Src-mediated RGS16 Tyrosine Phosphorylation Promotes RGS16 Stability*

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The amplitude of signaling evoked by stimulation of G protein-coupled receptors may be controlled in part by the GTPase accelerating activity of the regulator of G protein signaling (RGS) proteins. In turn, subcellular targeting, protein-protein interactions, or post-translational modifications such as phosphorylation may shape RGS activity and specificity. We found previously that RGS16 undergoes tyrosine phosphorylation on conserved tyrosine residues in the RGS box. Phosphorylation on Tyr168 was mediated by the epidermal growth factor receptor (EGFR). We show here that endogenous RGS16 is phosphorylated after epidermal growth factor stimulation of MCF-7 cells. In addition, p60-Src or Lyn kinase phosphorylated recombinant RGS16 in vitro, and RGS16 underwent phosphorylation in the presence of constitutively active Src (Y529F) in EGF–CHO-K1 cells. Blockade of endogenous Src activity by selective inhibitors attenuated RGS16 phosphorylation induced by pervanadate or receptor stimulation. Furthermore, the rate of RGS16 degradation was reduced in cells expressing active Src or treated with pervanadate or a G protein-coupled receptor ligand (CXCL12). Induction of RGS16 tyrosine phosphorylation was associated with increased RGS16 protein levels and enhanced GAP activity in cell membranes. These results suggest that Src mediates RGS16 tyrosine phosphorylation, which may promote RGS16 stability.

G protein-coupled receptors (GPCRs),1 the largest family of proteins in the human genome (1), mediate extracellular signals that control such diverse processes as sensation, cognition, cell growth and proliferation, cell migration, and hormone secretion. GPCRs signal through a common element, the heterotrimeric G protein, whose α subunit undergoes agonist-evoked GTP binding and dissociation from its tonic repressor, βγ. Each G protein component elicits diverse outcomes including increased enzyme activity or concentration, gene transcription, or cellular movement. The intrinsic GTPase activity of the α subunit allows re-formation of an αβγ trimeric complex, terminating signaling. Regulator of G protein signaling (RGS) proteins, which are GTPase-activating proteins (GAPs) for Gα subunits, may down-regulate G protein signaling cascades (2–3); however, their physiological roles have only begun to be elucidated. Some pathological conditions have been linked to abnormal RGS expression, suggesting that pharmaceutical modulation of RGS activity may impact the treatment of diseases associated with abnormal GPCR output (4). How specific RGS proteins modify discrete GPCRs or G proteins remains an unclear but intensely studied question (5). For these reasons, it is of vital importance to understand how individual RGS proteins are regulated.

One means by which RGS proteins may be modified is phosphorylation (6–17). Sst2 undergoes signal-dependent phosphorylation by MAP kinase, which slows Sst2 degradation (6). 14-3-3 proteins interact with phosphorylated RGS7, which reduces RGS7 GAP activity (7). Protein kinase C phosphorylates RGS2 on a serine residue in the RGS box, resulting in decreased GAP activity toward Gαs (8). By contrast, ERK2 phosphorylation of RGS-Gαs-interacting protein enhances GAP activity toward Gαs (9, 10). RGS16 is phosphorylated on Ser194 after epinephrine stimulation of cells expressing the a2b-adenosine receptor (11). Mutation of this serine residue impairs RGS16 GAP activity and its regulation of epinephrine-stimulated MAP kinase activation.

In addition to direct effects on RGS enzymatic activity, Ser phosphorylation may alter intracellular localization of certain RGS proteins. Protein kinase A phosphorylates RGS10, and mutation of the protein kinase A target Ser residue prevents RGS10 translocation from the plasma membrane and cytosol to the nucleus (12). Upon illumination of rod outer segments, RGS9-1 translocates to lipid rafts (13), where it undergoes Ser phosphorylation by an endogenous kinase (14).

RGS proteins also contain conserved tyrosine residues in the RGS box that appear to affect their function. Phosphorylation of the RGS domain-containing protein PDZ-RhoGEF by focal adhesion kinase enhances Rho activation after stimulation of Gαi2/3-coupled GPCRs (15). We reported previously that RGS16 undergoes epidermal growth factor receptor (EGFR)-mediated tyrosine phosphorylation on a conserved tyrosine residue in the RGS box, Tyr168, which enhances RGS16 GAP activity in single turnover assays (16). However, when cellular tyrosine phosphatase activity is inhibited by pervanadate,
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RGS16 lacking Tyr^{125} is also phosphorylated, indicating that Tyr^{177} (the only other tyrosine residue in RGS16) is a possible site of phosphorylation. Moreover, although mutation of Tyr^{177} does not affect RGS16 GAP activity in vitro, the mutant protein is unable to modulate G_{i}-coupled adenyl cyclase inhibition or G_{o}-coupled MAP kinase activation in cells. These results suggest that phosphorylation of this residue is of critical importance to the biological activity of RGS16.

