RGS16 Function Is Regulated by Epidermal Growth Factor Receptor-mediated Tyrosine Phosphorylation*

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Gαi-coupled receptor stimulation results in epidermal growth factor receptor (EGFR) phosphorylation and MAPK activation. Regulators of G protein signaling (RGS proteins) inhibit G protein-dependent signal transduction by accelerating Gαi GTP hydrolysis, shortening the duration of G protein effector stimulation. RGS16 contains two conserved tyrosine residues in the RGS box, Tyr168 and Tyr177, which are predicted sites of phosphorylation. RGS16 underwent phosphorylation in response to m2 muscarinic receptor or EGFR stimulation in HEK 293T or COS-7 cells, which required EGFR kinase activity. Mutational analysis suggested that RGS16 was phosphorylated on both tyrosine residues (Tyr168 >> Tyr177) after EGFR stimulation. RGS16 co-immunoprecipitated with EGFR, and the interaction did not require EGFR activation. Purified EGFR phosphorylated only recombinant RGS16 wild-type or Y177F in vitro, implying that EGFR-mediated phosphorylation depended on residue Tyr168. Phosphorylated RGS16 demonstrated enhanced GTPase accelerating (GAP) activity on Gαi. Mutation of Tyr168 to phenylalanine resulted in a 30% diminution in RGS16 GAP activity but completely eliminated its ability to regulate Gαi-mediated MAPK activation or adenylyl cyclase inhibition in HEK 293T cells. In contrast, mutation of Tyr177 to phenylalanine had no effect on RGS16 GAP activity but also abolished its regulation of Gαi-mediated signal transduction in these cells. These data suggest that tyrosine phosphorylation regulates RGS16 function and that EGFR may potentially inhibit Gαi-dependent MAPK activation in a feedback loop by enhancing RGS16 activity through tyrosine phosphorylation.

RGS1 proteins have emerged as important contributors to the kinetic precision associated with heterotrimeric G protein signaling (reviewed in Ref. 1). Genetic evidence in lower eukaryotes suggests that RGS proteins are negative regulators of G protein-coupled receptor (GPCR) pathway. RGS proteins may also be modified by Ser/Thr phosphorylation. The yeast RGS protein Sst2 undergoes pheromone-induced phosphorylation by MAPK, which slows its degradation (21). RGS7 is phosphorylated on a conserved Ser residue by protein kinase C, which promotes interaction with 14-3-3 proteins and inhibition of GRS7 GAP activity (22). Similarly, protein kinase C-induced phosphorylation inhibits RGS2 GAP activity on Gαq (23).

In contrast, ERK2 phosphorylates RGS-Gαi-interacting protein, which enhances its capacity to act as a GAP on Gαq (24). Protein kinase A phosphorylates RGS10, which does not affect GAP activity but correlates with RGS10 translocation from the plasma membrane and cytosol to the nucleus (25). RGS9–11 undergoes Ser phosphorylation by an unidentified endogenous kinase in rod outer segments (26). Finally, RGS16 is phosphorylated on Ser-194 after epinephrine stimulation of cells expressing the α2A-adrenergic receptor (27). Mutation of this serine residue to alanine impairs RGS16 GAP activity and its regulation of epinephrine-stimulated MAPK activity in these cells, but the direct effect of RGS16 Ser phosphorylation on its GAP activity has not been determined.

