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**MOL #15073**

# A key serine for the GTPase-activating protein function of Regulators of G protein Signalling proteins is not a general target for 14-3-3 interactions

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Running title: RGS GAP activity and 14-3-3 interactions

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[1] **Abbreviations**: GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; RGS, Regulator of G protein Signalling.

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## ABSTRACT

Mammalian Regulator of G protein Signalling (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 while 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.

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Regulator of G protein Signalling (RGS)[1] 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 hetero-trimeric G proteins (Ross and Wilkie, 2000, Zhong and Neubig, 2001, Chidiac and Roy, 2003). The first isolated members of the mammalian RGS family, for example 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). By 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 signalling networks (Burchett, 2000). A series of studies have 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., 1999, 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

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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., 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, based on the available structural information, is a direct Gα subunit contact residue (Tesmer et al., 1997). Not surprisingly, therefore, this serine is entirely conserved across other members of the mammalian RGS family. Despite this, although RGS3 has also been reported to interact with14-3-3 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

[$\gamma$-$^{32}$P]GTP and [$\gamma$-$^{32}$P]ATP were from Amersham Biosciences. Pertussis toxin and PKCα were from Sigma.

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## Constructs

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

Human 14-3-3$\tau$ and $\zeta$, subcloned into pGEX-2T, were obtained from Dr. T. Dubois (University of Edinburgh). The 14-3-3$\tau$ was used as a PCR template with primers designed to add a *BamH1* site and a HA-tag (YPYDVPDYA) at the N-terminal and an *EcoR1* site at the C-terminal. The fragment was subcloned into pcDNA3 (Invitrogen). 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, 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 $\alpha_{2A}$-adrenoceptor-RGS16 ($\alpha_{2A}$-AR-RGS16) fusion construct was a modification of the $\alpha_{2A}$-AR-RGS4 fusion described previously (Bahia et al., 2003). RGS4 was excised with *BamH1* and *Xba1* 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 *Kpn1* site onto the N-terminal with an *Xba1* site and a VSV-G tag (YTDIEMNRLGK) onto the C-termini. The fragments were subcloned into pcDNA3. N-($RGS3^{1-370}$) and

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C-terminal ($RGS3^{371-519}$) fragments of RGS3 were PCR amplified with primers designed to add a *Kpn1* site onto the N-terminal and an *Xba1* 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 *EcoR1* site onto the N-terminal and an *Xho1* site onto the C-terminal. 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 *EcoR1*/*Xho1*. The *EcoR1* site was converted to a blunt end and ligated to a blunt-ended *BamH1* site in pQE-32, while the *Xho1* site was ligated to a *Sal1* site to make a hexa-histidine (6xHis)-tagged RGS3 construct. Serine to alanine and serine to aspartate mutants were also introduced as described above.

Human RGS7 was obtained from the UMR cDNA Resource Centre (University of Missouri-Rolla) via Dr A. Tinker (University College London) and was PCR amplified with primers designed to add an N-terminal *BamH1* site and a C-terminal *Sal1* site which was subcloned into pQE-30, or an N-terminal *BamH1* site and C-terminal *Not1* 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 was carried out using standard techniques and constructs were fully sequenced before use.

**Cell culture, transient transfection and cell lysates**

HEK293 cells were maintained in Dulbeccos' modification of Eagles' medium supplemented with 10% (v/v) newborn calf serum and 2mM glutamine. Before

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transfection cells were seeded onto 10cm dishes at 50-70% confluency. Constructs were transfected into the cells using the Lipofectamine reagent (Invitrogen) according to the manufacturers' protocol. 48 h after transfection medium was removed and the cells washed twice with 1x PBS (120mM NaCl, 25mM KCl, 10mM $Na_2HPO_4$, 3mM $KH_2PO_4$ pH 7.4). Cells intended for membrane preparation were scraped into 5ml 1xPBS, pelleted and stored at -80°C. Cells for lysates were harvested with 1ml lysis buffer (1% Triton X-100, 20mM TrisHCl pH 7.5, 50mM NaCl, 50mM NaF, 15mM $Na_4P2O_7$, 2mM $Na_3VO_4$, 1mM EDTA and 1 complete protease inhibitor tablet, (Roche)) and incubated, rotating, at 4°C for 1 h. Lysed cells were centrifuged at 12000xg for 10 min at 4°C and the supernatant 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 1xTE buffer (1mM TrisHCl, 0.1mM EDTA pH 7.5) and disrupted with a Teflon on glass homogeniser for 2 min, before being passed through a 25 gauge needle 20 times. Unbroken cells were removed by centrifugation at 500xg for 5 min at 4ºC. The supernatant fraction was centrifuged at 90000xg for 30 min at 4ºC to recover the membranes, which were resuspended in 1xTE buffer and the protein concentration determined by BCA assay before being stored at -80ºC.

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**Purification of 6xHis-tagged proteins**

pQE-30/32 plasmids (Qiagen) containing full length cDNAs of RGS3 or RGS7 fused to the 6xHis tag were transformed into *Escherichia coli* strain BL21 DE3. 400ml of LB media containing 100μg/ml of ampicillin was inoculated with a 10ml starter culture of the transformed cells and grown to an $OD_{600}$ of 0.5. Expression of the 6xHis-tagged protein was induced by the addition of 1mM isopropyl-β, D-thiogalactopyranoside for 5 h before harvesting the cells by centrifugation at 6000xg for 15min at 4°C. Pellets were resuspended in denaturing lysis buffer (containing 6M urea) and the 6xHis-tagged protein purified using Ni-NTA agarose (Qiagen) according to the manufacturers' instructions. The eluted proteins were analysed by SDS-PAGE and BCA protein assay before being dialysed against 3 changes of 1xPBS containing 5% glycerol at 4°C over 2 days, before storage at -80°C.

**Purification of GST-fusion proteins**

pGEX plasmids (Amersham BioSciences) 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 20ml 1xPBS (with protease inhibitors) containing 0.5mg/ml lysozyme followed by incubation at 4°C with rotation for 1 h. The resuspended cells were sonicated for 4x30 s each on ice, with 30 s for cooling between bursts. Dithiothreitol was added to a final concentration of 5mM 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



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material removed by centrifugation at 12000xg for 15 min at 4°C. 1ml of a suspension of washed (3x with 10 volumes 1xPBS with protease inhibitors) glutathione Sepharose was added to each cleared lysate and incubated at 4°C overnight with rotation. The lysates were spun at 500xg for 5 minutes at 4°C and the supernatant removed from the pellet of glutathione Sepharose beads. The beads were washed with 3x10ml 1xPBS with protease inhibitors and the GST fusion proteins eluted with 5x1.5ml glutathione solution (10mM reduced glutathione in 50mM TrisHCl, pH 8.0). The eluted proteins were analysed by SDS-PAGE and BCA protein assay before being dialysed against 3 changes of 1xPBS containing 5% glycerol at 4°C over 2 days, before storage at -80°C.

**PKCα phosphorylation of recombinant RGS protein**

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

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**Pull-down experiments**

100-400µl aliquots of HEK293 cell lysate (dependent upon protein expression from the construct) were mixed with 200µl dialysed GST-fusion protein (0.5-2 µM) and 40ml 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 500xg for 5 min at 4°C and the supernatant removed. The beads were then washed with 4x1ml aliquots each lysis buffer and 40µl each 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 prior to 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 microtitre 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 addition to the assay mix. The volumes and concentrations of the components added to the assay were adjusted to allow a constant reaction volume of 100µl to be maintained in all cases.