In this study we investigated further mechanisms of RGS16 tyrosine phosphorylation and their possible effects on RGS16 function. We found that RGS16 was a substrate for Src family kinases in vitro and in mammalian cells. Src-mediated RGS16 phosphorylation did not affect RGS16 intracellular localization or GAP activity, but instead appeared to slow RGS16 protein degradation. Moreover, sustained phosphorylation correlated with an expanded pool of RGS16 and increased GAP activity in cell membranes. These results demonstrate that RGS16 function is modulated by tyrosine phosphorylation in mammalian cells, suggesting a novel form of feedback regulation of G protein signaling by tyrosine kinases.

EXPERIMENTAL PROCEDURES

Reagents—ATP, sodium orthovanadate, hydrogen peroxide (H_{2}O_{2}), doxorubicin, EGF, and carbachol were purchased from Sigma. PP2, PP3, and the proteasome inhibitor MG132 were obtained from Calbiochem. Pervanadate consisted of a mixture of 0.1 mM sodium orthovanadate and 10 mM H_{2}O.

Cells, Proteins, and Plasmids—HEK 293T and CHO-K1 cells were maintained in supplemented Dulbecco’s modified Eagle’s medium (in vitro) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 units/ml streptomycin, and 20 ng/ml gentamicin, in a humidified 5% CO_{2} incubator at 37 °C.

C. BaF3 cells stably expressing mouse HA-RGS16, the gift of Cheryl Miller (Millennium Pharmaceuticals), were plated in 100-mm tissue culture dishes and transfected with appropriate plasmids for 18 h using LipofectAMINE reagent (Invitrogen) or for 5 h using Superfect (Qiagen) according to the manufacturer’s instructions. After removal of the DNA mixture, the cells were incubated in serum-free medium for the indicated times in the presence or absence of the indicated agonist or inhibitor. Cells were lysed by buffer P and immunoprecipitated with either anti-RGS16 antibodies or HA-coupled agarose (Roche Molecular Biochemicals) as indicated. Immunoprecipitates were resolved by SDS-PAGE and exposed to autoradiography at ~70 °C.

Preparation of Membranes—Generation of HEK 293 cells stably expressing a 5-hydroxytryptamine 1A receptor (5-HT_{1A}R) or Grb2 fusion protein have been described previously (22). 30 h post-transfection, cells were treated overnight with pertussis toxin (PTX, 50 ng/ml, Calbiochem) to eliminate endogenous G_{oA} activity. The next day, cells were scraped and harvested in Tris-buffered saline and centrifuged at 4,000 × g for 10 min at 4 °C. Pellets were resuspended in ice-cold Tris-HCl-EDTA (TE), pH 7.4, and homogenized with 30–50 passages through a Dounce homogenizer followed by 15 passages through a 25-gauge needle. Unbroken cells and debris were pelleted by centrifugation at 4,000 × g for 5 min at 4 °C. The supernatant was centrifuged at 100,000 × g for 30 min at 4 °C to pellet the membrane fraction. Membranes were resuspended in TE buffer to ~2 mg/ml and stored as aliquots at ~−80 °C.

High Affinity GTPase Assays—Steady-state GTPase activity was determined in membranes expressing the fusion protein. Membrane preparations (10 μg of total protein) were stimulated with the indicated concentrations of 5-HT in an ATP-regenerating buffer system (20 mM creatine phosphate, 0.1 unit/ml creatine kinase, 200 μM AMP-PNP, 50 μM GTP, 2 mM MgCl_{2}, 10 μM GTP, 2 mM GDP) and phosphorylated by GTPyS (10 μM MgCl_{2}, 4 mM dithiothreitol, 200 μM EDTA, pH 7.5, 80 mM Tris, pH 7.5), spiked with 50,000 cpm [γ^{32P}]GTP (Amersham Biosciences, 3000 Ci/mmol), and incubated for 20 min at 30 °C. Reactions were subjected to SDS-PAGE, and gels were dried prior to autoradiography. The band containing RGS was quantitated by liquid scintillation spectrometry.

In Vitro Phosphorylation—We determined RGS phosphorylation by incubating 10–25 pmol of His_{6}RGS16 for 1 h at 30 °C with recombinant Lyn, Csk, or Ab1 kinases (1 μl) in buffer containing 50 mM HEPES, pH 7.5, 5 mM MgCl_{2}, 5 mM MnCl_{2}, 1 mg/ml bovine serum albumin, 0.1% Triton X-100, 1 mM Na_{3}VO_{4}, 5 μM cold ATP, and 1 μCi [γ^{32P}]ATP (100 mCi/mmol, 30 Ci mmol⁻¹) for 1 h at 30 °C. Reactions were subjected to SDS-PAGE, and gels were dried prior to autoradiography. The band containing RGS was quantitated by liquid scintillation spectrometry.

Stoichiometry of phosphorylation was calculated based on the specific activity of [γ^{32P}]ATP and RGS16 concentration determined by Bradford assay.