The current study shows that RGS16 GAP activity is regulated by phosphorylation of conserved tyrosine residues (Tyr168 and Tyr177) in the RGS box. EGFR phosphorylated RGS16 directly, which depended on the presence of residue Tyr168.
because mutation of this residue to phenylalanine abolished EGFR-mediated phosphorylation in vitro. The Y168F mutation also diminished RGS16 GAP activity. RGS16 (Y177F) underwent phosphorylation similar to WT RGS16 after EGF stimulation and demonstrated normal GAP activity. However, neither tyrosine mutant effectively regulated Gq-GPCR-mediated MAPK activation or inhibition of adenyl cyclase in vitro, suggesting that tyrosine phosphorylation is important for RGS16 function. EGFR-mediated phosphorylation of RGS16 suggests a novel form of cross-talk between receptor tyrosine kinase and G protein-coupled signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cells, Proteins, and Plasmids—**HEK 293T cells were maintained in supplemented Dulbecco’s modified Eagle’s medium (Invitrogen/Life Technologies) containing 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 20 ng/ml gentamicin, in a humidified 5% CO2 incubator at 37 °C. For transfections, 2 \times 10^6 cells were plated in 100-mm tissue culture dishes and transfected with appropriate plasmids for 18 h using LipofectAMINE reagent (Invitrogen). We washed the beads three times prior to addition of antigen-antibody complexes.

To generate recombinant RGS16, we subcloned an EcoRI/XhoI fragment from pcDNA3-RGS16 into pET-28a (Novagen) in-frame with an s modified Eagle’s medium/glutamine/
fected with appropriate plasmids for 18 h using LipofectAMINE reagent (Invitrogen/Life Technologies). After removal of the DNA mixture, the cells were incubated in Dulbecco’s modified Eagle’s medium/glutamine/ 0.1% Nonidet P-40 along with 0.2 mg/ml concanavalin A (washed 3 times) until harvest 48 h after transfection.

To generate recombinant RGS16, we subcloned an EcoRI/XhoI fragment from pcDNA3-RGS16 into pET-28a (Novagen) in-frame with an amino-terminal hexahistidine tag. This plasmid was used as a PCR template for the production of the mutants described below. The pcDNA3-RGS16 and pcDNA15-RGS2 were transformed into the Escherichia coli BL21(DE3)/pLyS2 strain (Strategene), and proteins were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Recombinant proteins were purified by Ni²⁺/nitriiloacetic acid-Sepharose (Qiagen) affinity chromatography per the manufacturer’s instructions.

pcDNA3-HA-RGS16 (human) was the kind gift of Drs. Carol Beadling and Kendall Smith (Cornell University Medical College). We introduced tyrosine mutations into this plasmid using the QuickChange site-directed mutagenesis kit (Strategene). We used the Y160F plasmid as a template for constructing Y168F/Y177F. This plasmid was also used as a template to generate the complete RGS16 coding region by PCR, which was inserted into TOPO-pcDNA3-V5/His (Invitrogen) per the manufacturer’s protocol. Constructs were confirmed by automated sequencing. The EGFR receptor construct was a gift of Dr. Amyn Habib (Harvard University), and the plasmid encoding m2R was a kind gift of Dr. Silvio Gutkind (NIDCR, National Institutes of Health). Native EGFR kinase purified from A431 cells by lectin chromatography was obtained from Dr. Silvio Gutkind (NIDCR, National Institutes of Health). The plasmid encoding m2R was the kind gift of Dr. Beadling (Cornell University Medical College). We introduced RGS16 phosphorylation was related to EGFR overexpression.

**RESULTS**

**RGS Proteins Are Tyrosine-phosphorylated in Mammalian Cells—**Analysis of the amino acid sequence of mammalian RGS proteins revealed the presence of two highly conserved tyrosine residues that were potential sites of phosphorylation (Table I).

To determine if RGS proteins underwent tyrosine phosphorylation in mammalian cells, we expressed HA-RGS2, RGS4, and RGS16 in HEK 293T cells and treated the cells with the tyrosine-phosphatase inhibitor sodium pervanadate. We immunoprecipitated RGS proteins with anti-HA antibody and immunoblotted them with anti-phosphotyrosine. Each of the three RGS proteins was phosphorylated on tyrosine residues in these cells after pervanadate treatment (Fig. 1). We studied the tyrosine phosphorylation of RGS16 in detail, because it contains only these two tyrosine residues.