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## Results

A HA-tagged version of the τ isoform of 14-3-3 was expressed in HEK293 cells and after SDS-PAGE of cell lysates was detected with the anti-HA antibody 12CA5 predominantly as a 33kDa polypeptide (**Figure 1A**). This polypeptide interacted with, and was pulled down by, a hexa-histidine (6xHis) tagged form of the Regulator of G protein signalling, RGS3 (**Figure 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. As 14-3-3 binding routinely requires a phosphorylated serine residue (Mackintosh, 2004) we altered this serine ($Ser^{496}$ in RGS3) to either alanine (Ala) or aspartic acid (Asp). Neither of these modifications altered the ability of 14-3-3τ to interact with 6xHis RGS3 (**Figure 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 371-519, and the N-terminal domain consisting of amino acids 1-370 (**Figure 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. Following expression in HEK293 cells full length RGS3-VSV-G migrated through SDS-PAGE predominantly as a 75kDa 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 23kDa species with lower levels of a 46kDa species whilst, in SDS-PAGE, the N-terminal fragment was predominantly a 52kDa species with evidence of some capacity to dimerise (**Figure 2**). Full length

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RGS3-VSV-G was pulled down by GST-fusions of both 14-3-3τ and 14-3-3ζ (**Figure 3A**). Again, this was not prevented by the introduction of $Ser^{496}$Ala or $Ser^{496}$Asp mutations into RGS3 (**Figure 3A**). Interestingly, although the VSV-G-tagged C-terminal 23kDa RGS domain fragment of RGS3 did not interact with either 14-3-3τ or14-3-3ζ, no matter the identity of the amino acid at position 496, the VSV-G-tagged N-terminal 52kDa fragment of RGS3 did interact with both 14-3-3 isoforms (**Figure 3B**). Recent studies have suggested a role for RGS3$Ser^{264}$, 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, mutation of $Ser^{264}$ to either Ala or Asp did eliminate interactions with both14-3-3τ and 14-3-3ζ (**Figure 3C**).

We have previously constructed a fusion protein ($\alpha_{2A}$-AR-$Cys^{351}$Ile$G_{o1}$) between the $\alpha_{2A}$-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 (25ng/ml, 24h) 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 addition of $\alpha_{2A}$-adrenoceptor agonists such as the natural ligand adrenaline (**Figure 4**). This reflected an increase in the $V_{max}$ of GTPase activity without an alteration in the apparent $K_m$ for GTP (**Figure 4A**). Addition of recombinantly expressed 6xHis-RGS3 to membranes of pertussis toxin-treated HEK293 cells expressing $\alpha_{2A}$-AR-$Cys^{351}$Ile$G_{o1}$ did not modify basal GTPase activity but resulted in a large elevation of adrenaline-stimulated high

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affinity GTPase activity (**Figure 4B(i)**) that was not modified by the co-addition of GST-14-3-3τ (**Figure 4B (i)**). Equally, 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 (**Figures 4B(ii)**, **4B(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 $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ than wild type RGS3 (**Figure 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, pulldown experiments were carried out as shown in (**Figure 4C**). The 6xHis-RGS3 was able to pulldown GST-14-3-3τ and this interaction was unaffected by treatment with the PKCα isoform. PKCα was, however, able to cause phosphorylation of the 6xHis-RGS3 but Ser$^{496}$ was not a key site for phosphorylation as the extent of incorporation of [$^{32}$P] into 6xHis-RGS3 was unaffected by the Ser$^{496}$Asp mutation (**Figure 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 $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ and examined effects on high affinity GTPase activity stimulated by a maximally effective concentration (10μM) 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 (**Figure 4E**).

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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 (**Figure 4E**) and hence further insights into the role of $Ser^{496}$, particularly as RGS3 was not a highly potent GAP ($EC_{50}$ above 1μM) for $\alpha_{2A}$-AR-$Cys^{351}IleG_{o1}$ (**Figure 4E**). However, as noted earlier, all mammalian 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 Ser equivalent to $Ser^{496}$ in RGS3 ($Ser^{173}$ in RGS1 and $Ser^{166}$ in RGS16) was modified to either Ala or Asp were also produced and purified. Addition of increasing concentrations of GST-wild type RGS1 to membranes of pertussis toxin-treated HEK293 cells expressing $\alpha_{2A}$-AR-$Cys^{351}IleG_{o1}$ resulted in a concentration-dependent elevation of adrenaline-stimulated high affinity GTPase activity with $EC_{50}$ = 4.6 nM (**Figure 5A**). $Ser^{173}$AlaRGS1 also functioned as an effective GAP for $\alpha_{2A}$-AR-$Cys^{351}IleG_{o1}$ but with potency decreased by some 30 fold ($EC_{50}$ = 140 nM). By contrast, $Ser^{173}$AspRGS1 had virtually no activity at concentrations up to 30μM (**Figure 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 Ser to Ala reduced the potency of the RGS to function as a GAP by a factor of 20 and conversion to Asp greatly reduced function (**Figure 5B**).

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As with RGS3, 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, where we reasoned that the Asp might act as a phosphoserine mimetic (**Figure 6**) and this was true when the molar ratio of GST-RGS16 to GST-14-3-3τ was varied between 1:1 to 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 (**Figure 7**). Again, however, this was unchanged by alteration of $Ser^{166}$ of RGS16 to either Ala or Asp (**Figure 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 Ser would modify interactions with HA-14-3-3τ. However, alteration of $Ser^{53}$ to either Ala or Glu did not inhibit the interaction and indeed, appeared to improve the interaction as monitored in pull-down studies (**Figure 7**).

As an alterative to adding recombinantly expressed forms of RGS proteins to membranes of HEK293 cells expressing the $\alpha_{2A}$-AR-$Cys^{351}IleG_{o1}$ target we generated a series of fusion proteins between the $\alpha_{2A}$-adrenoceptor and various wild type and mutant forms of the RGS proteins (Bahia et al., 2003, Ward and Milligan, 2004). These were then co-expressed in HEK293 cells along with the pertussis toxin-insensitive $Cys^{351}IleG_{o1}$ and membranes prepared from pertussis toxin-treated cells. When examining membranes co-expressing $\alpha_{2A}$-AR-RGS16 and $Cys^{351}IleG_{o1}$, as in membranes expressing $\alpha_{2A}$-AR-$Cys^{351}IleG_{o1}$ (**Figure 3**), addition of adrenaline



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resulted in a large increase in high affinity GTPase activity. However, when GTPase activity was analysed at a wide range of GTP concentrations, the characteristics of GTP hydrolysis were very distinct from those in membranes expressing only $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$. Now, as well as the large increase in GTPase $V_{max}$, a substantial increase in the measured $K_m$ for GTP was recorded (**Figure 8A**). As has been 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 $\alpha_{2A}$-adrenoceptor in this construct as a GAP for Cys$^{351}$Ile$G_{o1}$ (Bahia et al., 2003, Ward and Milligan, 2004). This effect required co-expression of the G protein. When membranes prepared from pertussis toxin-treated HEK293 cells expressing only $\alpha_{2A}$-AR-RGS16 were used to measure high affinity GTPase activity, no effect of adrenaline could be observed (**Figure 8B**). To extend this work we also generated $\alpha_{2A}$-AR-Ser$^{166}$AlaRGS16 and $\alpha_{2A}$-AR-Ser$^{166}$AspRGS16 fusions. When $\alpha_{2A}$-AR-Ser$^{166}$AlaRGS16 was co-expressed with Cys$^{351}$Ile$G_{o1}$, addition of adrenaline generated data equivalent to $\alpha_{2A}$-AR-RGS16 in that both GTPase $V_{max}$ and the measured $K_m$ for GTP increased greatly (**Figure 9A**). In total contrast, when Cys$^{351}$Ile$G_{o1}$ was co-expressed with $\alpha_{2A}$-AR-Ser$^{166}$AspRGS16, adrenaline was able to stimulate GTPase $V_{max}$ only weakly and there was no increase in measured $K_m$ for GTP (**Figure 9B**), confirming the virtual lack of GAP activity of Ser$^{166}$AspRGS16 noted earlier for the recombinant version of this protein. Expression levels of the$\alpha_{2A}$-AR-RGS16 fusions varied e between 12 to 15 pmol mg$^{-1}$ protein in individual transfections (for the actual values, see **Figure legends 8 and 9**). As

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expected from the above, the addition of recombinant 14-3-3τ to membranes co-expressing $\alpha_{2A}$-AR-RGS16 and Cys$^{351}$Ile$G_{o1}$ was without effect on either basal or adrenaline-stimulated GTPase activity (**Figure 10A**) and this was also the case for the limited effect of adrenaline in membranes co-expressing $\alpha_{2A}$-AR-Ser$^{166}$AspRGS16 and Cys$^{351}$Ile$G_{o1}$ (**Figure 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 $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ 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 co-addition of either 14-3-3τ or14-3-3ζ (**Figure 11**). Ser$^{379}$Asp RGS7 was far less effective as a GAP for adrenaline-activated $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ (**Figure 11**).