Proteolytic Cleavage—Protease digestion with endopeptidase Lys-C (500 ng, Roche Molecular Biochemicals) was carried out after phosphorylation in 30 μl of incubation buffer (25 mM Tris, pH 8.5, 1 mM EDTA) for 2 h at 37 °C. His-tagged RGS16 was phosphorylated as described for 1 h before affinity purification by nickel-agarose chromatography. Beads were washed twice in detergent-containing buffer P-Ni and once in protease incubation buffer before the addition of protease. Digests were separated on 10% Tricine gels and run alongside Mark 12 protein ladder (Invitrogen).

Single Turnover GAP Assays—We performed single turnover GTPase assays by loading recombinant, myristoylated Grb2 (Calbiochem) with [γ^{32P}]GTP (1 μM, 50–100 cpm fmol⁻¹) at 30 °C in buffer containing 50 mM HEPES, pH 8, 5 mM EDTA, 1 mM dithiothreitol, 0.1% C_{12}E_{50} (buffer C). The reaction was chilled on ice before gel filtration on a G-25 Sepharose column that was equilibrated with buffer C plus bovine serum albumin (0.1 mg ml⁻¹). GTP hydrolysis was initiated by adding G protein to a tube containing unlabeled GTP (100 μM) and MgSO_{4} (10 mM) with or without RGS. Aliquots were removed at various time points from 10 s prior to the start of the reaction (arbitrarily set as time 0) to 5 min and quenched in a chilled, activated charcoal-phosphate slurry. Inorganic phosphate in supernatants was measured by liquid scintillation spectrometry after centrifugation at 1500 × g. Net release of P, was determined by subtracting the amount at time 0.

Metabolic Labeling and Pulse-Chase Analysis—BaF3/RGS16 cells (5 × 10^{6} cells/ml) were washed once in methionine-free RPMI (Biofluids) and then incubated for an additional 30 min in this medium to deplete intracellular methionine pools. [^{35}S]Met/Leu (Amersham Biosciences, >1000 Ci/mmol, 10 μCi/ml) was added at a final concentration of 150 μCi/ml for 30 min at 37 °C. Cells were then pelleted, washed in chase medium (serum-free RPMI supplemented with cold methionine (Sigma, 15 mg/liter)), and incubated at 37 °C in chase medium for the indicated times in the presence or absence of the additional agonist or inhibitor. Cells were lysed in buffer P and immunoprecipitated with either anti-RGS16 antibodies or HA-coupled agarose (Roche Molecular Biochemicals) as indicated. Immunoprecipitates were resolved by SDS-PAGE and exposed to autoradiography at ~70 °C.

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Phosphorylation of Endogenous RGS16 in MCF-7 Cells—Our previous studies demonstrated EGF-mediated RGS16 tyrosine phosphorylation in transfected cells. To explore RGS16 phosphorylation further, we first investigated whether endogenous RGS16 underwent tyrosine phosphorylation in response to EGF stimulation in MCF-7 breast carcinoma cells. Rgs16 was discovered as a target of the p53 tumor suppressor gene and shown to be up-regulated by genotoxic stress in these cells (23). Its expression is difficult to test directly in cells that express both Src and RGS16. We next determined whether Src plays a role in RGS16 phosphorylation in mammalian cells. This hypothesis was supported by experiments showing that RGS16 was phosphorylated in vitro by recombinant p60-Src and Lyn but not by Btk or Abl kinases, and RGS2 was not phosphorylated by Src or Btk kinase (Fig. 2, A and B). Interestingly, purified Csk kinase was able to phosphorylate RGS16 in vitro; however, this phenomenon was not explored further. We determined the stoichiometry of phosphorylation by measuring counts/min of the RGS16 band excised from gels divided by the specific activity of [$\gamma$-32P]ATP. Based on this measurement, the stoichiometry of phosphorylation was 0.6 ± 0.1 mol of phosphate per mol of RGS16 (mean ± S.E. of 5 independent experiments), suggesting that Src phosphorylated RGS16 at a single site in vitro.

In Vivo RGS16 Phosphorylation in the Presence of Constitutively Active Src—We next determined whether Src plays a role in RGS16 phosphorylation in mammalian cells. This hypothesis is difficult to test directly in cells that express both Src and RGS16. In this study, we used an in vivo system to explore this relationship. We incubated RGS2 or RGS16 with recombinant kinase and [$\gamma$-32P]ATP and measured ATP incorporation into the protein by autoradiography. RGS16 was phosphorylated by recombinant p60-Src and Lyn but not by Btk or Abl kinases, and RGS2 was not phosphorylated by either Src or Btk kinase (Fig. 2, A and B). Interestingly, purified Csk kinase was able to phosphorylate RGS16 in vitro; however, this phenomenon was not explored further. We determined the stoichiometry of phosphorylation by measuring counts/min of the RGS16 band excised from gels divided by the specific activity of [$\gamma$-32P]ATP. Based on this measurement, the stoichiometry of phosphorylation was 0.6 ± 0.1 mol of phosphate per mol of RGS16 (mean ± S.E. of 5 independent experiments), suggesting that Src phosphorylated RGS16 at a single site in vitro.

