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A Key Serine for the GTPase-Activating Protein Function of Regulator of G Protein Signaling Proteins Is Not a General Target for 14-3-3 Interactions

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ABSTRACT

Mammalian regulator of G protein signaling (RGS) proteins are highly conserved within the RGS domain. Of amino acids that are universal, a serine residue at the C terminus of this domain has been described as the binding site in RGS7 for 14-3-3 proteins. However, studies with the related RGS3 indicate that the site of interaction is not within the RGS domain. We confirm that the interaction of RGS3 with 14-3-3τ and 14-3-3ζ requires Ser^{264} and not the RGS domain and show both that mutation of the conserved RGS domain serine, Ser^{496} in RGS3, to either alanine or aspartate does not prevent binding of 14-3-3 proteins and that 14-3-3 proteins do not inhibit GTPase-activating protein (GAP) activity against receptor-activated $G\alpha_{o1}$. However, mutation of Ser^{496} does directly impair the action of RGS3 as a GAP against receptor-activated $G\alpha_{o1}$. We mutated the equivalent serine residue in the family B/R4 RGS proteins RGS1 and RGS16. Using two distinct assay formats, conversion to aspartate virtually abolished GAP activity, whereas conversion to alanine decreased potency 20-fold. Neither alteration modulated interactions with 14-3-3τ or 14-3-3ζ, but the 14-3-3 proteins did not modulate the GAP activity of the wild-type or mutant RGS proteins. Although interactions between 14-3-3 proteins and many RGS proteins can be observed, this does not involve this conserved serine and does not inherently modify GAP function.

Regulator of G protein signaling (RGS) proteins represent a substantial family of polypeptides named for their capacity to accelerate the GTPase activity, and hence the deactivation, of the α subunit of members of the G_i and G_q subfamilies of heterotrimeric G proteins (Ross and Wilkie, 2000; Zhong and Neubig, 2001; Chidiac and Roy, 2003). The first isolated members of the mammalian RGS family, RGS1 and RGS4, consist of little more than the RGS domain of some 128 amino acids that defines the family and a short N-terminal amphipathic helix (Ross and Wilkie, 2000). In contrast, other family members such as RGS3 and RGS7 have long extensions N-terminal to the RGS domain, and in many cases these extensions include well-defined protein-protein interaction motifs that can provide scaffolds for the generation of G protein-mediated signaling networks (Burchett, 2000). A series of studies has also indicated that RGS proteins are targets for a range of post-translational modifications, including phosphorylation and palmitoylation, and that these can modulate their GTPase-activating protein (GAP) function (Tu et al., 1997; Rose et al., 2000; Chen et al., 2001; Cunningham et al., 2001; Derrien and Druey, 2001; Benzing et al., 2002; Hollinger et al., 2003; Jones, 2004; Takida et al., 2005). Such modifications may also modulate interactions between RGS proteins and other cellular polypeptides (Benzing et al., 2002; Bahia et al., 2003). Both RGS3 (Benzing et al., 2000; Niu et al., 2002) and RGS7 (Benzing et al., 2000, 2002) have been reported to interact with isoforms of the 14-3-3 protein. 14-3-3 isoforms are widely distributed and highly expressed cytosolic proteins that interact with a significant number of cellular polypeptides (Fu et al., 2000; Mackintosh, 2004). Such interactions generally require phosphorylation of the interacting protein on specific residues (Muslin et al., 1996; Mackintosh, 2004), and indeed, previous studies on RGS7 have indicated that interactions between this polypeptide and 14-3-3 requires phosphorylation of a specific serine residue in the RGS domain (Benzing et al.,

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ABBREVIATIONS: RGS, regulator of G protein signaling; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; PCR, polymerase chain reaction; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline; Ni-NTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PKCα, protein kinase Cα; 6xHis, hexahistidine; RS-79948-197, (8a*R*,12a*S*,13a*S*)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(ethylsulphonyl)-6*H*-isoquino[2,1-*g*][1,6]naphthyridine.

2000, 2002). This residue is close to the C-terminal end of the RGS domain, at the junction of α-helices 7 and 8 and, on the basis of the available structural information, is a direct Gα subunit contact residue (Tesmer et al., 1997). It is not surprising, therefore, this serine is entirely conserved across other members of the mammalian RGS family. Despite this, although RGS3 has also been reported to interact with 14-3-3 proteins (Benzing et al., 2000; Niu et al., 2002), mapping of this interaction has indicated it to occur outside the RGS domain and instead to require Ser^{264} in the long N-terminal extension (Niu et al., 2002).

Given these contrasting observations, we have re-examined interactions between 14-3-3 isoforms and both RGS3 and RGS7 and further examined the effects on GAP activity against receptor-activated $G\alpha_{o1}$ of mutating the highly conserved serine residue in both RGS3 and RGS7 and in a pair of RGS proteins that do not contain the long N-terminal extensions.

Materials and Methods

Materials. [γ-^{32}P]GTP and [γ-^{32}P]ATP were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Pertussis toxin and PKCα were from Sigma Chemical (St. Louis, MO).

Constructs. A fusion protein between the α_{2A}-adrenoceptor and the α subunit of the G protein G_{o1} that was rendered-insensitive to the ADP-ribosyltransferase activity of pertussis toxin (α_{2A}-AR-$Cys^{351}IleG_{o1}$) was generated previously (Cavalli et al., 2000).

Human 14-3-3τ and ζ, subcloned into pGEX-2T, were obtained from Dr. T. Dubois (University of Edinburgh, Edinburgh, Scotland). The 14-3-3τ was used as a PCR template with primers designed to add a BamHI site and an HA-tag (YPYDVPDYA) at the N terminus and an EcoRI site at the C terminus. The fragment was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). RGS1 (subcloned into pGEX-4T1) and RGS16 (subcloned into pGEX-2TK) were obtained from Dr. A. Meyerdierks (Department of Medical Microbiology, Medizinischer Hochschule, Hannover, Germany) and Dr. C. W. Fong (Institute of Molecular and Cell Biology, Proteos, Singapore), respectively. Serine-to-alanine and serine-to-aspartate mutants of these RGS constructs were generated by a dual overlapping PCR fragment amplification approach to produce mutant fragments of the RGS proteins that were then subcloned into the original constructs with appropriate flanking restriction sites. The α_{2A}-adrenoceptor-RGS16 (α_{2A}-AR-RGS16) fusion construct was a modification of the α_{2A}-AR-RGS4 fusion described previously (Bahia et al., 2003). RGS4 was excised with BamHI and XbaI and replaced with PCR-amplified RGS16 with these sites at the N and C termini, respectively.

Human RGS3 and murine RGS16 were PCR amplified with primers designed to add a KpnI site onto the N-terminal with an XbaI site and a VSV-G tag (YTDIEMNRLGK) onto the C termini. The fragments were subcloned into pcDNA3. N-($RGS3^{1–370}$) and C-terminal ($RGS3^{371–519}$) fragments of RGS3 were PCR-amplified with primers designed to add a KpnI site onto the N-terminal and an XbaI site with a VSV-G tag onto the C-terminal of each fragment. Human RGS3 was also PCR-amplified with primers designed to add an EcoRI site onto the N terminus and an XhoI site onto the C terminus. This fragment was then subcloned into pGEX-4T3 to make the GST-RGS3 fusion construct. The fragment was further subcloned into the vector pQE-32 by excising it with EcoRI/XhoI. The EcoRI site was converted to a blunt end and ligated to a blunt-ended BamHI site in pQE-32, whereas the XhoI site was ligated to a SalI site to make a hexahistidine (6xHis)-tagged RGS3 construct. Serine-to-alanine and serine-to-aspartate mutants were also introduced as described above.

Human RGS7 was obtained from the University of Missouri–Rolla cDNA Resource Centre via Dr. A. Tinker (University College London) and was PCR-amplified with primers designed to add an N-terminal BamHI site and a C-terminal SalI site, which was subcloned into pQE-30, or an N-terminal BamHI site and C-terminal NotI site with a VSV-G tag, which was subcloned into pcDNA3. Serine-to-alanine and serine-to-aspartate mutants were also introduced. All subcloning and PCR were carried out using standard techniques and constructs were fully sequenced before use.