## Discussion

In the current studies we demonstrate and quantitate the effects of mutating a totally conserved serine residue in the RGS domain of mammalian RGS proteins. Based on the atomic level structure of the RGS domain of RGS4 complexed with $G\alpha_{i1}$ (Tesmer et al., 1997) this serine (RGS4Ser$^{164}$) is in direct contact with the G protein and is 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. Firstly, akin to other studies, wild type or mutated RGS proteins were produced

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recombinantly in bacteria and following purification and quantitation were added to membranes of HEK293 cells expressing a $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ fusion protein. Addition of adrenaline to membranes expressing the fusion protein resulted in a substantial stimulation of high affinity GTPase activity, reflecting activation of the receptor-associated G protein, and 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 Ser residue to Asp almost eliminated GAP activity whilst mutation to Ala reduced the potency of the proteins as RGS GAPs some 20 fold. The Ser to Asp alterations could be related to Asp 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 and thus the second assay utilized co-expression of proteins in HEK293 cells. When heterologously expressed in mammalian cells substantial amounts of RGS proteins are not effectively targeted to the plasma membrane (Chidaic and Roy, 2003). Equally, 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 $\alpha_{2A}$-adrenoceptor, akin to the $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ construct, and as we have previously done for RGS4 (Bahia et al., 2003). Equivalent fusions incorporating Ser$^{166}$AlaRGS16 and Ser$^{166}$AspRGS16 were also produced and expressed in HEK293 cells with or without Cys$^{351}$Ile$G_{o1}$which was anticipated to act as a target for both elements of the receptor-RGS fusions, the $\alpha_{2A}$-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

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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 $\alpha_{2A}$-AR-Ser$^{166}$AspRGS16 fusion was able to act as a GEF for Cys$^{351}$Ile$G_{o1}$ as 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, based on the x-ray structure of RGS4 complexed with $G\alpha_{i1}$ (Tesmer et al., 1997) this may be due to the pivotal position that this residue occupies. Although the RGS Ser to Ala mutants made in the course of this study all displayed reduced potency, unlike the Ser to Asp 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 $G\alpha$ to improve efficacy.

A limitation of the studies employing 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 was insufficient to generate enough protein to perform full concentration-response curves to measure their potency as GAPs against Cys$^{351}$Ile$G_{o1}$. It was clear, however, that RGS3 was significantly less potent as a GAP in such assays than either RGS1 or RGS16. We have previously reported differences in the

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capacities of RGS1, RGS16 and RGS-GAIP to enhance $\alpha_{2A}$-adrenoreceptor agonist-stimulated GTPase activity of $G\alpha_{o1}$ (Hoffmann et al., 2001) and in a similar vein, Hooks et al., (2003) have recently noted that RGS6, RGS7, RGS9, and RGS11 stimulate GTPase activity of $G_i$ family G-proteins with differential selectivity and maximal activity whilst Tovey and Willars (2003) have noted that RGS 2, 3, and 4 differentially regulate signalling via the M3 muscarinic acetylcholine receptor. This is not inherently surprising and although many RGS proteins are co-expressed (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 (Cho et al, 2004, Zhong and Neubig, 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). Interestingly, 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 $\alpha$ 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, as 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 14-3-3$\tau$ and 14-3-3$\zeta$. 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-

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terminal extension of the RGS3 and outside 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, 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 $\alpha_{2A}$-AR-$Cys^{351}$Ile$G_{o1}$ 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, again there was no effect of the co-addition 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 appears that the reported effect of 14-3-3 proteins on RGS GAP activity is not general and that as previously concluded 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 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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## Figure Legends

### Figure 1

**Interaction of 14-3-3t 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 HEK293cells expressing the HA-14-3-3τ construct were used in pull-down experiments employing 6xHis-tagged forms of RGS3. (1) no RGS3, (2) wild type RGS3, (3) $Ser^{496}$Asp RGS3, (4) $Ser^{496}$AlaRGS3. Samples were resolved by SDS-PAGE and immunoblotted to detect HA-14-3-3τ.

### Figure 2

**Production of N and C terminal fragments of RGS3**

**A** RGS3 was split to generate N-terminal 1-370 and C terminal 371-519 (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.

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**Figure 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 1μM GST-tagged 14-3-3τ or 14-3-3ζ were probed with anti-VSV-G antibody. **B**. Equivalent pull-downs from lysates of HEK293cell 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.

**Figure 4**

**Effects on RGS3 on adrenaline-stimulated GTPase activity of a $\alpha_{2A}$-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 $\alpha_{2A}$-AR-$Cys^{351}IleG_{o1}$ (**closed symbols, basal activity, open symbols, stimulated with 10μM adrenaline**). Data are presented as an Eadie-Hofstee transformation. In the

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experiment shown $V_{max}$ values were 18.6 and 100.5 pmol $mg^{-1}min^{-1}$ for basal and stimulated respectively. Corresponding $K_m$ values were 321 and 367 nM. **B**. High affinity GTPase activitymeasured at 0.5μM GTP of membranes prepared from cells expressing an $\alpha_{2A}$-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 0.5μM). 1. basal, 2. stimulated with 10μM adrenaline, 3. basal + RGS, 4. 10μM adrenaline + RGS, 5. 10μM adrenaline + RGS + GST-14-3-3τ. GST-14-3-3τ was present at 1μM. **C**. Western blot of a pulldown 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 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 5μCi [$\gamma$-$^{32}$P]ATP to allow identification of the phosphorylated bands. **E**. GTPase activity measured at 0.5μM GTP of membranes as in **B** with varying amounts of 6xHis-tagged RGS3 (closed squares), $Ser^{496}$Asp RGS3 (**open squares**) or $Ser^{496}$Ala RGS3 (**open circles**). Stimulation with 10μM adrenaline.

**Figure 5**

**Alteration of $Ser^{173}$ in RGS1 and $Ser^{166}$ in RGS16: effects on GAP activity**

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Adrenaline (10μM) stimulated high affinity GTPase activity was measured at 0.5μM GTP in membranes prepared from cells expressing the $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ fusion construct. Samples contained differing concentrations of **A**. GST-wild-type RGS1 (**open circles**), GST-Ser$^{173}$AspRGS1 (**closed circles**) and GST-Ser$^{173}$Ala RGS1 (**open squares**). **B**. GST-wild-type RGS16 (**open circles**), GST-Ser$^{166}$Asp RGS16 (**closed circles**) and GST-Ser$^{166}$Ala RGS16 (**open squares**).

**Figure 6**

**14-3-3τ does not inhibit the GAP effect of RGS16**

Adrenaline (10μM) stimulated high affinity GTPase activity was measured at 0.5μM GTP in membranes prepared from cells expressing the $\alpha_{2A}$-AR-Cys$^{351}$Ile$G_{o1}$ 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 (2μM each) of the RGS and 14-3-3τ.

**Figure 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 0.5μM GST-fusions of wild type RGS16 or point mutations at Ser$^{53}$ or

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Ser[166]. GST-pull downs were resolved by SDS-PAGE and the presence of 14-3-3τ monitored using an anti-HA antibody.

**Figure 8**

**An $\alpha_{2A}$-adrenoceptor-RGS16 fusion protein acts as both a GEF and a GAP for Cys[351]IleG$_{o1}$**

Basal (**squares**) and adrenaline (10μM) stimulated (**diamonds**) high affinity GTPase assays was measured at varying concentrations of GTP in membranes of pertussis toxin-treated HEK293 cells expressing $\alpha_{2A}$-AR-RGS16 with (**A**) or without (**B**) co-expressed Cys[351]IleG$_{o1}$. The inset in **A** represents an Eadie-Hofstee transformation of the data to allow visual inspection of the alteration in the apparent $K_m$ for GTP. The $V_{max}$ and $K_m$ values calculated by linear regression are **A**, basal activity $V_{max}$ 23.8 +/- 1.7 pmol.mg$^{-1}$min$^{-1}$ and $K_m$ 331 +/- 43 nM, adrenaline stimulated 116.2 +/- 10.2 pmol.mg$^{-1}$min$^{-1}$, $K_m$ 712 +/- 94 nM: B basal activity $V_{max}$ 8.6 +/- 0.9 pmol.mg$^{-1}$min$^{-1}$ and $K_m$ 306 +/- 60 nM, adrenaline stimulated 9.6 +/- 0.8 pmol.mg$^{-1}$min$^{-1}$, $K_m$ 386 +/- 60 nM. Expression levels of the fusion constructs in these membranes were determined by ligand binding studies employing [$^3$H]RS-79948-197 (Ward and Milligan, 2004) (**A**) $\alpha_{2A}$-AR-RGS16 / Cys[351]IleG$_{o1}$ 11.9 pmol.mg$^{-1}$ (**B**) $\alpha_{2A}$-AR-RGS16 15.0 pmol.mg$^{-1}$