**RGS16 Undergoes Tyrosine Phosphorylation in Response to m2R or EGFR Stimulation—**To determine which extracellular stimuli induced RGS16 tyrosine phosphorylation, we transfected HEK 293T cells with a GPCR (m2 muscarinic), an RTK (EGFR), and HA-RGS16 and immunoblotted cell lysates with anti-phosphotyrosine. We detected tyrosine phosphorylation of HA-RGS16 in cells stimulated with carbachol (Fig. 2A). Surprisingly, EGFR treatment induced strong RGS16 phosphorylation over a 1- to 15-min time period (Fig. 2B) and data not shown). To determine whether RGS16 phosphorylation was specific, we used a selective inhibitor of EGFR kinase activity, Compound 32 (Cp32), which completely blocked EGF-induced RGS16 phosphorylation. To exclude the possibility that EGF-induced RGS16 phosphorylation was related to EGF overexpression or the HA epitope tag, we utilized another cell line with high levels of endogenous EGFR, COS-7, and transfected
the cells with RGS16-V5/His. Recombinant RGS16 was extracted from detergent cell lysates by nickel affinity chromatography. In COS-7 cells, we observed phosphorylation of WT RGS16 after stimulation of endogenous EGFR, which was blocked by Cp32 (Fig. 2C, lane 4) and was not observed when RGS16 contained mutations of both tyrosine residues to phenylalanine (Y168/Y177F, lane 5). EGFR phosphorylation induced by EGF treatment was verified by immunoblotting.

**EGFR-mediated RGS16 Tyrosine Phosphorylation.** A, carbachol-induced RGS16 tyrosine phosphorylation. HEK 293T cells were transfected with m2R (0.2 μg), EGFR (0.2 μg), and HA-RGS16 (3 μg) and stimulated with carbachol (1 mM) for 5 min. A, or EGF (2 ng ml⁻¹) for 0, 3, 7, and 15 min (B). Whole cell lysates were immunodetected with anti-phosphotyrosine (top panels) or anti-HA or anti-RGS16 (bottom panels). In lanes 2–5, cells were pre-treated with Cp32 (500 pM) for 30 min prior to EGF stimulation. C, stimulation of endogenous EGFR in COS-7 cells induces RGS16 tyrosine phosphorylation. Cells transfected with RGS16-V5/His were stimulated with EGF (20 ng ml⁻¹) for 5 min, and RGS16 was purified from detergent lysates by Ni²⁺ affinity chromatography before immunoblotting with anti-phosphotyrosine (top panel) or anti-V5 (second panel from top). To verify EGFR stimulation, cell lysates (50 μg of total protein) were resolved by SDS-PAGE and immunodetected with anti-phosphotyrosine (third panel from top) or anti-EGFR (bottom panel). D, cells transfected with EGFR, m2R, and HA-RGS16 were stimulated with EGF (lanes 2 and 3) or carbachol (lanes 4 and 5) for 7 min. In lanes 3 and 5, cells were pre-treated with Cp32 (500 pM) for 30 min prior to stimulation.

**EGFR-mediated RGS16 Tyrosine Phosphorylation.** B, carbachol-induced RGS16 tyrosine phosphorylation was inhibited ~70% by RGS16 (WT). In contrast, there was no significant difference in agonist-induced MAPK activation levels in cells transfected with empty vector or each of the three mutant proteins (Fig. 4B). Differences between the activity of WT RGS16 and the tyrosine mutants were not likely due to a gross defect in RGS16 stability or localization, because all mutants were expressed at similar levels in detergent lysates (Fig. 4B, bottom) and in 100,000 × g supernatants (data not shown).