Cell Culture, Transient Transfection, and Cell Lysates. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum and 2 mM glutamine. Before transfection, cells were seeded onto 10-cm dishes at 50 to 70% confluence. Constructs were transfected into the cells using the Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the medium was removed, and the cells washed twice with 1× PBS (120 mM NaCl, 25 mM KCl, 10 mM Na_2HPO_4, and 3 mM KH_2PO_4, pH 7.4). Cells intended for membrane preparation were scraped into 5 ml of 1× PBS, pelleted, and stored at −80°C. Cells for lysates were harvested with 1 ml of lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1 mM EDTA, and 1 complete protease inhibitor tablet; Roche Diagnostics, Indianapolis, IN) and incubated, rotating, at 4°C for 1 h. Lysed cells were centrifuged at 12,000*g* for 10 min at 4°C, and the supernatant was transferred to fresh tubes. Protein concentration was determined by BCA assay, and the lysates were stored at −80°C.

Membrane Preparation. Cells were resuspended in 1× 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5, and disrupted with a Teflon-on-glass homogenizer for 2 min before being passed through a 25-gauge needle 20 times. Unbroken cells were removed by centrifugation at 500*g* for 5 min at 4°C. The supernatant fraction was centrifuged at 90,000*g* for 30 min at 4°C to recover the membranes, which were resuspended in 1× 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5, and the protein concentration was determined by BCA assay before being stored at −80°C.

Purification of 6xHis-Tagged Proteins. pQE-30/32 plasmids (QIAGEN, Valencia, CA) containing full-length cDNAs of RGS3 or RGS7 fused to the 6xHis tag were transformed into *Escherichia coli* strain BL21 DE3. LB medium (400 ml) containing 100 μg/ml ampicillin was inoculated with a 10-ml starter culture of the transformed cells and were grown to an optical density at 600 nm of 0.5. Expression of the 6xHis-tagged protein was induced by the addition of 1 mM isopropyl-β, D-thiogalactopyranoside for 5 h before harvesting the cells by centrifugation at 6000*g* for 15 min at 4°C. Pellets were resuspended in denaturing lysis buffer (containing 6 M urea), and the 6xHis-tagged protein was purified using Ni-NTA agarose (QIAGEN) according to the manufacturer's instructions. The eluted proteins were analyzed by SDS-PAGE and BCA protein assay before being dialysed against three changes of 1× PBS containing 5% glycerol at 4°C over 2 days, before storage at −80°C.

Purification of GST-Fusion Proteins. pGEX plasmids (GE Healthcare) containing the appropriate cDNA fused to glutathione *S*-transferase were transformed and grown up as described in the previous section. Cell pellets were lysed by resuspending in 20 ml of 1× PBS (with protease inhibitors) containing 0.5 mg/ml lysozyme followed by incubation at 4°C with rotation for 1 h. The resuspended cells were sonicated for 4 × 30 s each on ice, with 30 s for cooling between bursts. Dithiothreitol was added to a final concentration of 5 mM and Triton X-100 (as a 10% stock) to a final concentration of 1%. The lysates were then incubated at 4°C with rotation for 1 h, and the insoluble material was removed by centrifugation at 12,000*g* for 15 min at 4°C. One milliliter of a suspension of washed (three times with 10 volumes of 1× PBS with protease inhibitors) glutathione Sepharose was added to each cleared lysate and incubated at 4°C overnight with rotation. The lysates were spun at 500*g* for 5 min at 4°C, and the supernatant was removed from the pellet of glutathione Sepharose beads. The beads were washed with 3 × 10 ml of 1× PBS with protease inhibitors, and the GST fusion proteins were eluted

with 5 × 1.5 ml of glutathione solution (10 mM concentration of reduced glutathione in 50 mM Tris-HCl, pH 8.0). The eluted proteins were analyzed by SDS-PAGE and BCA protein assay before being dialyzed against three changes of 1× PBS containing 5% glycerol at 4°C over 2 days before storage at −80°C.

PKCα Phosphorylation of Recombinant RGS Protein. Recombinant RGS protein (5–10 μg) was incubated for 30 min at 37°C in a total volume of 100 μl with 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 μM ATP, 20 μg/ml diacylglycerol, 100 μg/ml phosphatydylserine, 0.03% Triton X-100, and 0.5 U of PKCα (or equivalent volume enzyme dilution buffer). Reactions were then placed on ice until added to pull-down assays. Phosphorylation was monitored by including 5 μCi of [γ-^{32}P]ATP, followed by SDS-PAGE and detection by autoradiography.

Pull-Down Experiments. Aliquots (100–400 μl) of HEK293 cell lysate (dependent on protein expression from the construct) were mixed with 200 μl of dialyzed GST fusion protein (0.5–2 μM) and 40 μl of washed glutathione Sepharose slurry. Lysis buffer was added to give a constant final volume. After overnight incubation at 4°C with rotation, the beads were centrifuged at 500*g* for 5 min at 4°C, and the supernatant was removed. The beads were then washed with 4 × 1-ml aliquots each of lysis buffer, and 40 μl each of SDS-PAGE sample buffer was added. The proteins were allowed to elute for 15 min at room temperature and were then heated to 65°C for 5 min before analysis by SDS-PAGE. Experiments with 6xHis-tagged proteins were essentially similar, but Ni-NTA agarose was used in place of glutathione Sepharose.

High-Affinity GTPase Assays. High-affinity GTPase assays were carried out as described by Hoffmann et al. (2001) and Ward and Milligan (2004), using the procedure adapted to a 96-well microtiter plate format. In the case of assays requiring the addition of GST-RGS, GST-14-3-3, or 6xHis-RGS proteins, these were combined with the membranes and allowed to incubate on ice for 30 min before the addition to the assay mix. The volumes and concentrations of the components added to the assay were adjusted to allow for a constant reaction volume of 100 μl to be maintained in all cases.

Results

An HA-tagged version of the τ isoform of 14-3-3 was expressed in HEK293 cells, and after SDS-PAGE analysis of cell lysates, it was detected with the anti-HA antibody 12CA5 predominantly as a 33-kDa polypeptide (Fig. 1A). This polypeptide interacted with, and was pulled down by, a 6xHis-tagged form of the regulator of G protein signaling, RGS3 (Fig. 1B). All mammalian RGS proteins contain a serine residue within a conserved Asp-Ser-Tyr-Xaa-Arg motif at the C-terminal end of the RGS domain that defines this protein family. Previous work (Benzing et al., 2000) has indicated that this serine is central for the binding of 14-3-3 proteins to both RGS3 and RGS7. Because 14-3-3 binding routinely requires a phosphorylated serine residue (Mackintosh, 2004) we altered this serine (Ser^{496} in RGS3) to either alanine or aspartic acid. Neither of these modifications altered the ability of 14-3-3τ to interact with 6xHis RGS3 (Fig. 1B). To examine the role of the RGS box of RGS3 in interactions with 14-3-3τ, RGS3 was split into the RGS domain and C terminus, consisting of amino acids 371 to 519, and the N-terminal domain consisting of amino acids 1 to 370 (Fig. 2). The VSV-G epitope tag sequence was added to the C terminus of each of these fragments and to full-length RGS3 to facilitate detection. After expression in HEK293 cells, full-length RGS3-VSV-G migrated through SDS-PAGE predominantly as a 75-kDa polypeptide with a small amount of a higher molecular mass species that might represent an SDS-PAGE-resistant dimer. The C-terminal fragment migrated predominantly as a 23-kDa species with lower levels of a 46-kDa species, whereas in SDS-PAGE, the N-terminal fragment was predominantly a 52-kDa species with evidence of some capacity to dimerize (Fig. 2). Full-length RGS3-VSV-G was pulled down by GST fusions of both 14-3-3τ and 14-3-3ζ (Fig. 3A). Again, this was not prevented by the introduction of Ser^{496}Ala or Ser^{496}Asp mutations into RGS3 (Fig. 3A). It is interesting that although the VSV-G-tagged C-terminal 23-kDa RGS domain fragment of RGS3 did not interact with either 14-3-3τ or 14-3-3ζ, no matter the identity of the amino acid at position 496, the VSV-G-tagged N-terminal 52-kDa fragment of RGS3 did interact with both 14-3-3 isoforms (Fig.

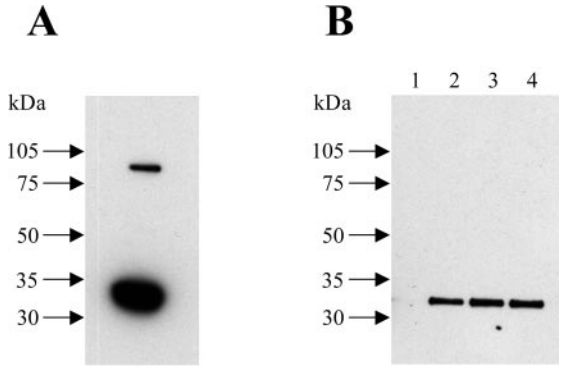

Fig. 1. Interaction of 14-3-3τ with RGS3. A, an HA-14-3-3τ construct expressed in HEK293 cells was subjected to SDS-PAGE and immunoblotted with anti-HA antibody. B, lysates of HEK293 cells expressing the HA-14-3-3τ construct were used in pull-down experiments using 6xHis-tagged forms of RGS3: no RGS3 (1), wild-type RGS3 (2), Ser^{496}Asp RGS3 (3), and Ser^{496}AlaRGS3 (4). Samples were resolved by SDS-PAGE and immunoblotted to detect HA-14-3-3τ.