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**Figure 9**

**Altering Ser166 in RGS16: effects on GAP activity against $Cys^{351}IleG_{o1}$**

Basal (**squares**) and adrenaline (10μM) stimulated (**diamonds**) 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 $\alpha_{2A}$-AR-$Ser^{166}$AlaRGS16 (**A**) or $\alpha_{2A}$-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 are **A**, basal activity $V_{max}$ 21.2 +/- 1.3 pmol.$mg^{-1}min^{-1}$ and $K_m$ 343 +/- 37 nM, adrenaline stimulated 156.5 +/- 11.2 pmol.$mg^{-1}min^{-1}$, $K_m$ 1140 +/- 111 nM, **B** basal activity $V_{max}$ 27.5 +/-1.9 pmol.$mg^{-1}min^{-1}$ and $K_m$ 323 +/- 40 nM, adrenaline stimulated 34.4 +/- 2.0 pmol.$mg^{-1}min^{-1}$, $K_m$ 277 +/- 30 nM. Expression levels of the fusion constructs in these membranes were determined as in Figure 8 (**A**) $\alpha_{2A}$-AR-$Ser^{166}$AlaRGS16 / $Cys^{351}IleG_{o1}$ 15.7 pmol $mg^{-1}$ (**B**) $\alpha_{2A}$-AR-$Ser^{166}$AspRGS16 / $Cys^{351}IleG_{o1}$ 14.3 pmol $mg^{-1}$.

**Figure 10**

**14-3-3τ does not regulate the GAP activity of $\alpha_{2A}$-AR-RGS16**

Basal and adrenaline (10μM) stimulated high affinity GTPase assays were performed at 0.5μM GTP in membranes prepared from HEK293 cells co-expressing $G\alpha_{o1}C^{351}I$

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with either $\alpha_{2A}$-AR-wild type RGS16 (upper panel) or $\alpha_{2A}$-AR-Ser$^{166}$AspRGS16 (lower panel). GST-14-3-3τ was present at 2.6μM

**Figure 11**

**RGS7 is a GAP for Cys$^{351}$IleG$_{o1}$ but this is unaffected by 14-3-3τ**

Basal (**1,3**) and adrenaline (10μM) (**2, 4-6**) stimulated high affinity GTPase assays were performed at 0.5μM GTP in membranes prepared from HEK293 cells expressing the $\alpha_{2A}$-AR-Cys$^{351}$IleG$_{o1}$ fusion protein. Assays also contained forms of 6xHis-RGS7 (0.4μM)(**3-6**) and GST-14-3-3τ (**5**) or GST-14-3-3ζ (**6**) (each 0.5μM). Upper panel: 6xHis-wild type RGS7, middle panel 6xHis-Ser$^{379}$Asp RGS7, lower panel 6xHis-Ser$^{379}$Ala RGS7.

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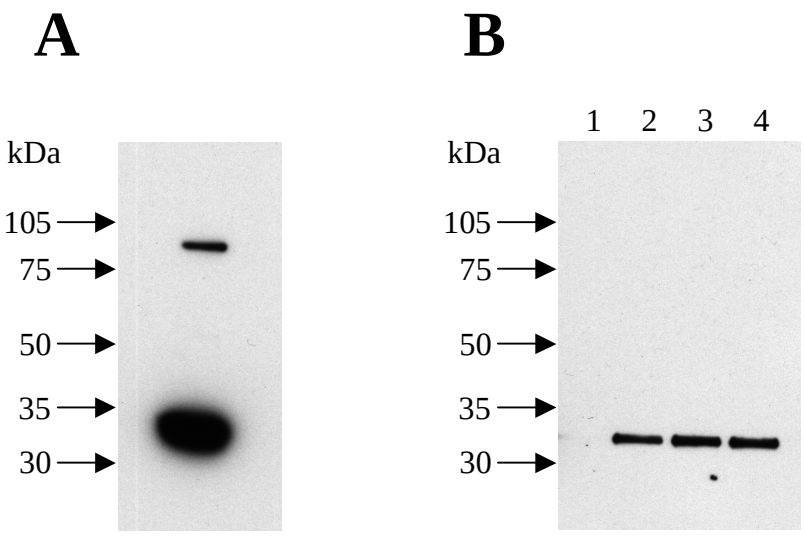
# Figure 1

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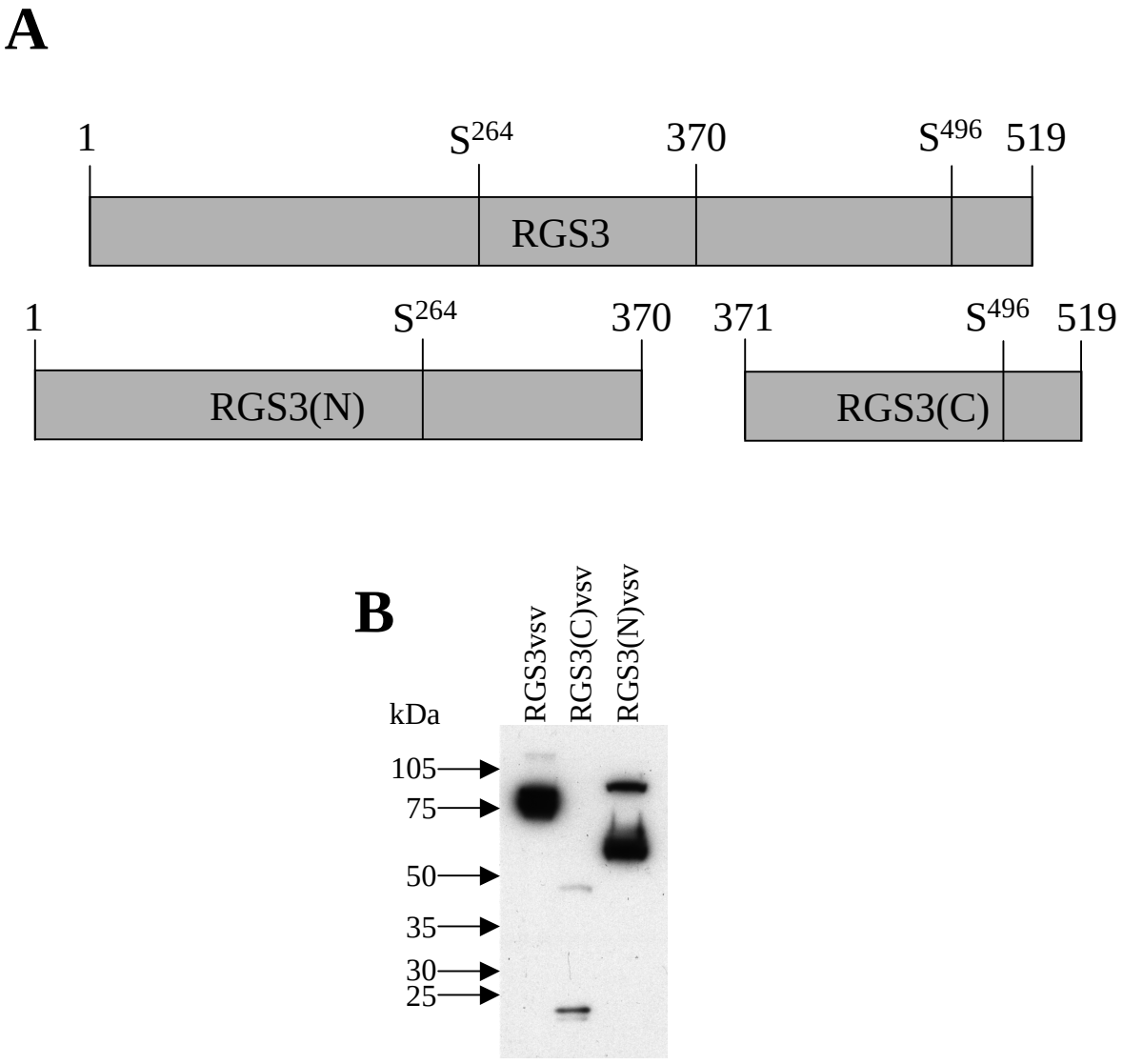
# Figure 2