RESULTS

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kDa band only in anti-RGS16 immunoprecipitates (Fig. 1A, bottom). Finally, to determine whether endogenous RGS16 underwent tyrosine phosphorylation, we treated the cells with either medium alone or EGF, and we immunoblotted cell lysates with either anti-phosphotyrosine (Fig. 1B, top) or anti-RGS16 (Fig. 1B, bottom). RGS16 was not phosphorylated under basal conditions but was significantly phosphorylated after EGF treatment. These results suggest that RGS16 tyrosine phosphorylation occurs in response to a physiological stimulus in MCF-7 cells.

Src Phosphorylates RGS16 in Vitro—To determine which, if any, additional kinase(s) mediate RGS16 tyrosine phosphorylation, we tested the capacity of purified RGS16 to act as a substrate for various kinases in vitro. We incubated RGS2 or RGS16 with recombinant kinase and [$\gamma$-32P]ATP and measured ATP incorporation into the protein by autoradiography. RGS16 was phosphorylated by recombinant p60-Src and Lyn but not by Btk or Abl kinases, and RGS2 was not phosphorylated by either Src or Btk kinase (Fig. 2, A and B). Interestingly, purified Csk kinase was able to phosphorylate RGS16 in vitro; however, this phenomenon was not explored further. We determined the stoichiometry of phosphorylation by measuring counts/min of the RGS16 band excised from gels divided by the specific activity of [$\gamma$-32P]ATP. Based on this measurement, the stoichiometry of phosphorylation was 0.6 ± 0.1 mol of phosphate per mol of RGS16 (mean ± S.E. of 5 independent experiments), suggesting that Src phosphorylated RGS16 at a single site in vitro.

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EGFR because EGFR stimulates Src kinase activity (24), and in turn Src enhances EGFR activity (25). To minimize a possible effect of EGFR kinase, we examined RGS16 phosphorylation in CHO-K1 cells, which do not express functional EGFR but do express a closely related EGFR family member ErbB2 (26, 27). To determine whether these cells respond to EGFR, we measured MAP kinase activation after EGF or carbachol stimulation in cells transfected with m2R by immunoblotting with anti-phospho-ERK and ERK1 antibodies. Similar to previous studies, EGF treatment of CHO-K1 cells did not induce ERK activation, whereas increased ERK activity was observed after carbachol treatment (28) (Fig. 3A). To evaluate RGS16 phosphorylation in the presence of Src activation, we expressed HA-RGS16 with control or constitutively active Src (Y529F) plasmids, and we assessed RGS16 phosphorylation by immunoprecipitation with anti-HA and detection with anti-phosphotyrosine antibodies. RGS16 underwent pronounced tyrosine phosphorylation in the presence of active Src (Fig. 3B). To determine whether endogenous Src kinases(s) in CHO-K1 cells mediate RGS16 phosphorylation, we expressed RGS16-V5/His and treated cells with pervanadate, an inhibitor of tyrosine phosphatases. We affinity-purified recombinant His-tagged proteins with Ni²⁺/nitriloacetic acid-coupled agarose and immunoblotted with anti-phosphotyrosine and anti-V5. There were no tyrosyl-phosphorylated proteins in affinity precipitations from vector-transfected or RGS16-transfected cells without pervanadate stimulation. In contrast, we observed strong RGS16 phosphorylation in pervanadate-treated cells, which was blocked by the Src-specific inhibitor PP2 (Fig. 3C). This result indicates that pervanadate-treated cells mediate Src-dependent RGS16 phosphorylation.

Receptor-induced RGS16 Tyrosine Phosphorylation Is Sensitive to Src Kinase Blockade—We have shown previously (16) that GPCR stimulation (m2R) induces RGS16 tyrosine phosphorylation. To examine whether Src plays a role in GPCR-induced RGS16 tyrosine phosphorylation, we utilized BaF3 B-lymphoblastoid cells lacking endogenous RGS16 that stably overexpress HA-RGS16. We stimulated cells with CXCL12, which activates endogenous CXCR4 chemokine receptors coupled to Gαi. Activation of Gαi is associated with increased Src activity (17). We immunoprecipitated RGS16 using anti-RGS16 antibodies and immunodetected with anti-phosphotyrosine or anti-HA. Treatment with the CXCR4 ligand CXCL12 induced RGS16 tyrosine phosphorylation, which was blocked by PP2 (Fig. 4A). To test whether Src-dependent RGS16 tyrosine phosphorylation could occur independently of GPCR stimulation, we stimulated BaF3/RGS16 cells with anti-IgM + IgG to cross-link the B-cell receptor, which activates the endogenous Src family kinase Lyn (21). In these cells, we observed robust RGS16 tyrosine phosphorylation in response to Ig re-
Fig. 5. Src-mediated RGS16 phosphorylation depends on Tyr177. A, recombinant RGS16 WT or mutants were incubated with recombinant p60-Src as in Fig. 2. Reactions were stopped by the addition of Laemmli buffer, and samples were resolved by SDS-PAGE. Gels were immunoblotted sequentially with anti-phosphotyrosine (PY) and anti-RGS16 antibodies. RGS16 phosphorylation is indicated by the arrow and Src autophosphorylation by the arrowhead. B, His-tagged RGS16 WT or Y177F was incubated with Src kinase (100 ng) and 1 μCi of [γ-32P]ATP for 90 min. Ni2+/nitriloacetic acid beads (20 μl) were added for an additional 30 min. Beads were then washed twice in detergent-containing buffer and once in phosphate incubation buffer. EndoLys-C (500 ng) was added for an additional 30 min. Beads were then washed twice in detergent-containing buffer and once in phosphate incubation buffer. EndoLys-C is predicted to generate a C-terminal fragment consisting of residues 174–202 (containing Tyr177) plus an additional 13 linker residues contained in the plasmid used to generate recombinant RGS16. Together, this polypeptide has an expected mass of 4.2 kDa. After incubation with Src kinase plus [γ-32P]ATP, we affinity-purified His-RGS16 WT or Y177F by nickel chromatography before digestion with EndoLys-C. We visualized a radioactive band at 4 kDa in reactions containing RGS16 WT but not Y177F (Fig. 5C). There was also a radioactive band at ~10 kDa, which we hypothesize to be a partial digestion product. Coomassie Blue staining demonstrated a similar cleavage pattern for both RGS16 WT and Y177F (Fig. 5C), indicating that the C-terminal peptide was not phosphorylated in the Y177F mutant. This result suggests Src phosphorylates native RGS16 at residue Tyr177 in vitro.