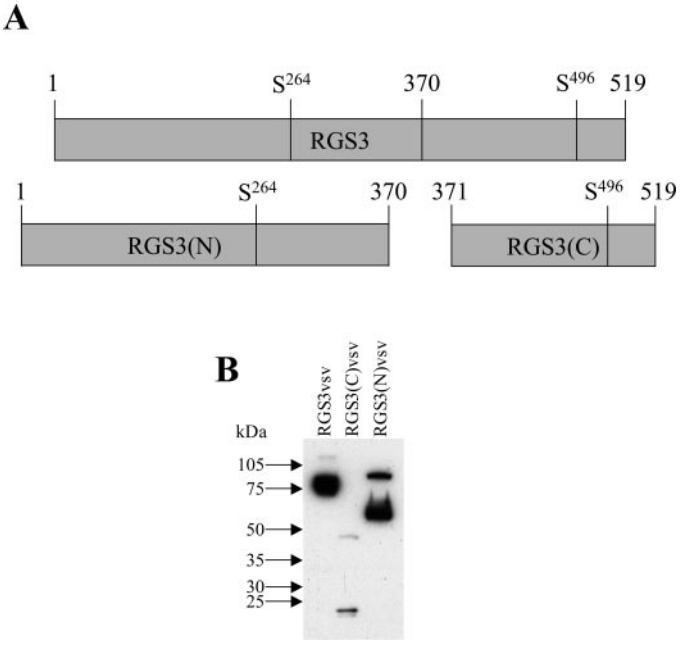

Fig. 2. Production of N- and C-terminal fragments of RGS3. A, RGS3 was split to generate N-terminal 1 to 370 and C-terminal 371 to 519 (containing the RGS domain sequence) fragments. Each of these was C-terminally tagged with the VSV-G epitope sequence to assist detection. Two serine residues that were mutated in the studies are highlighted. B, Full-length and the N- and C-terminal RGS3-VSV-G constructs were expressed in HEK293 cells, resolved by SDS-PAGE, and detected with anti-VSV-G antibody.

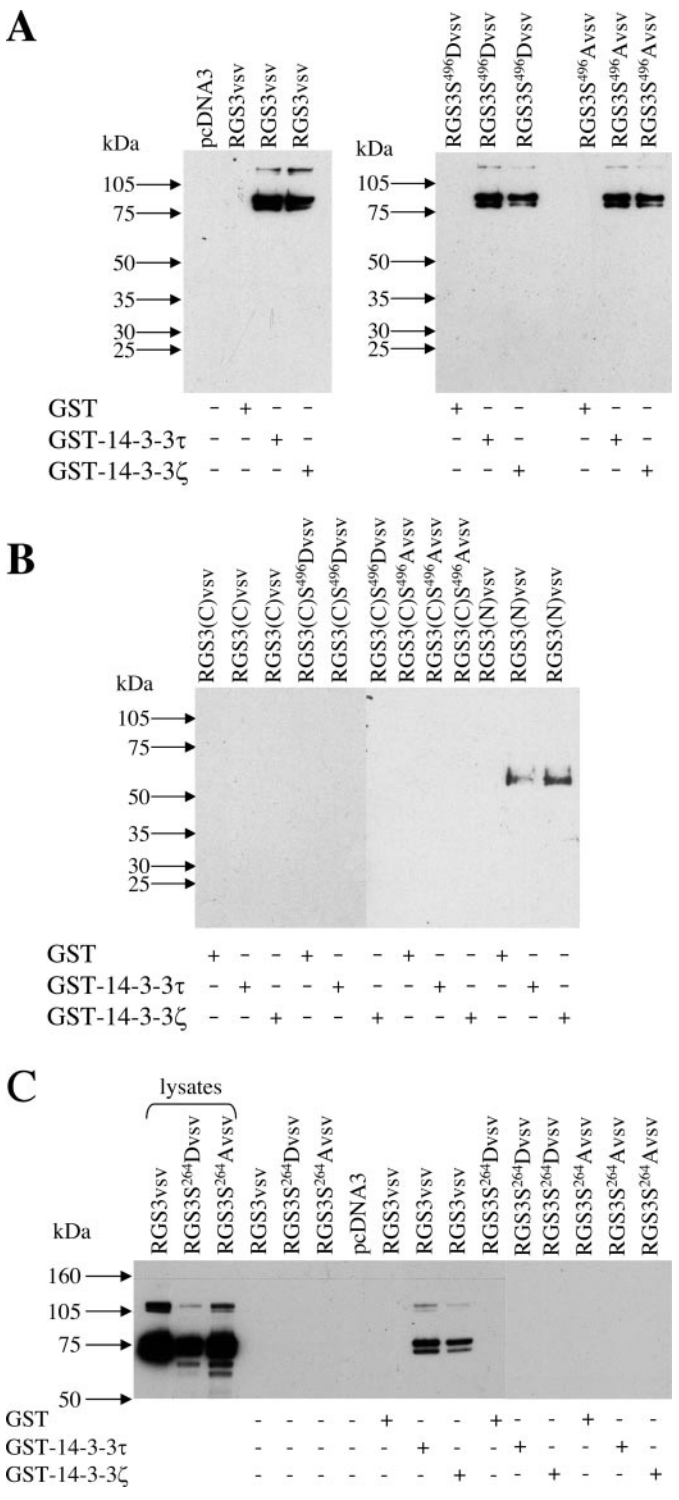

Fig. 3. Interactions between 14-3-3 isoforms and RGS3 are not defined by the RGS domain. A, Western blots of pull-down experiments using lysates of HEK293 cells expressing RGS3-VSV-G, Ser^{496}Asp RGS3-VSV-G, or Ser^{496}Ala RGS3-VSV-G with 1 μM GST-tagged 14-3-3τ or 14-3-3ζ were probed with anti-VSV-G antibody. B, equivalent pull downs from lysates of HEK293 cells expressing the RGS3-VSV-G N- and C-terminal (incorporating Ser^{496}Asp and Ser^{496}Ala mutations) fragments with GST-tagged 14-3-3τ or 14-3-3ζ were probed with anti-VSV-G antibody. C, equivalent pull downs from lysates of HEK293 cells expressing wild-type RGS3-VSV-G and Ser^{264}Asp and Ser^{264}Ala mutants of RGS3-VSV-G. Input levels of the forms of RGS3 are shown as lysates.

3B). Recent studies have suggested a role for RGS3Ser^{264} within the N-terminal domain and outside the RGS domain in interactions with 14-3-3 isoforms (Niu et al., 2002). In agreement with this, the mutation of Ser^{264} to either alanine or aspartic acid did eliminate interactions with 14-3-3τ and 14-3-3ζ (Fig. 3C).