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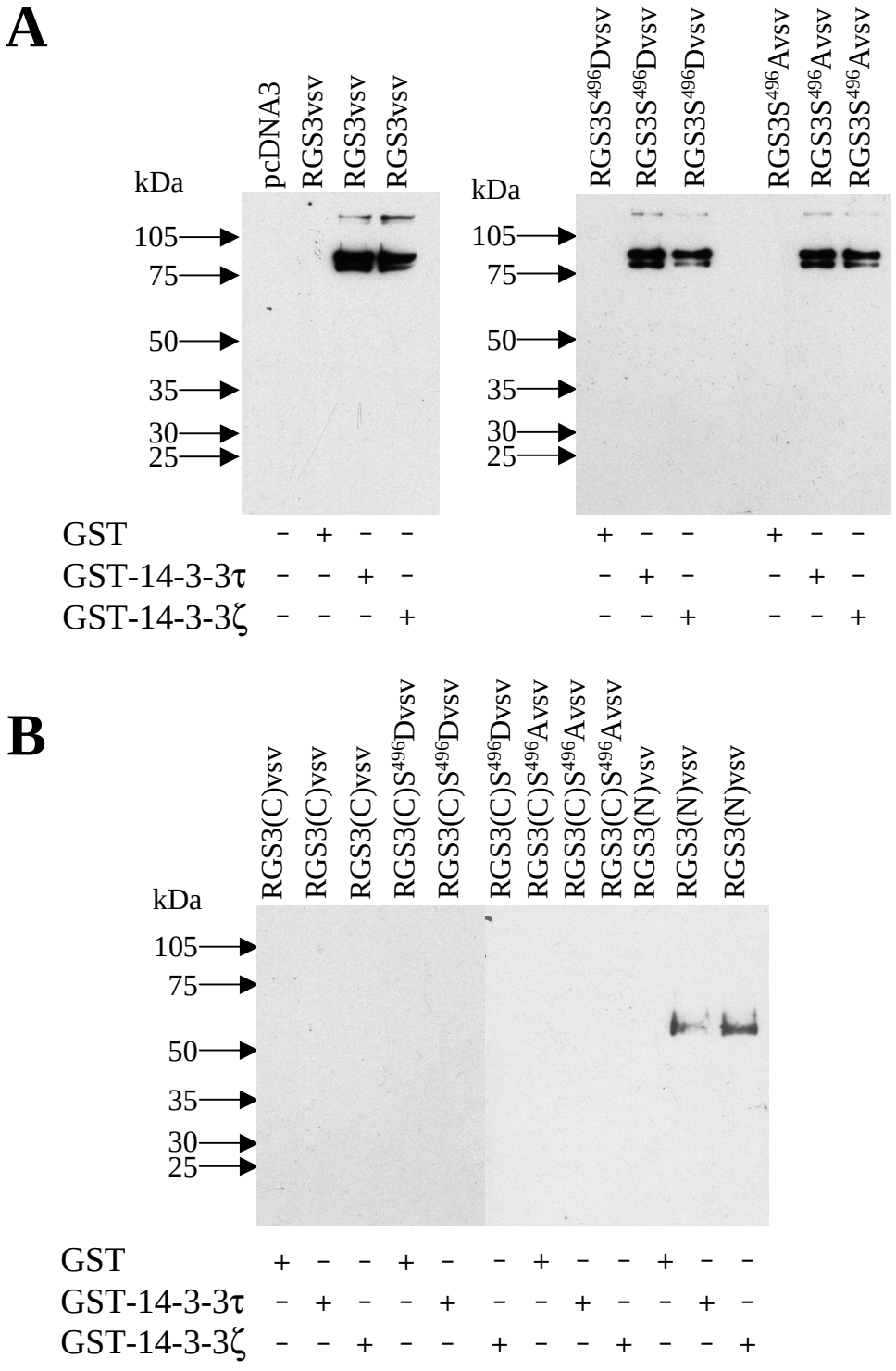


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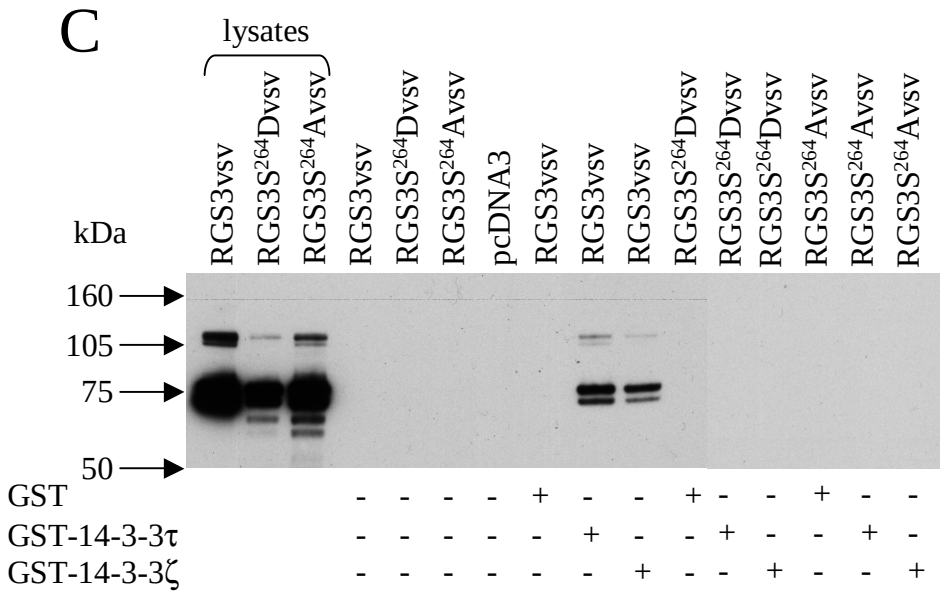


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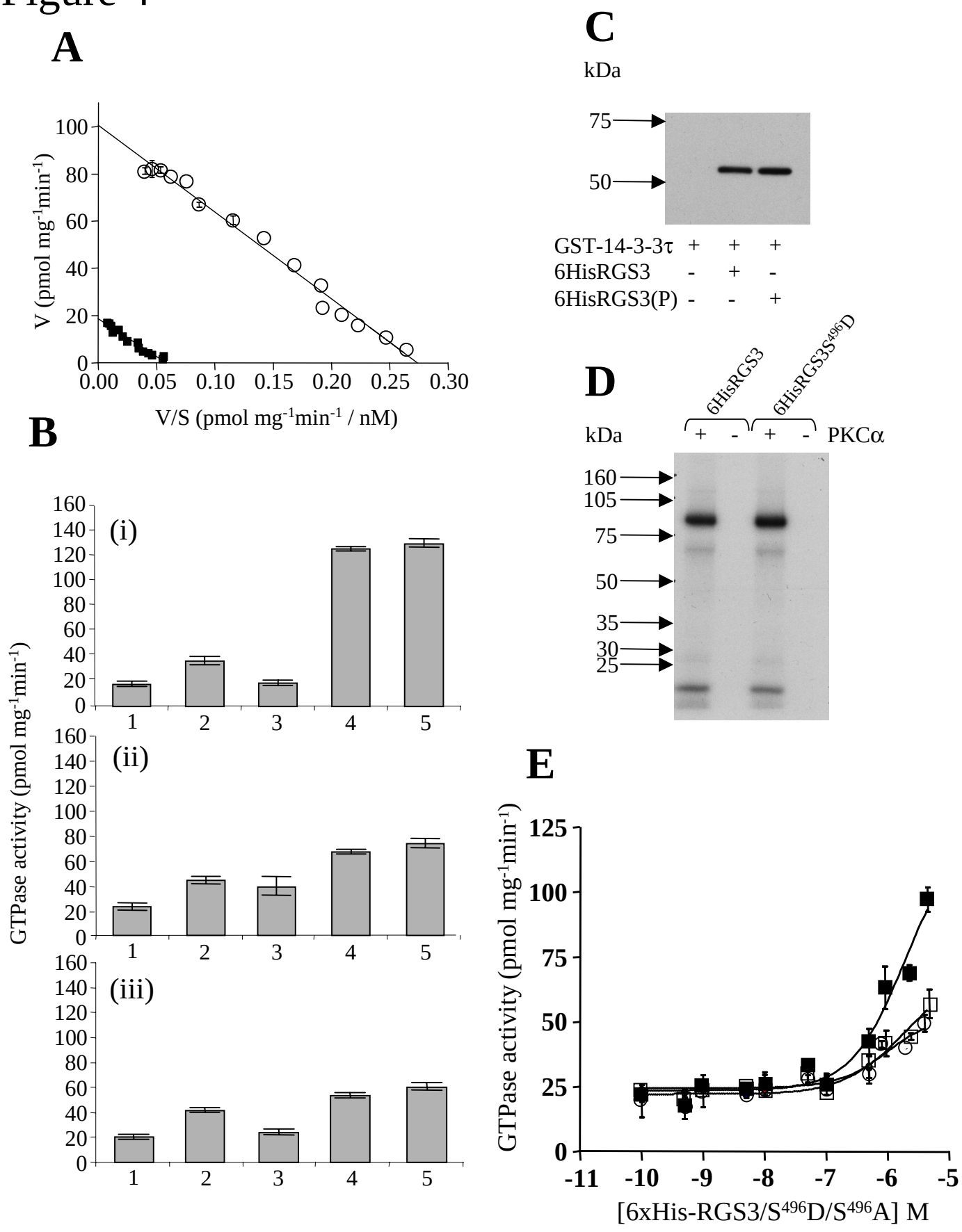