To determine the site of phosphorylation of RGS16 in cells, we expressed HA-RGS16 WT or mutants with active Src (Y529F) in CHO-K1 cells. We immunoprecipitated RGS16 with anti-HA and blotted with anti-phosphotyrosine or anti-HA to confirm equal RGS16 levels. Whereas phosphorylation of RGS16 (Y168F) was similar to WT, the level of phosphorylation of RGS16 (Y177F) was reduced by at least 50% (Fig. 5D), and this phosphorylation reduces GAP activity of the recombinant protein because of altered protein folding or conformation, as this mutation reduces GAP activity of the recombinant protein in vitro, independently of phosphorylation (16). To map the phosphorylation site in the WT protein, we digested phosphorylated RGS16 WT with endoproteinase Lys-C (EndoLys-C). Together, this polypeptide has an expected mass of 4.2 kDa. After incubation with Src kinase plus [γ-32P]ATP, we affinity-purified His-RGS16 WT or Y177F by nickel chromatography before digestion with EndoLys-C. We visualized a radioactive band at 4 kDa in reactions containing RGS16 WT but not Y177F (Fig. 5C). There was also a radioactive band at ~10 kDa, which we hypothesize to be a partial digestion product. Coomassie Blue staining demonstrated a similar cleavage pattern for both RGS16 WT and Y177F (Fig. 5C), indicating that the C-terminal peptide was not phosphorylated in the Y177F mutant. This result suggests Src phosphorylates native RGS16 at residue Tyr177 in vitro.

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To determine the site of phosphorylation of RGS16 in cells, we expressed HA-RGS16 WT or mutants with active Src (Y529F) in CHO-K1 cells. We immunoprecipitated RGS16 with anti-HA and blotted with anti-phosphotyrosine or anti-HA to confirm equal RGS16 levels. Whereas phosphorylation of RGS16 (Y168F) was similar to WT, the level of phosphorylation of RGS16 (Y177F) was reduced by at least 50% (Fig. 5D), and
Src Phosphorylates RGS16

Fig. 7. Phosphorylation affects RGS16 stability. A, CHO-K1 cells were transfected with RGS16 (no epitope tag) together with lacZ or Src (Y529F) plasmids. 24 h post-transfection, cells were treated with cycloheximide (10 μg/ml) before lysis at the indicated times. Equal volume aliquots from lysates were separated by SDS-PAGE and immunoblotted with anti-RGS16. The levels of a more stable protein, ERK1, did not vary substantially over the indicated period except at 4 h as determined by immunoblotting with anti-ERK1 (data not shown). ID, immunodetection. β-Gal, β-galactosidase. B, graphic representation of RGS16 levels (percent of the initial value, mean ± S.E. of 2–4 independent experiments) in the presence or absence of active Src, as determined by densitometry. C, CHO-K1 cells were transfected with RGS16-V5/His. 24 h post-transfection, cells were incubated with cycloheximide (15 μg/ml) with or without pervanadate. Cells were lysed at the indicated times, and lysates were subjected to SDS-PAGE and immunoblotting with anti-V5 antibodies. D, cells transfected with RGS16-V5/His (Y168F/Y177F) together with lacZ or active Src were processed as in A. E, graphic representation of RGS16 (Y168F/Y177F) levels in the presence of control or active Src plasmids determined by densitometry. Values represent mean ± S.E. of two independent experiments. F, same experiment as C, except that the Y168F/Y177F mutant was transfected.

