after transfection, the cells were serum-starved overnight before stimulation with epinephrine (100 μM) for 15 min. ERK2 kinase was immunoprecipitated and separated on a 10% SDS–PAGE gel before immobilization onto PVDF blotting membrane. The level of ERK phosphorylation was detected by immunoblotting with phospho-p44/p42 MAPK (Thr202/Tyr204) E10 monoclonal antibody (Cell Signaling Technology) according to the manufacturer's instructions. The amount of kinase loaded was further determined by probing with anti-ERK polyclonal antibody (Santa Cruz) after stripping the same membrane with Restore™ Western Blot Stripping Buffer (Pierce). The expression levels of RGS16 proteins in cells were examined by immunoblotting the cell lysates with anti-RGS16 polyclonal antibody as previously described [4].

3. Results

3.1. RGS16 is constitutively phosphorylated at serine 194

We found that RGS16 is a phosphoprotein based on in vivo orthophosphate labeling experiments (Fig. 1A). These were performed by transfecting HEK293T cells with the C-terminal FLAG-tagged mouse RGS16 (the N-terminal tagging attenuated RGS16 function, data not shown). After 36 h, free phosphate in the medium was removed by rinsing and incubating the cells with phosphate-free medium. After 5–6 h, [^{32}P]-labeled orthophosphate was added to the cells for at least 4 h. The cells were then lysed, and the FLAG-RGS16 protein was immunoprecipitated with M2 anti-FLAG beads. Autoradiography of the SDS–PAGE gel of the immunoprecipitated proteins showed a distinct band corresponding to FLAG-tagged RGS16 at around 25 kDa (Fig. 1A), which is not present in proteins immunoprecipitated from vector (pCMV5)-transfected HEK293T cells. A polyclonal anti-RGS16 antibody further confirmed the 25 kDa [^{32}Pi]-labeled band as the full-length RGS16. The antibody also detected a break-down fragment of RGS16, which is not [^{32}Pi]-labeled.

Analysis of the phosphorylated protein by phosphoamino acid mapping indicated that serine residues of RGS16 are phosphorylated (Fig. 1B). A preliminary assessment of the site of phosphorylation was then carried out by digesting the [^{32}Pi]-labeled RGS16 with cyanogen bromide. As cyanogen bromide cleaves a polypeptide at the C-terminus of methionines, it is expected to digest FLAG-RGS16 at methionine 1 and 162 to give a 18.5 kDa N-terminal fragment and a 5.4 kDa C-terminal fragment (including the FLAG tag sequence). Subsequently, the C-terminal fragment (residues 163–201) was found to be [^{32}Pi]-labeled (Fig. 1C).

Serine residues in the C-terminal fragment were individually mutated to alanine and subjected to in vivo orthophosphate

