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Domains Necessary for G α 12 Binding and Stimulation of PP2A: is G α 12 a Novel Regulatory
Subunit of PP2A?

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Abbreviations: PP2A, protein phosphatase-2A; MDCK, Madin Darby canine kidney; dox, doxycycline; HEAT repeat, 39 amino acid motif named for huntingtin, elongation, A subunit, TOR (target of rapamycin); HEK, human embryonic kidney

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Abstract

Many cellular signaling pathways share regulation by PP2A, a widely expressed serine/threonine phosphatase, and the heterotrimeric G protein, G α 12. PP2A activity is altered in carcinogenesis and in some neurodegenerative diseases. We previously identified binding of G α 12 with the A α subunit of PP2A, a trimeric enzyme composed of A (scaffolding), B (regulatory), and C (catalytic) subunits, and demonstrated G α 12 stimulated phosphatase activity (Zhu et al., 2004 JBC 279; 54983). We now show in substrate-velocity analysis using purified PP2A that V_{\max} was stimulated 3-4 fold by GST-G α 12 with little effect on K_m . To identify the binding domains mediating the A α -G α 12 interaction, an extensive mutational analysis was performed. Well-characterized mutations of A α were expressed in vitro and tested for binding to GST-G α 12 in pull-down assays. G α 12 binds to A α along repeats 7-10, and PP2A B subunits are not necessary for binding. To identify where A α binds to G α 12, a series of 61 G α 12 mutants were engineered to contain the sequence asn-ala-ala-ile-arg-ser (NAAIRS) in place of 6 consecutive amino acids. Mutant G α 12 proteins were individually expressed in HEK cells, and analyzed for interaction with GST or GST-A α in pull-downs assays. The A α binding sites were localized to regions near the N and C terminus of G α 12. The expression of constitutively activated G α 12 (QL α 12) in MDCK cells stimulated PP2A activity as determined by decreased phosphorylation of tyrosine-307 on the catalytic subunit. Based upon crystal structures of G α 12 and PP2A A α , a model describing the binding surfaces and potential mechanisms of G α 12-mediated PP2A activation is presented.

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Heterotrimeric G proteins regulate fundamental processes in all eukaryotic cells. The canonical signaling pathway in which a membrane-bound G-protein-coupled-receptor activates $G\alpha$ and $G\beta\gamma$ subunits to stimulate downstream effectors has been significantly extended in recent years. In particular, G protein signaling has been shown to regulate unique cellular functions in specialized membrane domains, and interact with numerous protein-kinase signaling pathways (for example, (El-Shewy et al., 2006; Luttrell and Luttrell, 2004)). Novel regulatory mechanisms of G protein signaling have also been identified through interactions of $G\alpha$ subunits with proteins that modulate $G\alpha$ function. These include RGS proteins (Regulator of G protein signaling) that stimulate $G\alpha$ GTPase activity and GPR proteins (G protein regulatory) proteins that inhibit GDP release (Hollinger and Hepler, 2002; Lanier, 2004). $G\alpha_{12}$ and $G\alpha_{13}$ comprise one of the four heterotrimeric G protein families that also include $G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_q$. $G\alpha_{12/13}$ regulate the actin cytoskeleton (Buhl et al., 1995) and epithelial cell junctions (Meigs et al., 2002; Meyer et al., 2003) in addition to other cellular processes including transformation of fibroblasts (Jiang et al., 1993), stimulation of apoptosis (Berestetskaya et al., 1998), neurite retraction in PC12 cells (Katoh et al., 1998), directed cell movement within the developing embryo (Lin et al., 2005), and cell migration (Goulimari et al., 2005). $G\alpha_{12}$ and $G\alpha_{13}$ interact with the RGS domain of the Rho exchange factor p115-RhoGEF and $G\alpha_{12}$ binds the RGS domain of axin (Kozasa et al., 1998; Stemmler et al., 2006). In addition, $G\alpha_{12}$ and/or $G\alpha_{13}$ bind to a diverse group of other signaling and structural proteins including Bruton's tyrosine kinase, Hsp90, the tight junction protein ZO-1, and E-cadherin (Jiang et al., 1998; Meigs et al., 2001; Meyer et al., 2002; Vaiskunaite et al., 2001; Waheed and Jones, 2002).

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PP2A is composed of three subunits; A (scaffolding), B (regulatory) and C (catalytic). There are two isoforms for the A and C subunits (α and β). There are 4 families of B subunits (B/PR55, B'/B56/PR61, B'', and B''') encoding at least 14 separate gene products and several alternatively spliced variants that regulate localization of the core enzyme (A-C) and C subunit activity (reviewed in (Janssens and Goris, 2001)). Catalytic subunits are not found as free monomers in cells, but always in complex with the A subunit forming an A-C core enzyme. B subunits reversibly interact with the core enzyme to regulate PP2A function, and other proteins can mimic (replace) B subunits. We previously demonstrated G α 12 stimulation of PP2A phosphatase activity in vitro and in COS cells (Zhu et al., 2004). Many signaling pathways share involvement of G α 12 and PP2A, although until recently a direct link between these two proteins has been missing. Some cellular processes modulated by both G α 12/13 and PP2A include cellular transformation, growth, apoptosis, and stress responses. Furthermore, PP2A has been directly implicated in carcinogenesis and neurodegenerative diseases (including Alzheimer's; reviewed in (Janssens and Goris, 2001)). PP2A is also localized in epithelial cell tight junctions and regulates the phosphorylation state of numerous proteins during maintenance and assembly of the junction (Nunbhakdi-Craig et al., 2002). We and others have observed G α 12 in the tight junction (Dodane and Kachar, 1996; Meyer et al., 2003; Meyer et al., 2002) suggesting that both G α 12 and PP2A are localized within the same sub-cellular microdomain. Taken together, we hypothesize that G α 12 regulation of PP2A is a critical signaling pathway important to diverse cellular functions and in the pathophysiology of certain disease states. In order to define the mechanism(s) of G α 12 regulation of PP2A and provide the basis for pharmacologic modulation of these signaling pathways, we have performed extensive mutagenesis and in vitro binding analyses to identify the requisite binding domains of A α and

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G α 12. In addition, we provide evidence that G α 12 activation in cells stimulates PP2A, and in vitro kinetic analysis reveals that G α 12 stimulates V_{\max} without significantly affecting K_m .

