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Domains Necessary for $G\alpha_{12}$ Binding and Stimulation of Protein Phosphatase-2A (PP2A): Is $G\alpha_{12}$ a Novel Regulatory Subunit of PP2A?

Deguang Zhu, Robert I. Tate, Ralf Ruediger, Thomas E. Meigs, and Bradley M. Denker

Renal Division, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts (B.M.D., D.Z.); Department of Biology, University of North Carolina at Asheville, Asheville, North Carolina (R.I.T., T.E.M.); and Department of Pathology, University of California at San Diego, La Jolla, California (R.R.)

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ABSTRACT

Many cellular signaling pathways share regulation by protein phosphatase-2A (PP2A), a widely expressed serine/threonine phosphatase, and the heterotrimeric G protein $G\alpha_{12}$. PP2A activity is altered in carcinogenesis and in some neurodegenerative diseases. We have identified binding of $G\alpha_{12}$ with the $A\alpha$ subunit of PP2A, a trimeric enzyme composed of A (scaffolding), B (regulatory), and C (catalytic) subunits and demonstrated that $G\alpha_{12}$ stimulated phosphatase activity (*J Biol Chem* **279:** 54983–54986, 2004). We now show in substrate-velocity analysis using purified PP2A that V_{max} was stimulated 3- to 4-fold by glutathione transferase (GST)-$G\alpha_{12}$ with little effect on K_m values. To identify the binding domains mediating the $A\alpha$-$G\alpha_{12}$ interaction, an extensive mutational analysis was performed. Well-characterized mutations of $A\alpha$ were expressed in vitro and tested for binding to GST-$G\alpha_{12}$ in pull-down assays. $G\alpha_{12}$ binds to $A\alpha$ along repeats 7 to 10, and PP2A B subunits are not necessary for binding. To identify where $A\alpha$ binds to $G\alpha_{12}$, a series of 61 $G\alpha_{12}$ mutants were engineered to contain the sequence Asn-Ala-Ala-Ile-Arg-Ser (NAAIRS) in place of 6 consecutive amino acids. Mutant $G\alpha_{12}$ proteins were individually expressed in human embryonic kidney cells and analyzed for interaction with GST or GST-$A\alpha$ in pull-down assays. The $A\alpha$ binding sites were localized to regions near the N and C termini of $G\alpha_{12}$. The expression of constitutively activated $G\alpha_{12}$ ($QL\alpha_{12}$) in Madin Darby canine kidney cells stimulated PP2A activity as determined by decreased phosphorylation of tyrosine 307 on the catalytic subunit. Based on crystal structures of $G\alpha_{12}$ and PP2A $A\alpha$, a model describing the binding surfaces and potential mechanisms of $G\alpha_{12}$-mediated PP2A activation is presented.

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 (Luttrell and Luttrell, 2004; El-Shewy et al., 2006). 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 regulator of G protein signaling (RGS) proteins that stimulate $G\alpha$ GTPase activity and G protein regulatory (GPR) 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

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ABBREVIATIONS: RGS, regulator of G protein signaling; PP2A, protein phosphatase-2A; MDCK, Madin Darby canine kidney; dox, doxycycline; HEAT repeat, 39 amino acid motif named for huntingtin, elongation, A subunit, target of rapamycin; HEK, human embryonic kidney; GST, glutathione transferase; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; GTPγS, guanosine 5[prime]-3-*O*-(thio)triphosphate; WT, wild type.

$G\alpha_{12}$ binds the RGS domain of axin (Kozasa et al., 1998; Stemmle 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, heat shock protein-90, the tight junction protein ZO-1, and E-cadherin (Jiang et al., 1998; Meigs et al., 2001; Vaiskunaite et al., 2001; Meyer et al., 2002; Waheed and Jones, 2002).