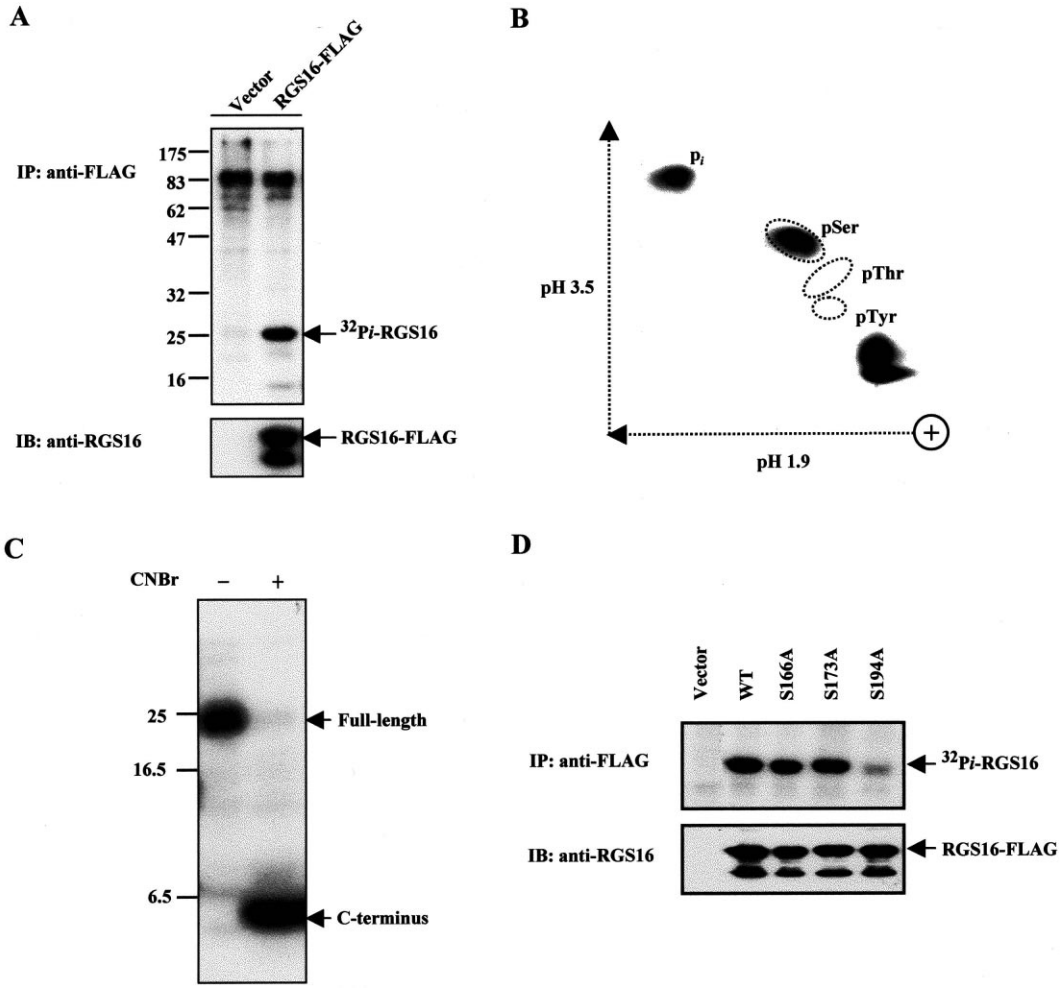

Fig. 1. RGS16 is a phosphoprotein when expressed in HEK293T cells. A: The FLAG-tagged RGS16 was transiently expressed in HEK293T cells, in vivo labeled with [^{32}P]orthophosphate, immunoprecipitated with anti-FLAG antibody, and was resolved on a 12% SDS–PAGE gel before transferring to the PVDF membrane. Top panel is an autoradiograph showing the incorporation of [^{32}Pi] on RGS16 (right lane), but not on vector-transfected cells (left lane). Bottom panel is a blot of the same membrane probed with anti-RGS16 antibody. B: Phosphoamino acid mapping of the [^{32}Pi]RGS16 band in (A) using 2D-TLC electrophoresis indicated that serine residues were phosphorylated. The relative positions of the ninhydrin-stained amino acid standards are indicated within dash-lined circles. C: Cyanogen bromide (CNBr) cleavage of [^{32}Pi]RGS16 protein showed that only the shorter 5.4 kDa C-terminal fragment was [^{32}Pi]-labeled (right lane). D: FLAG-tagged alanine mutant constructs of RGS16 were expressed and subjected to in vivo orthophosphate labeling as in (A). Top panel is an autoradiograph of the [^{32}Pi]-labeled proteins. Bottom panel shows Western blotting of the same membrane with anti-RGS16 antibody, indicating similar expression of the various alanine mutants.

labeling. The alanine mutant of serine 194 clearly prevented the incorporation of [^{32}Pi] onto the RGS protein (Fig. 1D). The dramatic loss of constitutive phosphorylation on the RGS16 (S194A) mutant precludes the possibility of phosphorylation at other serine residues on the C-terminal.

3.2. Stimulation of α2A adrenergic receptor expressing cells with epinephrine induces phosphorylation at serine 53

Stimulation of GPCRs frequently results in the activation of various kinases including protein kinase A, protein kinase C, G-protein-regulated kinases and the MAPK. These kinases mediate the responses of GPCR activation or feedback to regulate the intensity and duration of signaling. As RGS proteins are negative regulators of G-protein signaling, we are keen to explore whether RGS proteins may themselves also be regulated in a ligand-dependent manner.