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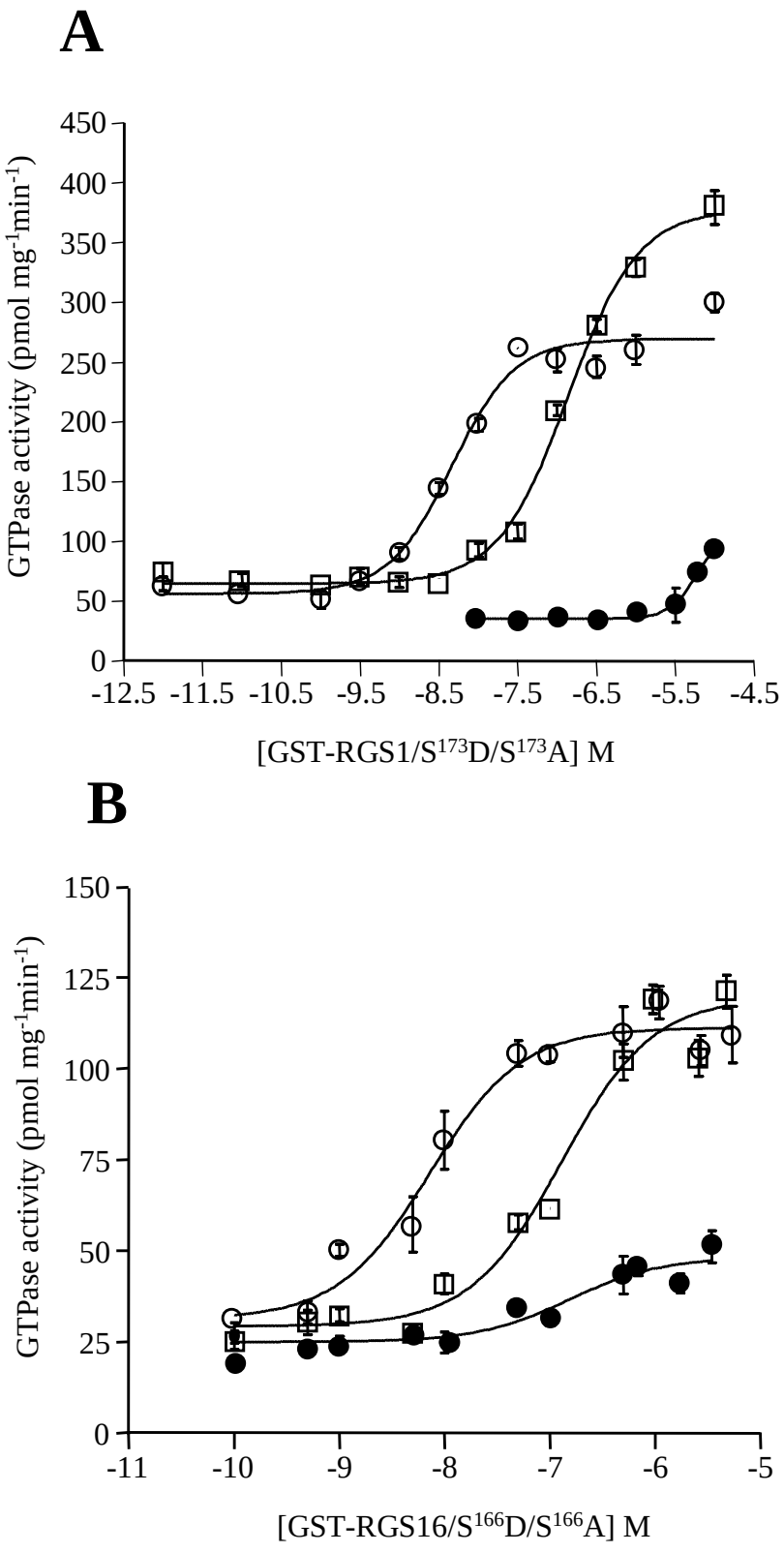


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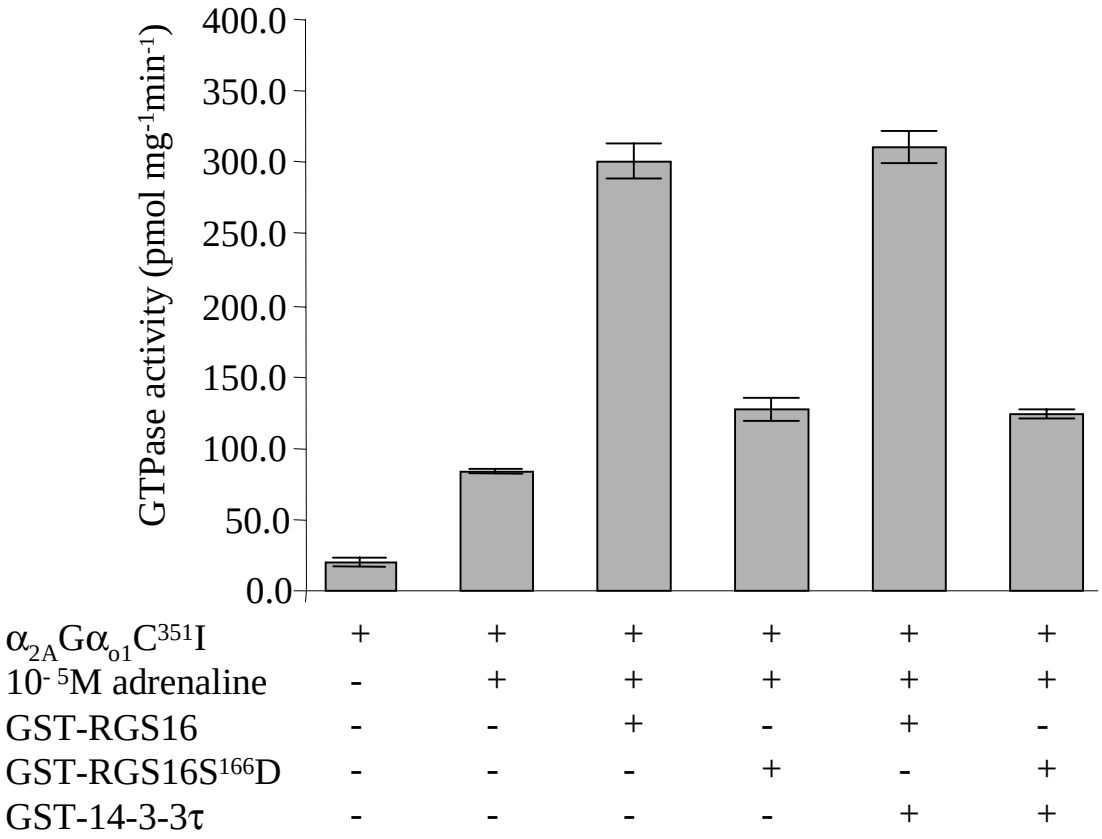


