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A Glutamate-Substituted Mutant Mimics the Phosphorylated and Active Form of Guanylyl Cyclase-A

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Abbreviations: cGMP, cyclic guanosine monophosphate; GC, guanylyl cyclase; Km, Michaelis constant;

NP, natriuretic peptide; Vmax, maximal velocity; WT, wild type

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ABSTRACT

Multisite phosphorylation is required for activation of guanylyl cyclase (GC)-A, also known as NPR-A or NPR1, by cardiac natriuretic peptides (NPs). Seven chemically identified sites (Ser-487, Ser-497, Thr-500, Ser-502, Ser-506, Ser-510, Thr-513) and one functionally identified putative site (Ser-473) were reported. Single alanine substitutions for Ser-497, Thr-500, Ser-502, Ser-506 and Ser-510 reduced maximal velocity (V_{max}), while glutamate substitutions had no effect or increased V_{max} . Ala but not Glu substitution for Ser-497 increased the Michaelis constant (K_m) ~400%. A GC-A mutant containing Glu substitutions for all 7 chemically identified sites (GC-A-7E) had a K_m ~10-fold higher than phosphorylated wild type (WT) GC-A but one additional substitution for Ser-473 to make GC-A-8E resulted in the same V_{max} , K_m , and EC_{50} as the phosphorylated WT enzyme. Adding more glutamates to make GC-A-9E or GC-A-10E had little effect on activity and sequential deletion of individual glutamates in GC-A-8E progressively increased the K_m . Double Ala substitutions for Ser-497 and either Thr-500, Ser-510 or Thr-513 in WT-GC-A increase the K_m 23 to 70-fold but the same mutations in GC-A-8E only increased the K_m 8-fold, consistent with one site affecting the phosphorylation of other sites. Phosphate measurements confirmed that single site Ala substitutions reduced receptor phosphate levels more than expected for the loss of a single site. We conclude that a concentrated region of negative charge, not steric properties, resulting from multiple interdependent phosphorylation sites is required for a GC-A conformation capable of transmitting the hormone-binding signal to the catalytic domain.

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INTRODCUTION

Atrial natriuretic peptide (NPs) and B-type NP are cardiac hormones that reduce blood pressure and volume by activating guanylyl cyclase (GC)-A, the target of two FDA approved drugs (Hubers and Brown, 2016). GC-A is a homo-multimeric, single membrane-spanning enzyme that catalyzes the conversion of GTP to the second messenger, cGMP and pyrophosphate. GC-A contains a glycosylated, extracellular ligand binding domain, a single membrane-spanning region and multi-domain intracellular region consisting of a kinase homology domain, dimerization domain and C-terminal GC catalytic domain (Potter and Hunter, 2001). The homologous enzyme called GC-B is 78% identical to GC-A at the intracellular amino acid level but is activated by C-type NP (CNP), which regulates long bone growth, axonal bifurcation and resumption of meiosis in the oocyte (Kuhn, 2016; Potter, 2011).

GC-A is maximally phosphorylated in serum-starved 293 cells (Joubert et al., 2001; Koller et al., 1993; Potter and Garbers, 1992). Prolonged ANP exposure or brief exposure to chemical activators of protein kinase C like lysophosphatidic acid cause GC-A dephosphorylation, which reduces ANP-dependent GC activity (Joubert et al., 2001; Koller et al., 1993; Muller et al., 2006; Potter and Garbers, 1992; Potter and Garbers, 1994). Prolonged CNP exposure, or brief exposure to PKC activators or intracellular calcium elevating agents results in GC-B dephosphorylation and inactivation as well (Abbey and Potter, 2002; Abbey and Potter, 2003; Abbey-Hosch et al., 2004; Egbert et al., 2014; Potter, 1998; Potter and Hunter, 2000). Endogenous GC-A and GC-B isolated from normal mouse and rat tissues are also phosphorylated (Bryan et al., 2006). Congestive failure is associated with increased GC-B and reduced GC-A activity but the role of dephosphorylation in the latter process is unclear (Bryan et al., 2007; Dickey et al., 2012; Dickey et al., 2007). In contrast, luteinizing hormone-dependent inactivation of GC-B was correlated with dephosphorylation of GC-B in rat ovarian follicles (Egbert et al., 2014), and CNP-dependent GC activity in ovarian follicle membranes from a “knockin” mouse expressing a mutant form of GC-B containing glutamate substitutions for all known phosphorylation sites was resistant to luteinizing hormone-dependent inactivation (Shuhaibar et al., 2015).

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Seven GC-A phosphorylation sites and six homologous phosphorylation sites in GC-B were chemically identified in the N-terminus of the kinase homology domain (Fig. 1) (Potter and Hunter, 1998a; Schroter et al., 2010; Yoder et al., 2012; Yoder et al., 2010). Alanine substitutions for all individual sites but Ser-487 in GC-A decrease ANP-dependent activity in GC assays using 1 mM GTP substrate concentrations. Strikingly, the mutation of four or more phosphorylation sites to alanine yielded enzymes that were completely unresponsive to ANP (Potter and Hunter, 1998b). Conversely, glutamate substitution mimicked the functional effect of a single phosphorylation site (Potter and Hunter, 1998b), but mutating all six sites to glutamate yielded an enzyme with only about 20% of the activity of the phosphorylated WT enzyme (Potter and Hunter, 1999; Yoder et al., 2012). In subsequent studies, the substitution of Glu but not Ala for Ser-473 in GC-A or Ser-489 in GC-B produced near WT like enzymatic activity in single substrate assays but no peptides containing these juxtamembrane sites were identified from GC-A or GC-B by mass spectrometry or tryptic phosphopeptide labeling (Yoder et al., 2012).

Surprisingly, the effect of each individual phosphorylation site on the V_{max} and K_m of GC-A has not been examined. Here, we performed substrate-velocity assays on membranes prepared from 293 cells expressing Ala or Glu substitutions for individual or multiple phosphorylation sites to determine how each site or multiple sites affects enzymatic activity. As a result, we created a phosphomimetic mutant of GC-A that has the same enzymatic characteristics as phosphorylated WT enzyme.

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MATERIALS AND METHODS

Reagents: 125 I-cGMP radioimmunoassay kits were from Perkin Elmer (Waltham, MA) and the NPs were from Sigma (St. Louis, MO).

Mutagenesis: The Ala or Glu substitutions for each or multiple phosphorylation sites were created by QuikChange II system (Stratagene, Cedar Creek, TX) on the CMV3-GC-A plasmid as previously described (Potter and Hunter, 1998b).

Transient transfection: Human embryonic kidney 293T cells were transiently transfected with 5 μ g of pCMV3-GC-A plasmids containing single or multiple phosphorylation site mutations by the HEPES-calcium-phosphate precipitation method as previously described (Yoder et al., 2012).

