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Domains Necessary for Gα12 Binding and Stimulation of PP2A: is Gα12 a Novel Regulatory Subunit of PP2A?

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Running Title: Gα12 Regulation 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, Gα12. PP2A activity is altered in carcinogenesis and in some neurodegenerative diseases. We previously identified binding of Gα12 with the Aα subunit of PP2A, a trimeric enzyme composed of A (scaffolding), B (regulatory), and C (catalytic) subunits, and demonstrated Gα12 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-Gα12 with little effect on K_m. To identify the binding domains mediating the Aα-Gα12 interaction, an extensive mutational analysis was performed. Well-characterized mutations of Aα were expressed in vitro and tested for binding to GST-Gα12 in pull-down assays. Gα12 binds to Aα along repeats 7-10, and PP2A B subunits are not necessary for binding. To identify where Aα binds to Gα12, a series of 61 Gα12 mutants were engineered to contain the sequence asn-ala-ala-ile-arg-ser (NAAIRS) in place of 6 consecutive amino acids. Mutant Gα12 proteins were individually expressed in HEK cells, and analyzed for interaction with GST or GST-Aα in pull-downs assays. The Aα binding sites were localized to regions near the N and C terminus of Gα12. The expression of constitutively activated Gα12 (QLα12) in MDCK cells stimulated PP2A activity as determined by decreased phosphorylation of tyrosine-307 on the catalytic subunit. Based upon crystal structures of Gα12 and PP2A Aα, a model describing the binding surfaces and potential mechanisms of Gα12-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 Gα and Gβγ 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 Gα subunits with proteins that modulate Gα function. These include RGS proteins (Regulator of G protein signaling) that stimulate Gα GTPase activity and GPR proteins (G protein regulatory) proteins that inhibit GDP release (Hollinger and Hepler, 2002; Lanier, 2004). Gα12 and Gα13 comprise one of the four heterotrimeric G protein families that also include Gαs, Gαi/o, and Gαq. Gα12/13 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). Gα12 and Gα13 interact with the RGS domain of the Rho exchange factor p115-RhoGEF and Gα12 binds the RGS domain of axin (Kozasa et al., 1998; Stemmle et al., 2006). In addition, Gα12 and/or Gα13 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 Gα12 stimulation of PP2A phosphatase activity in vitro and in COS cells (Zhu et al., 2004). Many signaling pathways share involvement of Gα12 and PP2A, although until recently a direct link between these two proteins has been missing. Some cellular processes modulated by both Gα12/13 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 Gα12 in the tight junction (Dodane and Kachar, 1996; Meyer et al., 2003; Meyer et al., 2002) suggesting that both Gα12 and PP2A are localized within the same sub-cellular microdomain. Taken together, we hypothesize that Gα12 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 Gα12 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 Aα and

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Gα12. In addition, we provide evidence that Gα12 activation in cells stimulates PP2A, and in vitro kinetic analysis reveals that Gα12 stimulates V_{max} without significantly affecting K_m. Based on crystal structures of PP2A Aα and Gα12 subunits and the results of binding studies with mutant proteins, it is possible to propose a model of the mechanism by which Gα12 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 Gβγ purified from baculovirus was kindly provided by Dr. Thomas Michel (Brigham and Women's Hospital). Gα12 antibodies were purchased from Santa Cruz. Gα12 Tet-off MDCK cells and GST-Gα12 are previously described (Meyer et al., 2002). Glutathione-sepharose was from Amersham Pharmacia Biotech. Full-length mouse Aα cDNA was subcloned into pGEX using standard techniques and GST-Aα fusion protein was expressed and purified from *E.coli* as previously described (Luo and Denker, 1999). Substitution mutants within myc-tagged QLα12, 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-Gα12 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. Aα, Bα and mutant Aα cDNAs in pBS or pcDNA3 were in vitro translated using 0.5-1µg 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 Aα Subunits. ^{35}S-labeled Aα subunits were pre-cleared by incubation with glutathione agarose beads for 1h at 4^{o}C. Translates (5-20µl; depending upon translation efficiency) were incubated with GST or GST-Gα12 (0.5-1µg) 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 Aα was expressed as a fraction of the total and normalized to the wild type Aα control.

GST-Gα12 Pull-Downs of ^{35}S-labeled Aα and Bα Subunits. Aα and Bα 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 1µg of GST or GST-Gα12 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 Gα12 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% CO_2 atmosphere. For each Gα12 mutant, 7 µg 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 N_2 and stored at –80°C.