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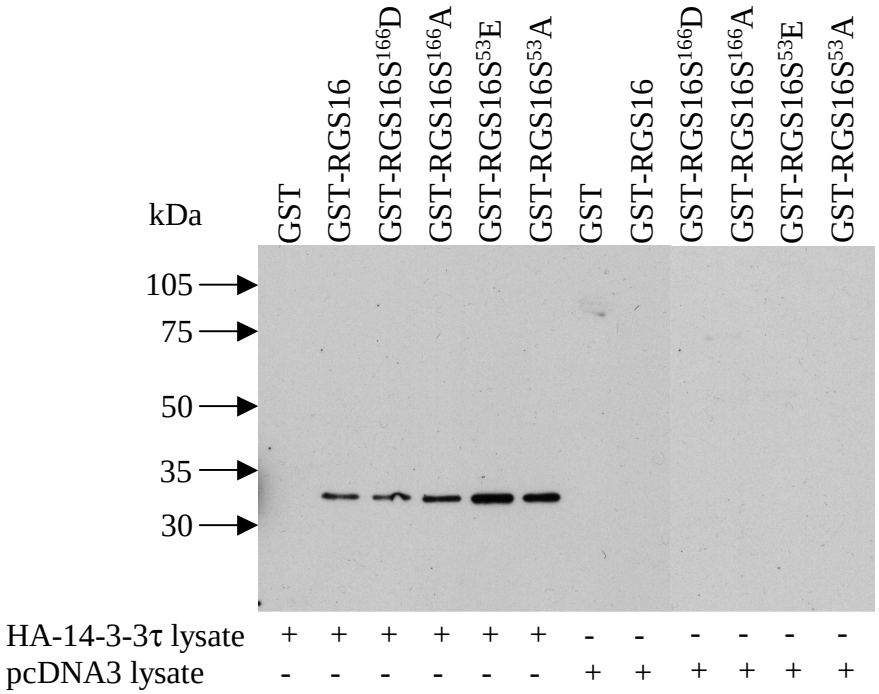


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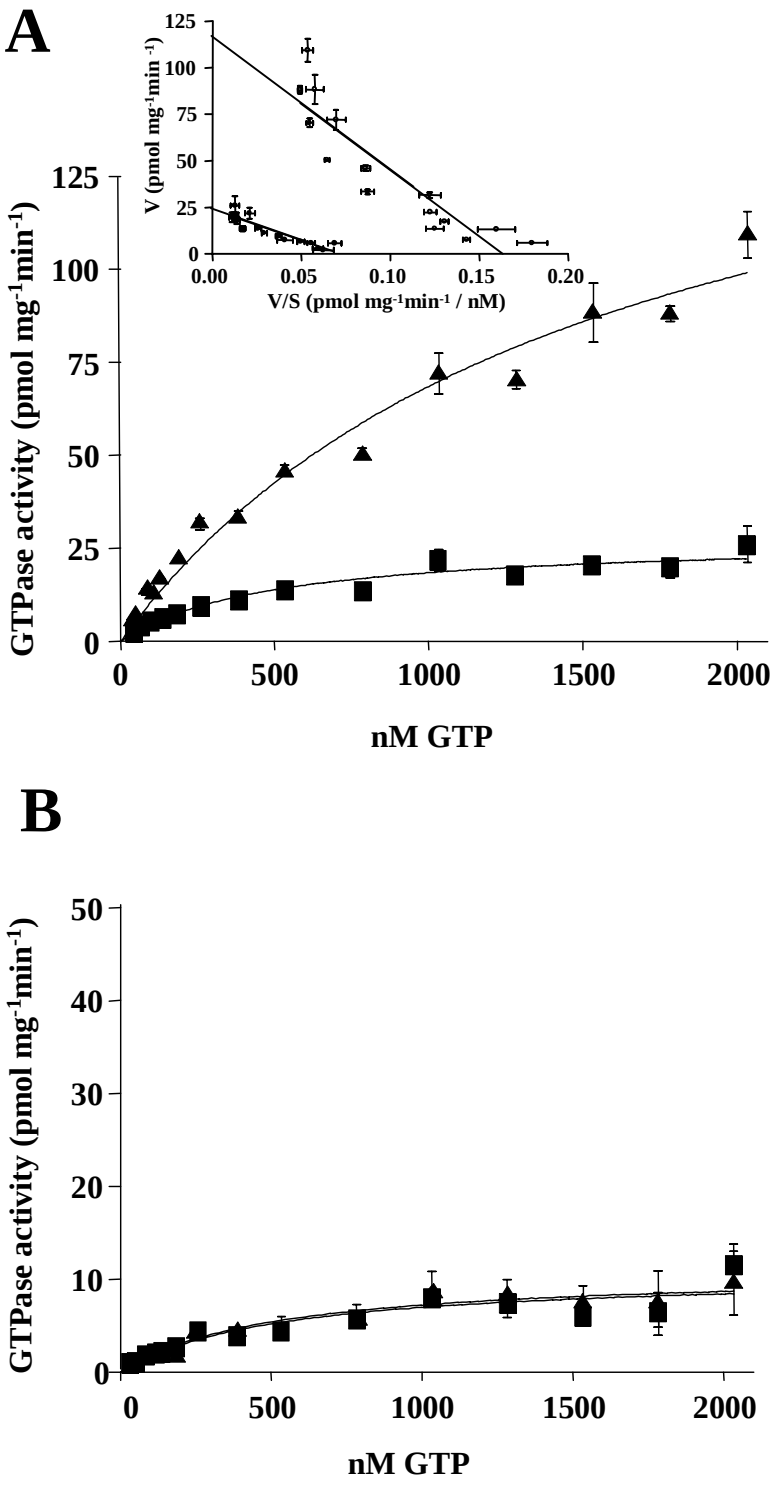


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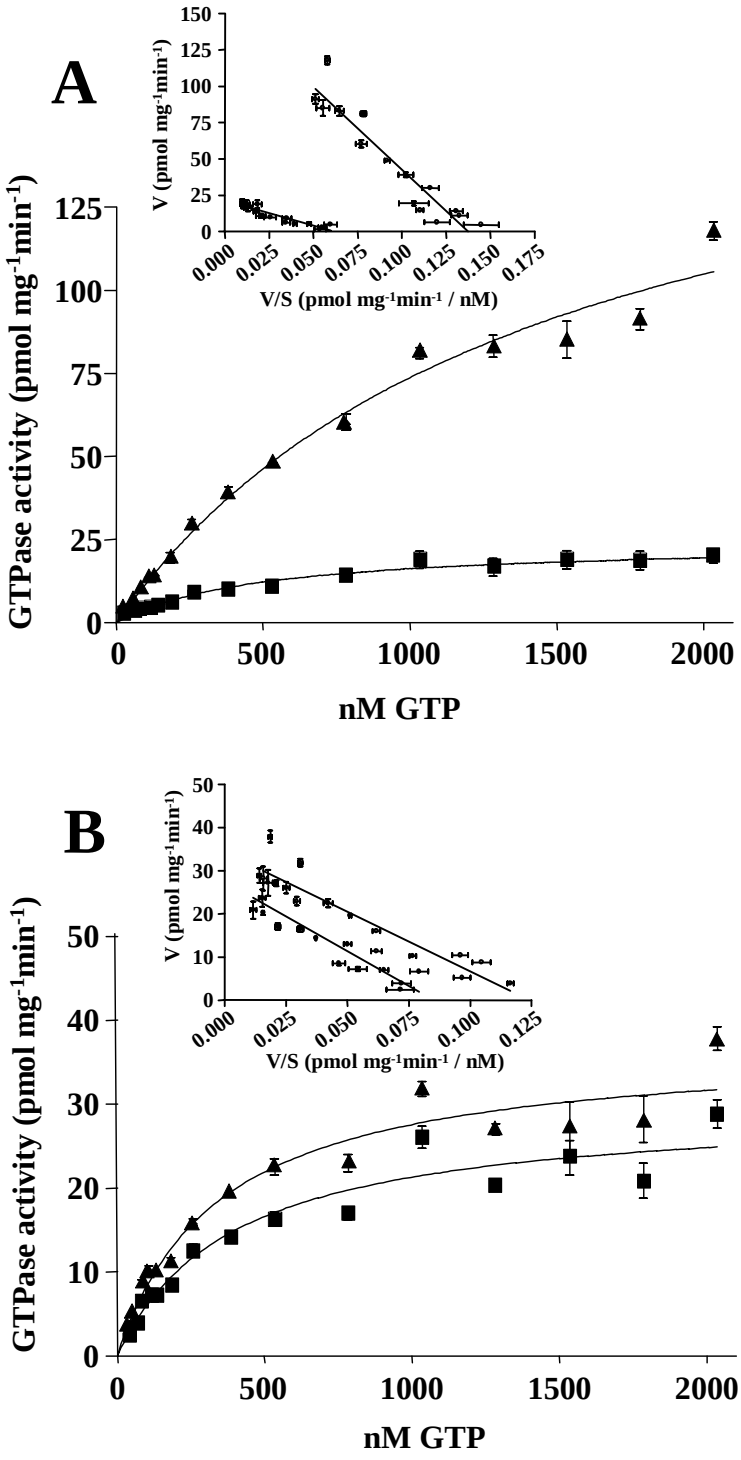