We have constructed previously a fusion protein (α_{2A}-AR-Cys^{351}IleG$_{o1}$) between the α_{2A}-adrenoceptor and the α subunit of the G protein G$_{o1}$ that has been rendered insensitive to the ADP-ribosyltransferase activity of pertussis toxin by altering the reactive cysteine residue to isoleucine (Cavalli et al., 2000). When expressed in HEK293 cells that were treated with pertussis toxin (25 ng/ml for 24 h) to produce ADP-ribosylation and inactivation of endogenously expressed members of the family of pertussis toxin-sensitive G$_i$/G$_o$ G proteins, this fusion protein generated a large stimulation of high-affinity GTPase activity upon the addition of α_{2A}-adrenoceptor agonists such as the natural ligand adrenaline (Fig. 4). This reflected an increase in the V_{max} of GTPase activity without an alteration in the apparent K_m value for GTP (Fig. 4A). The addition of recombinantly expressed 6xHis-RGS3 to membranes of pertussis toxin-treated HEK293 cells expressing α_{2A}-AR-Cys^{351}IleG$_{o1}$ did not modify basal GTPase activity but resulted in a large elevation of adrenaline-stimulated high-affinity GTPase activity (Fig. 4Bi) that was not modified by the coaddition of GST-14-3-3τ (Fig. 4Bi). Likewise, the presence of GST-14-3-3τ did not modulate the stimulatory GAP effects of either 6xHis-Ser^{496}Ala RGS3 or 6xHis-Ser^{496}Asp RGS3 (Fig. 4B, ii and iii). However, both Ser^{496}Ala RGS3 and Ser^{496}Asp RGS3 were significantly less able to enhance the effects of adrenaline on the GTPase activity of α_{2A}-AR-Cys^{351}IleG$_{o1}$ than wild-type RGS3 (Fig. 4B). These studies suggested a direct role of Ser^{496} in RGS3 in the GAP activity of this RGS rather than an indirect role involving interactions with 14-3-3 isoforms. To demonstrate that the 6xHis-RGS3 and GST-14-3-3τ recombinant proteins were able to interact when both were present in the GTPase assay, pull-down experiments were carried out as shown in (Fig. 4C). The 6xHis-RGS3 was able to pull down GST-14-3-3τ, and this interaction was unaffected by treatment with the PKCα isoform. PKCα, however, was able to cause phosphorylation of the 6xHis-RGS3, but Ser^{496} was not a key site for phosphorylation because the extent of incorporation of ^{32}P into 6xHis-RGS3 was unaffected by the Ser^{496}Asp mutation (Fig. 4D). To further explore the role of Ser^{496}, we added different amounts of purified 6xHis wild-type RGS3, Ser^{496}Ala RGS3, and Ser^{496}Asp RGS3 to membranes of pertussis toxin-treated HEK293 cells expressing α_{2A}-AR-Cys^{351}IleG$_{o1}$ and examined the effects on high-affinity GTPase activity stimulated by a maximally effective concentration (10 μM) of adrenaline. Each of the forms of RGS3 enhanced adrenaline-stimulated GTPase activity in a concentration-dependent manner, but wild-type RGS3 was clearly more effective than equimolar amounts of either Ser^{496}Ala RGS3 or Ser^{496}Asp RGS3 (Fig. 4E).

Because RGS3 is a large polypeptide, it was difficult to achieve sufficient levels of expression to allow purification of amounts of protein required to generate full concentration-response curves (Fig. 4E) and hence further insights into the role of Ser^{496}, particularly because RGS3 was not a highly potent GAP (EC$_{50}$ value greater than 1 μM) for α_{2A}-AR-Cys^{351}IleG$_{o1}$ (Fig. 4E). However, as noted earlier, all mam-

malian RGS proteins contain the conserved Asp-Ser-Tyr-Xaa-Arg sequence within the RGS domain. We thus used other members of the B/R4 group (Ross and Wilkie, 2000) of RGS proteins that do not contain the long N-terminal extension present in RGS3. GST fusions of both RGS1 and RGS16 were generated and purified. Equivalent GST fusions in which the serine equivalent to Ser^{496} in RGS3 (Ser^{173} in RGS1 and Ser^{166} in RGS16) was modified to either alanine or aspartic acid were also produced and purified. The addition of increasing concentrations of GST wild-type RGS1 to membranes of pertussis toxin-treated HEK293 cells expressing α_{2A}-AR-$Cys^{351}IleG_{o1}$ resulted in a concentration-dependent elevation of adrenaline-stimulated high-affinity GTPase activity with an EC_{50} value of 4.6 nM (Fig. 5A). Ser^{173}AlaRGS1 also functioned as an effective GAP for α_{2A}-AR-$Cys^{351}IleG_{o1}$ but with a potency decreased by some 30-fold (EC_{50} = 140 nM). In contrast, Ser^{173}AspRGS1 had virtually no activity at concentrations up to 30 μM (Fig. 5A). Similar results were obtained using GST fusions of wild-type RGS16 (EC_{50} = 7.3 nM) and both Ser^{166}AlaRGS16 (EC_{50} = 130 nM) and Ser^{166}AspRGS16. Alteration of this serine to alanine reduced the potency of the RGS to function as a GAP by a factor of 20, and conversion to aspartic acid greatly reduced function (Fig. 5B).

As with RGS3, the addition of GST-14-3-3τ along with the GST-RGS16 proteins did not modify the ability of GST-RGS16 to function as a GAP, and this was true whether measuring the high-level GAP activity of GST wild-type RGS16 or the much lesser effect of Ser^{166}AspRGS16, in which we reasoned that the aspartic acid might act as a phosphoserine mimetic (Fig. 6), and this was true when the molar ratio of GST-RGS16 to GST-14-3-3τ was varied be-