GST Pull-down of Gα12 Mutants. GST-Aα 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 Gα12 tested, 45 µL of HEK293 supernatant (prepared as described above in buffer A + 1% detergent) was diluted to 450 µL 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-Aα 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-Gα12 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 Gα12-expressing MDCK cells. Wild type Gα12 and QLα12-MDCK cells were cultured +/- doxycycline (dox) for 72h to induce Gα12 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 Na_3VO_4, 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 Gα12 crystal structure (1ZCA (Kreutz et al., 2006)) and Aα 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-Gα12 or recombinant Gα12, but not with several other purified Gα subunits (Zhu et al., 2004). In addition, there were no detectable differences in affinity of GDP-, GTPγS-, or AlF_4^- liganded Gα12 for Aα in immunoprecipitation studies, and recombinant Gα12 stimulated phosphatase activity similarly in the inactive and active conformations. Based upon the similar binding and stimulation of PP2A by GST-Gα12 and active and inactive purified Gα12, GST-Gα12 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-Gα12. Figure 1 shows the initial velocity versus substrate plot of mean PP2A activity +/- pre-incubation with GST (control) or GST-Gα12 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-Gα12. 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-Gα12 compared to GST was observed, with no significant effect on K_m. Preincubation of GST-Gα12 with GTPγS or Gβγ subunits had no demonstrable effect on the kinetics (results not shown). However, it is not known whether GST-Gα12 exchanges guanine nucleotides or interacts with Gβγ. Nevertheless, these findings are consistent with our previous observations with purified Gα12. Preincubation of Gα12 with GTPγS {Zhu, 2004 #773}, or Gβγ (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 Gα12 conformation or affected by Gα12 complexing with Gβγ.

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To gain additional insights into how Gα12 stimulates PP2A phosphatase activity, we sought to identify the amino acid regions of Aα and Gα12 necessary for the interaction. A series of well-characterized mutants of Aα (Ruediger et al., 1994; Ruediger et al., 1992) were [^{35}S]methionine labeled, incubated with GST or GST-Gα12 and analyzed in GST pull-down assays (Fig. 2). GST-Gα12 and GST proteins are shown in Figure 2A, and the [^{35}S]methionine labeled Aα mutants are shown in Figure 2B. The results of the pull-downs are shown in Figure 2C next to a schematic of each Aα mutant protein. Wild type Aα, 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 Aα 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 Aα 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 Aα 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 Aα-B56γ1-Cα confirms C subunit interaction with Aα repeats 11-15 but reveals B' subunit binding to repeats 2-7 (Cho and Xu, 2006). In mutational studies with various B subunits, Aα repeat 5 was required for all known B subunit interactions (Ruediger et al., 1999). The analysis shown in Figure 2 indicates that Gα12 binds to HEAT repeats 7-10 within Aα. There was a partial reduction in binding (25-50% of control) seen with deletion of repeat 7 (Δ7) or repeat 8 (Δ8), and there was complete loss

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of binding with deletion of repeat 9 (Δ9) or repeat 10 (Δ10). Deletion of N-terminal repeats had no effect on Gα12 binding (Δ1, Δ1-2, Δ1-3), and mutants with single repeats 3, 4, or 5 deleted also bound normally. The normal interaction of Gα12 with Δ5 indicates that B subunit binding is not required for the Gα12 interaction. Deletion of any single repeat between 11 and 15 prevents binding of the C-subunit (Ruediger et al., 1992), yet Gα12 interacted normally with Δ15 and Δ14-15, and was only slightly reduced with the larger Δ11-15 deletion. Consistent with the single repeat deletions, deletion of repeats 9-15 (Δ9-15) resulted in complete loss of binding to Gα12.