The α2A adrenergic receptor is widely distributed in mammalian cells [20] and is promiscuous in activating various Gα subunits [21]. It is also known to activate the MAPK pathways, especially the ERK. Stimulation by epinephrine (100 μM) for 30 min in HEK293T cells expressing the α2A adrenergic receptor resulted in [^{32}Pi]-labeling of the RGS16 (S194A) mutant protein (Fig. 2A). This effect was not apparent in wild-type RGS16, due to the constitutive phosphorylation at serine 194. Phosphoamino acid mapping again showed that the serine residue, but not tyrosine or threonine, was phosphorylated (Fig. 2B). Cyanogen bromide cleavage showed that the N-terminal fragment (2–162) was phosphorylated upon ligand stimulation (result not shown). Various serine to alanine mutants were constructed and subjected to in vivo labeling assays. After an extensive search, mutation of serine 53 to alanine was found to abolish epinephrine-stimulated [^{32}Pi] incorporation onto the RGS16 (S194A) protein (Fig. 2C).

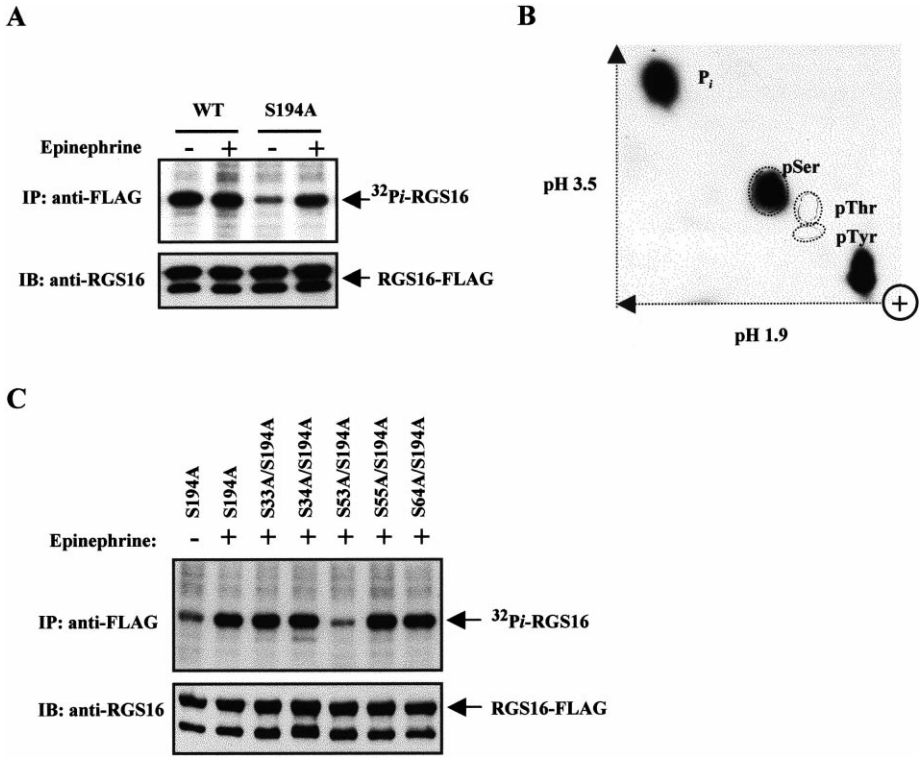

Fig. 2. RGS16 is phosphorylated upon ligand stimulation. A: FLAG-tagged wild-type (WT) RGS16 or FLAG-tagged mutant RGS16 (S194A) was co-expressed with α2A adrenergic receptor in HEK293T cells (using 1 μg of RGS16 cDNA +0.5 μg α2A adrenergic receptor cDNA) and subjected to in vivo [^{32}P]orthophosphate labeling. Epinephrine (100 μM) was added 30 min before harvest. The RGS16 proteins were immunoprecipitated and resolved on SDS–PAGE gel as in Fig. 1A. Top panel shows the ligand-dependent incorporation of [^{32}Pi] into RGS16 proteins; bottom panel shows anti-RGS16 probe analysis of the same membrane, indicating similar expression of the proteins. B: Phosphoamino acid analysis was performed on the epinephrine-stimulated [^{32}Pi]-labeled RGS16 (S194A) as described in (A). The relative positions of the ninhydrin-stained amino acid standards are indicated within dash-lined circles. C: Various FLAG-tagged alanine mutant constructs of RGS16 were expressed and subjected to in vivo orthophosphate labeling under the stimulation of epinephrine as described in (A). Top panel is an autoradiograph of the [^{32}Pi]-labeled proteins. Bottom panel shows Western blotting of the same membrane with anti-RGS16 antibody, indicating similar expression of the alanine mutants.