To ascertain whether the effect of the tyrosine mutations on RGS16 inhibition of Gα-mediated signaling was specific to the MAPK pathway, we tested the capacity of each mutant to...
regulate another G, effector, adenylyl cyclase. We stimulated HEK 293T cells simultaneously through endogenous G, (β-adrenergic) and G,-coupled (somatostatin) receptors and measured inhibition of isoproterenol-induced cAMP by somatostatin. In vector-transfected cells, somatostatin inhibited the isoproterenol response by 36.5 ± 1.9% (Fig. 4C). In cells transfected with WT RGS16, cAMP generation was only inhibited by 8.1 ± 2.4%, indicating that RGS16 blocked G, -mediated adenylyl cyclase inhibition (p < 0.0001). In contrast, we observed no significant diminution in somatostatin inhibition of the isoproterenol-induced response in cells transfected with each of the three mutant proteins. These data provide further evidence for the inability of Y168F, Y177F, or Y168/Y177F to regulate Gi-mediated signaling.

**Effect of Tyrosine Mutations on RGS16 GAP Activity**—To determine the biochemical mechanism underlying the impaired function of the mutants in *vivo*, we tested the ability of recombinant RGS16 (WT) and mutant proteins to act as GAPs for G, GTP hydrolysis by G, (200 nM) was determined during a single catalytic turnover in the presence or absence of RGS protein. We measured *k* for GTPase reactions containing a range of RGS WT or mutant concentrations (5–200 nM). When these values were expressed as a percentage of *k* for reactions containing the WT protein, Y168F exhibited 73% and Y168/Y177F 60.3% of WT activity, whereas Y177F had GAP activity similar to WT (Fig. 5). These data suggest that Tyr177 was not required for RGS16 GAP activity, whereas mutation of Tyr168 to phenylalanine impaired the GAP activity of RGS16.

**EGFR Phosphorylates RGS16 in Vitro**—Because RGS16 tyrosine phosphorylation in 293T and COS-7 cells depended on EGFR kinase activity, we hypothesized that EGFR kinase phosphorylated RGS16 directly. To test this possibility, we incubated recombinant His,RGS16 with purified EGFR and [γ-32P]ATP. EGFR phosphorylated RGS16 (WT) but not RGS16 (Y168/177F) or His,RGS2 (Fig. 6A). To determine which residue might be phosphorylated by purified EGFR, single- or double-tyrosine mutants were incubated with the kinase and immunoblotted with anti-phosphotyrosine. WT RGS16 and Y177F were phosphorylated to a similar extent, whereas neither Y168F nor Y168/Y177F were phosphorylated (Fig. 6B). This result suggests that EGFR phosphorylates RGS16 specifically on Tyr168, although these studies must be confirmed by phosphoamino acid mapping.

**Co-immunoprecipitation of EGFR and RGS16**—To determine whether RGS16 interacted with EGFR in cells, we expressed the two proteins in 293T cells and immunoprecipitated EGFR with a specific antibody. A band corresponding to HA-RGS16 was observed in EGFR immunoprecipitates regardless of whether the cells were stimulated with EGF (Fig. 7A). This result suggested an association between RGS16 and EGFR that was independent of EGFR activation. To exclude the possibility that co-immunoprecipitation was a result of EGFR overexpression, we transfected COS-7 cells with RGS16 V5/His and extracted RGS16 protein with Ni2+ beads. After extensive
washing, we immunoblotted for endogenous EGFR. EGFR bound only to beads containing RGS16 (Fig. 7B).

**EGFR-mediated Phosphorylation Stimulates RGS16 GAP Activity**

To test whether EGFR-mediated RGS16 phosphorylation affected RGS16 GAP activity, we measured G_{i1} GTPase rates in the presence of RGS16 preincubated or not with purified EGFR. 