These results indicate that B subunit interactions are not required for Gα12 binding to the core enzyme. However, this does not exclude the possibility that Gα12 can interact with the PP2A holoenzyme (Aα-B-C). To test whether Gα12 may interact with the holoenzyme, Aα and Bα 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 Aα and/or Bα with GST or GST-Gα12 were analyzed for detection of labeled subunits with the precipitated protein complex. As shown in Figure 3, there is readily detectable Aα and Bα subunits in the GST-Gα12 pull-down that was not seen with GST. This finding suggests that Gα12 may interact with a holoenzyme in this assay, and we have not excluded the possibility of a direct Gα12-Bα interaction. The Bα signal was stronger than for Aα in this experiment, but because the amounts of endogenous Aα and Bα 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 Gα12 that are important for its interaction with PP2A Aα, we first engineered a series of amino acid substitution mutants that span the full length of Gα12. To produce each mutant, a sextet of consecutive amino acids in the primary sequence of myc-tagged QLα12 (constitutively active Gα12) was replaced by the sequence asn-ala-ala-ile-arg-ser ("NAAIRS"). This series of mutations was created in the QLα12 protein to facilitate the study of Gα12 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 Gα12 that was selectively uncoupled from a known binding partner, p115RhoGEF (Meigs et al., 2005). It was previously shown that wild type and QLα12 interact with PP2A Aα (Zhu et al., 2004). The myc-tagged QLα12 was used as starting material so that the subsequently engineered NAAIRS mutants could be distinguished from endogenous Gα12 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-Aα as described in Materials and Methods.

Next, an Aα GST fusion protein (GST-Aα) was constructed and purified as described in Materials and Methods. GST-Aα migrates at its predicted molecular weight of ~80kDa during SDS-PAGE (Fig. 5A). GST-Aα was tested for its ability to precipitate myc-tagged QLα12 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 QLα12 was included as an internal control and was precipitated by Aα in all experiments (Fig. 5B). While most of the NAAIRS mutants were precipitated by GST-Aα, several mutants displayed severely impaired or abolished binding to Aα (Fig. 5B, and summarized in Fig. 4). To provide a quantitative analysis of the relative affinity of these mutants for Aα, 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-QLα12 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 Aα. 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 Gα12 (see Fig. 4). As an additional control, myc-QLα12 and all NAAIRS mutants were tested for precipitation by glutathione-sepharose bound GST; this complex did not precipitate myc-QLα12 or any of its mutant variants (Fig. 5B).

Our next goal was to determine whether Gα12 regulates PP2A activity in cells. To address this question, we used well-characterized MDCK cells with inducible expression of Gα12 and QLα12 (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 Gα12 in MDCK cells is not readily detectable by Western blot with available anti-Gα12 antibodies. However, after 72h in the absence of doxycycline, QLα12 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 QLα12 expressing MDCK cells (-dox) that was not seen in + dox QLα12 cells (no QLα12 expression) or in wild type Gα12-MDCK cells +/- dox (Fig. 6). This finding is consistent with Gα12 activation of PP2A phosphatase activity in MDCK cells and our prior finding of Gα12/PP2A stimulated dephosphorylation of tau in transfected COS cells (Zhu et al., 2004).

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Discussion

We previously reported that Gα12 binds to the Aα subunit of PP2A and stimulates its activity (Zhu et al., 2004). We now identify the specific regions of Gα12 and Aα necessary for this interaction, and show that Gα12 binding to PP2A core enzyme stimulates catalytic activity with little effect on substrate affinity. Analysis of well-characterized deletions of individual HEAT repeats in Aα identifies repeats 7-10 as the critical region for Gα12 interaction. Gα12 binding does not require the B subunit to be bound, but in vitro, Gα12 may interact with the PP2A holoenzyme. Analysis of 61 distinct Gα12 mutants identifies regions near the N and C termini as important for Gα12 binding to Aα. Furthermore, using a cell culture model we provide evidence for Gα12-dependent activation of PP2A. Based upon these results and crystal structures of Gα 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 Aα 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 Aα deletion mutants shown in Figure 2 reveal a unique pattern of Gα12 interaction. Gα12 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. Gα12 binds Δ5 and wild type Aα with similar affinity, indicating that B subunit binding to the A-C core enzyme complex is not required for Gα12 interaction. Gα12 binding does not overlap on Aα with C subunit binding (repeats 11-15), a finding consistent with simultaneous binding of C subunit and Gα12 to Aα. This proximity is likely to be important for inducing conformational changes resulting in enhanced phosphatase activity. The B56γ1 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 Gα12 to the site immediately adjacent to the C subunit may permit direct interaction(s) of Gα12 with the C subunit.