Based on crystal structures of PP2A A α and G α 12 subunits and the results of binding studies with mutant proteins, it is possible to propose a model of the mechanism by which G α 12 regulates PP2A function.

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Materials and Methods

Chemicals, Antibodies and cDNA Constructs. Purified PP2A core enzyme (A-C) was obtained from Upstate Biotechnology. Mouse β -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO), p-Y307 antibody from Epitomics (Burlingame, CA). The rabbit C-subunit antibody was kindly provided by Dr. David Virshup (University of Utah). Recombinant G $\beta\gamma$ purified from baculovirus was kindly provided by Dr. Thomas Michel (Brigham and Women's Hospital). G α 12 antibodies were purchased from Santa Cruz. G α 12 Tet-off MDCK cells and GST-G α 12 are previously described (Meyer et al., 2002). Glutathione-sepharose was from Amersham Pharmacia Biotech. Full-length mouse A α cDNA was subcloned into pGEX using standard techniques and GST-A α fusion protein was expressed and purified from *E.coli* as previously described (Luo and Denker, 1999). Substitution mutants within myc-tagged QL α 12, in which regions of the cDNA encoding consecutive sextets of amino acids are replaced with DNA sequence encoding the sextet NAAIRS, were engineered using oligonucleotide-directed mutagenesis as described previously (Meigs et al., 2005). All constructs were verified by sequencing.

PP2A Phosphatase Activity. Phosphatase activity was determined as previously described (Zhu et al., 2004). Briefly, phosphatase activity was determined by Malachite Green phosphatase assay (Upstate, Lake Placid, NY). The phospho-peptide (K-R-pT-I-R-R) was used as substrate to determine PP2A activity alone or PP2A in combination with GST, or GST-G α 12 at 1:1 molar ratios. A standard curve was generated in each assay and PP2A activity determined in duplicate for each concentration. The reaction was initiated by addition of substrate at t=0, and after 30 min the reaction was terminated, absorbance measured at 624nm, and enzyme activity

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determined (nmol phosphate/min/U). K_m and V_{max} were calculated from substrate-velocity curve analysis and Lineweaver Burk plots generated in GraphPad Prism (GraphPad Software, San Diego, CA).

In Vitro Translation. $A\alpha$, $B\alpha$ and mutant $A\alpha$ cDNAs in pBS or pcDNA3 were in vitro translated using 0.5-1 μ g of plasmid, the appropriate RNA polymerase in a coupled rabbit reticulocyte translation system (TNT system, Promega, Madison WI) plus [35 S] methionine (NEN Life Science Products) as previously described (Denker et al., 1995). Protein expression was analyzed by SDS-PAGE and autoradiography.

GST Pull-Downs of $A\alpha$ Subunits. 35 S-labeled $A\alpha$ subunits were pre-cleared by incubation with glutathione agarose beads for 1h at 4 $^{\circ}$ C. Translates (5-20 μ l; depending upon translation efficiency) were incubated with GST or GST-G α 12 (0.5-1 μ g) for 3h in buffer A (50 mM HEPES, pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM MgSO $_4$, 0.05% polyoxyethylene-10-lauryl ether). Samples were centrifuged, washed three times with buffer A and eluted with SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and autoradiography. Band intensity was analyzed using NIH-Image 1.63 (Wayne Rasband) and relative intensity of precipitated $A\alpha$ was expressed as a fraction of the total and normalized to the wild type $A\alpha$ control.

GST-G α 12 Pull-Downs of 35 S-labeled $A\alpha$ and $B\alpha$ Subunits. $A\alpha$ and $B\alpha$ subunits were in vitro translated and 35 S-labeled in rabbit reticulocyte lysate as described above. Equivalent amounts (determined by densitometry) of the labeled proteins were mixed together and incubated

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with 1 μ g of GST or GST-G α 12 for 3h in buffer A. Samples were centrifuged, washed three times with buffer A and eluted with SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and autoradiography.

Preparation of G α 12 mutants from HEK cell lysates. Human embryonic kidney (HEK293) cells were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin, and were maintained at 37°C in a 5.0% CO₂ atmosphere. For each G α 12 mutant, 7 μ g of plasmid DNA were used to transfect a 10-cm dish of HEK293 cells at 60-80% confluency, using Lipofectamine reagent (Invitrogen) in accordance with the manufacturer's instructions. At 48 h post-transfection, cells were rinsed twice with phosphate-buffered saline (PBS), scraped from the dish, pelleted at 800 x g, and then 0.5 mL of ice-cold Buffer A containing 1 % polyoxyethylene-10-lauryl ether supplemented with protease inhibitors was used to resuspend each cell pellet. Samples were mixed by inversion at 4°C for 30 min, and then centrifuged at 100,000 x g, 4°C, for 1 h. Supernatants were snap-frozen in liquid N₂ and stored at -80°C.

GST Pull-down of G α 12 Mutants. GST-A α and GST were expressed in *E.coli* and purified by immobilization onto glutathione-sepharose as described previously (Luo and Denker, 1999; Meigs et al., 2001). For each mutant form of G α 12 tested, 45 μ L of HEK293 supernatant (prepared as described above in buffer A + 1% detergent) was diluted to 450 μ L using buffer A with no detergent (final detergent concentration 0.1%). 5% of each diluted lysate was set aside as "lysate load" and the remainder was equally divided and incubated with glutathione-sepharose bound to either GST-A α or GST. Samples were incubated for 2 h at 4°C, pelleted, and the

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supernatant was discarded. Beads were washed three times with buffer A + 0.05% polyoxyethylene-10-lauryl ether followed by resuspension of the sepharose pellets in SDS sample buffer and heating to 72°C for 10 min. For each sample, 80% of the volume was subjected to SDS-PAGE and subsequent blot transfer. Immunoblots were blocked in TBST [50 mM Tris, pH 7.7, 150 mM NaCl, 0.05 % Tween-20] supplemented with 5% powdered milk for 2 h, and then incubated with rabbit anti-G α 12 antibody (at 1:500 dilution) for 2h. Following three 10-min washes using TBST, alkaline phosphatase-conjugated anti-rabbit antibody was diluted 1:7500 in TBST+milk and applied for 1 h. Three additional washes were performed using TBST, and blots were developed using NBT/BCIP reagent (Promega) according to the manufacturer's instructions. Results were documented using a Kodak Gel Logic 100 scanner and band intensities were quantified using Kodak 1D image analysis software. The remaining 20% of each sample volume was subjected to SDS-PAGE, and gels were stained overnight with Coomassie blue, destained, and photographed.