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 four 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 (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 demonstrated previously $G\alpha_{12}$ stimulation of PP2A phosphatase activity in vitro and in COS cells (Zhu et al., 2004). Many signaling pathways share involvement of $G\alpha_{12}$ and PP2A, although until recently a direct link between these two proteins has been missing. Some cellular processes modulated by both $G\alpha_{12/13}$ and PP2A include cellular transformation, growth, apoptosis, and stress responses. Furthermore, PP2A has been directly implicated in carcinogenesis and neurodegenerative diseases (including Alzheimer's) (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 $G\alpha_{12}$ in the tight junction (Dodane and Kachar, 1996; Meyer et al., 2002, 2003) suggesting that both $G\alpha_{12}$ and PP2A are localized within the same subcellular microdomain. Taken together, we hypothesize that $G\alpha_{12}$ regulation of PP2A is a critical signaling pathway important to diverse cellular functions and in the pathophysiology of certain disease states. To define the mechanism(s) of $G\alpha_{12}$ regulation of PP2A and provide the basis for pharmacological modulation of these signaling pathways, we have performed extensive mutagenesis and in vitro binding analyses to identify the requisite binding domains of Aα and $G\alpha_{12}$. In addition, we provide evidence that $G\alpha_{12}$ activation in cells stimulates PP2A, and in vitro kinetic analysis reveals that $G\alpha_{12}$ stimulates V_{max} without significantly affecting K_m values. Based on crystal structures of PP2A Aα and $G\alpha_{12}$ subunits and the results of binding studies with mutant proteins, it is possible to propose a model of the mechanism by which $G\alpha_{12}$ regulates PP2A function.

Materials and Methods

Chemicals, Antibodies, and cDNA Constructs. Purified PP2A core enzyme (A–C) was obtained from Upstate Biotechnology (Lake Placid, NY). Mouse β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO), and p-Tyr307 antibody was from Epitomics (Burlingame, CA). The rabbit C-subunit antibody was kindly provided by Dr. David Virshup (University of Utah, Salt Lake City, UT). Recombinant $G\beta\gamma$ purified from baculovirus was kindly provided by Dr. Thomas Michel (Brigham and Women's Hospital, Boston, MA). $G\alpha_{12}$ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). $G\alpha_{12}$ Tet-off MDCK cells and GST-$G\alpha_{12}$ were described previously (Meyer et al., 2002). Glutathione-Sepharose was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Full-length mouse Aα cDNA was subcloned into pGEX using standard techniques, and GST-Aα fusion protein was expressed and purified from *Escherichia coli* as described previously (Luo and Denker, 1999). Substitution mutants within myc-tagged QLα_{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 described previously (Zhu et al., 2004). In brief, phosphatase activity was determined by Malachite Green phosphatase assay (Upstate). 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-$G\alpha_{12}$ at 1:1 M ratios. A standard curve was generated in each assay, and PP2A activity was determined in duplicate for each concentration. The reaction was initiated by the addition of substrate at $t = 0$, and after 30 min, the reaction was terminated, absorbance was measured at 624 nm, and enzyme activity was determined (expressed in nanomoles of phosphate per minute per unit). K_m and V_{max} values were calculated from substrate-velocity curve analysis, and Lineweaver-Burk plots were generated using Prism software (GraphPad Software, San Diego, CA).

In Vitro Translation. Aα, Bα, and mutant Aα cDNAs in pBluescript or pcDNA3 were in vitro translated using 0.5 to 1 μg of plasmid, the appropriate RNA polymerase in a coupled rabbit reticulocyte translation system (TNT system; Promega, Madison, WI) plus [^{35}S]methionine (PerkinElmer Life and Analytical Sciences, Boston, MA) as described previously (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 precleared by incubation with glutathione agarose beads for 1 h at 4°C. Translates (5–20 μl, depending on translation efficiency) were incubated with GST or GST-$G\alpha_{12}$ (0.5–1 μg) for 3 h in buffer A (50 mM HEPES, pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM $MgSO_4$, and 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 (http://rsb.info.nih.gov/nih-image/) (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-$G\alpha_{12}$ 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 with 1 μg of GST or GST-$G\alpha_{12}$ for 3 h 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 $G\alpha_{12}$ Mutants from Human Embryonic Kidney Cell Lysates. Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and penicillin/streptomycin and were maintained at 37°C in a 5.0% CO_2 atmosphere. For each $G\alpha_{12}$ mutant, 7 μg of plasmid DNA were used to transfect a 10-cm dish of HEK293 cells at 60 to 80% confluence using Lipofectamine reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. At 48 h after transfection, cells were rinsed twice with phosphate-buffered saline, scraped from the dish, pelleted at 800g, 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 100,000g at 4°C for 1 h. Supernatants were snap-frozen in liquid N_2 and stored at −80°C.