Membrane Preparation: Cells cultured on 10 cm plates were placed in serum-free media for 4 hours before membranes were prepared. Membranes were harvested at 4°C by washing the plates twice with phosphate buffered saline, scraping the cells off the plates in 0.6 ml phosphatase inhibitor buffer (PIB) (Antos et al., 2005). Cells were lysed by sonication and the lysates were centrifuged at 20,000 x g for 15 min at 4°C. The supernatant was aspirated and the membrane pellet was resuspended with 0.5 ml PIB and centrifuged again at 20,000 x g for 15 min at 4°C. The supernatant was aspirated, and the pellet was resuspended in PIB to yield a protein concentration between 1-3 mg/ml. Crude membranes were assayed for GC activity without freezing.

Guanylyl cyclase assays: Crude membranes were assayed for GC activity at 37°C in a buffer containing 0.5 mM isomethylxanthine to inhibit phosphodiesterases, 5 mM MgCl_2 , and a nucleotide regenerating system as reported (Robinson and Potter, 2012). 0.02 ml of crude membranes were added per assay. A solution of 100 mM GTP and 100 mM MgCl_2 was diluted to the appropriate substrate concentrations used for each assay. Because enzymatic activity was not completely linear with time, the kinetic parameters are considered “apparent.”

Immunoblot analysis: 10 μ g of membrane protein prepared in PIB from transiently transfected 293 cells was fractionated on an 8% gel by SDS-PAGE and blotted to a PVDF membrane for 2 hours at 4°C. The

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amount GC-A was detected with rabbit polyclonal antiserum 6325 at a dilution of 1/10,000 using a Li-Cor detections system as described.

Experimental Protocol: Plasmids encoding wild type (WT) GC-A and corresponding Ala and Glu mutants were transiently transfected into 293 cells (Yoder et al., 2010). Two days later, crude membranes were prepared from sonicated cells in a buffer designed to maximally inhibit GC-A dephosphorylation (Antos et al., 2005) and assayed for cGMP production for 5 min in the presence of 1 μ M ANP, 1 mM ATP, and increasing concentrations of Mg^{2+} GTP to determine the V_{max} and K_m of the enzymes. The data from each set of WT, Ala mutant and Glu single mutant enzymes are presented in Table 1, which represents three separate experiments where each substrate concentration was measured in duplicate for an n of 3. The three individual membrane preparations were also western blotted and the intensities for all enzymes were compared by a two-tailed t-test. None of the mutants were expressed at higher or lower levels than the other mutant or wild type GC-A (data not shown). Since membranes from 293 cells transfected with a plasmid encoding GFP or empty vector contain less than 2% of the activity detected in membranes from cells transfected with WT-GC-A, the activities and protein levels detected were attributed to the transfected plasmids for each experiment (Yoder et al., 2010). Because the results for each phosphorylation site was generated from a separate transfection with differing transfection efficiencies, the data are presented as the percentage of WT activity for each experiment.

Whole cell cGMP Elevation Assays: Transiently transfected 293 cells grown in 48-well plates to 90% confluency were pretreated for 10 min at 37°C in DMEM containing 25mM HEPES, pH 7.4 and 0.5mM 1-methyl-3-isobutylxanthine to inhibit phosphodiesterases (Dickey et al., 2008). This medium was aspirated and replaced with the same medium containing increasing concentrations of rat ANP for 1 min. The reaction was stopped by aspiration of the medium and addition of 0.2 ml ice-cold 80% ethanol. Cyclic GMP concentrations in the extract were determined by cGMP RIA (Abbey and Potter, 2002).

Gel Staining - Eight percent resolving gels were fixed in a 30 mL solution of 50% methanol and 10% acetic acid for 30 min with gentle rocking. The solution was changed two times for a total of three washes in the fixing solution. The gels were then washed twice in 100 mL of water for 10 min. Ten milliliters of

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Pro-Q Diamond phosphoprotein gel stain was added, and the gels were incubated with gentle rocking for 1.5 h in the dark. The gels were then destained with 80 mL of a solution of 20% acetonitrile and 50 mM sodium acetate (pH 4.0) for 15 min. This wash was repeated two times for a total of three washes. The gels were then rinsed in water, scanned with a 532 nm laser, and imaged with FUJI FLA 5000 software. After imaging, the same gels were stained with 50% Methanol, 7% Acetic Acid, and 0.1% Coomassie Brilliant Blue for 10 minutes and destained in a solution of 50% methanol and 7% acetic acid.

Statistical analysis: Statistical analyses were performed with GraphPad Prism 7 software. Substrate-velocity curves were analyzed by non-linear regression using a Michaelis-Menten model to determine V_{max} and K_m . For each pair of enzymes and each model parameter, we are comparing the full model fit against a null model where that parameter set to be equal. Because of this, we used the extra sum of squares F test to generate p-values. P-values were then adjusted using the Bonferroni correction for each set of four comparisons. Dose-response curves were analyzed by non-linear regression using a sigmoidal dose-response curve to determine EC_{50} .

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RESULTS

Effect of Single Phosphorylation Site Substitutions. Ala mutants of the first six reported GC-A phosphorylation sites (Ser-497, Thr-500, Ser-502, Ser-506, Ser-510 and Thr-513) have reduced ANP-dependent GC activity using single substrate concentration assays (Potter and Hunter, 1998b), but how the mutations affect the activity of the enzyme is not known. Here, we measured the effects of single Ala and Glu mutants with increasing substrate concentrations to characterize the effects on maximal velocity (V_{max}) and the Michaelis constant (K_m) as well as to determine the ability of the corresponding Glu mutation to mimic the functional effects of each individual phosphorylation site (Table I). Every Ala substitution for the first six discovered sites reduced the V_{max} of GC-A, although the substitution at Thr-513 was not statistically significant. The reduction in V_{max} ranged from 13% to 55%. In contrast, V_{max} values for the Glu substitutions for the corresponding residues were either not lower or higher than the V_{max} values obtained for WT-GC-A. Five of the six Ala substitutions increased the K_m , but only the Ala mutations at Ser-497 and Ser-506 reached statistical significance. However, since both the Ala and Glu substitutions for Ser-506 increased the K_m , the change in activity cannot be due to a charge difference.

The seventh GC-A phosphorylation site identified by mass spectrometry was Ser-487 (Schroter et al., 2010; Yoder et al., 2010). Initial single substrate concentration assays on the Ser-487-Glu mutant indicated reduced ANP-dependent activity (Schroter et al., 2010). Subsequent assays confirmed the reduced activity for Glu-487 but found no difference in activity between the WT enzyme and the Ala-487 mutant (Yoder et al., 2010). Here, substrate-velocity assays on mutants with single Ala or Glu substitutions for Ser-487 revealed no significant differences in the K_m or V_{max} between either mutant and phosphorylated WT GC-A (Table I).