Y168/Y177F was not phosphorylated (data not shown). Collectively, although these experiments suggest that Tyr177 may be the site of Src-mediated RGS16 phosphorylation, it also appears that Tyr168 is phosphorylated in the presence of constitutively active Src in vivo. As mentioned above, Tyr168 phosphorylation could be an indirect consequence of Src activation, which is discussed below.

Src-induced RGS16 Tyrosine Phosphorylation Does Not Affect GAP Activity in Vitro or Localization in Transfected Cells—In a previous study, we found that EGFR-mediated tyrosine phosphorylation of RGS16 increased its GAP activity on Go1. To determine whether Src-mediated RGS16 phosphorylation also affected its catalytic activity, we phosphorylated RGS16 with Src prior to measuring GTP hydrolysis by Go1. However, we observed no difference in the GAP activity of RGS16 in the presence or absence of Src (Fig. 6).

We hypothesized that because Src-mediated tyrosine phosphorylation did not appear to affect RGS16 GAP activity, it could play a role in protein localization. To address this possibility, we expressed RGS16-GFP in CHO-K1 cells together with lacZ or active Src and examined the cells by fluorescence microscopy. Transfected RGS16-GFP was distributed throughout the cytoplasm and at the membrane, consistent with its distri-
Src-mediated RGS16 Phosphorylation Enhances RGS16 Protein Stability—Because phosphorylation was shown to increase stability of another RGS protein, Stat2 (6), we hypothesized that RGS16 tyrosine phosphorylation could affect RGS16 degradation. To test this possibility, we expressed RGS16 in CHO-K1 cells in the presence of either lacZ or Src (Y529F) plasmids. 24 h after transfection, we added cycloheximide to prevent de novo protein synthesis and collected cells at various time points to evaluate RGS16 levels by immunoblotting. The rate of RGS16 degradation was decreased in the presence of activated Src, suggesting enhanced protein stability (Fig. 7A). The half-life of RGS16 in the absence of Src was ~2 h, but in the presence of active Src, RGS16 levels diminished more slowly over a period of 4 h (Fig. 7B). To determine whether RGS16 stability in the presence of active Src was related to phosphorylation, we stimulated cycloheximide-treated cells with pervanadate, and we assessed the rate of RGS16 degradation by immunoblotting. The half-life of RGS16 in the presence of pervanadate was significantly prolonged, from less than 30 min to greater than 60 min after pervanadate treatment (Fig. 7C).

We hypothesize that the variability in the estimated half-life of RGS16 in our experiments is most likely the result of differing concentrations of cycloheximide or the presence or location of epitope tags used in different experiments. To ascertain whether the enhanced RGS16 stability we observed with Src co-expression was dependent on RGS16 tyrosine phosphorylation, we expressed the phosphorylation-resistant RGS16 mutant (Y168F/Y177F) and assessed RGS16 degradation after pervanadate treatment or in the presence of co-transfected Src (Y529F) by immunoblotting. In either case, RGS16 (Y168F/Y177F) showed a similar half-life and did not undergo phosphorylation (Fig. 7, D–F, and data not shown). Taken together, these results suggest that Src-mediated RGS16 phosphorylation promotes RGS16 stability in CHO-K1 cells.

To determine whether RGS16 protein turnover was slowed by physiological Src activation, we analyzed RGS16 levels in BaF3-RGS16 cells stimulated through CXCR4. We performed pulse-chase analysis of radioactively labeled RGS16 to exclude the possibility that cycloheximide affected RGS6 degradation in previous experiments and to measure protein levels more precisely than by quantitative immunoblotting. Cells were metabolically labeled with [35S]methionine and then treated with medium alone or medium containing CXCL12 for various times. Detergent lysates were then immunoprecipitated with anti-RGS16, electrophoresed, and visualized by autoradiography. We did not detect RGS16 in immunoprecipitates of vector-transfected cells (Fig. 8A, lane 1), but we observed a doublet of ~30 kDa in anti-RGS16 immunoprecipitates. The identity of the lower band is uncertain, but it has been observed previously in immunoprecipitates of endogenous RGS16 from rat liver using this antiserum (29). We hypothesize that the lower molecular weight band could represent either a proteolytic cleavage product or alternative start site because it is not recognized by anti-HA antibody (see below). Treatment with chemokine appeared to decrease the rate of RGS16 degradation over a 3-h period. Interestingly, at the 3-h time point, RGS16 immunoprecipitates contained several bands of both higher and lower molecular weight. Because RGS16 has been shown to be an N-end rule substrate in reticulocyte lysates, signifying proteasome-mediated degradation, we hypothesized that these bands could represent mult ubiquitinilated RGS16 (30). To confirm that RGS16 degradation was proteasome-dependent in mammalian cells, we performed pulse-chase in the presence or absence of the proteasome inhibitor MG132. In this experiment, lysates were immunoprecipitated with agaroose coupled to HA and immunoblotted with anti-RGS16; thus, a single band of ~30 kDa was observed by autoradiography (Fig. 8B) or by immunoblotting (not shown). With MG132 treatment, the rate of RGS16 degradation appeared to be significantly reduced, comparable with the rate induced by GPCR stimulation. These results suggest that GPCR-evoked phosphorylation slows RGS16 turnover and that RGS16 degradation is dependent on the proteasome.