Analysis of Y-307 phosphorylation in G α 12-expressing MDCK cells. Wild type G α 12 and QL α 12-MDCK cells were cultured +/- doxycycline (dox) for 72h to induce G α 12 expression using previously described conditions (Meyer et al., 2002). Briefly, MDCK cell lysates were prepared by scraping cells in buffer B (1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH7.5, 150 mM NaCl, 4 mM EDTA, 1 mM Na₃VO₄, 25 mM NaF and protease inhibitor cocktail), briefly sonicated, centrifuged and the resulting supernatant saved for analysis. Protein concentration was determined by BCA Protein Assay Kit (Pierce) and equivalent protein amounts analyzed by SDS-PAGE and Western as described above.

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Modeling of Crystal Structures. Cnd4 version 4.1 was obtained from

www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml and G α 12 crystal structure (1ZCA (Kreutz et al., 2006)) and A α crystal structure (1B3U_A) retrieved from the protein database (PDB) and independently studied using Cnd4. Mutations were highlighted in yellow and various aspects of the crystal structure were hidden. Images were exported as PNG files and resolution/size adjusted in Adobe Photoshop and then labeled and assembled in Adobe Illustrator (Adobe, San Jose, CA).

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Results

We previously demonstrated increased PP2A phosphatase activity when core enzyme (A-C) was incubated with GST-G α 12 or recombinant G α 12, but not with several other purified G α subunits (Zhu et al., 2004). In addition, there were no detectable differences in affinity of GDP-, GTP γ S-, or AlF $_4^-$ liganded G α 12 for A α in immunoprecipitation studies, and recombinant G α 12 stimulated phosphatase activity similarly in the inactive and active conformations. Based upon the similar binding and stimulation of PP2A by GST-G α 12 and active and inactive purified G α 12, GST-G α 12 was used to determine K_m and V_{max} for PP2A core enzyme from substrate-velocity and Lineweaver-Burk plots in the presence of equimolar GST or GST-G α 12. Figure 1 shows the initial velocity versus substrate plot of mean PP2A activity +/- pre-incubation with GST (control) or GST-G α 12 in 3 separate experiments each performed in duplicate over a range of substrate concentrations. Figure 1 (inset) shows the Lineweaver-Burk plot of the velocity versus substrate data for PP2A alone, PP2A+GST and PP2A+GST-G α 12. The K_m and V_{max} for each condition were determined in GraphPad Prism as described in Material and Methods and are summarized in Table 1. A 3- to 4-fold stimulation of V_{max} by GST-G α 12 compared to GST was observed, with no significant effect on K_m . Preincubation of GST-G α 12 with GTP γ S or G $\beta\gamma$ subunits had no demonstrable effect on the kinetics (results not shown). However, it is not known whether GST-G α 12 exchanges guanine nucleotides or interacts with G $\beta\gamma$. Nevertheless, these findings are consistent with our previous observations with purified G α 12. Preincubation of G α 12 with GTP γ S {Zhu, 2004 #773}, or G $\beta\gamma$ (not shown) had no effect on steady state phosphatase activities. Together, these findings suggest that the stimulation of PP2A phosphatase activity in vitro is not dependent upon the activated G α 12 conformation or affected by G α 12 complexing with G $\beta\gamma$.

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To gain additional insights into how G α 12 stimulates PP2A phosphatase activity, we sought to identify the amino acid regions of A α and G α 12 necessary for the interaction. A series of well-characterized mutants of A α (Ruediger et al., 1994; Ruediger et al., 1992) were [³⁵S]methionine labeled, incubated with GST or GST-G α 12 and analyzed in GST pull-down assays (Fig. 2). GST-G α 12 and GST proteins are shown in Figure 2A, and the [³⁵S]methionine labeled A α mutants are shown in Figure 2B. The results of the pull-downs are shown in Figure 2C next to a schematic of each A α mutant protein. Wild type A α , consisting of 589 amino acids (WT589) was analyzed in parallel with each mutant, and the binding of each mutant is expressed relative to the wild type control in that experiment. The A α subunit is composed of 15 tandem HEAT repeats (huntingtin, elongation, A subunit, TOR (target of rapamycin) each composed of approximately 39-amino acids. The repeats in the A α subunit were proposed to be composed of two superimposed α -helices yielding an elongated molecule often described as a hook (see Fig. 7) (Ruediger et al., 1992). This structure was subsequently confirmed and molecular details extended by crystal structure analysis (Groves et al., 1999). Mutational analysis of A α has identified the domains required for B and C subunit binding (Ruediger et al., 1994; Ruediger et al., 1992). C-subunits interact with repeats 11-15 while the heterogeneous regulatory B subunits require repeats 1-10 for binding. The recent crystal structure of trimeric A α -B56 γ 1-C α confirms C subunit interaction with A α repeats 11-15 but reveals B' subunit binding to repeats 2-7 (Cho and Xu, 2006). In mutational studies with various B subunits, A α repeat 5 was required for all known B subunit interactions (Ruediger et al., 1999). The analysis shown in Figure 2 indicates that G α 12 binds to HEAT repeats 7-10 within A α . There was a partial reduction in binding (25-50% of control) seen with deletion of repeat 7 (Δ 7) or repeat 8 (Δ 8), and there was complete loss

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of binding with deletion of repeat 9 ($\Delta 9$) or repeat 10 ($\Delta 10$). Deletion of N-terminal repeats had no effect on G α 12 binding ($\Delta 1$, $\Delta 1-2$, $\Delta 1-3$), and mutants with single repeats 3, 4, or 5 deleted also bound normally. The normal interaction of G α 12 with $\Delta 5$ indicates that B subunit binding is not required for the G α 12 interaction. Deletion of any single repeat between 11 and 15 prevents binding of the C-subunit (Ruediger et al., 1992), yet G α 12 interacted normally with $\Delta 15$ and $\Delta 14-15$, and was only slightly reduced with the larger $\Delta 11-15$ deletion. Consistent with the single repeat deletions, deletion of repeats 9-15 ($\Delta 9-15$) resulted in complete loss of binding to G α 12.