No data containing the expected mass to charge ratio of the predicted phosphopeptide containing Ser-473 was identified in tryptic digests of GC-A (Schroter et al., 2010; Yoder et al., 2010). However, in single substrate concentration assays, Ala but not Glu substitutions for Ser-473 resulted in about a 20% reduction in ANP-dependent activity when normalized to detergent-dependent activity (Yoder et al., 2012). In contrast, when GC activities of the individual 473-Ala and 473-Glu mutants were assessed by

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substrate-velocity assays, no difference in the V_{max} or K_m values were observed between either mutant and phosphorylated WT-GC-A (Table I).

Enzymatic characterization of GC-A mutants with multiple glutamate substitutions. GC-A-6E, an enzyme containing Glu substitutions for Ser-497, Thr-500, Ser-502, Ser-506, Ser-510 and Thr-513 was stimulated by ANP about 10-fold but was only about 20% as active as the phosphorylated WT enzyme (Potter and Hunter, 1999). Substituting an additional Glu for Ser-487 slightly increased V_{max} and decreased the K_m . Adding the Ser-473-Glu substitution to GC-A-6E increased V_{max} 63% and decreased the K_m 4-fold, but the K_m of GC-A-6E-S473E (GCA-7E) was still 2-fold higher than the phosphorylated WT enzyme (Fig. 2A and B). Converting all eight sites to Glu to generate GC-A-6E-S473E-S487E, now named GC-A-8E, resulted in an enzyme that mimicked the enzymatic activity of the phosphorylated WT enzyme (Fig. 2C).

Phosphorylation Site Mutants Have WT-Like Concentration Responses. The effect of multiple phosphorylation site substitutions on ANP concentration responses was also determined. GC assays performed on membrane preparations from 293 cells transiently transfected with WT-GC-A, GC-A-6E or GC-A-8E determined that the EC_{50} was not significantly different between either of the mutants and WT-GC-A (Fig. 3 A, B). The EC_{50} for ANP was also determined for WT-GC-A, GC-A-6E, GC-A-6E-S473E, GC-A-6E-S487E and GC-A-8E in live, transiently transfected 293 cells (Fig. 3 C and D). No differences in EC_{50} were observed between any of the GC-A enzymes, although cells expressing GC-A-6E and GC-A-6E-S487E had lower maximal cGMP levels and cells expressing GC-A-6E-S473E and GC-A-8E had higher maximal cGMP levels than cells expressing WT-GC-A.

A negative charge of eight in the N-terminus of the KHD provides WT-like GC-A responsiveness. The effect of sequentially removing negative charge by substituting Ala for individual Glu in GC-A-8E was investigated. The substitutions were started at the C-terminal most phosphorylation site. For instance, in Fig. 4A, 7E = GC-A-8E with an E513A substitution, whereas 6E = GC-A-8E with a E513A and a E510A substitution. Substrate-velocity assays indicated that the first four substitutions resulted in a step-wise increase in the K_m without significantly affecting V_{max} (Fig 4A). However, one

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additional Ala substitution to make GC-A-3E increased the K_m 4.6-fold and the next Ala substitution reduced the V_{max} 6-fold.

The effect of increasing negative charge to greater than -8 was also investigated by substituting Glu for Ser-494 or Ser-494 and Ser-514, which are not naturally phosphorylated, in GC-A-8E to make GC-A-9E and GC-A-10E, respectively. However, the additional negative charge did not significantly affect the K_m or V_{max} values of GC-A (Fig. 4B).

Double alanine substitutions dramatically increased the K_m and modestly decreased V_{max} .

Next, we compared the effect of double Ala mutations for known phosphorylation sites to the effects of single Ala substitutions for GC-A phosphorylation sites shown in Table 1. Three double Ala or Glu mutants containing Ser-497 and either Thr-500, Ser-510 or Thr-513 substitutions were analyzed (Fig. 5 A, B, C). Ala substitutions at Ser-497 and Thr- 500 reduced the V_{max} 75% but increased the K_m 23-fold compared to the WT enzyme. From Table 1, Ala substitutions for Ser-497 and Thr-500 reduced V_{max} to 59% and 45% of the V_{max} for WT-GC-A. However, V_{max} and K_m values of the double Ala mutant were 25% and 23.3-fold more respectively of WT-GC-A. Adding the effect on V_{max} brought by single Ala substitutions at Ser-497 and Thr- 500 results in a 96% reduction in V_{max} compared to WT (Table1). Thus, the double Ala mutant has a close additive effect on V_{max} . However, adding the effect on K_m brought by single Ala substitutions at Ser-497 and Thr- 500 results in a 5-fold increase in K_m (Table1). Thus, the double Ala substitution at Ser-497 and Thr- 500 has a synergistic effect on the K_m of GC-A. In contrast, the corresponding double Glu mutant had no effect on V_{max} and only increased the K_m about two-fold. Ala substitutions at Ser-497 and Ser-510 reduced V_{max} 86% and increased the K_m 46-fold but in this case, the corresponding Glu mutations did not completely restore activity to WT levels, although it was much more active than the corresponding Ala mutant. Ala substitutions for Ser-497 and Thr-513 did not significantly reduce the V_{max} but increased the K_m 70-fold. However, the corresponding double Glu mutant produced V_{max} and K_m values that were not different from the WT enzyme. Since the double Ala mutations had dramatically reduced V_{max} values, we loaded equal amount of total protein and performed western blots but found no differences in GC-A protein levels between the mutants and the WT enzyme

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(Fig. 5). Coomassie staining was performed on the same membrane following western blotting to demonstrate equal protein loading (data not shown). In summary, all double Ala mutations had a synergistic effect on the K_m compared the increase in the K_m observed with the single Ala substitutions shown in Table 1. In contrast, the effects on V_{max} were closer to additive.