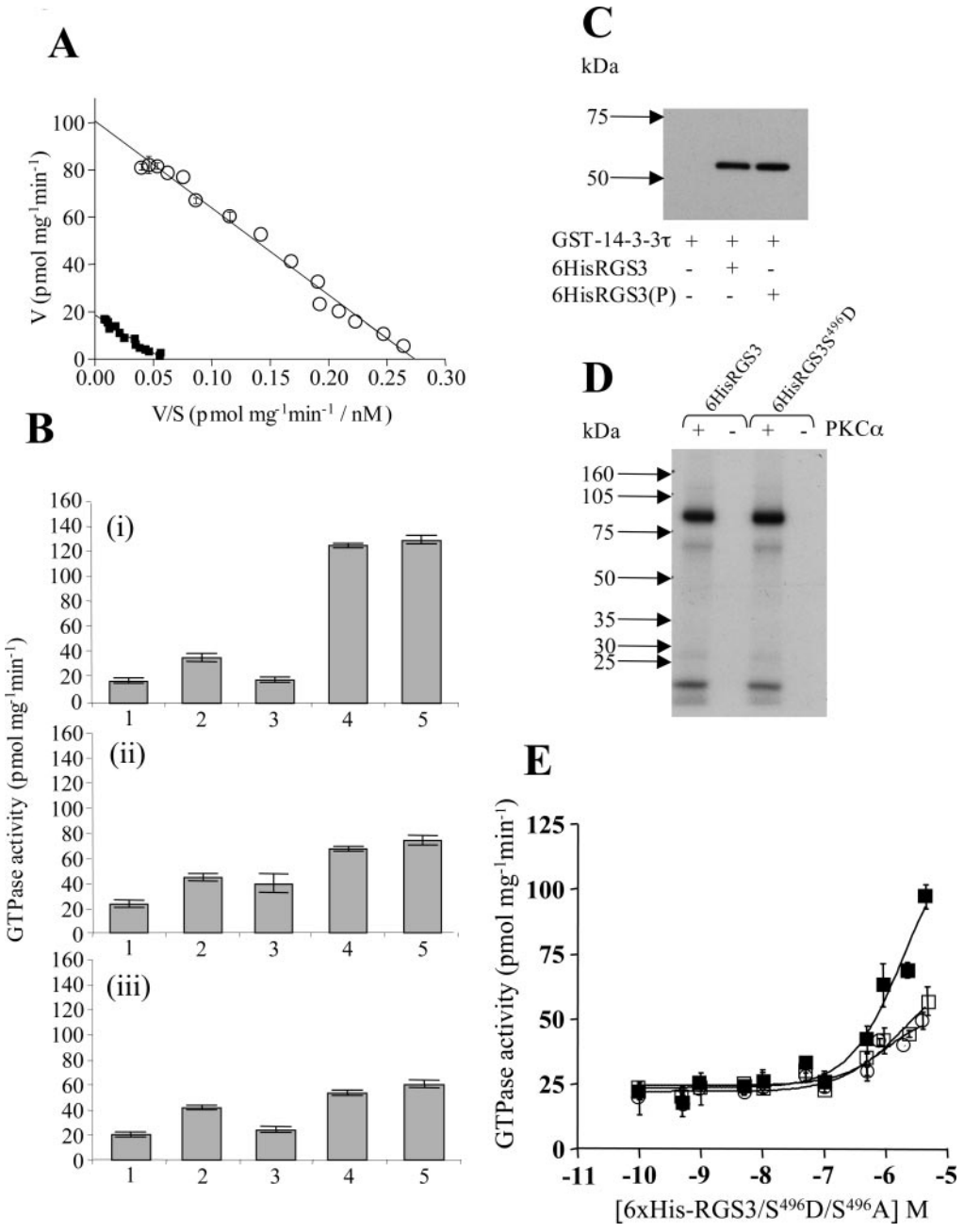

Fig. 4. Effects on RGS3 on adrenaline-stimulated GTPase activity of a α_{2A}-AR-$Cys^{351}IleG_{o1}$ fusion protein. A, high-affinity GTPase assays were performed at varying concentrations of GTP on membranes prepared from HEK293 cells expressing α_{2A}-AR-$Cys^{351}IleG_{o1}$ (closed symbols, basal activity; open symbols, stimulated with 10 μM adrenaline). Data are presented as an Eadie-Hofstee transformation. In the experiment shown, V_{max} values were 18.6 and 100.5 pmol/mg/min for basal and stimulated activity, respectively. Corresponding K_m values were 321 and 367 nM, respectively. B, high-affinity GTPase activity measured at 0.5 μM GTP of membranes prepared from cells expressing an α_{2A}-AR-$Cys^{351}IleG_{o1}$ fusion construct plus added 6xHis-RGS3 (i), 6xHis-Ser^{496}Asp RGS3 (ii), and 6xHis-Ser^{496}AlaRGS3 (iii) (each at 0.5 μM). 1, basal; 2, stimulated with 10 μM adrenaline; 3, basal + RGS; 4, 10 μM adrenaline + RGS; 5, 10 μM adrenaline + RGS + GST-14-3-3τ. GST-14-3-3τ was present at 1 μM. C, Western blot of a pull-down experiment between GST-14-3-3τ and 6xHis-RGS3 or 6xHis-RGS3 phosphorylated by PKCα [6His-RGS3(P)]. The 6xHis tags were pulled down with Ni-NTA agarose, and the blot was probed with anti-GST antibody. D, autoradiograph of a PKCα phosphorylation experiment using 6xHis-RGS3 or 6xHis-Ser^{496}Asp RGS3 as substrates. Reactions were initiated by the addition of enzyme dilution buffer (−) or diluted PKCα (+) and were performed in the presence of 5 μCi [γ-^{32}P]ATP to allow for the identification of the phosphorylated bands. E, GTPase activity measured at 0.5 μM GTP of membranes as in B with varying amounts of 6xHis-tagged RGS3 (■), Ser^{496}Asp RGS3 (□), or Ser^{496}Ala RGS3 (○). Stimulation was done with 10 μM adrenaline.

tween 1:1 and 1:10 (data not shown). Despite this and the lack of interactions between GST-14-3-3τ and the C-terminal domain of RGS3-VSV-G containing the RGS box, interactions could be observed between HA-14-3-3τ and GST wild-type RGS16 in pull-down studies (Fig. 7). Again, however, this was unchanged by the alteration of Ser^{166} of RGS16 to either alanine or aspartic acid (Fig. 7). Previous studies on RGS16 have suggested an important role for Ser^{53} in function (Chen et al., 2001). We thus also tested whether alteration of this serine would modify interactions with HA-14-3-3τ. However, alteration of Ser^{53} to either alanine or glutamic acid did not inhibit the interaction and, indeed, seemed to improve the interaction as monitored in pull-down studies (Fig. 7).

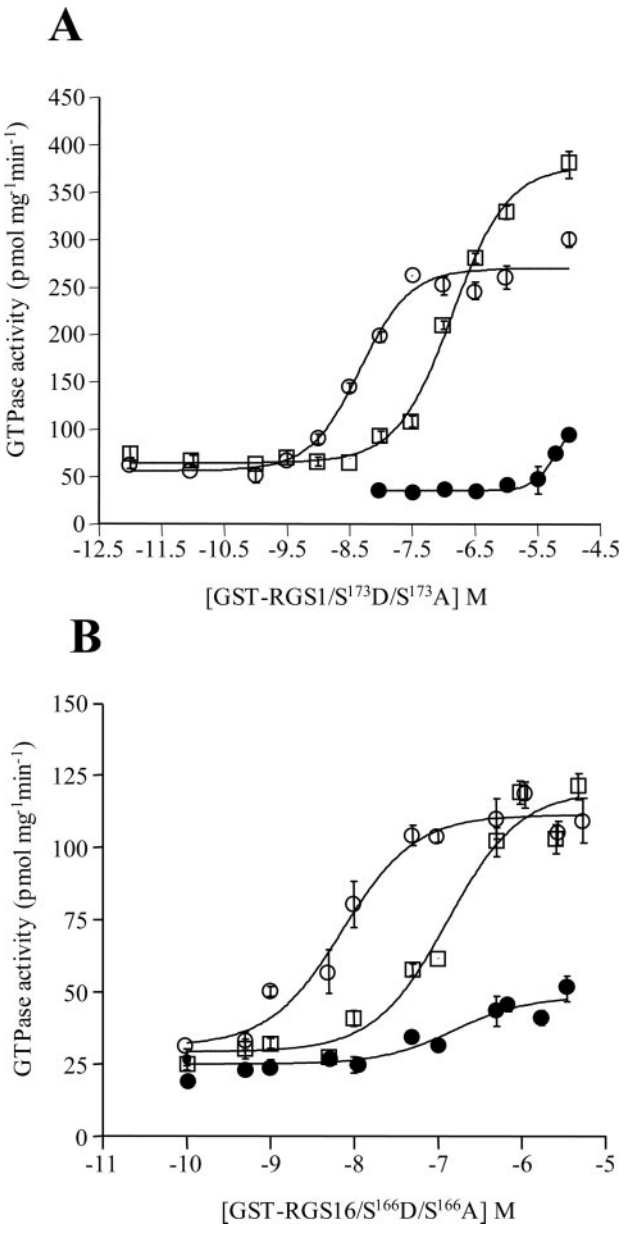

Fig. 5. Alteration of Ser^{173} in RGS1 and Ser^{166} in RGS16: effects on GAP activity. Adrenaline (10 μM)-stimulated high-affinity GTPase activity was measured at 0.5 μM GTP in membranes prepared from cells expressing the α_{2A}-AR-$Cys^{351}IleG_{o1}$ fusion construct. Samples contained differing concentrations of GST-wild-type RGS1 (○), GST-Ser^{173}AspRGS1 (●), and GST-Ser^{173}Ala RGS1 (□) (A); and GST-wild-type RGS16 (○), GST-Ser^{166}Asp RGS16 (●), and GST-Ser^{166}Ala RGS16 (□) (B).

As an alterative to adding recombinantly expressed forms of RGS proteins to membranes of HEK293 cells expressing the α_{2A}-AR-$Cys^{351}IleG_{o1}$ target, we generated a series of fusion proteins between the α_{2A}-adrenoceptor and various wild-type and mutant forms of the RGS proteins (Bahia et al., 2003; Ward and Milligan, 2004). These were then coexpressed in HEK293 cells along with the pertussis toxin-insensitive $Cys^{351}IleG_{o1}$ and membranes prepared from pertussis toxin-treated cells. When examining membranes coexpressing α_{2A}-AR-RGS16 and $Cys^{351}IleG_{o1}$, as in membranes expressing α_{2A}-AR-$Cys^{351}IleG_{o1}$ (Fig. 3), the addition of adrenaline resulted in a large increase in high-affinity GTPase activity. However, when GTPase activity was analyzed at a wide range of GTP concentrations, the characteristics of GTP hydrolysis were very distinct from those in membranes expressing only α_{2A}-AR-$Cys^{351}IleG_{o1}$. Now, in addition to the large increase in GTPase V_{max}, a substantial

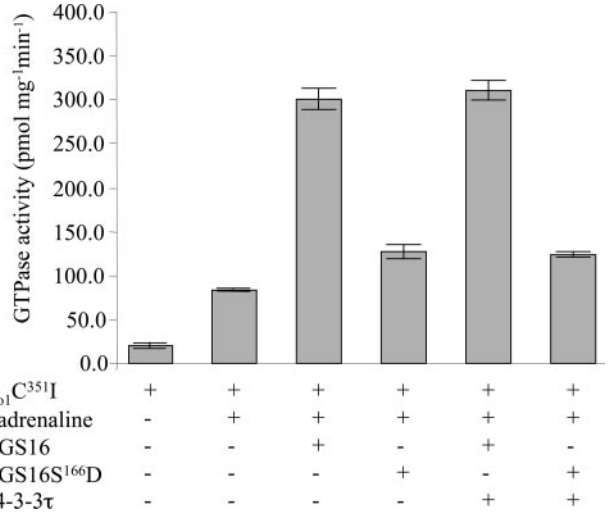

Fig. 6. 14-3-3τ does not inhibit the GAP effect of RGS16. Adrenaline (10 μM)-stimulated high-affinity GTPase activity was measured at 0.5 μM GTP in membranes prepared from cells expressing the α_{2A}-AR-$Cys^{351}IleG_{o1}$ fusion. The effect of GST-14-3-3τ on wild-type and Ser^{166}Asp RGS16-stimulated activity was assessed at a 1:1 molar ratio (2 μM each) of the RGS and 14-3-3τ.