3.3. *Double-glutamate mutants of serine 53 and serine 194 diminished RGS16 GAP activity on $G_o\alpha$*

As previously shown, RGS16 binds to the transition state of Gα subunits and acts as a GAP to enhance the intrinsic GTPase activity of $G_o\alpha$ [23]. We therefore examined the GAP activities of these mutants. RGS16 mutants at serine 53 and serine 194, which mimic loss (serine to alanine) or gain (serine to glutamate) of phosphorylation, were constructed and assayed for their interactions with G proteins. These RGS16 mutant proteins were generated and expressed in bacteria cells, and first subjected to G-protein binding assays as previously described [14]. All mutants tested retained similar

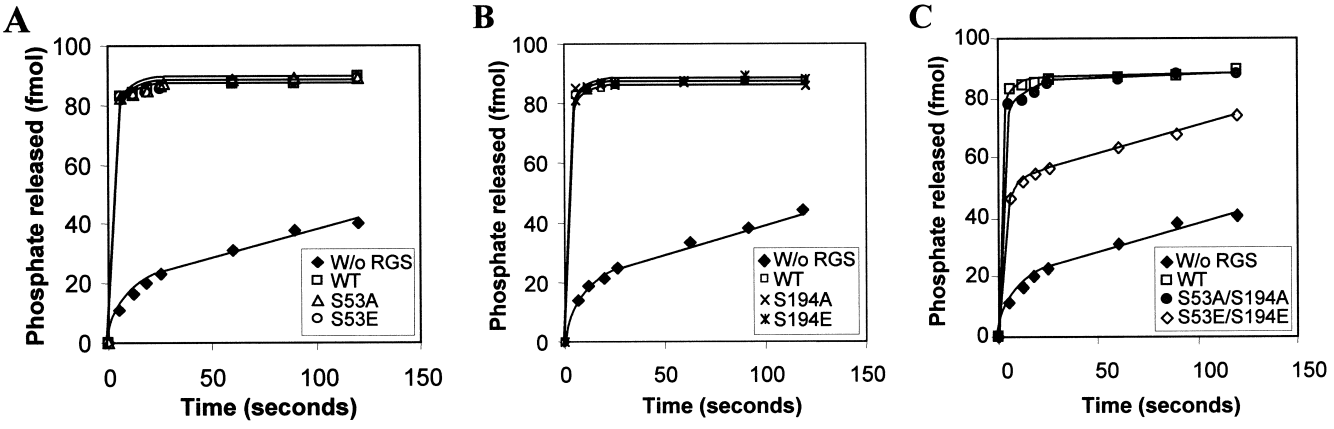

Fig. 3. GAP activity of various RGS16 proteins. The single-turnover assay was carried out to assess the GAP activity of each RGS16 protein. The time curves of GTP hydrolysis for $G_o\alpha$ (0.25 μM) were obtained by preloading $G_o\alpha$ with [γ-^{32}P]GTP at 30°C, and then incubating with the reaction buffer (w/o RGS) or with 2 μM of wild-type RGS16 (WT), or the same amount of RGS16 mutant proteins at 8°C. The GAP activities on $G_o\alpha$ of Ser-53 mutants (S53A, S53E), Ser-194 mutants (S194A, S194E), and Ser-53/Ser-194 mutants (S53A/S194A, S53E/S194E), together with the WT RGS16, are shown in panels A, B, and C, respectively.