RGS16 Phosphorylation Enhances GAP Activity in Cell Membranes—We hypothesized that, over time, induction of RGS16 phosphorylation would lead to a larger pool of available GAP due to reduced degradation and would thus increase GAP activity in cell membranes. To explore this possibility, we measured 5-HT-evoked GTPase activity in HEK 293 membranes expressing a fusion protein between the 5-hydroxytryptamine 1A receptor (5-HT1A) and Gα13. In this construct, Gα contains a pertussis-toxin (PTX) resistance mutation, enabling PTX treatment of cells prior to membrane extraction to ablate GTPase activity by endogenous Gαi. We have employed this strategy previously to measure the GAP activity of co-transfected RGS proteins in vivo (31). In this case, this method was ideal to determine the extent of RGS16 phosphorylation induced by pervanadate, because the activity of the receptor/G protein fusion protein was unlikely to be directly modulated by tyrosine phosphorylation, in contrast to activation of downstream signaling components that could be strongly affected by tyrosine kinase activity. We treated cells stably expressing 5-HT1A/Gα13 and transiently expressing WT RGS16 with or without pervanadate for 30 min before preparing membranes and measuring 5-HT-induced GTPase activity. As expected, GTPase rates were greater in membranes expressing WT RGS16 than in vector-transfected cells (Fig. 9A).
However, GTPase activity in cells expressing RGS16 and exposed to pervanadate was nearly 2.5-fold higher than the activity of untreated membranes containing RGS16. This enhanced GAP activity correlated with a nearly 2-fold increase in RGS16 protein levels in the membrane as determined by immunoblotting (inset). By contrast, levels of the fusion protein (detected with a 5-HT1A receptor antibody) were not significantly affected by pervanadate treatment. To determine whether the increase in activity was attributable to the enlarged pool of RGS16 and to exclude the possibility that pervanadate stimulated the GTPase activity of the fusion protein directly, we treated cells expressing the phosphorylation-resistant RGS16 mutant (Y168F/Y177F) with pervanadate and measured agonist-evoked GTPase activity. Although GTPase activity in cells expressing the mutant was higher than in vector-transfected cells, there was no difference in activity in untreated and pervanadate-stimulated cells. Moreover, the levels of RGS16 (Y168F/Y177F) did not change with pervanadate stimulation (Fig. 9B). The fold increase in GTPase activity between no agonist and the highest 5-HT concentration was significantly greater for pervanadate-exposed membranes expressing WT RGS16 than for untreated cells. In contrast, the increase was nearly identical in membranes expressing the phosphorylation-resistant mutant with or without pervanadate (p = 0.01, WT+/−/V04−/+).

**Fig. 9.** Effect of RGS16 stabilization by phosphorylation on GAP activity in cellular membranes. A, HEK 293 cells stably expressing a 5-HT1A/GOα1 fusion protein were transiently transfected with lacZ or HA-RGS16 plasmids (5 μg). After PTX treatment (50 ng/ml overnight) to eradicate activity of endogenous Gαi proteins, cells were treated with serum-free medium or medium containing pervanadate for 30 min before harvest and membrane extraction. Membranes were stimulated with the indicated concentrations of 5-HT for 20 min at 37 °C to measure agonist-induced GTPase activity. An aliquot of membrane protein (50 μg) was separated by SDS-PAGE and immunoblotted with anti-RGS16 (inset, bottom) and anti-5-HT1A receptor (inset, top). B, same experiment as A, except that cells were transfected with HA-RGS16 (Y168F/Y177F). C, bar graph showing the fold increase in GTPase activity in unstimulated cells versus cells treated with 10−3 M 5-HT. Values are mean ± S.E. of 3–4 experiments performed in triplicate (*, p = 0.01 WT−/+ versus WT+, 2-factor analysis of variance).
pervanadate) (Fig. 9C). Because the levels of RGS16 (Y168F/Y177F) did not change appreciably with pervanadate stimulation, increases in RGS16 protein levels due to up-regulated transcription or membrane translocation over a relatively short time (30 min) seem unlikely. In summary, induction of RGS16 tyrosine phosphorylation is associated with increased steady-state RGS16 levels and augmented GAP activity in cell membranes.