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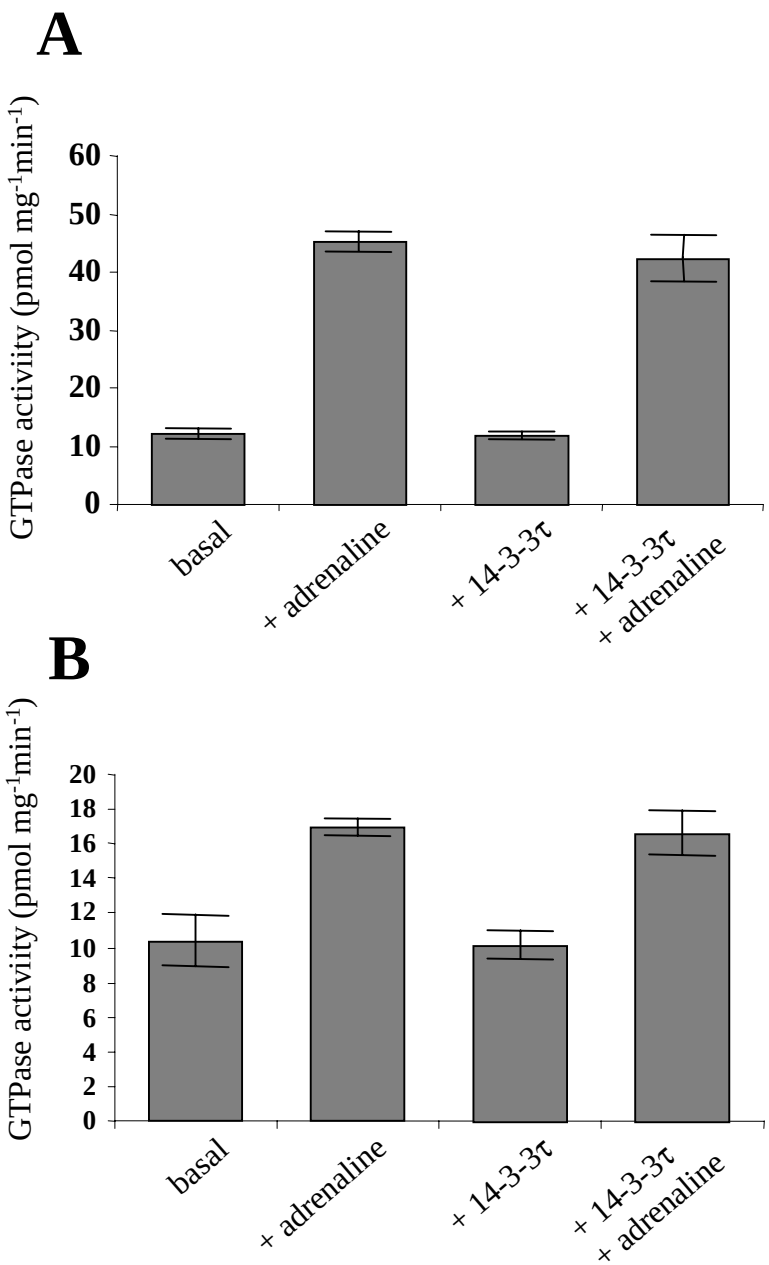


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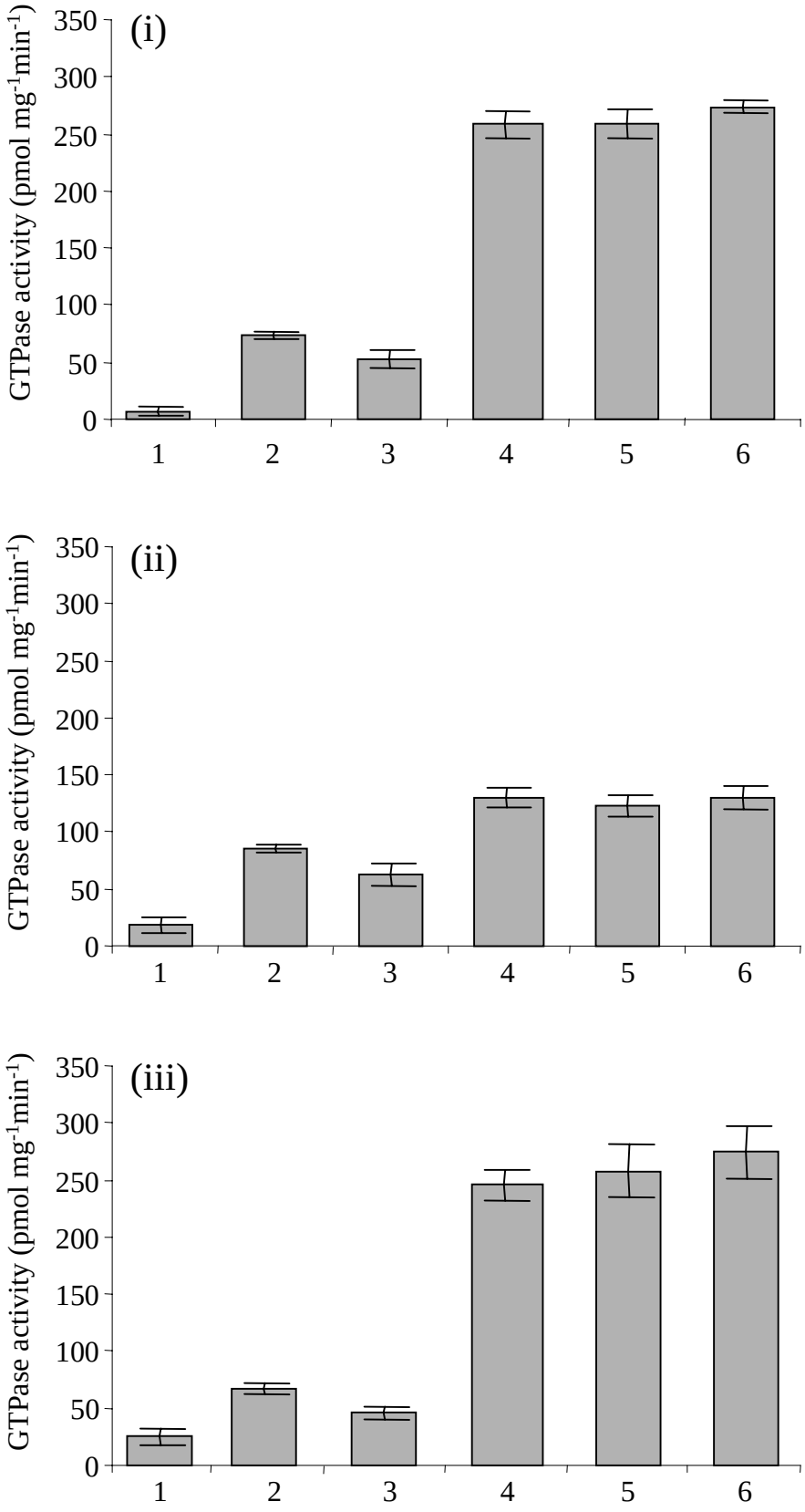


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