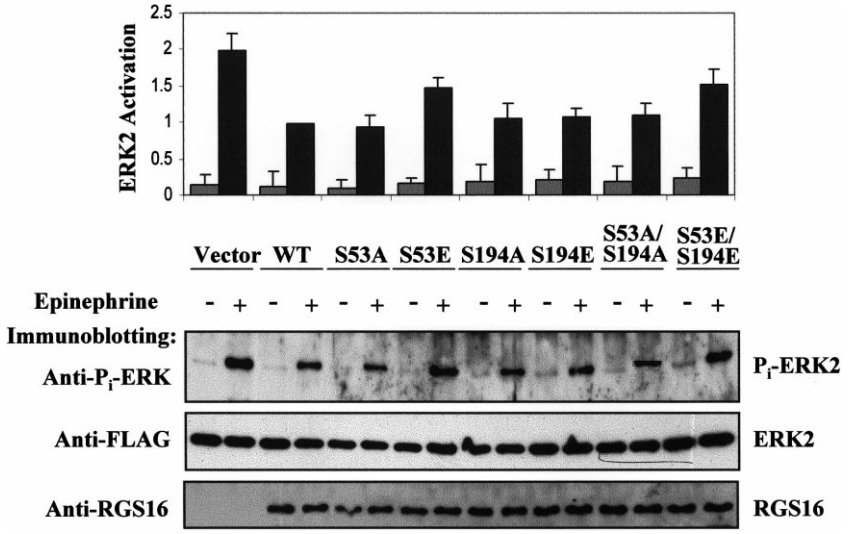

Fig. 4. Inhibitory effect of RGS mutants on the epinephrine-stimulated ERK2 activation. RGS16 wild-type, alanine, or glutamate mutants of Ser-53 and Ser-194 were expressed in HEK293T cells, together with the α2A adrenergic receptor and FLAG-tagged ERK2. The cells were serum-starved overnight before stimulation with epinephrine for 15 min. Kinase activity of each FLAG-tagged immunoprecipitate was detected by immunoblotting with anti phospho-ERK antibody. The histogram shows the fold activation of ERK2 for the various RGS16 constructs in the absence (−) or presence (+) of epinephrine stimulation, based on the phosphorylation of ERK2 protein ([Pi]-ERK2, indicated on the right), normalized to the amount of precipitated FLAG-ERK2 in each sample (ERK2, indicated on the right). The expression of different RGS16 mutants (bottom panel) was noted to be similar. WT: wild-type.

binding capacity towards in vitro generated [^{35}S]-labeled Gα subunits ($G_o\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$; data not shown), indicating that these proteins are properly folded. Single-turnover GTPase activity of $G_o\alpha$ was used to determine the GAP activity of individual RGS16 mutants at 8°C. In the absence of RGS protein, $G_o\alpha$ displayed an intrinsic GTPase activity to hydrolyze bound [γ-^{32}P]GTP to [^{32}Pi] and GDP (Fig. 3). In the presence of excess amounts (eight-fold) of recombinant RGS16 protein, maximal [Pi] release was observed in a much shorter time (less than 8 s). Single mutants of serine 53 or serine 194 (S53A, S53E, S194A, and S194E) retained their full capacity as wild-type RGS16 to stimulate GTP hydrolysis by $G_o\alpha$ (Fig. 3A,B). Surprisingly, only the double-glutamate mutant (S53E/S194E), but not the double-alanine mutant (S53A/S194A), exhibited a significantly reduced GAP function (Fig. 3C). This suggests that the phosphorylation of RGS16 on both serine residues diminished its capacity as a GAP for Gα subunit.

3.4. Epinephrine-stimulated phosphorylation of RGS16 attenuates its ability to inhibit MAPK activation

RGS16 was previously shown to interact with $G_i\alpha$, $G_o\alpha$, and $G_q\alpha$ proteins [4,14] and attenuate $G_q\alpha$-mediated p38-MAPK activation by the platelet-activating factor [4]. We therefore further studied the ability of RGS16 to inhibit ERK2 activation by the α2A adrenergic receptor. Time-course determination of ERK2 activity was performed by stimulating the cells with epinephrine at various time points (5, 15, and 30 min), followed by cell lysis and immobilization of FLAG-tagged ERK2 onto M2 beads, and Western blotting with specific phospho-p44/p42 MAPK antibody. Upon stimulation, the level of phospho-ERK2 protein increased rapidly and reached a maximum within 5 min; this high activity was maintained until 30 min (data not shown). Co-expression of the RGS16 protein attenuated epinephrine-stimulated ERK2 activation at all the time points tested, with the most obvious effect at 15 min. We therefore used this time point to test the various RGS16 proteins.