Differential effects of phosphorylation sites. The double Ala mutants had a synergistic negative effect on the K_m of the enzyme and an additive or slightly greater than additive effect on the V_{max} . One explanation for these drastic effects is that double Ala mutations promote the dephosphorylation of other sites besides the mutated sites. To test this possibility, Ala substitutions at both Ser-497 and Thr-500 were introduced into either GC-A-8E or into the phosphorylated WT enzyme. Consistent with the Ala substitutions affecting more than the modified site, the Ala-substituted version of WT-GC-A had much less activity than the Ala-substituted version of GC-A-8E (Fig. 6 A). The effect of single Ala mutations was also compared between the phosphorylated WT form of GC-A or GC-A-8E, and again, the GC-A-8E mutants had higher enzymatic activity than the corresponding mutations introduced into WT-GC-A, which is consistent with the idea that one site affects the phosphorylation of other sites (Fig. 6B). In agreement with the synergistic effect on protein phosphorylation, Pro-Q phosphate and Coomassie protein analysis indicated that Ala substitutions for Ser-497, Ser-506, Thr-513, and the double mutant Ser-497 and Thr-513 reduced the phosphate content of GC-A more than would be expected if the mutations only eliminated only one phosphate. For instance, GC-A has seven chemically identified phosphorylation sites but the single S506A mutation decreased the phosphate content of GC-A by about half, which suggests that the loss of phosphorylation of Ser-506 is affecting the phosphorylation of other sites as well.

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DISCUSSION

Phosphorylation affects proteins by facilitating dockings interactions that involve steric and charge interactions between different proteins or by causing unimolecular structural changes through salt bridge formation that result solely from charge interactions (Hunter, 2012). Here, we report that replacement of phosphorylated Ser and Thr in GC-A with Glu, a sterically smaller moiety with a negative charge, completely mimics the functional characteristics of the phosphorylated WT enzyme. Hence, we conclude that phosphorylation is required for hormonal activation of GC-A solely due to charge interactions. Furthermore, we suggest that the critical amount of negative charge in and around subdomain I of the KHD is approximately -8 and that reductions in negative charge in this area reduce activity primarily by raising the K_m .

Reductions in enzyme activity were observed for all Ala substitutions of phosphorylation sites with the exception of Ser-473 and Ser-487. Initial single substrate assays revealed relatively small reductions in activity for the 487-Glu and no effect for 487-Ala. It is unclear why no significant differences were observed in the substrate-velocity assays. However, previous assays evaluated activity differences when expressed as percent of detergent-dependent GC activity (Yoder et al., 2010), and detergent normalization of activity was not employed in the current study. No change in the substrate concentration curves was observed for Ala or Glu mutants of Ser-473 either. This differs from the Ala substitution at the corresponding residue in GC-B, Ser-489, which increased the K_m of the GC-B mutant five-fold (Yoder et al., 2012). The reason for the differing effects of the single Ala substitution of this juxta-membrane site in GC-A and GC-B is not known but the Ser-489-Ala mutant was compared to the Ser-489-Glu mutant, which has a slightly lower K_m than the WT enzyme and likely exaggerated the difference. It is also possible that phosphorylation of Ser-487 in GC-A may be a factor, since this site is not conserved in GC-B.

For the first time, we present evidence for phosphorylation site interdependence in GC-A. Single or double Ala mutations for known phosphorylation sites had a much greater effect in the WT phosphorylated enzyme than in the glutamate-substituted form of GC-A (Fig. 6), which is consistent with

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one phosphorylation site affecting other phosphorylation sites. For example, Ala substitution at Ser-506 had a much greater effect on WT-GCA than GC-A-8E, suggesting that negative charge at Ser-506 is important for the phosphorylation of other sites. In agreement with this observation, Pro-Q analysis revealed that the S506A mutation decreased receptor phosphate content by about 50%, which is more than could be explained by the loss of a single site. However, the location of the charge is also important since the mutation of Ser-497 to Ala markedly reduced the activity of both WT-GC-A and GC-A-8E.

Based on the data reported here and elsewhere, we propose a model where the phosphorylation of GC-A causes a conformational change in the KHD that is required for transmission of the hormone binding signal. We predict that when GC-A is in its dephosphorylated state, its KHD is unordered due to the lack of negatively charged residues that normally form salt bridges with positively charged Arg and Lys residues (Fig. 7A). When these salt bridges are formed (Fig. 7B), a ridged and ordered structure is formed. Crystal structure studies comparing unbound receptor to ANP bound receptor reveals that ANP binding causes the two receptor monomers to undergo an intermolecular twist of about 30 degrees (Ogawa et al., 2004). We hypothesize that phosphorylation provides a rigid kinase homology domain downstream of the extracellular domain that allows the rotational force caused by ANP binding to be transmitted to the catalytic domain to increase substrate affinity.

In conclusion, we describe for the first time a dephosphorylated form of GC-A called GC-A-8E that has the same activity as the phosphorylated WT enzyme. Although much has been reported regarding the effects of phosphorylation of GC-A in cell culture (Potter et al., 2006), nothing is known about the role of phosphorylation in the control of GC-A in a physiologic setting. Recent “knock-in” studies revealed a critical role of GC-B dephosphorylation in the resumption of meiosis in the oocyte (Shuhaibar et al., 2015). To identify physiologic functions that are regulated by changes in GC-A phosphorylation, we collaborated with Dr. Laurinda Jaffe’s group to generate homozygous GC-A^{8E/8E} mice. Potential phenotypic differences in the GC-A^{8E/8E} compared to GC-A^{WT/WT} mice are reduced blood pressure (Lopez et al., 1995), reduced cardiac hypertrophy (Knowles et al., 2001) and/or increased fat metabolism (Bordicchia et al., 2012). Finally, whether changes in GC-A phosphorylation affect disease processes is

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not known. However, we recently found that the Ser-488-Pro mutation in GC-B (Miura et al., 2014) only activates the phosphorylated form of the enzyme (Dickey et al, manuscript in preparation), which suggest that similar scenarios may exist for GC-A as well.