The results shown in Figure 3 suggest that a tetrameric complex of Gα12 with the holoenzyme may form in vitro. Consistent with this model, the crystal structure of Aα-B56γ1-Cα reveals binding of B' to Aα repeats 2-7 (Cho and Xu, 2006) indicating that for this specific holoenzyme the Gα12 binding sites at repeats 8-10 should be accessible to Gα12 for binding. However, we have not excluded the possibility that Gα12 binds to Bα in the absence of Aα or C subunits. Furthermore, our analysis is unable to distinguish Gα12 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 Aα. Thus, the regulation of PP2A by B subunits and Gα12 may depend on the specific B subunit. A more refined mutational analysis using purified proteins will be necessary to determine whether Gα12 binds to Bα in the absence of Aα and C subunits whether Gα12 interacts with PP2A holoenzymes.

Molecular Pharmacology Fast Forward. Published on February 15, 2007 as DOI: 10.1124/mol.106.033555
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The mutational analysis of Gα12 indicates that regions near the N and C termini are the domains critical for binding Aα. This analysis was performed with a comprehensive set of mutations created in the QL (activated) Gα12 protein. The crystal structure of the Gα12 N terminus is not known, and the unique N-terminal structure of Gα12 is thought to inhibit Gα12 protein expression and crystallization (Kreutz et al., 2006). To crystallize Gα12, it was necessary to generate a chimeric Gα12 missing the first 46 amino acids and containing the first 28 amino acids of Gαi1 (Kreutz et al., 2006). The N-terminal Gα12 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 Aα. The N terminus of Gα subunits contributes to protein binding and lipid modifications important for membrane attachment (Busconi et al., 1997; Busconi and Denker, 1997); Gα12/13 are palmitoylated within this region (Jones and Gutkind, 1998). The N terminus of Gα subunits also binds to Gβγ (Denker et al., 1992). However, QLα12 has a low affinity for Gβγ, and the addition of Gβγ did not affect stimulation of PP2A phosphatase activity. This indicates that Gβγ and the N-terminal sites used for binding Gβγ are not critical for the interaction of Gα12 with Aα. 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 Gα12 N terminus is unknown, it is predicted to lie in close proximity to the C terminus (see Figure 7). The C terminus of Gα12 contained the largest region important for binding to Aα. Most of the region necessary for Aα binding comprises the Gα12 α4 helix, β6 sheet and α5 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 Aα. 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 Aα, but may induce secondary conformational changes in the α4-β6-α5 region. These in vitro observations do not exclude the possibility that Gα12 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 Gα12-expressing MDCK cells (Fig. 6), there was no change in tyrosine 307 phosphorylation with wild type Gα12, but with expression of QLα12 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 Gα12 stimulate PP2A in cells. The lack of an effect for wild type Gα12 on PP2A activity could result from the protein being denatured, although in other studies Gα12 reversibly interacts with the basolateral membrane (Meyer et al., 2002) and modulates paracellular flux (Meyer et al., 2003).

Activated Gα12 and Gα13 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 Gα12/13 may be important regulators of cellular phosphatases. Many studies have linked G protein signaling to protein kinase pathways, and the interactions of Gα12 and Gα13 with protein phosphatases suggest that G protein signaling through Gα12/13 may counter-regulate protein phosphorylation occurring in response to activation of other G protein signaling pathways. While Gα12/13 stimulation of protein phosphatases may be a broad regulatory mechanism, the modes of activation of PP5 vs PP2A by Gα12 appear to be distinct. PP5 is a single subunit phosphatase stimulated by both Gα12 and

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Gα13. In contrast, we did not detect Gα13 stimulation of PP2A (Zhu et al., 2004), although preliminary results indicate that Gα13 can bind to the GST-Aα fusion protein (not shown). In addition, there is no sequence homology between the TPR domain of PP5 and the HEAT repeats 7-10 of Aα.

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 Gα12 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 Gα12 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 Gα12 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 Gα12-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. Gα12 Stimulates V_{max} of PP2A Core Enzyme.

PP2A core enzyme (A-C) was pre-incubated with GST or GST-Gα12 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. Gα12 Binds to Repeats 7-10 on Aα.

A. Coomassie blue stained gel of GST-Gα12 and GST fusion proteins. B. Autoradiogram of [^{35}S] methionine labeled and in vitro translated wild type Aα and Aα mutants. 0.5-2µl of translate were analyzed by SDS-PAGE and autoradiography. Exposure time was 4-12h. White vertical lines indicate separate gels. C. Wild type (WT) Aα 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-Gα12 and GST is shown for each mutant in the adjacent panel. The input of labeled Aα 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. Gα12 Binds to labeled Aα and Bα Subunits In Vitro. Autoradiogram of pull-down with GST-Gα12 or GST of [^{35}S] methionine labeled Aα and Bα that were in vitro translated (IVT) separately, then mixed together (Aα+Bα) and equilibrated with endogenous proteins in the rabbit reticulocyte lysate for 30 minutes at 23^{o}C prior to analysis as described in Materials and Methods. Exposure time is 48h.