From Fig. 4, we can see that the level of phospho-ERK2 was strongly elevated upon epinephrine stimulation of the cells expressing the α2A adrenergic receptor (Fig. 4, vector control). Expressing wild-type RGS16 in these cells significantly reduced the magnitude of ERK2 phosphorylation (Fig. 4, WT). In addition, both the single-alanine mutants (S53A, or S194A) and the double-alanine mutant (S53A/S194A) did not exhibit any difference from wild-type RGS16 in attenuating epinephrine-stimulated ERK phosphorylation. Similar to the alanine mutants, alteration of serine 194 to glutamate (S194E) also had little effect. In contrast, the single-glutamate mutant (S53E) and double-glutamate mutant (S53E/S194E) partially lost their ability to inhibit ERK2 phosphorylation by 50% (Fig. 4). As serine 194 is constitutively phosphorylated in mammalian cells (as shown in Fig. 1), it was presumed that the single-glutamate mutant (S53E), when expressed in 293T cells, should mimic the effect of the double-glutamate mutant (S53E/S194E). Although we failed to notice a reduction in GAP activity by the single-glutamate mutant (S53E), this is likely due to the absence of constitutive phosphorylation on serine 194 for bacteria-expressed proteins. Moreover, the double-glutamate mutant (S53E/S194E), which mimics phosphorylation on both serine residues, consistently reduced the GAP and MAPK attenuation capacity of RGS16. Therefore, it appears that in mammalian cells, epinephrine stimulation can regulate the function of RGS16 via phosphorylation.

4. Discussion

Our current study shows that serine residues of RGS16 are phosphorylated constitutively (S194) and in a ligand-dependent manner (S53). Serine 194 is unique to mouse RGS16 and is not found in the human homolog, while serine 53 is com-

mon to RGS16 from both species and is located at the extreme N-terminus of the RGS domain, within the first α-helix. Our results indicate that the double-glutamate mutant (S53E/S194E) significantly reduced the GAP capacity of RGS16 protein, and consistently exhibited diminished ability to attenuate the α2A adrenergic receptor-stimulated ERK activation. As epinephrine-stimulated phosphorylation of RGS16 on serine 53 was observed at least after 15 min (data not shown), it appears that a sustained stimulation of the α2A adrenergic receptor was needed to downregulate RGS16 activity. This is a novel observation of the effect of sustained stimulation of a GPCR on the RGS16 protein.

Ligand-dependent phosphorylation of RGS proteins was previously shown for the yeast RGS member (Sst2) on serine 539 within a consensus MAPK phosphorylation sequence [12]. Phosphorylation of Sst2p by the MAPK Fus3p does not affect desensitization of yeast pheromone response, but instead decreases the rate of Sst2 degradation, due to a feedback regulation of the RGS protein in response to pheromone stimulation. Among the mammalian RGS proteins, RGS7 has been shown to be phosphorylated by the stress-activated protein kinase p38 upon tumor necrosis factor-α treatment, which prevents the proteasome-dependent degradation of RGS7 [22]. In our current study on RGS16, the phosphorylation sites at S53 and S194 bear little resemblance to the consensus MAPK phosphorylation sequence (PXSP), which is in accordance with the observation that addition of MAPK inhibitors SB203580 or PD98059 failed to block the constitutive and ligand-dependent in vivo phosphorylation of RGS16 (result not shown). This also indicates that the kinases responsible for RGS16 phosphorylation do not belong to the MAPK family. Furthermore, treatment of HEK293T cells expressing the various RGS16 mutants with cycloheximide (for up to 8 h) did not indicate any change in stability of these proteins compared to wild-type RGS16 (result not shown).