Authorship Contributions

Participated in Research Design: Otto and Potter

Conducted Experiments: Otto, Dickey, and McDowell

Performed Data Analysis: Otto, Dickey, and Potter

Wrote the manuscript: Otto and Potter

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REFERENCE

- Abbey SE and Potter LR (2002) Vasopressin-dependent inhibition of the C-type natriuretic peptide receptor, NPR-B/GC-B, requires elevated intracellular calcium concentrations. *The Journal of biological chemistry* **277**(45): 42423-42430.
- Abbey SE and Potter LR (2003) Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. *Endocrinology* **144**(1): 240-246.
- Abbey-Hosch SE, Cody AN and Potter LR (2004) Sphingosine-1-phosphate inhibits C-type natriuretic peptide activation of guanylyl cyclase B (GC-B/NPR-B). *Hypertension* **43**(5): 1103-1109.
- Antos LK, Abbey-Hosch SE, Flora DR and Potter LR (2005) ATP-independent activation of natriuretic peptide receptors. *The Journal of biological chemistry* **280**(29): 26928-26932.
- Bordicchia M, Liu D, Amri EZ, Ailhaud G, Dessì-Fulgheri P, Zhang C, Takahashi N, Sarzani R and Collins S (2012) Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J Clin Invest* **122**(3): 1022-1036.
- Bryan PM, Smirnov D, Smolenski A, Feil S, Feil R, Hofmann F, Lohmann S and Potter LR (2006) A sensitive method for determining the phosphorylation status of natriuretic peptide receptors: cGK-Ialpha does not regulate NPR-A. *Biochemistry* **45**(4): 1295-1303.
- Bryan PM, Xu X, Dickey DM, Chen Y and Potter LR (2007) Renal hyporesponsiveness to atrial natriuretic peptide in congestive heart failure results from reduced atrial natriuretic peptide receptor concentrations. *American journal of physiology Renal physiology* **292**(5): F1636-1644.
- Dickey DM, Burnett JC, Jr. and Potter LR (2008) Novel bifunctional natriuretic peptides as potential therapeutics. *The Journal of biological chemistry* **283**(50): 35003-35009.
- Dickey DM, Dries DL, Margulies KB and Potter LR (2012) Guanylyl cyclase (GC)-A and GC-B activities in ventricles and cardiomyocytes from failed and non-failed human hearts: GC-A is inactive in the failed cardiomyocyte. *Journal of molecular and cellular cardiology* **52**(3): 727-732.
- Dickey DM, Flora DR, Bryan PM, Xu X, Chen Y and Potter LR (2007) Differential regulation of membrane guanylyl cyclases in congestive heart failure: natriuretic peptide receptor (NPR)-B, Not NPR-A, is the predominant natriuretic peptide receptor in the failing heart. *Endocrinology* **148**(7): 3518-3522.
- Egbert JR, Shuhaibar LC, Edmund AB, Van Helden DA, Robinson JW, Uliasz TF, Baena V, Geerts A, Wunder F, Potter LR and Jaffe LA (2014) Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes. *Development* **141**(18): 3594-3604.
- Hubers SA and Brown NJ (2016) Combined Angiotensin Receptor Antagonism and Neprilysin Inhibition. *Circulation* **133**(11): 1115-1124.
- Hunter T (2012) Why nature chose phosphate to modify proteins. *Philos Trans R Soc Lond B Biol Sci* **367**(1602): 2513-2516.
- Joubert S, Labrecque J and De Lean A (2001) Reduced activity of the npr-a kinase triggers dephosphorylation and homologous desensitization of the receptor. *Biochemistry* **40**(37): 11096-11105.
- Knowles JW, Esposito G, Mao L, Hagaman JR, Fox JE, Smithies O, Rockman HA and Maeda N (2001) Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A-deficient mice. *J Clin Invest* **107**(8): 975-984.
- Koller KJ, Lipari MT and Goeddel DV (1993) Proper glycosylation and phosphorylation of the type A natriuretic peptide receptor are required for hormone-stimulated guanylyl cyclase activity. *The Journal of biological chemistry* **268**(8): 5997-6003.
- Kuhn M (2016) Molecular Physiology of Membrane Guanylyl Cyclase Receptors. *Physiol Rev* **96**(2): 751-804.

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- Lopez MJ, Wong SK, Kishimoto I, Dubois S, Mach V, Friesen J, Garbers DL and Beuve A (1995) Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide. *Nature* **378**(6552): 65-68.
- Miura K, Kim OH, Lee HR, Namba N, Michigami T, Yoo WJ, Choi IH, Ozono K and Cho TJ (2014) Overgrowth syndrome associated with a gain-of-function mutation of the natriuretic peptide receptor 2 (NPR2) gene. *Am J Med Genet A* **164A**(1): 156-163.
- Muller D, Cortes-Dericks L, Budnik LT, Brunswig-Spickenheier B, Pancratius M, Speth RC, Mukhopadhyay AK and Middendorff R (2006) Homologous and lysophosphatidic acid-induced desensitization of the atrial natriuretic peptide receptor, guanylyl cyclase-A, in MA-10 leydig cells. *Endocrinology* **147**(6): 2974-2985.
- Ogawa H, Qiu Y, Ogata CM and Misono KS (2004) Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain: rotation mechanism for transmembrane signal transduction. *J Biol Chem* **279**(27): 28625-28631.
- Potter LR (1998) Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptor B: dephosphorylation is a mechanism of desensitization. *Biochemistry* **37**(8): 2422-2429.
- Potter LR (2011) Guanylyl cyclase structure, function and regulation. *Cellular signalling* **23**: 1921-1926.
- Potter LR, Abbey-Hosch S and Dickey DM (2006) Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocrine reviews* **27**(1): 47-72.
- Potter LR and Garbers DL (1992) Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization. *The Journal of biological chemistry* **267**(21): 14531-14534.
- Potter LR and Garbers DL (1994) Protein kinase C-dependent desensitization of the atrial natriuretic peptide receptor is mediated by dephosphorylation. *The Journal of biological chemistry* **269**(20): 14636-14642.
- Potter LR and Hunter T (1998a) Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. *The Journal of biological chemistry* **273**(25): 15533-15539.
- Potter LR and Hunter T (1998b) Phosphorylation of the kinase homology domain is essential for activation of the A-type natriuretic peptide receptor. *Molecular and cellular biology* **18**(4): 2164-2172.
- Potter LR and Hunter T (1999) A constitutively "phosphorylated" guanylyl cyclase-linked atrial natriuretic peptide receptor mutant is resistant to desensitization. *Molecular biology of the cell* **10**(6): 1811-1820.
- Potter LR and Hunter T (2000) Activation of protein kinase C stimulates the dephosphorylation of natriuretic peptide receptor-B at a single serine residue: a possible mechanism of heterologous desensitization. *The Journal of biological chemistry* **275**(40): 31099-31106.
- Potter LR and Hunter T (2001) Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation. *The Journal of biological chemistry* **276**(9): 6057-6060.
- Robinson JW and Potter LR (2012) Guanylyl cyclases a and B are asymmetric dimers that are allosterically activated by ATP binding to the catalytic domain. *Science signaling* **5**(240): ra65.
- Schroter J, Zahedi RP, Hartmann M, Gassner B, Gazinski A, Waschke J, Sickmann A and Kuhn M (2010) Homologous desensitization of guanylyl cyclase A, the receptor for atrial natriuretic peptide, is associated with a complex phosphorylation pattern. *FEBS J* **277**(11): 2440-2453.
- Shuhaibar LC, Egbert JR, Edmund AB, Uliasz TF, Dickey DM, Yee SP, Potter LR and Jaffe LA (2015) Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase is required for rapid resumption of oocyte meiosis in response to luteinizing hormone. *Dev Biol* **409**(1): 194-201.
- Yoder AR, Robinson JW, Dickey DM, Andersland J, Rose BA, Stone MD, Griffin TJ and Potter LR (2012) A Functional Screen Provides Evidence for a Conserved, Regulatory, Juxtamembrane Phosphorylation Site in Guanylyl Cyclase A and B. *PLoS ONE* **7**(5): e36747.
- Yoder AR, Stone MD, Griffin TJ and Potter LR (2010) Mass spectrometric identification of phosphorylation sites in guanylyl cyclase a and B. *Biochemistry* **49**(47): 10137-10145.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Comparison of the primary amino acid sequence of the intracellular juxta-membrane region of rat and human GC-A and GC-B. Red residues are phosphorylation sites that have been identified by mass spectrometry or tryptic phosphopeptide mapping of $^{32}\text{PO}_4$ label enzymes isolated from living cells. The blue residue is a conserved serine and putative phosphorylation site, which was identified based on enzymatic changes associated with phosphomimetic substitutions. Sequences were aligned with CLUSTAL version 2.0.1. Numbers correspond to residues from rat GC-A. Abbreviations are: rGC-A, rat GC-A; hGC-A, human GC-A; rGC-B, rat GC-B; hGC-B, human GC-B.