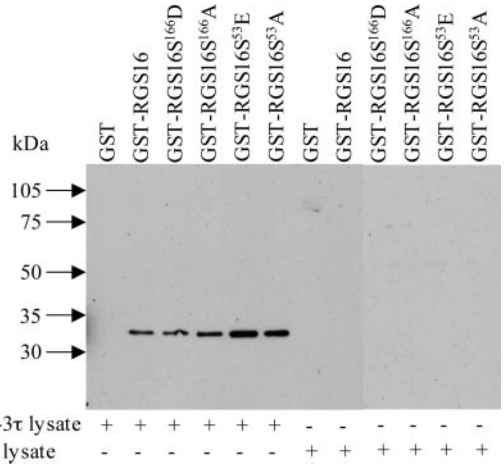

Fig. 7. 14-3-3τ interacts with RGS16: pull-down studies. Lysates of HEK293 cells expressing HA-14-3-3τ or vector-transfected controls were incubated with 0.5 μM GST fusions of wild-type RGS16 or point mutations at Ser^{53} or Ser^{166}. GST pull downs were resolved by SDS-PAGE, and the presence of 14-3-3τ was monitored using an anti-HA antibody.

increase in the measured K_m value for GTP was recorded (Fig. 8A). As discussed previously (Cavalli et al., 2000; Hoffmann et al., 2001; Cladman and Chidiac, 2002), these are exactly the characteristics expected when agonist stimulation of G protein GTPase activity is enhanced by the action of an RGS and proves the functionality of the RGS fused to the α_{2A}-adrenoceptor in this construct as a GAP for $Cys^{351}IleG_{o1}$ (Bahia et al., 2003; Ward and Milligan, 2004). This effect required coexpression of the G protein. When membranes prepared from pertussis toxin-treated HEK293 cells expressing only α_{2A}-AR-RGS16 were used to measure high-affinity GTPase activity, no effect of adrenaline could be observed (Fig. 8B). To extend this work, we also generated α_{2A}-AR-Ser^{166}AlaRGS16 and α_{2A}-AR-Ser^{166}AspRGS16 fusions. When α_{2A}-AR-Ser^{166}AlaRGS16 was coexpressed with $Cys^{351}IleG_{o1}$, the addition of adrenaline generated data equivalent to α_{2A}-AR-RGS16 in that both GTPase V_{max} and the measured K_m values for GTP increased greatly (Fig. 9A). In total contrast, when $Cys^{351}IleG_{o1}$ was coexpressed with α_{2A}-AR-Ser^{166}AspRGS16, adrenaline was able to stimulate GTPase V_{max} only weakly, and there was no increase in the measured K_m value for GTP (Fig. 9B), confirming the virtual

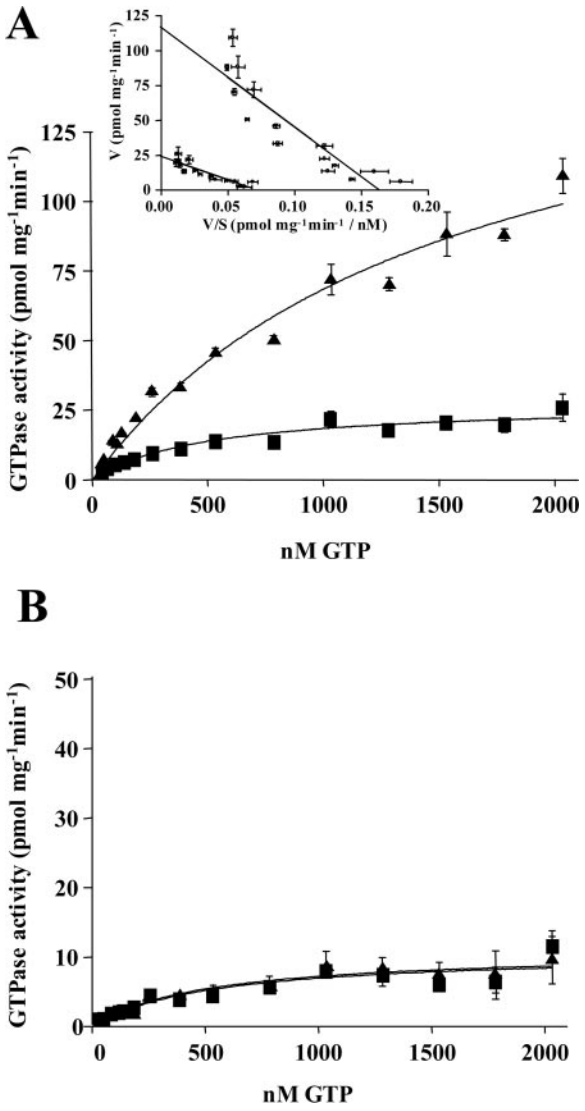

Fig. 8. An α_{2A}-adrenoceptor-RGS16 fusion protein acts as both a GEF and a GAP for $Cys^{351}IleG_{o1}$. Basal (■) and adrenaline (10 μM)-stimulated (▲) high-affinity GTPase assays was measured at varying concentrations of GTP in membranes of pertussis toxin-treated HEK293 cells expressing α_{2A}-AR-RGS16 with (A) or without (B) coexpressed $Cys^{351}IleG_{o1}$. The inset in A represents an Eadie-Hofstee transformation of the data to allow the visual inspection of the alteration in the apparent K_m value for GTP. The V_{max} and K_m values calculated by linear regression were: for A, basal activity, V_{max} = 23.8 ± 1.7 pmol/mg/min, and K_m = 331 ± 43 nM; adrenaline-stimulated, V_{max} = 116.2 ± 10.2 pmol/mg/min, and K_m = 712 ± 94 nM; for B, basal activity, V_{max} = 8.6 ± 0.9 pmol/mg/min, and K_m = 306 ± 60 nM; adrenaline-stimulated, V_{max} = 9.6 ± 0.8 pmol/mg/min, and K_m = 386 ± 60 nM. Expression levels of the fusion constructs in these membranes were determined by ligand binding studies using [^{3}H]RS-79948-197 (Ward and Milligan, 2004) α_{2A}-AR-RGS16/$Cys^{351}IleG_{o1}$, 11.9 pmol/mg (A) or α_{2A}-AR-RGS16, 15.0 pmol/mg (B).

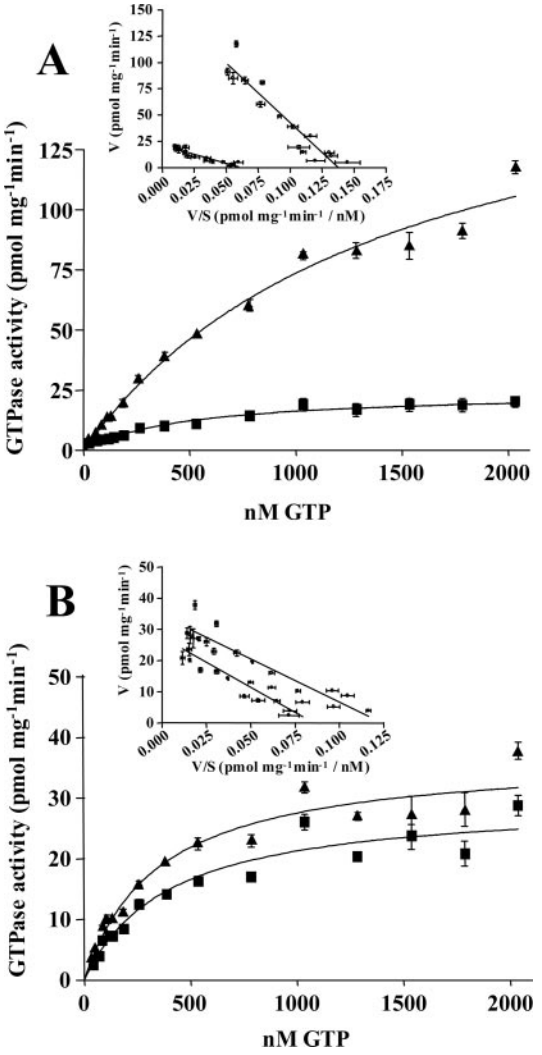

Fig. 9. Altering Ser^{166} in RGS16: the effects on GAP activity against $Cys^{351}IleG_{o1}$. Basal (■) and adrenaline (10 μM)-stimulated (▲) high-affinity GTPase assays was measured at varying concentrations of GTP in membranes of pertussis toxin-treated HEK293 cells expressing $Cys^{351}IleG_{o1}$ and either α_{2A}-AR-Ser^{166}AlaRGS16 (A) or α_{2A}-AR-Ser^{166}AspRGS16 (B). The insets represent Eadie-Hofstee transformations of the data to allow visual inspection of any alteration in the apparent K_m for GTP. The V_{max} and K_m values calculated by linear regression were: for A, basal activity, V_{max} = 21.2 ± 1.3 pmol/mg/min, and K_m = 343 ± 37 nM; adrenaline-stimulated, V_{max} = 156.5 ± 11.2 pmol/mg/min, and K_m = 1140 ± 111 nM; for B, basal activity, V_{max} = 27.5 ± 1.9 pmol/mg/min, and K_m = 323 ± 40 nM; adrenaline-stimulated, V_{max} = 34.4 ± 2.0 pmol/mg/min, and K_m = 277 ± 30 nM. Expression levels of the fusion constructs in these membranes were determined as in Fig. 8: α_{2A}-AR-Ser^{166}AlaRGS16/$Cys^{351}IleG_{o1}$, 15.7 pmol/mg (A), and α_{2A}-AR-Ser^{166}AspRGS16/$Cys^{351}IleG_{o1}$, 14.3 pmol/mg (B).