*Activity*—To test whether EGFR-mediated RGS16 phosphorylation affected RGS16 GAP activity directly, we measured G_{i1} GTPase rates in the presence of RGS16 preincubated or not with purified EGFR. 

$$k_{\text{cat}}$$ for G_{i1} GTP hydrolysis at 4°C was 0.006 ± 0.001 s⁻¹. Rate constants for reactions containing RGS16 from 1 to 50 nM increased linearly, from 2- to 20-fold over basal rates. Surprisingly, at each RGS16 concentration, $$k_{\text{cat}}$$ values for reactions containing phosphorylated RGS16 (pRGS16) were nearly 2-fold higher than those containing unmodified RGS16, which was statistically significant (Fig. 8A, p < 0.05, paired t test). To eliminate variability in rate constants due to different RGS16 preparations and differing stoichiometries of phosphorylation from individual reactions, we determined $$k_{\text{cat}}$$ values for reactions containing pRGS16 from independent phosphorylation assays. We normalized these values to rate constants for reactions containing unmodified RGS16. The average $$k_{\text{cat}}$$ for reactions containing 25 and 50 nM pRGS16 was 180% ± 0.3% of the control values (Fig. 8B, p < 0.05 between RGS16 and pRGS16; no statistical difference between the two concentrations). There was no effect of EGFR phosphorylation on RGS16 GAP activity. EGFR enzyme phosphorylated RGS16 at Tyr168. Single turnover GTPase assays were performed using G_{i1} (200 nM) in the absence or presence of RGS16 WT, Y168F, Y177F, or Y168/Y177F mutant proteins (5–200 nM). $$k_{\text{cat}}$$ was determined for each RGS16 concentration and expressed as a percentage of WT values (100%). The graph represents mean ± S.E. of the percentage WT GAP activity for each of the mutants (for all concentrations). Y168F exhibited 73 ± 4.9%, Y177F 101 ± 8.7%, and Y168/Y177F 60.3 ± 7.3% of the WT activity (*p < 0.05, WT versus Y168F and Y168/Y177F; no significant difference between Y168F and Y168/Y177F or between WT and Y177F).
Tyrosine phosphorylation may also alter protein conformation to modulate activity, as is the case for src kinase (32).

We found that EGFR-mediated phosphorylation increased RGS16 GAP activity in vitro, suggesting that it might affect RGS16 conformation directly. NMR analysis of “free” RGS4 versus RGS4 complexed with AlF4-Gαi1 indicates that a kink in helix 6 between residues Lys116 and Tyr119 (Lys165 to Tyr168 in RGS16) is more pronounced upon G protein binding (33). Because Tyr168 is situated in this region and is located between RGS residues critical for Gαi interaction (34), its phosphorylation could potentially play a role in this conformational change and modification of the RGS-Gαi interface. Notably, the increase in pRGS16 activity is probably underestimated by our studies, because of possible sub-maximal stoichiometry of RGS16 phosphorylation and because of inherent limitations in single turnover GTPase assays. For example, RGS2 GAP activity toward Gαi is undetectable in traditional single turnover assays but becomes readily apparent when agonist-stimulated GTPase activity of Gαi is measured in proteoliposomes reconstituted with both a GPCR and heterotrimeric G protein (35). A similar strategy might uncover larger differences in RGS16 GAP activity in the presence and absence of phosphorylation.

Interestingly, although RGS16 phosphorylation after stimulation of the Gαi-linked m2R was relatively low, mutation of either tyrosine residue almost completely abolished the activity of RGS16 as an inhibitor of Gαi-mediated signal transduction. This discrepancy might be explained by several factors. First, the Y168F mutation alone diminished RGS16 GAP activity in vitro and, thus, may contribute to the absent activity of RGS16(Y168F) in cells independent of phosphorylation. Second, fractional RGS16 phosphorylation after m2R stimulation may have been artificially low due to the high amounts of available. Third, RGS16 overexpression may block m2R-induced EGFR transactivation, resulting in diminution of carbachol-induced RGS16 phosphorylation, because it depends on EGFR activation. In any case, the fact that significant differences in the activity of RGS16 versus phosphorylated RGS16 exist in the context of relatively weak phosphorylation argues for the vital importance of tyrosine phosphorylation to RGS16 biological function. Confirmation of these data awaits studies of endogenous RGS16 in native mammalian systems.