Figure 2. Glutamate Substitutions at Ser-473 and Ser-487 in GC-A-6E produce enzymes with similar characteristics to phosphorylated WT-GCA. 293 cells were transiently transfected with the indicated form of GC-A and assayed for GC activity for 5 min in the presence of 1 μM ANP, 1 mM ATP, and increasing concentrations of GTP. (A) Comparison between GC-A-6E, GC-A-6E-S473E, and GC-A-6E-487E where $n = 2$ from two experiments. (B) Comparison between WT-GC-A, GC-A-6E-487E, and GC-A-6E-473E where $n = 3$ from three experiments. (C) Comparison between WT-GC-A, GC-A-7E (GC-A-6E-473E), and GC-A-8E (GC-A-7E-487E) where $n = 4$ from four experiments. * indicates significantly different from WT-GC-A (B) and (C) or GC-A-6E (A) at $p < 0.0125$. Vertical bars in the symbols represent SEM.

Figure 3. GC-A-8E has a similar ANP dose response to phosphorylated WT-GCA. (A) 293 cells were transiently transfected with the indicated form of GC-A and assayed for GC activity for 3 min in the presence of 0.1 mM GTP, 1 mM ATP, and increasing concentrations of ANP where $n = 6$ from 3 experiments. (B) Percent of maximum activity for each construct where $n = 6$ from 3 experiments. (C) 293 cells were transiently transfected with the indicated form of GC-A. 119,000 cells per well were plated and assayed for ANP-dependent cGMP elevations in the medium after 1 min in the presence of increasing concentrations of ANP where $n = 2$ from 2 experiments. (D) Percent of maximum activity is shown for

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each construct where $n = 2$ from 2 experiments. Vertical bars in the symbols represent the range of duplicate determinations.

Figure 4. A threshold negative charge of eight is required for maximal GC-A activity. 293 cells were transiently transfected with the indicated form of GC-A and membranes from these cells were assayed for GC activity for 5 min in the presence of 1 μ M ANP, 1 mM ATP, and increasing concentrations of GTP. (A) Comparison between GC-A-8E, GC-A-7E (GC-A-8E with 513A), GC-A-6E (GC-A-7E with 510A), GC-A-5E (GC-A-6E with 506A), GC-A-4E (GC-A-5E with 502A), GC-A-3E (GC-A-4E with 500A), GC-A-2E (GC-A-3E with 497A), GC-A-1E (GC-A-2E with 487A), and GC-A-7A (WT-GC-A with 513A, 510A, 506A, 502A, 500A, 497A, 487S, and 473A) where $n = 2$ from two experiments. (B) Comparison between GC-A-8E, GC-A-8E-494E, and GC-A-8E-494E and 514E, where $n = 2$ from 2 experiments. Vertical bars in the symbols represent the range of duplicate determinations.

Figure 5. Ala substitutions at Ser-497 and either Thr-500, Ser-510 or Thr-513 have synergistic effects on the K_m for GC-A. 293 cells were transiently transfected with the indicated form of GC-A and membranes from these cells were assayed for GC activity for 5 min in the presence of 1 μ M ANP, 1 mM ATP, and increasing concentrations of GTP. (A) Comparison between WT-GC-A, GC-A-S497A-T500A, and GC-A-S497E-T500E where $n = 3$ from three experiments. (B) Comparison between WT-GC-A, GC-A-S497A-S510A, and GC-A-S497E-S510E where $n = 3$ from three experiments. (C) Comparison between WT-GC-A, GC-A-S497A-T513A, and GC-A-S497E-T513E where $n = 2$ from 2 experiments. *

indicates significantly different from WT-GC-A at $p < 0.0125$. Western blots indicate the amount of protein for each form of GC-A for each experiment. Vertical bars in the symbols represent the SEM.

Figure 6. Differential effects of phosphorylation site mutations on phosphorylated WT-GC-A and GC-A-8E. 293 cells were transiently transfected with the indicated form of GC-A and assayed for GC activity

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for 5 min in the presence of 1 μ M ANP, 1 mM ATP, and increasing concentrations of GTP. (A)

Comparison between GC-A-8E-S497A and T500A and GC-A-WT-S497A and T500A where $n = 2$ from 2 experiments. Vertical bars in the symbols represent the range of duplicate determinations.

(B) Comparison between WT-GC-A-S497A, WT-GC-A-S506A, WT-GC-A-T513A, GC-A-7E-S497A, GC-A-7E-S506A and GC-A-7E-T513A, where $n = 2$ from 2 experiments. Vertical bars in the symbols represent the range of duplicate determinations. (C) Pro-Q diamond and Coomassie staining comparing phosphate and protein values for WT-GC-A, WT-GC-A-S497A, WT-GC-A-S506A, WT-GC-A-T513A, WT-GC-A-S497A and T513A and WT-GC-A-7A. Ratio of the intensity of Pro-Q diamond staining to Coomassie staining was normalized to WT. $n = 4$ from four independent immunoprecipitations. * indicates significantly different from WT-GC-A at $p < 0.05$. Vertical bars in the symbols represent the SEM.

Figure 7. Model of phosphorylation-dependent ANP-stimulation of GC-A. (A) When GC-A is in its dephosphorylated state, the kinase homology domain is unordered and does not allow transmission of the ANP binding to the catalytic domain. (B) The increased negative charge resulting from the phosphorylation sites provide a concentrated region of negative charge that interacts with positive residues within the kinase homology domain, which provides a ridged and ordered structure that allows the rotational force elicited by ANP binding to be transferred to the catalytic domain to decrease the K_m and increase V_{max} .