These results indicate that B subunit interactions are not required for G α 12 binding to the core enzyme. However, this does not exclude the possibility that G α 12 can interact with the PP2A holoenzyme (A α -B-C). To test whether G α 12 may interact with the holoenzyme, A α and B α subunits were in vitro translated in rabbit reticulocyte lysate, labeled with [35 S]methionine, and allowed to equilibrate with the endogenous subunits to form holoenzymes (as previously shown (Ruediger et al., 1992)). Pull-down experiments of labeled A α and/or B α with GST or GST-G α 12 were analyzed for detection of labeled subunits with the precipitated protein complex. As shown in Figure 3, there is readily detectable A α and B α subunits in the GST-G α 12 pull-down that was not seen with GST. This finding suggests that G α 12 may interact with a holoenzyme in this assay, and we have not excluded the possibility of a direct G α 12-B α interaction. The B α signal was stronger than for A α in this experiment, but because the amounts of endogenous A α and B α in the rabbit reticulocyte lysate are unknown it is not possible to compare the relative binding of these subunits.

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In order to map the determinants within G α 12 that are important for its interaction with PP2A A α , we first engineered a series of amino acid substitution mutants that span the full length of G α 12. To produce each mutant, a sextet of consecutive amino acids in the primary sequence of myc-tagged QL α 12 (constitutively active G α 12) was replaced by the sequence asn-ala-ala-ile-arg-ser (“NAAIRS”). This series of mutations was created in the QL α 12 protein to facilitate the study of G α 12 binding proteins that would be expected to be downstream effectors in canonical G protein signaling. The NAAIRS sequence is believed to be a well-tolerated substitution in proteins due to its appearance in both α -helical and β -sheet secondary structures (Wilson et al., 1985) and was recently used to successfully engineer a mutant of G α 12 that was selectively uncoupled from a known binding partner, p115RhoGEF (Meigs et al., 2005). It was previously shown that wild type and QL α 12 interact with PP2A A α (Zhu et al., 2004). The myc-tagged QL α 12 was used as starting material so that the subsequently engineered NAAIRS mutants could be distinguished from endogenous G α 12 when expressed in cultured mammalian cells (Meigs et al., 2005). The NAAIRS mutants were given consecutive alphabetical designations: *a* to *z* followed by *aa* to *zz*, ending with *aaa* to *kkk* (see Fig. 4). We were able to express 61 of these 63 mutants in HEK293 cells, as determined by immunoblot analysis (data not shown). Mutant *w* was not engineered due to the positioning of the myc epitope tag within it, and mutant *zz* was undetectable in cell lysates. Mutants were detergent-extracted from HEK293 lysates and then screened for binding to GST-A α as described in Materials and Methods.

Next, an A α GST fusion protein (GST-A α) was constructed and purified as described in Materials and Methods. GST-A α migrates at its predicted molecular weight of ~80kDa during SDS-PAGE (Fig. 5A). GST-A α was tested for its ability to precipitate myc-tagged QL α 12 as

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well as the full panel of NAAIRS substitution mutants. Seven or eight mutants were screened in each experiment, and myc-tagged QL α 12 was included as an internal control and was precipitated by A α in all experiments (Fig. 5B). While most of the NAAIRS mutants were precipitated by GST-A α , several mutants displayed severely impaired or abolished binding to A α (Fig. 5B, and summarized in Fig. 4). To provide a quantitative analysis of the relative affinity of these mutants for A α , and also to account for differing levels of expression of these mutants in HEK293 cells, we calculated a normalized ratio using the densities of the bands on Western blots for the starting material and the pull-down. This was compared to the same ratio determined for myc-QL α 12 within the same experiment. These results are summarized in Figure 4. The majority of the NAAIRS mutants showed a normalized ratio that was greater than 50% of the control value (no marking in Figure 4), but several mutants displayed a dramatic loss of interaction with A α . Mutants showing moderate-to-severe impairment of interaction (**t**, **rr**, **bbb**, **eee**, **fff**, **jjj**; 10-25% of control) are shown in clear boxes in Figure 4, and those with abolished interactions (<10% of control) are highlighted in the gray boxes in Figure 4. These latter mutants (**a**, **f**, **i**, **aaa**, **ccc**, **ddd**, and **hhh**), with the exception of mutant **u**, were localized near the N and C termini of G α 12 (see Fig. 4). As an additional control, myc-QL α 12 and all NAAIRS mutants were tested for precipitation by glutathione-sepharose bound GST; this complex did not precipitate myc-QL α 12 or any of its mutant variants (Fig. 5B).

Our next goal was to determine whether G α 12 regulates PP2A activity in cells. To address this question, we used well-characterized MDCK cells with inducible expression of G α 12 and QL α 12 (Meyer et al., 2003; Meyer et al., 2002). PP2A activity can be assessed by the relative tyrosine phosphorylation on the catalytic subunit (Y-307), and decreased

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phosphorylation is associated with increased PP2A activity (Chen et al., 1992). Figure 6 shows that endogenous G α 12 in MDCK cells is not readily detectable by Western blot with available anti-G α 12 antibodies. However, after 72h in the absence of doxycycline, QL α 12 was readily detectable. The Western blots were stripped and reprobed for actin, total C subunit, and phosphotyrosine 307 levels. There was a small but consistent reduction in normalized phosphotyrosine 307 levels in QL α 12 expressing MDCK cells (-dox) that was not seen in + dox QL α 12 cells (no QL α 12 expression) or in wild type G α 12-MDCK cells +/- dox (Fig. 6). This finding is consistent with G α 12 activation of PP2A phosphatase activity in MDCK cells and our prior finding of G α 12/PP2A stimulated dephosphorylation of tau in transfected COS cells (Zhu et al., 2004).

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Discussion

We previously reported that G α 12 binds to the A α subunit of PP2A and stimulates its activity (Zhu et al., 2004). We now identify the specific regions of G α 12 and A α necessary for this interaction, and show that G α 12 binding to PP2A core enzyme stimulates catalytic activity with little effect on substrate affinity. Analysis of well-characterized deletions of individual HEAT repeats in A α identifies repeats 7-10 as the critical region for G α 12 interaction. G α 12 binding does not require the B subunit to be bound, but in vitro, G α 12 may interact with the PP2A holoenzyme. Analysis of 61 distinct G α 12 mutants identifies regions near the N and C termini as important for G α 12 binding to A α . Furthermore, using a cell culture model we provide evidence for G α 12-dependent activation of PP2A. Based upon these results and crystal structures of G α subunits and PP2A, their interacting surfaces are modeled and possible mechanism(s) of regulation are discussed (Fig. 7).