Figure 4. Gα12 Amino Acid Sequence, NAAIRS Mutants, and Summary of Binding Assays.

Amino acid sequence of Gα12 indicating sites of NAAIRS substitutions. The starting point for the mutants was myc-tagged QLα12 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 Aα 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 Gα12 Western Blots of NAAIRS Mutants with Intact and Disrupted Aα Binding. **A.** Purification and immobilization of GST-fused Aα. GST-Aα 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 Gα12 precipitation.

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Lysates from HEK293 cells expressing either myc-tagged QLα12 or each indicated "NAAIRS" substitution mutant of this protein (e.g. *I-myc*, *DDD-myc*) were pulled down with GST alone or GST-Aα as described in Materials and Methods. For each individual panel, the left lane (GST-Aα) indicates Gα12 that was precipitated by GST-Aα, the middle lane (GST) indicates Gα12 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. QLα12 Expression in MDCK cells Reduces Y-307 Phosphorylation. Previously characterized, stably transfected MDCK II cells with inducible expression of Gα12 (wild type, WT) or a constitutively active mutant of Gα12 (QL) were cultured for 72h +/- doxycycline and analyzed by Western blotting as described in Materials and Methods. Equivalent amounts of total protein (50μg) 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 QLα12-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 Aα and Gα12 binding domains. (A,B) The PP2A-Aα crystal structure was analyzed using Cn3d as described in Materials and Methods. The N and C termini are noted. In A, the Gα12 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 Gα12 (amino acids 47-379), with the N terminus denoted by a yellow dash. The α4 and α5 helices and the β6 sheet are noted with arrows, and the α5 helix ends at the C terminus. The NAAIRS mutants with reduced or absent binding to Aα 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^{o} and up, so that now the C terminus (α5 helix) is projecting directly out of the image.

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	K_m (μM)	V_{max} (nmol Phos/Min)
PP2A	242 +/- 109	1.7 +/- 0.3
PP2A+GST	337 +/-246	3.3 +/-1.2
PP2A+Gα12	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.

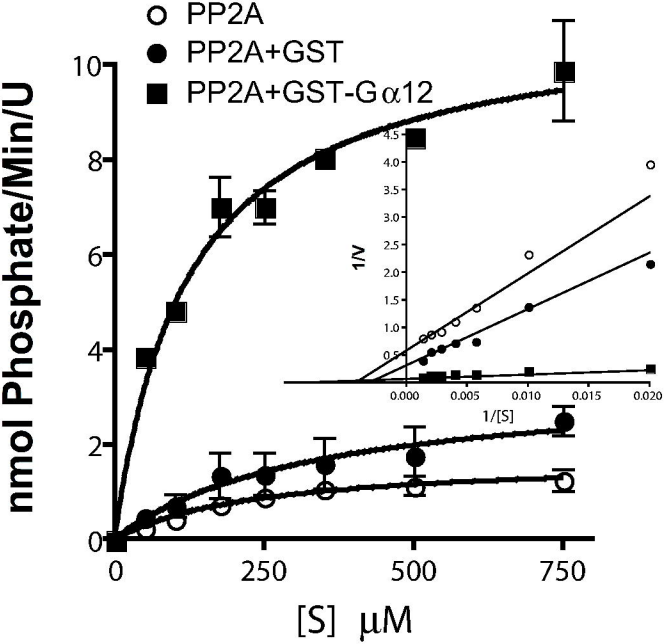
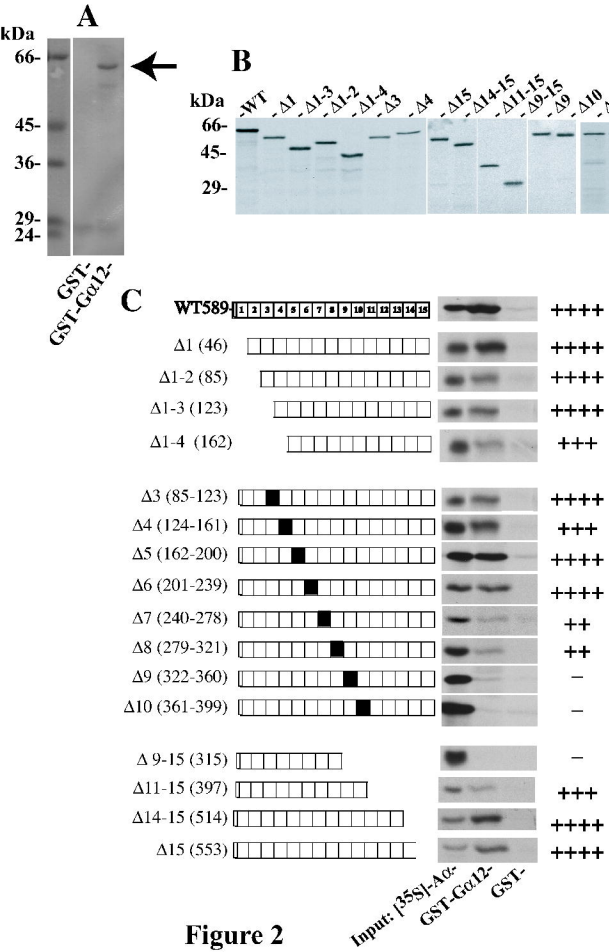