The phosphorylation of another mammalian RGS member, GAIP, results in the acceleration of its GAP activity and autophagy in human colon cancer HT-29 cells [13]. Serine 151 on GAIP, which is phosphorylated by the MAPK ERK1/2, is located in the inter-helical region of helix 5 and 6, and is believed to be part of the binding region with Gα [3]. In our present study, the RGS16 serine residues that are phosphorylated are located either in the extreme N-terminus of the RGS box (S53) or outside of it (S194). We had previously shown that the core domain of RGS16, in which both of these two phosphorylation sites were deleted, still retains its binding and GAP activity towards G proteins [23]. Consistent with this conjecture, our experiments with the alanine mutants at serine 53 and serine 194 of RGS16 (S53A, S194A, and S53A/S194A) indicated no effect on G-protein binding (result not shown) or disruption of RGS GAP activity (Figs. 3 and 4).

Interestingly, double-glutamate mutant (S53E/S194E) significantly reduced RGS GAP activity, although it can still bind to the G protein (Figs. 3 and 4, and result not shown). Furthermore, determination of the soluble structures of RGS4 by nuclear magnetic resonance (NMR) has revealed significant differences in both the N- and C-terminal regions of the free RGS4 NMR structure with the RGS4-$G_{i1}\alpha$ X-ray structure [24,25]. This suggests that in order to serve as an efficient GAP, RGS proteins in the presence of the Gα subunit, must undergo substantial conformational changes involving both the N- and C-terminal regions to form an optimal structure for binding to and stabilizing the transition state of the Gα subunit. Thus, the introduction of two negatively charged phosphate groups on the N- and C-terminal regions might interfere with the conformational changes, and possibly result in a reduced GAP ability as seen in the double-glutamate mutant of RGS16. As serine 194 is constitutively phosphorylated in mammalian HEK293T cells, expressing the single-glutamate mutant of serine 53 should therefore give a similar effect as the double-glutamate mutant (S53E/S194E) (Fig. 1). Hence, it is not surprising that in mammalian cells, the S53E mutant also has a reduced capacity to inhibit epinephrine-stimulated ERK2 activation. However, the S194E mutant, which mimics the constitutively phosphorylated wild-type RGS16, can inhibit ERK2 activation by epinephrine (Fig. 4). These observations suggest that phosphorylation of RGS16 on different sites can differentially modulate its GAP activity.

The recent observation that 14-3-3, a scaffolding protein, can directly interact with RGS3 and RGS7 [26], strongly suggests that RGS proteins may have a more diverse role than previously thought. The binding of RGS3 and RGS7 with 14-3-3 is dependent on the phosphorylation of a conserved serine located in the Gα-interacting portion of the RGS domain, and therefore also inhibited the RGS GAP activity [26]. Despite the presence of the conserved serine residue (S166) in RGS16, we did not observe any phosphorylation of this residue either constitutively or upon epinephrine stimulation. It is likely that the phosphorylation event is tightly regulated and specific to certain stimuli as well as to distinct RGS members, as in the case of the epinephrine-stimulated phosphorylation of RGS16. The ability of RGS proteins to interact with molecules in addition to Gα subunits was shown for the p115 RhoGEF [8], which interacts not only with $G_{12}\alpha$ and $G_{i3}\alpha$ subunits, but also with a guanine nucleotide exchange factor for Rho, a member of the small G-protein family. Recently, RGS12 is shown to interact with N-type calcium channels through its PTB domain in a tyrosine-kinase-dependent manner upon stimulation by γ-aminobutyric acid [27], which acts via a GPCR. These findings further extend the role of RGS proteins beyond acting as GAPs for Gα subunits. Therefore, it is conceivable that phosphorylation of RGS16 on serine 53 may generate a docking site for interaction with as-yet-identified molecules other than the G proteins, as in the case of RGS3 and RGS7 interactions with 14-3-3 [26], or RGS12 with N-type calcium channel [27]. This interaction with novel molecules may potentially inhibit the GAP function of RGS protein.

In summary, we have clearly shown that RGS16 is a phosphoprotein that can be phosphorylated on multiple serine sites. Phosphorylation of RGS16 upon stimulation of the α2A adrenergic receptor by epinephrine significantly reduced its GAP function and consequently its attenuation of the MAPK pathway. This is the first report of RGS phosphorylation and functional downregulation via a GPCR. It will be interesting to explore whether such a phenomenon extends to other RGS proteins.

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