PP2A is composed of three subunits; A (scaffolding), B (regulatory) and C (catalytic). Catalytic subunits are always found in complex with the A subunit forming an A-C core enzyme. B subunits reversibly interact with A-C to regulate PP2A function, and other proteins can mimic B subunits. For instance, tumor (T) antigens can displace B subunits to affect PP2A activity and this has been implicated in carcinogenesis (see (Janssens and Goris, 2001)). SV40-small T antigen requires A α repeats 3-6 and Polyoma small T and middle T antigens require repeats 2-8 (Ruediger et al., 1994; Ruediger et al., 1992). The results of the binding experiments with A α deletion mutants shown in Figure 2 reveal a unique pattern of G α 12 interaction. G α 12 binds to repeats 7-10, and a complete loss of binding occurs when either repeat 9 or repeat 10 is deleted. This pattern is distinct from B and C subunit binding and from binding of T antigens, allowing

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for potentially complex regulation of the enzyme. B subunits interact along the first 10 repeats, and all tested B subunits require repeat 5 to bind. G α 12 binds Δ 5 and wild type A α with similar affinity, indicating that B subunit binding to the A-C core enzyme complex is not required for G α 12 interaction. G α 12 binding does not overlap on A α with C subunit binding (repeats 11-15), a finding consistent with simultaneous binding of C subunit and G α 12 to A α . This proximity is likely to be important for inducing conformational changes resulting in enhanced phosphatase activity. The B56 γ 1 subunit makes direct contact with the C subunit and is postulated to affect substrate specificity (Cho and Xu, 2006). In an analogous manner, the binding of G α 12 to the site immediately adjacent to the C subunit may permit direct interaction(s) of G α 12 with the C subunit.

The results shown in Figure 3 suggest that a tetrameric complex of G α 12 with the holoenzyme may form in vitro. Consistent with this model, the crystal structure of A α -B56 γ 1-C α reveals binding of B' to A α repeats 2-7 (Cho and Xu, 2006) indicating that for this specific holoenzyme the G α 12 binding sites at repeats 8-10 should be accessible to G α 12 for binding. However, we have not excluded the possibility that G α 12 binds to B α in the absence of A α or C subunits. Furthermore, our analysis is unable to distinguish G α 12 binding to the intra-repeat loops (inner surface of hook) from binding to the inter-repeat loops (outer surface, Fig. 7B). The diversity in structure of B subunits makes it likely that there are differences in how B subunits interact with A α . Thus, the regulation of PP2A by B subunits and G α 12 may depend on the specific B subunit. A more refined mutational analysis using purified proteins will be necessary to determine whether G α 12 binds to B α in the absence of A α and C subunits whether G α 12 interacts with PP2A holoenzymes.

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The mutational analysis of G α 12 indicates that regions near the N and C termini are the domains critical for binding A α . This analysis was performed with a comprehensive set of mutations created in the QL (activated) G α 12 protein. The crystal structure of the G α 12 N terminus is not known, and the unique N-terminal structure of G α 12 is thought to inhibit G α 12 protein expression and crystallization (Kreutz et al., 2006). To crystallize G α 12, it was necessary to generate a chimeric G α 12 missing the first 46 amino acids and containing the first 28 amino acids of G α i1 (Kreutz et al., 2006). The N-terminal G α 12 sequence missing from the crystal structure contains 2 of 8 regions in which NAAIRS mutants (**a,f**; Fig. 4) resulted in nearly complete loss of binding to A α . The N terminus of G α subunits contributes to protein binding and lipid modifications important for membrane attachment (Busconi et al., 1997; Busconi and Denker, 1997); G α 12/13 are palmitoylated within this region (Jones and Gutkind, 1998). The N terminus of G α subunits also binds to G $\beta\gamma$ (Denker et al., 1992). However, QL α 12 has a low affinity for G $\beta\gamma$, and the addition of G $\beta\gamma$ did not affect stimulation of PP2A phosphatase activity. This indicates that G $\beta\gamma$ and the N-terminal sites used for binding G $\beta\gamma$ are not critical for the interaction of G α 12 with A α . NAAIRS mutant **b** (Fig. 4) mutates the palmitoylation site (cysteine 11), and the normal pull-down of mutant **b** suggests that palmitoylation is not required for the interaction. Although the structure of the G α 12 N terminus is unknown, it is predicted to lie in close proximity to the C terminus (see Figure 7). The C terminus of G α 12 contained the largest region important for binding to A α . Most of the region necessary for A α binding comprises the G α 12 α 4 helix, β 6 sheet and α 5 helix that ends at the C terminus. This region forms an exposed surface (Fig. 7 C-E) available for potential interaction with repeats 7-10 of A α . NAAIRS mutants **t** and **u** are located apart from the N- and C-terminal binding regions (Fig. 7 C-

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E) and are not likely to directly contribute to interactions with $A\alpha$, but may induce secondary conformational changes in the $\alpha 4$ - $\beta 6$ - $\alpha 5$ region. These in vitro observations do not exclude the possibility that $G\alpha 12$ activation is necessary for PP2A activation in cells. The high protein concentrations in vitro may permit stimulation of PP2A by virtue of the binding that occurs in both conformations. In the $G\alpha 12$ -expressing MDCK cells (Fig. 6), there was no change in tyrosine 307 phosphorylation with wild type $G\alpha 12$, but with expression of $QL\alpha 12$ there was loss of phosphorylation at tyrosine 307 on the C subunit, a finding consistent with increased PP2A catalytic activity. Nevertheless, it remains to be established whether both active and inactive $G\alpha 12$ stimulate PP2A in cells. The lack of an effect for wild type $G\alpha 12$ on PP2A activity could result from the protein being denatured, although in other studies $G\alpha 12$ reversibly interacts with the basolateral membrane (Meyer et al., 2002) and modulates paracellular flux (Meyer et al., 2003).