lack of GAP activity of Ser^{166}AspRGS16 noted earlier for the recombinant version of this protein. Expression levels of the α_{2A}-AR-RGS16 fusions varied between 12 and 15 pmol/mg protein in individual transfections (for the actual values, see figure legends 8 and 9). As expected from the above, the addition of recombinant 14-3-3τ to membranes coexpressing α_{2A}-AR-RGS16 and Cys^{351}IleG$_{o1}$ was without effect on either basal or adrenaline-stimulated GTPase activity (Fig. 10A) and this was also the case for the limited effect of adrenaline in membranes coexpressing α_{2A}-AR-Ser^{166}AspRGS16 and Cys^{351}IleG$_{o1}$ (Fig. 10B).

Given previous reports of an effect of 14-3-3 isoforms on the GAP activity of RGS7 (Benzing et al., 2000, 2002), we explored the effects of each of wild type, Ser^{379}Ala and Ser^{379}Asp RGS7, on basal and adrenaline-stimulated GTPase activity of the α_{2A}-AR-Cys^{351}IleG$_{o1}$ fusion protein. Mirroring other results, wild-type RGS7 and Ser^{379}Ala RGS7 produced a large enhancement of this activity that was unaffected by the coaddition of either 14-3-3τ or 14-3-3ζ (Fig. 11). Ser^{379}Asp RGS7 was far less effective as a GAP for adrenaline-activated α_{2A}-AR-Cys^{351}IleG$_{o1}$ (Fig. 11).

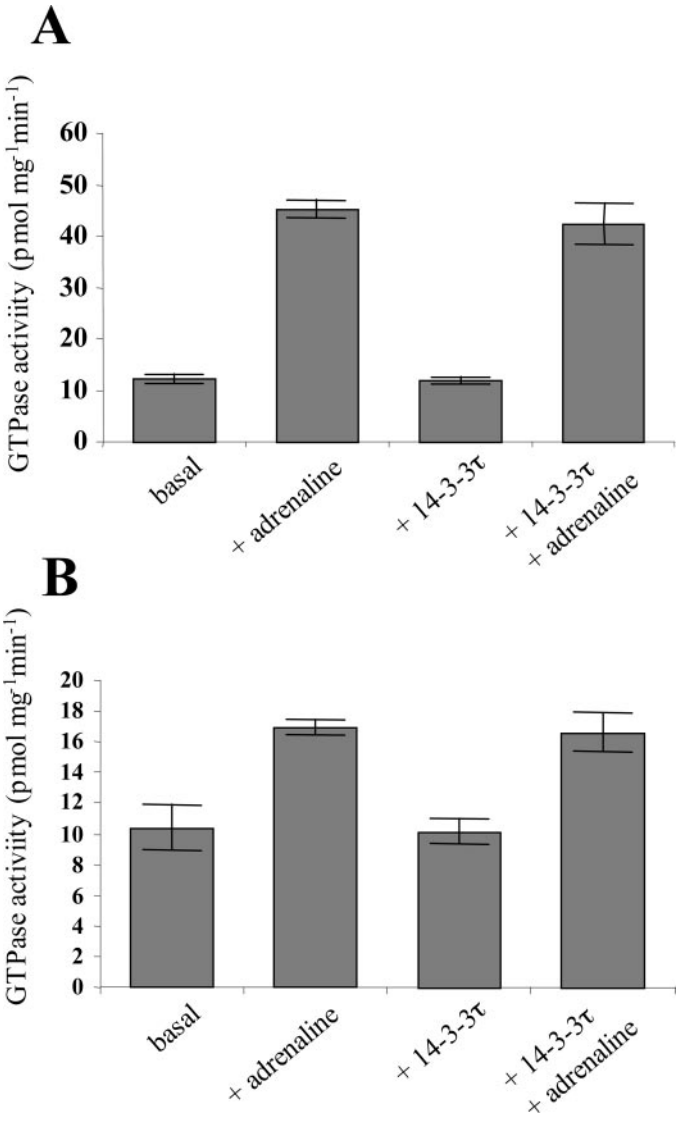

Fig. 10. 14-3-3τ does not regulate the GAP activity of α_{2A}-AR-RGS16. Basal and adrenaline (10 μM)-stimulated high-affinity GTPase assays were performed at 0.5 μM GTP in membranes prepared from HEK293 cells coexpressing Gα_{o1}C^{351}I with either α_{2A}-AR-wild-type RGS16 (top) or α_{2A}-AR-Ser^{166}AspRGS16 (bottom). GST-14-3-3τ was present at 2.6 μM.

Discussion

In the current studies, we demonstrate and quantify the effects of mutating a totally conserved serine residue in the RGS domain of mammalian RGS proteins. On the basis of the atomic level structure of the RGS domain of RGS4 complexed with Gα_{i1} (Tesmer et al., 1997), this serine (RGS4Ser^{164}) is in direct contact with the G protein and is

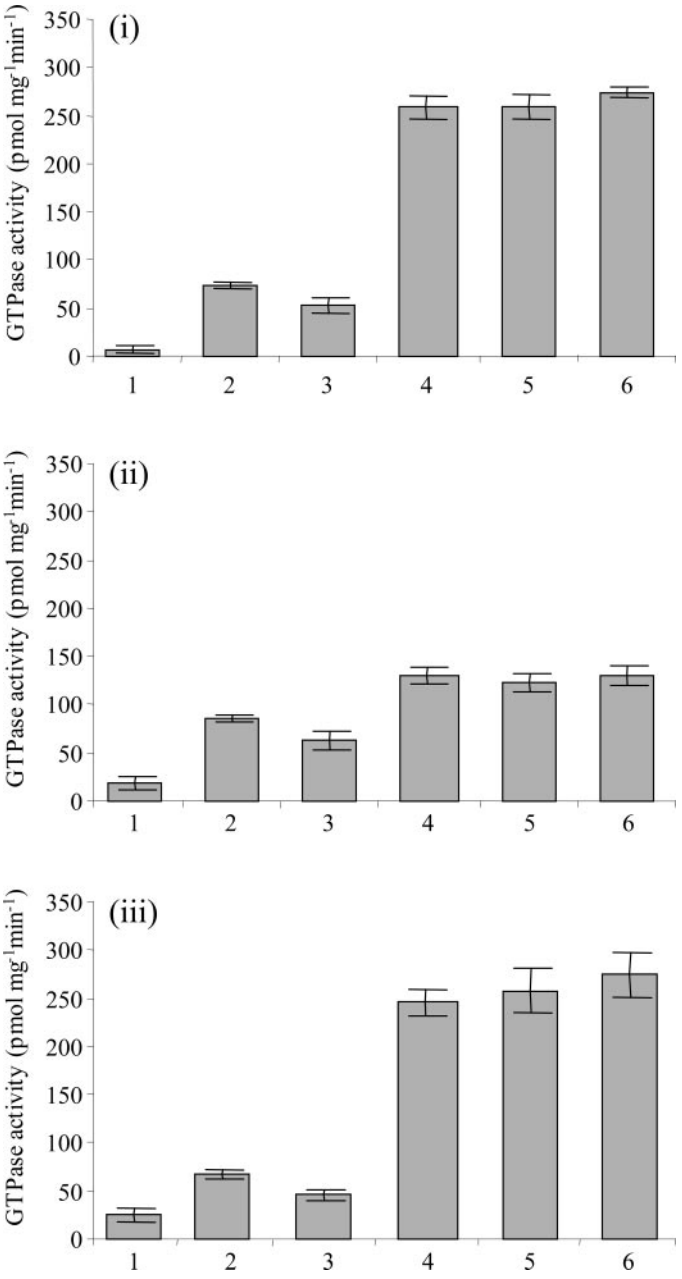

Fig. 11. RGS7 is a GAP for Cys^{351}IleG$_{o1}$, but this is unaffected by 14-3-3τ. Basal (1 and 3) and adrenaline (10 μM)-stimulated (2, 4–6) high-affinity GTPase assays were performed at 0.5 μM GTP in membranes prepared from HEK293 cells expressing the α_{2A}-AR-Cys^{351}IleG$_{o1}$ fusion protein. Assays also contained forms of 6xHis-RGS7 (0.4 μM)(3–6) and GST-14-3-3τ (5) or GST-14-3-3ζ (6) (each 0.5 μM). Top, 6xHis-wild-type RGS7; middle, 6xHis-Ser^{379}Asp RGS7; bottom, 6xHis-Ser^{379}Ala RGS7.