GST Pull-Down of $G\alpha_{12}$ 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 $G\alpha_{12}$ 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%). Five percent 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 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, and 0.05% Tween 20) supplemented with 5% powdered milk for 2 h and then incubated with rabbit anti-$G\alpha_{12}$ antibody (at 1:500 dilution) for 2 h. After 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 nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate 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 (Eastman Kodak, Rochester, NY). 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 Tyr307 Phosphorylation in $G\alpha_{12}$-Expressing MDCK Cells. Wild-type $G\alpha_{12}$ and QLα_{12}-MDCK cells were cultured ± doxycycline (dox) for 72 h to induce $G\alpha_{12}$ expression using conditions described previously (Meyer et al., 2002). In brief, MDCK cell lysates were prepared by scraping cells in buffer B (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 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, Rockford, IL), and equivalent protein amounts were analyzed by SDS-PAGE and Western blots as described above.

Modeling of Crystal Structures. Cnd4 version 4.1 was obtained from http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml, and $G\alpha_{12}$ crystal structure (1ZCA; Kreutz et al., 2006) and Aα crystal structure (1B3U_A) were retrieved from the Protein Data Base 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 was adjusted in Adobe Photoshop and then labeled and assembled in Adobe Illustrator (Adobe Systems, Mountain View, CA).

Results

We demonstrated previously increased PP2A phosphatase activity when core enzyme (A–C) was incubated with GST-$G\alpha_{12}$ or recombinant $G\alpha_{12}$ 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 $G\alpha_{12}$ for Aα in immunoprecipitation studies, and recombinant $G\alpha_{12}$ stimulated phosphatase activity similarly in the inactive and active conformations. Based on the similar binding and stimulation of PP2A by GST-$G\alpha_{12}$ and baculovirus-purified $G\alpha_{12}$, GST-$G\alpha_{12}$ was used to determine K_m and V_{max} values for PP2A core enzyme from substrate-velocity and Lineweaver-Burk plots in the presence of equimolar GST or GST-$G\alpha_{12}$. Figure 1 shows the initial velocity versus substrate plot of mean PP2A activity ± preincubation with GST (control) or GST-$G\alpha_{12}$ in three 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-$G\alpha_{12}$. The K_m and V_{max} values for each condition were determined in GraphPad Prism as described under *Materials and Methods* and are summarized in Table 1. A 3- to 4-fold stimulation of V_{max} by GST-$G\alpha_{12}$ compared with GST was observed, with no significant effect on K_m values. Preincubation of GST-$G\alpha_{12}$ with GTPγS or G$\beta\gamma$ subunits had no demonstrable effect on the kinetics (results not shown). However, it is not known whether GST-$G\alpha_{12}$ exchanges guanine nucleotides or interacts with G$\beta\gamma$. Nevertheless, these findings are consistent with our previous observations with purified $G\alpha_{12}$. Preincubation of $G\alpha_{12}$ with GTPγS (Zhu et al., 2004), or G$\beta\gamma$ (data 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 on the activated $G\alpha_{12}$ conformation or affected by $G\alpha_{12}$ complexing with G$\beta\gamma$.

To gain additional insights into how $G\alpha_{12}$ stimulates PP2A phosphatase activity, we sought to identify the amino acid regions of Aα and $G\alpha_{12}$ necessary for the interaction. A series of well-characterized mutants of Aα (Ruediger et al., 1992, 1994) were [^{35}S]methionine-labeled, incubated with GST or GST-$G\alpha_{12}$, and analyzed in GST pull-down assays (Fig. 2). GST-$G\alpha_{12}$ and GST proteins are shown in Fig. 2A, and the [^{35}S]methionine-labeled Aα mutants are shown in Fig. 2B. The results of the pull-downs are shown in Fig. 2C next to a schematic of each Aα mutant protein. Wild-type Aα, consisting of 589 amino acids, 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, target of rapamycin) each composed of approximately 39 amino acids. The repeats in the Aα subunit