Activated $G\alpha 12$ and $G\alpha 13$ have been shown to bind and stimulate the PP5 family of serine/ threonine phosphatases (Yamaguchi et al., 2002; Yamaguchi et al., 2003) through an interaction with the TPR (tetratricopeptide repeat) domain. This observation, in addition to the studies presented here, suggest that $G\alpha 12/13$ may be important regulators of cellular phosphatases. Many studies have linked G protein signaling to protein kinase pathways, and the interactions of $G\alpha 12$ and $G\alpha 13$ with protein phosphatases suggest that G protein signaling through $G\alpha 12/13$ may counter-regulate protein phosphorylation occurring in response to activation of other G protein signaling pathways. While $G\alpha 12/13$ stimulation of protein phosphatases may be a broad regulatory mechanism, the modes of activation of PP5 vs PP2A by $G\alpha 12$ appear to be distinct. PP5 is a single subunit phosphatase stimulated by both $G\alpha 12$ and

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G α 13. In contrast, we did not detect G α 13 stimulation of PP2A (Zhu et al., 2004), although preliminary results indicate that G α 13 can bind to the GST-A α fusion protein (not shown). In addition, there is no sequence homology between the TPR domain of PP5 and the HEAT repeats 7-10 of A α .

The numerous functions of PP2A require sophisticated regulatory mechanisms to allow for specific regulation. The diverse family of B subunits can modulate PP2A catalytic activity and direct the core enzyme to specific substrates. Our finding that G α 12 regulates PP2A provides a novel mechanism for regulation. Future studies are needed to determine how this occurs in cells, and it remains to be determined whether G α 12 functions as a novel "B" subunit or instead acts in concert with B subunits to regulate PP2A activity. However, these two mechanisms are not mutually exclusive and the mechanism(s) may depend upon the specific B subunit in the PP2A trimer. The question of whether there are differences in PP2A stimulation in cells by active and inactive G α 12 will require additional study. The findings in this report will provide the basis for future studies to dissect aberrant signaling seen in some pathologic conditions. For example, the G α 12-PP2A interaction can be disrupted by deletion of a single HEAT repeat that would not be expected to affect other subunit interactions.

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Footnotes

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FIGURE LEGENDS

Figure 1. $G\alpha 12$ Stimulates V_{max} of PP2A Core Enzyme.

PP2A core enzyme (A-C) was pre-incubated with GST or GST- $G\alpha 12$ at a 1:1 molar ratio and substrate velocity determined over a range of substrate concentrations as described in Materials and Methods. Results are the mean of 3 experiments each done in duplicate. The Lineweaver Burk plot was obtained using GraphPad Prism and is shown as inset. The summary of V_{max} and K_m values are shown in Table 1.

Figure 2. $G\alpha 12$ Binds to Repeats 7-10 on $A\alpha$.

A. Coomassie blue stained gel of GST- $G\alpha 12$ and GST fusion proteins. B. Autoradiogram of [35 S] methionine labeled and in vitro translated wild type $A\alpha$ and $A\alpha$ mutants. 0.5-2 μ l of translate were analyzed by SDS-PAGE and autoradiography. Exposure time was 4-12h. White vertical lines indicate separate gels. C. Wild type (WT) $A\alpha$ schematic with 15 boxed repeats is shown on top. The deletions of N and C-terminal repeats are shown as gaps and single repeat deletions are highlighted by a black box. A representative autoradiogram of GST pull-down with GST- $G\alpha 12$ and GST is shown for each mutant in the adjacent panel. The input of labeled $A\alpha$ protein is 1% of the total used in the binding assay. Wild type binding was set at 100% and in each experiment a wild type control was included for comparison of mutants. Binding for each mutant was scored from quantification of band intensity determined by densitometry and expressed relative to wild type control. +++++ is >75% of wild type, +++ (51-75%), ++ (25-50%), + (10-24%) and - if <10%. Each mutant was tested 2-4 times and scoring is the average value obtained from the individual experiments. Exposure times ranged from 1-7 days.

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Figure 3. G α 12 Binds to labeled A α and B α Subunits In Vitro. Autoradiogram of pull-down with GST-G α 12 or GST of [³⁵S] methionine labeled A α and B α that were in vitro translated (IVT) separately, then mixed together (A α +B α) and equilibrated with endogenous proteins in the rabbit reticulocyte lysate for 30 minutes at 23°C prior to analysis as described in Materials and Methods. Exposure time is 48h.

Figure 4. G α 12 Amino Acid Sequence, NAAIRS Mutants, and Summary of Binding Assays.

Amino acid sequence of G α 12 indicating sites of NAAIRS substitutions. The starting point for the mutants was myc-tagged QL α 12 cDNA and is described in Materials and Methods. The myc tag was inserted between proline 139 and valine 140 (mutant, *w*). The *w* sequence, therefore, was not converted to a NAAIRS mutant. Sextets replaced by the sequence NAAIRS are flanked by vertical bars. The activating Q-to-L substitution is indicated by a dashed square. Regions in which replacement by NAAIRS resulted in a 75-90% decrease in binding to A α are indicated by *clear boxes*, and those that resulted in a 90-100% decrease are indicated by *shaded boxes*. Mutants with >50% binding were tested twice and those with <50% binding (clear and shaded boxes) were tested three times.

Figure 5. Representative G α 12 Western Blots of NAAIRS Mutants with Intact and Disrupted A α Binding. **A.** Purification and immobilization of GST-fused A α . GST-A α and GST, previously expressed in bacteria and immobilized on glutathione-sepharose (see Materials and Methods) were subjected to SDS-PAGE and then stained using Coomassie blue. Molecular weight standards (in kDa) are indicated at right. **B.** Immunoblot analysis of G α 12 precipitation.

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Lysates from HEK293 cells expressing either myc-tagged QL α 12 or each indicated “NAAIRS” substitution mutant of this protein (e.g. *I-myc*, *DDD-myc*) were pulled down with GST alone or GST-A α as described in Materials and Methods. For each individual panel, the left lane (GST-A α) indicates G α 12 that was precipitated by GST-A α , the middle lane (GST) indicates G α 12 that was precipitated by GST alone, and the right lane (*load*) indicates a fraction (5%) of the HEK293 cell lysate that was set aside prior to precipitation.

Figure 6. QL α 12 Expression in MDCK cells Reduces Y-307 Phosphorylation.