**DISCUSSION**

We identified two tyrosine residues in the RGS box conserved in many mammalian RGS proteins that were potential sites of phosphorylation. Although our previous work (16) implied a role for EGFR-mediated phosphorylation of RGS16 on Tyr168 in the regulation of RGS16 GAP activity, under conditions of reduced tyrosine phosphatase activity both RGS16 Tyr residues appeared to be phosphorylated in HEK 293T cells. Therefore, we investigated the possible role of Tyr177 phosphorylation for RGS16 function. We found that Src family kinases phosphorylated WT RGS16 but not RGS16 (Y177F), and we observed in vitro phosphorylation of a peptide with the expected molecular mass of a proteolytic cleavage product containing Tyr177, suggesting that this residue was the site of Src-mediated RGS16 phosphorylation. Interestingly, however, when RGS16 (Y168F) or (Y177F) was expressed with constitutively active Src in CHO-K1 cells, each mutant was phosphorylated. We hypothesize that phosphorylation of RGS16 (Y177F) could result from Tyr168 phosphorylation by a receptor tyrosine kinase closely related to EGFR (ErbB2) expressed in these cells (25). There is evidence that Src mediates direct EGFR phosphorylation on tyrosine residues which augments EGFR activity (32, 33). In addition, Gα activation stimulates Src kinase (17) and leads to EGFR trans-activation (34, 35). Collectively, these studies emphasize the inter-relatedness of GPCR and RTK pathways, and RGS16 phosphorylation represents yet another potential link between these signaling cascades.

The importance of Tyr177 for RGS16 function was suggested by our previous study (16), which showed that mutation of this residue did not affect RGS16 GAP activity in vitro but eliminated the ability of RGS16 to regulate Gq-coupled MAP kinase activation or Gq-mediated inhibition of adenylyl cyclase. We extended these observations in the current work by demonstrating that Src-induced phosphorylation (putatively on Tyr177) did not alter the GAP activity of RGS16 toward Gαi in vitro. Although further studies will be required to determine whether Src-induced phosphorylation affects Gαi GAP activity or the ability of RGS16 to act as an effector antagonist of Gαi, these results suggest that Tyr177 phosphorylation does not induce a structural change in RGS16 that affects G protein binding. Extrapolation of the crystal structure of RGS4 in a complex with Gαi2 to RGS16 suggests that Tyr168, located in helix 8, is in closer proximity to the Gα-binding surface than Tyr177, which is located on helix 9 (Fig. 10A) (36). In fact, residues that directly contact Gα switch I region (Asp166, Ser167, and Arg170) surround Tyr168, and NMR analysis of unbound RGS4 suggests that this region undergoes a conformational change upon Gα binding (37). Therefore, the introduction of negative charge by phosphorylation could alter this G-binding surface through electrostatic interactions. In contrast, Tyr177 faces away from the G protein interface, in a short helix preceded by a flexible linker, which might easily facilitate interactions with other proteins (see below). Therefore, it appears more likely that phosphorylation of Tyr168 would directly affect GAP activity, in agreement with our previous results.

Because Src-mediated phosphorylation did not alter RGS16 GAP activity in vitro, we explored other possible effects of this modification on RGS16 biological function. We found that co-expression of activated Src or receptor activation of endogenous Src prolonged the half-life of RGS16 in cells. This increased longevity appeared to be related to RGS16 phosphorylation inasmuch as a comparable increase in RGS16 stability was induced by pervanadate treatment. Moreover, the pervanadate-induced reduction in RGS16 degradation was accompanied by a corresponding increase in membrane GAP activity. This result suggests that alterations in RGS16 protein turnover have a direct effect on its activity in cells.

In contrast to our findings, another protein with an RGS domain, GRK2, was shown to undergo Src-mediated phosphorylation, which promoted GRK2 proteolysis by the proteasome pathway (38). However, GRK2 phosphorylation appeared to occur on tyrosine residues (Tyr13, Tyr89, and Tyr225) distinct from the two phosphorylated residues in RGS16. Only two of three tyrosines in GRK2 that are targets of Src phosphorylation are located within the GRK2 RGS domain (on helix 4) (39). In addition, increased GRK2 degradation required recruitment
of an additional protein, β-arrestin, as well as GRK2 kinase activity, because a kinase-inactive GRK2 mutant underwent phosphorylation in cells co-expressing active Src but did not exhibit altered proteolysis. Thus, the mechanism whereby Src phosphorylation promotes stabilization of RGS16 is likely to be distinct from its effect on GRK2 turnover.

It is perhaps not surprising that a regulatory protein such as RGS16 is relatively unstable in order to allow greater flexibility in local RGS concentration at the site of G protein action before and after stimulation of receptors. In addition to Sst2, which undergoes pheromone-induced phosphorylation that stabilizes RGS16-GFP fusion protein in CHO-K1 cells co-transfected with markers for organelles or cytoskeletal elements and affects access to the proteasome. More detailed experiments or prevent RGS16 localization in lipid rafts, which might also affect access to the proteasome. Further experiments utilizing markers for organelles or cytoskeletal elements and better visualization methods (EM, confocal microscopy) as well as localization of endogenous RGS16 may be necessary to determine differences in RGS16 localization after phosphorylation.

Because Src activity is evoked by numerous stimuli, including, among others, cytokines (43), extracellular matrix proteins (44), steroids (45), ion channels, and integrins (46), Src-mediated RGS16 phosphorylation and the resultant stabilization of RGS16 levels may represent a mechanism by which diverse cell surface receptors down-regulate GPCR output in a complex cellular milieu. Our findings provide a framework to study the effects of tyrosine phosphorylation on RGS16 function and their generalization to closely related RGS proteins.

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