Figure 1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45-
36-
29-
24-
GST-
GST-Gα12-
B
kDa
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-Δ1-2
-Δ1-4
-Δ3
-Δ4
-Δ15
-Δ14-15
-Δ11-15
-Δ9-15
-Δ9
-Δ10
-Δ8
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Δ6 (201-239)
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Δ7 (240-278)
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Δ8 (279-321)
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Δ9 (322-360)
–
Δ10 (361-399)
–
Δ 9-15 (315)
–
Δ11-15 (397)
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Δ15 (553)
++++
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GST-Gα12-
GST-

Figure 2

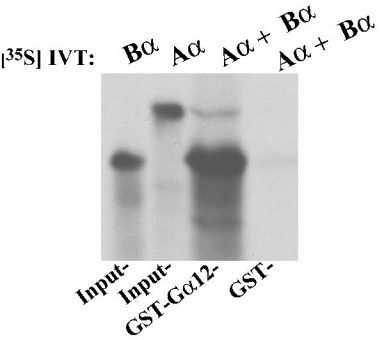
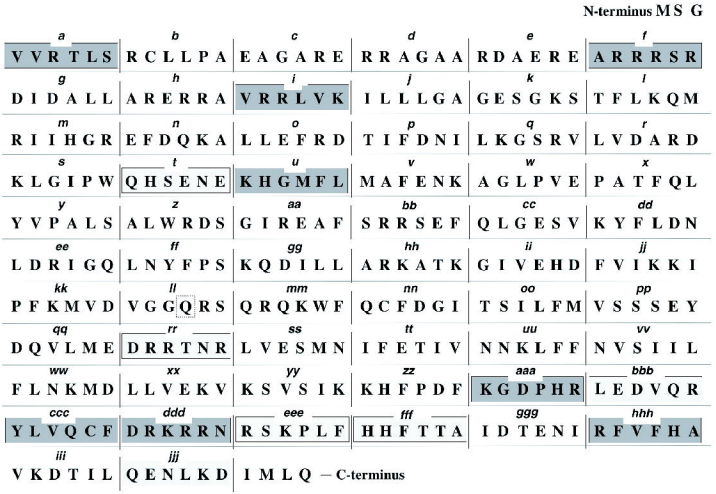


Figure 3


N-terminus M S G
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m n o p q r
R I I H G R E F D Q K A L L E F R D T I F D N I L K G S R V L V D A R D
s t u v w x
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Figure 4