Previously characterized, stably transfected MDCK II cells with inducible expression of G α 12 (wild type, WT) or a constitutively active mutant of G α 12 (QL) were cultured for 72h +/- doxycycline and analyzed by Western blotting as described in Materials and Methods. Equivalent amounts of total protein (50 μ g) were analyzed by SDS-PAGE and Western blotting with indicated antibodies. The nitrocellulose was sequentially stripped and reprobed. Band intensities were determined using NIH Image software and phosphotyrosine 307 density in - dox is compared to + dox control for WT and QL α 12-MDCK cells. Results are the mean of 4 separate experiments analyzed with GraphPad Prism software. Two-tailed T test indicated significant reduction (indicated with * p=0.03).

Figure 7. Crystal Structures of A α and G α 12 binding domains. (A,B) The PP2A-A α crystal structure was analyzed using Cn3d as described in Materials and Methods. The N and C termini are noted. In A, the G α 12 binding region (Repeats 7-10) is colored yellow within this hook-shaped structure. In B, the structure has been rotated to highlight an intra-repeat surface (the inside region where B and C subunits bind) and the inter-repeat loops on the outside. C. Crystal

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structure of G α 12 (amino acids 47-379), with the N terminus denoted by a yellow dash. The α 4 and α 5 helices and the β 6 sheet are noted with arrows, and the α 5 helix ends at the C terminus. The NAAIRS mutants with reduced or absent binding to A α are highlighted yellow. D. The identical structure in panel C revealing only the amino acids identified in the interaction sites. With the exception of *t-u*, the contact regions are predominantly within the C-terminal 60 amino acids and near the N terminus. Note that although the N-terminal structure is missing in the crystal, it is predicted to lie in close proximity to the C terminus. E. The structure in D has been rotated 90° and up, so that now the C terminus (α 5 helix) is projecting directly out of the image.

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	K_m (μ M)	V_{max} (nmol Phos/Min)
PP2A	242 +/- 109	1.7 +/- 0.3
PP2A+GST	337 +/-246	3.3 +/-1.2
PP2A+G α 12	123 +/-25	11.0 +/-0.6

Table 1. Summary of K_m and V_{max} values determined from substrate velocity analysis as described in Materials and Methods. Values are the mean +/- s.d. of three experiments performed in duplicate.

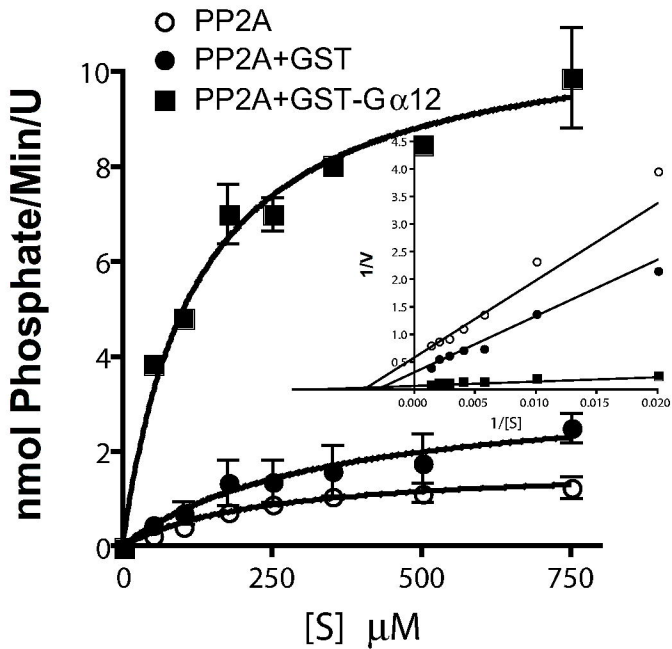
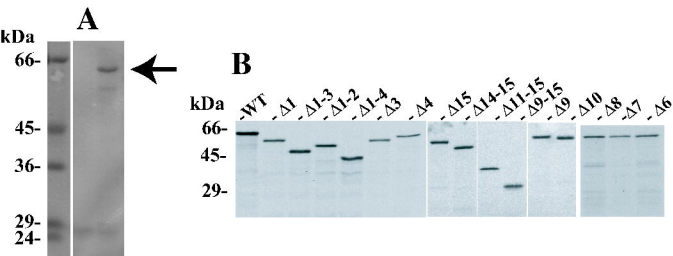


Figure 1



GST-
GST-G α 12-

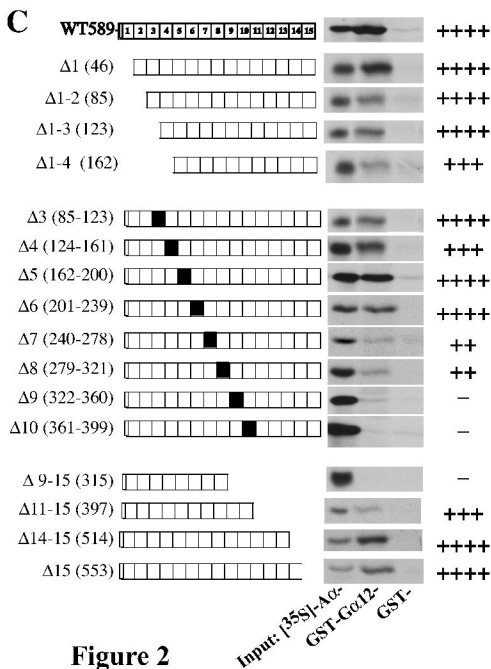
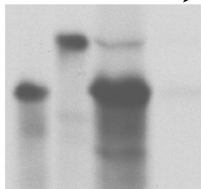


Figure 2

^{35}S] IVT: $\text{B}\alpha$ $\text{A}\alpha$ $\text{A}\alpha + \text{B}\alpha$ $\text{A}\alpha + \text{B}\alpha$