part of a contact interface at the junction of helices α7 and α8 of the RGS domain. The effect of mutating this residue on GAP activity was assessed in two distinct assays. First, akin to other studies, wild-type or mutated RGS proteins were produced recombinantly in bacteria, and after purification and quantification, these proteins were added to membranes of HEK293 cells expressing a α_{2A}-AR-Cys^{351}IleG$_{o1}$ fusion protein. The addition of adrenaline to membranes expressing the fusion protein resulted in a substantial stimulation of high-affinity GTPase activity, reflecting the activation of the receptor-associated G protein, and the addition of the purified RGS proteins markedly increased this agonist-induced activity as anticipated for an active RGS GAP. For RGS1 and RGS16, mutation of the serine residue to aspartic acid almost eliminated GAP activity, whereas mutation to alanine reduced the potency of the proteins as RGS GAPs some 20-fold. The serine-to-aspartic acid alterations could be related to aspartic acid acting as a phosphoserine mimetic, and if so, then phosphorylation of this serine would be anticipated to be highly detrimental to GAP activity. Of course, bacterially expressed proteins are not modified post-translationally in this way; thus, the second assay used the coexpression of proteins in HEK293 cells. When heterologously expressed in mammalian cells, substantial amounts of RGS proteins are not effectively targeted to the plasma membrane (Chidiac and Roy, 2003). Likewise, the mutation of proteins frequently alters steady-state expression levels. To limit both of these potential issues, we generated fusion proteins in which RGS16, as a model, was fused to the C-terminal tail of the α_{2A}-adrenoceptor, akin to the α_{2A}-AR-Cys^{351}IleG$_{o1}$ construct, and as we have done previously for RGS4 (Bahia et al., 2003). The equivalent fusions incorporating Ser^{166}AlaRGS16 and Ser^{166}AspRGS16 were also produced and expressed in HEK293 cells with or without Cys^{351}IleG$_{o1}$, which was anticipated to act as a target for both elements of the receptor RGS fusions, the α_{2A}-adrenoceptor as a guanine nucleotide exchange factor (GEF), and the RGS16 as a GAP. Analysis of agonist-stimulated GTP hydrolysis at different concentrations of GTP confirmed the functionality of both the receptor and wild-type RGS16 in this fusion because both GTPase V_{max} and apparent K_m for GTP increased greatly (Bahia et al., 2003; Ward and Milligan, 2004). As with the recombinant RGS studies, the receptor element of the α_{2A}-AR-Ser^{166}AspRGS16 fusion was able to act as a GEF for Cys^{351}IleG$_{o1}$ because adrenaline-stimulated high-affinity GTPase activity was present. However, again in this assay, Ser^{166}AspRGS16 GAP activity was negligible. These studies confirmed a key role for Ser^{166} in RGS16, and in this case, if Ser^{166}Asp is a phosphoserine mimetic, then Ser^{166} is not constitutively phosphorylated to a significant extent, despite the protein being expressed in mammalian cells. It was noted that the RGS1 Ser^{173}Ala mutant displayed a higher V_{max} value than the wild-type RGS1. Although a definitive explanation for this cannot be provided, on the basis of the X-ray structure of RGS4 complexed with Gα_{i1} (Tesmer et al., 1997), this may be caused by the pivotal position that this residue occupies. Although the RGS serine-to-alanine mutants made in the course of this study all displayed reduced potency, unlike the serine-to-aspartic acid mutants, at sufficiently high concentrations, they each display maximal activity at least as good as the wild-type RGS. In the case of RGS1, the mutation may therefore modify the contact between the RGS and Gα to improve efficacy.

A limitation of the studies using RGS3 and RGS7 is that they are relatively large proteins, and thus levels of expression of recombinant forms of these two polypeptides and the various mutants were insufficient to generate enough protein to perform full concentration-response curves to measure their potency as GAPs against Cys^{351}IleG$_{o1}$. It was clear, however, that RGS3 was significantly less potent as a GAP in such assays than either RGS1 or RGS16. We have reported previously differences in the capacities of RGS1, RGS16, and RGS-G$_\alpha$-interacting protein to enhance α_{2A}-adrenoreceptor agonist-stimulated GTPase activity of Gα_{o1} (Hoffmann et al., 2001), and in a similar vein, Hooks et al. (2003) recently noted that RGS6, RGS7, RGS9, and RGS11 stimulate GTPase activity of G$_i$ family G proteins with differential selectivity and maximal activity, whereas Tovey and Willars (2004) have noted that RGS2, RGS3, and RGS4 differentially regulate signaling via the M$_3$ muscarinic acetylcholine receptor. This is not inherently surprising, and although many RGS proteins are coexpressed (Mittmann et al., 2002), because there are selective expression patterns of RGS family members (Cho et al., 2003) and regulation of their levels by a range of cellular challenges (Grant et al., 2000; Taymans et al., 2003; Cho et al., 2004; Riddle et al., 2005), RGS proteins may be potential targets for therapeutic intervention (Zhong and Neubig, 2001; Cho et al., 2004; Riddle et al., 2005).

Recent studies have shown certain RGS proteins, including RGS3 and RGS7, to interact with 14-3-3 proteins, and at least for RGS7, this interaction has been reported to inhibit GAP function (Benzing et al., 2000). It is interesting that the amino acid reported to be key to this interaction is the serine equivalent to Ser^{166} in RGS16. If interactions of an RGS with a 14-3-3 protein and a G protein α subunit were both to require this serine, and hence, to be mutually exclusive, this would provide a potential explanation for the reported 14-3-3 protein inhibition of RGS GAP activity. Furthermore, because interactions with 14-3-3 proteins generally require a phosphorylated amino acid, then a serine is an obvious potential target. We confirmed in pull-down studies that RGS3 can interact with both 14-3-3τ and 14-3-3ζ. The work of Niu et al. (2002) indicates that the interaction of RGS3 with 14-3-3 is mediated by a residue within the N-terminal extension of the RGS3 and outside of the RGS box. This residue, Ser^{264}, was identified by yeast two-hybrid screening, followed by deletion and point mutational analysis. In agreement with this study, Niu et al. (2002) found that Ser^{264}Ala abolished binding of 14-3-3 to RGS3.

14-3-3τ did not alter the GAP activity of RGS3 against the α_{2A}-AR-Cys^{351}IleG$_{o1}$ fusion protein, and this was true also for the Ser^{496} RGS3 mutants, even when the molar ratio of 14-3-3 to RGS proteins was increased to 10:1, although such interactions are expected to be stoichiometric. Although pull-down studies did indicate a degree of interaction between the 14-3-3 and both RGS16 and RGS1, there was no effect of the coaddition of 14-3-3 to assays that measured the GAP activity of RGS16.

In conclusion, although a central role of this fully conserved serine residue for the GAP function of RGS proteins is now abundantly clear, it seems that the reported effect of 14-3-3 proteins on RGS GAP activity is not general, and that as concluded previously for RGS3, this conserved serine is

not the key amino acid for RGS/14-3-3 interactions. Examination of the atomic level structure of RGS4 complexed with $G\alpha_{i1}$ suggests that the effect of mutation of this conserved serine is to modulate interactions with both Thr^{183} of $G\alpha_{o1}$ and intermolecular contacts with a functionally important and conserved asparagine in the RGS box. Given the key role of Thr^{183} in the GTPase activity of $G\alpha_{o1}$, effects on GTPase activity and, hence, RGS GAP activity by modification of this serine, are easily explained at a molecular level and do not require interactions with 14-3-3.

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