Further evidence that tyrosine phosphorylation regulates RGS16 function is that residue Tyr177 also appears to be phosphorylated after EGF stimulation. Mutation of Tyr177 to phenylalanine prevented RGS16 from attenuating G protein-mediated MAPK activation or adenyl cyclase inhibition without affecting GAP activity in vitro and, thus, may contribute to the absent activity of RGS16(Y168F) in cells independent of phosphorylation. Second, fractional RGS16 phosphorylation after m2R stimulation may have been artificially low due to the high amounts of transacted RGS16 relative to the amount of tyrosine kinase available. Third, RGS16 overexpression may block m2R-induced EGFR transactivation, resulting in diminution of carbachol-induced RGS16 phosphorylation, because it depends on EGFR activation. In any case, the fact that significant differences in the activity of RGS16 versus phosphorylated RGS16 exist in the context of relatively weak phosphorylation argues for the vital importance of tyrosine phosphorylation to RGS16 biological function. Confirmation of these data awaits studies of endogenous RGS16 in native mammalian systems.

This study supports the notion that two conserved tyrosine residues in RGS16 are phosphorylated and necessary to regulate Gαi-mediated MAPK activation and inhibition of adenyl cyclase activity. Because EGFR and RGS16 co-immunoprecipitated in two different cell lines and because EGFR stimulation resulted in phosphorylation of RGS16 that depended on Tyr168 and, to a lesser extent, Tyr177, a functional interaction between EGFR and RGS16 likely exists. In addition, because EGFR stimulation induces the activation of other tyrosine kinases, another enzyme could be responsible for phosphorylation of residue Tyr177, because purified EGFR did not phosphorylate RGS16 (Y168F). Alternatively, phosphorylation could depend on the presence of both tyrosine residues.

Tyrosine phosphorylation regulates the activity of many G protein-signaling components. For example, a tyrosine-phosphorylated, G protein-dependent Ca2+ channel interacts with the phosphotyrosine binding domain of RGS12, resulting in enhanced G protein deactivation and attenuated channel activity (30). Phosphotyrosines in the cytoplasmic tail of RTKs serve as a scaffold to which other signaling proteins are recruited (31).
to RGS16 Tyr$^{168}$ in RGS5 is Leu, implying that RGS5 is not phosphorylated by EGFR at this site. Thus, tyrosine phosphorylation of select RGS proteins expressed in the same cell may determine their effect on a particular G protein or GPCR pathway.

Unexpectedly, we found that GPCR-induced RGS16 phosphorylation was dependent upon on EGFR kinase activity. GPCRs activate a number of cytoplasmic tyrosine kinases as well as RTKs such as EGFR (28, 29, 36–38). For instance, both GTP-bound Go$\alpha$ and Go$\beta$ stimulate src kinase activity directly (29). We hypothesized that m2R stimulation, which is coupled to Go$\alpha$, would induce strong RGS16 phosphorylation if src were the primary kinase involved. Instead, we found that m2R-mediated RGS16 phosphorylation required EGFR tyrosine phosphorylation and kinase activity. These results do not exclude a direct role for src or other kinases in RGS16 phosphorylation, however.

Cross-talk between GPCRs and RTKs is well documented (36–38). The mitogenic effects of G$\alpha_i$ or G$\alpha_q$-coupled receptors may require EGFR tyrosine phosphorylation and kinase activity (36, 38). Our results suggest that this RTK potentially inhibits G protein signaling in a feedback loop by enhancing the activity of a GAP. The rate of Go$\alpha_q$ deactivation might be faster in the presence of phosphorylated RGS16, resulting in decreased downstream G protein effector stimulation. It will be of interest to determine if other RTKs (e.g. insulin receptor or platelet-derived growth factor receptor) phosphorylate RGS16 and to study the effect of EGFR-induced RGS16 phosphorylation on the activation of both RTK- and GPCR-induced biological end points.

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