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Table 1

Mutation	Vmax (% of WT)	Km (% of WT)
S497A	59% ± 8%*	389% ± 174%*
S497E	119% ± 3%	184% ± 79%
T500A	45% ± 4%*	150% ± 56%
T500E	93% ± 7%	107% ± 38%
S502A	70% ± 9%*	70% ± 41%
S502E	107% ± 12%	113% ± 54%
S506A	87% ± 4%*	221% ± 44%*
S506E	119% ± 4%*	196% ± 28%*
S510A	58% ± 11%*	195% ± 151%
S510E	113% ± 14%	162% ± 82%
T513A	80% ± 8%	165% ± 70%
T513E	152% ± 10%*	111% ± 33%
S487A	107%± 14%	99%±58%
S487E	130%±16%	144%±77%
S473A	91%± 3%	88%± 16%
S473E	83%± 8%	116%± 58%

Average Vmax for WT 16.5 +/- 3.9

Average Km for WT 54.8 +/- 1.4

Table 1. Effect of individual alanine or glutamate substitutions at GC-A phosphorylation sites on maximal velocity and the Michaelis constant of the enzyme. 293 cells were transiently transfected with plasmids expressing the indicated mutant and assayed for GC activity in the presence 1μM ANP, 1mM ATP and increasing concentrations of Mg²⁺GTP. Values are presented as mean ± SEM where *n* = 3. Average Vmax and Km values for WT GC-A were 16.5 ± 3.9 nmol cGMP/mg protein/5 min) and 54.8 ± 1.4 μM GTP, respectively. * Indicates significantly different from WT-GC-A at *p* < 0.0125.

Figure 1

473 487 497 500 502 506 510 513

rGC-A- **S**ELWRVRWEDLQPS**S**LERHLRSAG**S**RL**T**LSGRGS**S**NYGS**L**L**T**

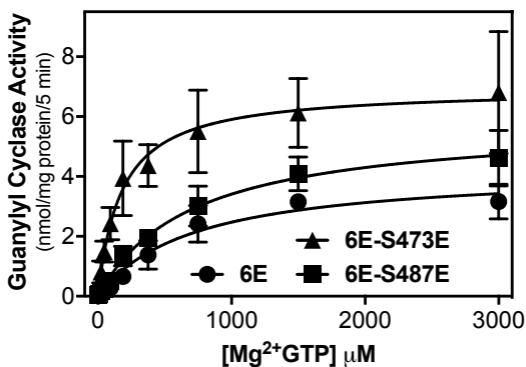
hGC-A- **S**ELWRVRWEDVEPS**S**LERHLRSAG**S**RL**T**LSGRGS**S**NYGS**L**L**T**

rGC-B- **S**MLWRIRWEELQFGNSDRYHKGAG**S**RL**T**LSLRGS**S**YG**S**LM**T**

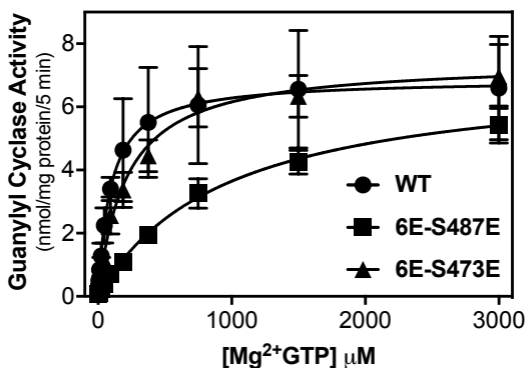
hGC-B- **S**MLWRIRWEELQFGNSERYHKGAG**S**RL**T**LSLRGS**S**YG**S**LM**T**

Figure 2

A



B



C

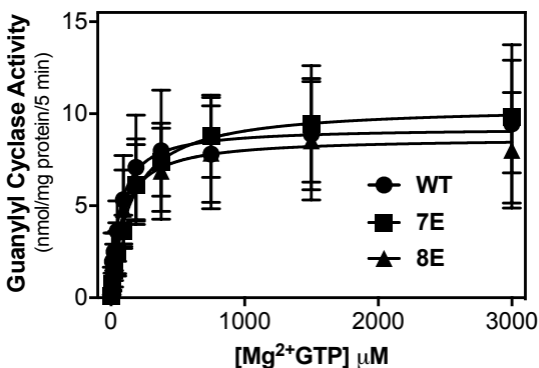


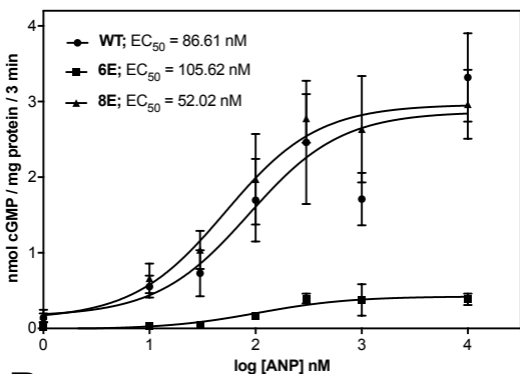
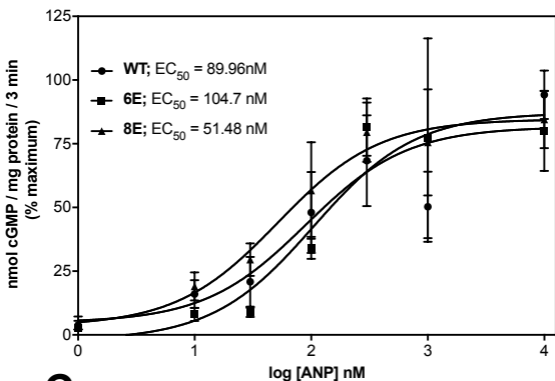
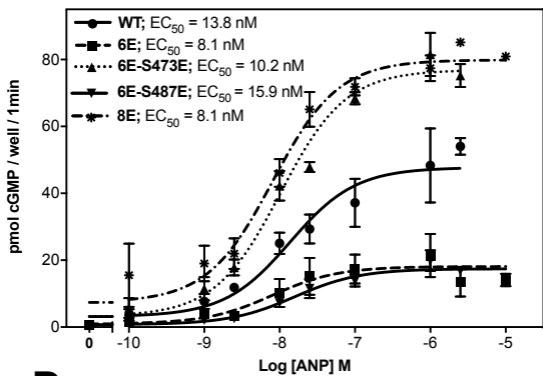
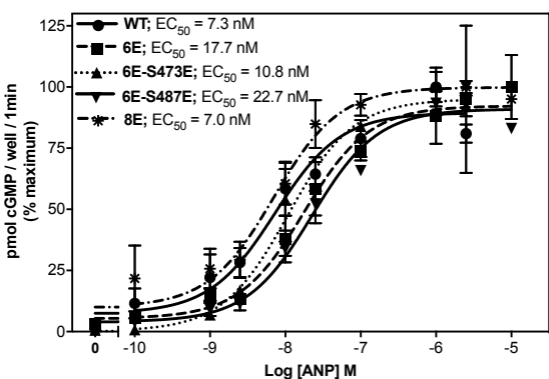
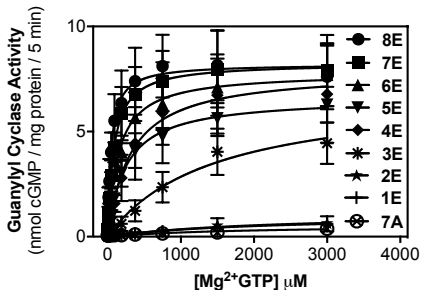
Figure 3**A****B****C****D**