Input-
Input-
GST-Gc12-
GST-

Figure 3

N-terminus MS G

<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>
VVRTLS	RCLLPA	EAGARE	RRAGAA	RDAERE	ARRRSR
<i>g</i>	<i>h</i>	<i>i</i>	<i>j</i>	<i>k</i>	<i>l</i>
DIDALL	ARERRA	VRRLVK	ILLLGA	GESGKS	TFLKQM
<i>m</i>	<i>n</i>	<i>o</i>	<i>p</i>	<i>q</i>	<i>r</i>
RIIHGR	EFDQKA	LLEFRD	TIFDNI	LKGSRV	LVDARD
<i>s</i>	<i>t</i>	<i>u</i>	<i>v</i>	<i>w</i>	<i>x</i>
KLGI PW	QHSENE	KHGMFL	MAFENK	AGLPVE	PATFQL
<i>y</i>	<i>z</i>	<i>aa</i>	<i>bb</i>	<i>cc</i>	<i>dd</i>
YVPALS	ALWRDS	GIREAF	SRRSEF	QLGESV	KYFLDN
<i>ee</i>	<i>ff</i>	<i>gg</i>	<i>hh</i>	<i>ii</i>	<i>jj</i>
LDRIGQ	LNRYFPS	KQDILL	ARKATK	GIVEHD	FVIKKI
<i>kk</i>	<i>ll</i>	<i>mm</i>	<i>nn</i>	<i>oo</i>	<i>pp</i>
PFKMVD	VGGQRS	QRQKWF	QCFDGI	TSILFM	VSSSEY
<i>qq</i>	<i>rr</i>	<i>ss</i>	<i>tt</i>	<i>uu</i>	<i>vv</i>
DQVLME	DRRTNR	LVESMN	IFETIV	NNKLF	NVSIIL
<i>ww</i>	<i>xx</i>	<i>yy</i>	<i>zz</i>	<i>aaa</i>	<i>bbb</i>
FLNKMD	LLVEKV	KSVSIK	KHF PPF	KGD PHR	LEDVQR
<i>ccc</i>	<i>ddd</i>	<i>eee</i>	<i>fff</i>	<i>ggg</i>	<i>hhh</i>
YLVQCF	DRKRRN	RSKPLF	HHFTTA	IDTENI	RFVFHA
<i>iii</i>	<i>lij</i>				
VKDTIL	QENLKD	I ML Q – C-terminus			

Figure 4

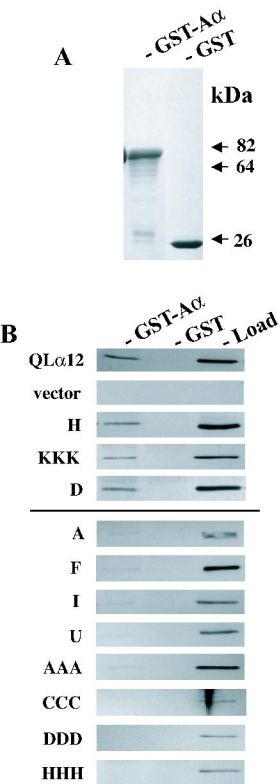
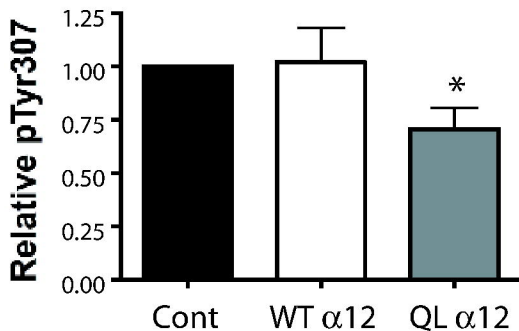
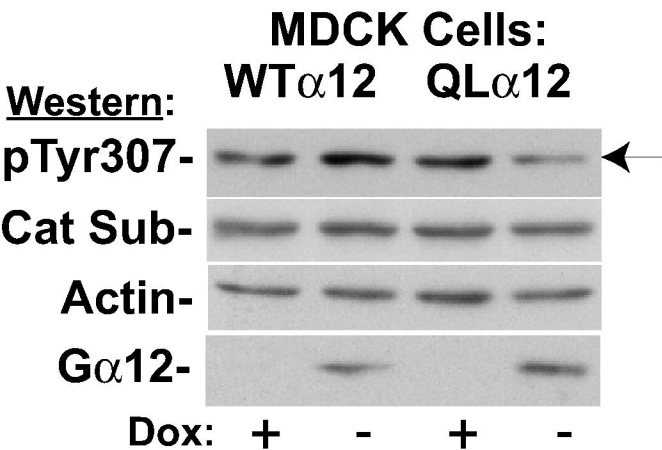


Figure 5



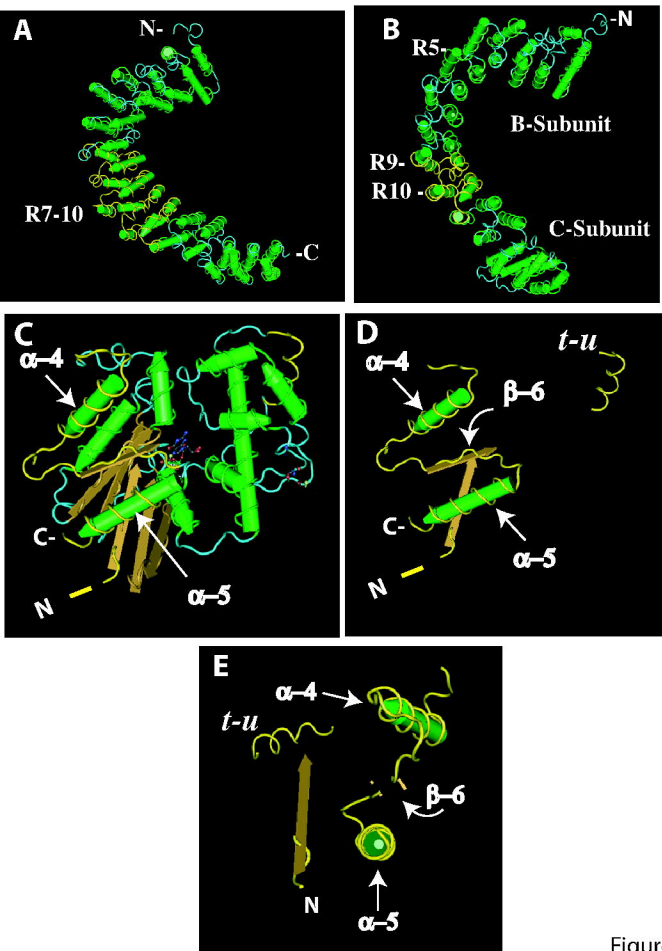


Figure 7