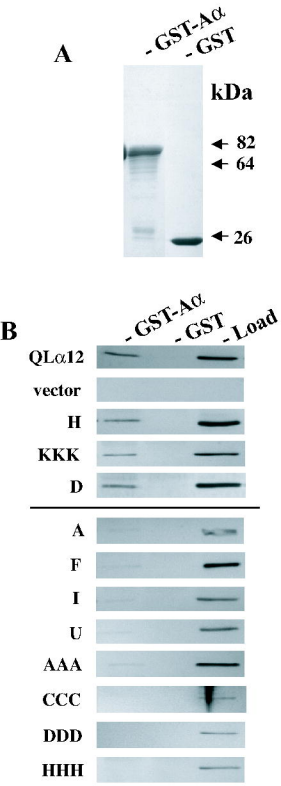
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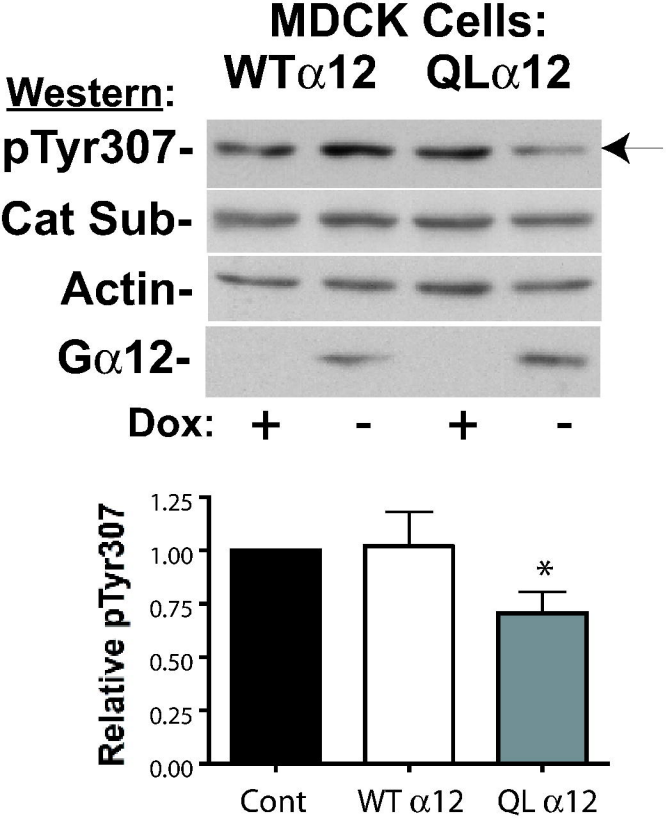
Figure 5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Relative pTyr307
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1.00
0.75
0.50
0.25
0.00
*
Cont
WT α12
QL α12

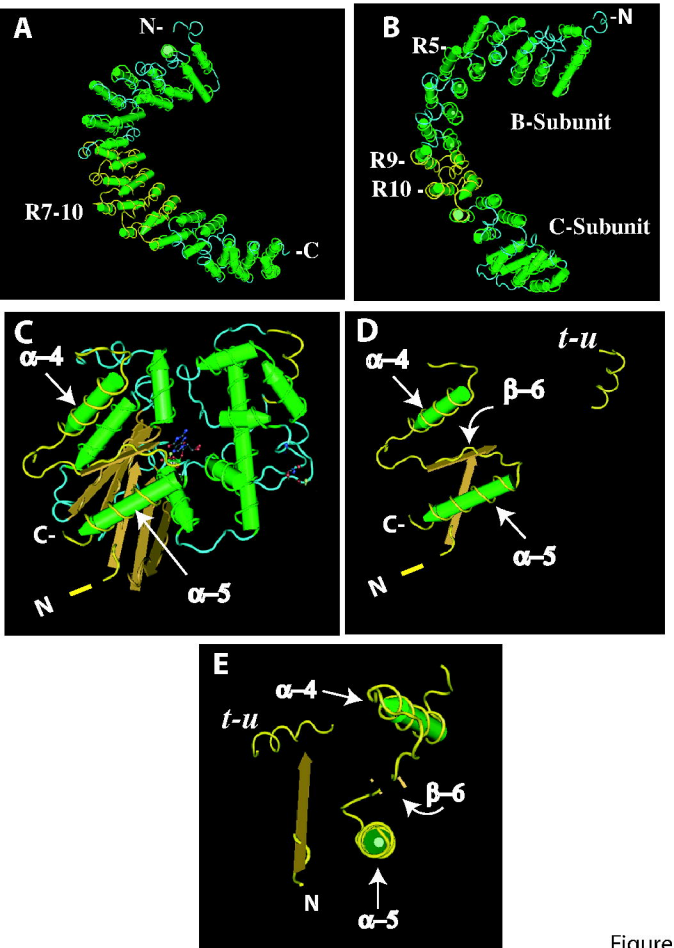
A
N-
R7-10
-C
B
-N
R5-
B-Subunit
R9-
R10 -
C-Subunit
C
α–4
C-
N
α–5
D
t-u
α–4
β–6
C-
α–5
N
E
α–4
t-u
β–6
N
α–5

Figure 7