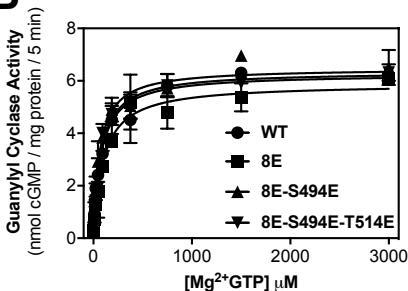
Figure 4

A



	8E	7E	6E	5E	4E	3E	2E	1E	7A
Vmax	8.215	8.257	7.809	6.63	7.892	6.93	1.138	1.011	0.5724
Km	46.18	82.9	143.5	245.1	313	1431	2197	2010	1867

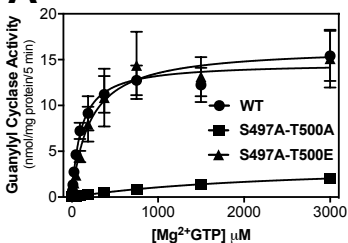
B



	WT	8E	8E-S494E	8E-S494E-T514E
Vmax	6.266	5.885	6.479	6.352
Km	76.77	99.9	63.54	74.42

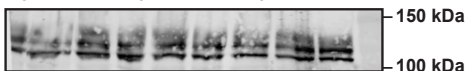
Figure 5

A

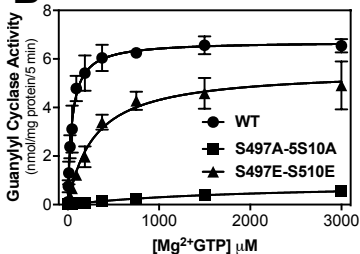


	WT	S497A-T500A	S497A-T500E
Vmax	14.62	3.703	16.33
Km	105.8	2468	207

1st 2nd 3rd
wt A E wt A E wt A E

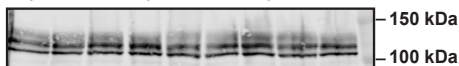


B

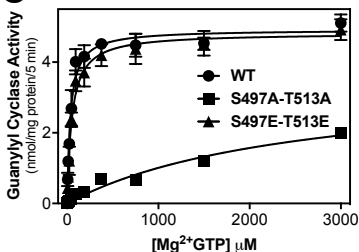


	WT	S497A-5S10A	S497E-S510E
Vmax	6.717	0.9165	5.551
Km	45.58	2032 *	288.2 *

1st 2nd 3rd
wt A E wt A E wt A E



C



	WT	S497A-T513A	S497E-T513E
Vmax	4.922	3.546	4.823
Km	36.07	2516 *	52.12

1st 2nd
wt A E wt A E

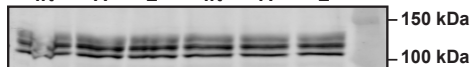
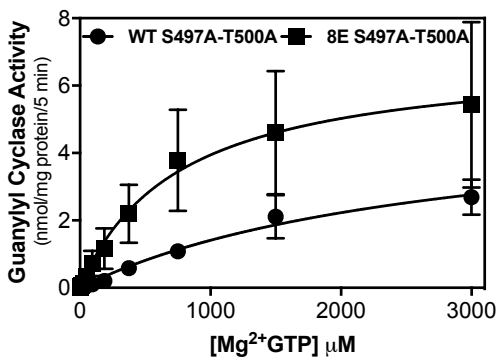
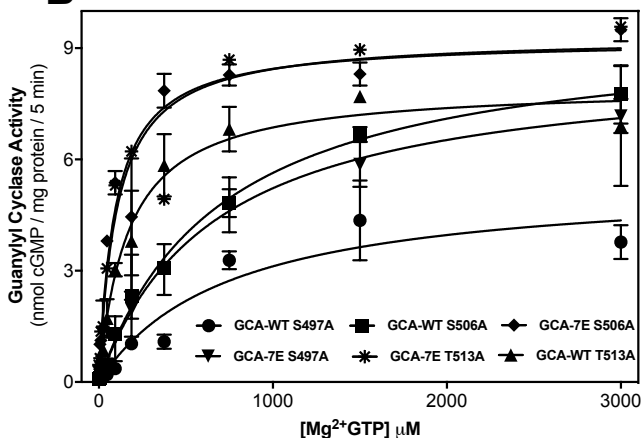


Figure6

A



B



C

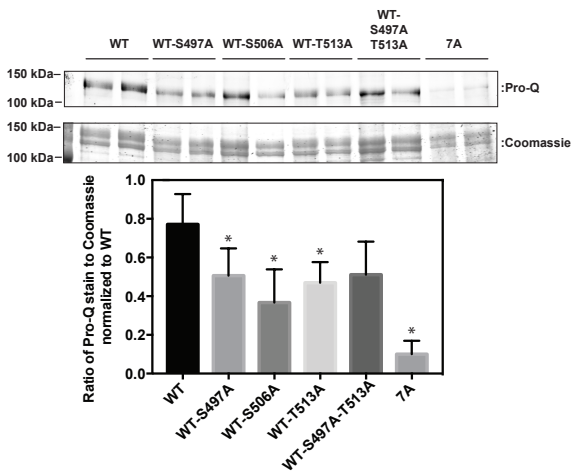
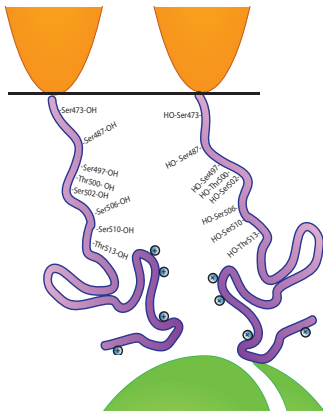
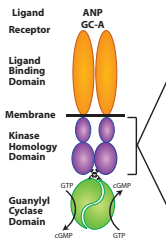


Figure 7

A

Membrane



B

Membrane