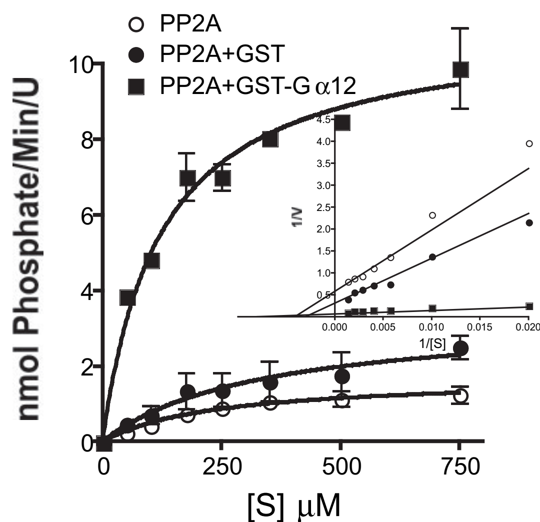

Fig. 1. $G\alpha_{12}$ stimulates the V_{max} value of PP2A core enzyme. PP2A core enzyme (A–C) was preincubated with GST or GST-$G\alpha_{12}$ at a 1:1 M ratio, and substrate velocity was determined over a range of substrate concentrations as described under *Materials and Methods*. Results are the mean of three experiments, each done in duplicate. The Lineweaver-Burk plot was obtained using GraphPad Prism and is shown as an inset. The summary of V_{max} and K_m values is shown in Table 1.

were proposed to be composed of two superimposed α-helices yielding an elongated molecule often described as a hook (Fig. 7) (Ruediger et al., 1992). This structure was subsequently confirmed, and molecular details were 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., 1992, 1994). C subunits interact with repeats 11 to 15 whereas the heterogeneous regulatory B subunits require repeats 1 to 10 for binding. The recent crystal structure of trimeric Aα-B56γ1-Cα confirms C subunit interaction with Aα repeats 11 to 15 but reveals B′ subunit binding to repeats 2 to 7 (Cho and Xu, 2007). 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 Fig. 2 indicates that $G\alpha_{12}$ binds to HEAT repeats 7 to 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 of binding with deletion of repeat 9 (Δ9) or repeat 10 (Δ10). Deletion of N-terminal repeats had no effect on $G\alpha_{12}$ binding (Δ1, Δ1–2, and Δ1–3), and mutants with single repeats 3, 4, or 5 deleted also bound normally. The normal interaction of $G\alpha_{12}$ with Δ5 indicates that B subunit binding is not required for the $G\alpha_{12}$ interaction. Deletion of any single repeat between 11 and 15 prevents binding of the C subunit (Ruediger et al., 1992), yet $G\alpha_{12}$ 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 to 15 (Δ9–15) resulted in complete loss of binding to $G\alpha_{12}$.

TABLE 1

Summary of K_m and V_{max} values determined from substrate velocity analysis as described under *Materials and Methods*

Values are the mean ± S.D. of three experiments performed in duplicate.

	K_m	V_{max}
	μM	*nmol phosphate/min*
PP2A	242 ± 109	1.7 ± 0.3
PP2A + GST	337 ± 246	3.3 ± 1.2
PP2A + $G\alpha_{12}$	123 ± 25	11.0 ± 0.6

These results indicate that B subunit interactions are not required for $G\alpha_{12}$ binding to the core enzyme. However, this does not exclude the possibility that $G\alpha_{12}$ can interact with the PP2A holoenzyme (Aα–B–C). To test whether $G\alpha_{12}$ 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 shown previously; Ruediger et al., 1992). Pull-down experiments of labeled Aα and/or Bα with GST or GST-$G\alpha_{12}$ were analyzed for detection of labeled subunits with the precipitated protein complex. As

Fig. 2. $G\alpha_{12}$ binds to repeats 7 to 10 on Aα. 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 Aα and Aα mutants. Translates (0.5–2 μl) were analyzed by SDS-PAGE and autoradiography. Exposure time was 4 to 12 h. 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-$G\alpha_{12}$ 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. ++++, >75% of wild type; +++, 51 to 75%; ++, 25 to 50%; +, 10 to 24%; and −, <10%. Each mutant was tested two to four times, and scoring is the average value obtained from the individual experiments. Exposure times ranged from 1 to 7 days.

shown in Fig. 3, there is readily detectable Aα and Bα subunits in the GST-Gα_{12} pull-down that was not seen with GST. This finding suggests that Gα_{12} may interact with a holoenzyme in this assay, and we have not excluded the possibility of a direct Gα_{12}-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.

To map the determinants within Gα_{12} 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 Gα_{12}. To produce each mutant, a sextet of consecutive amino acids in the primary sequence of myc-tagged QLα_{12} (constitutively active Gα_{12}) was replaced by the sequence NAAIRS. This series of mutations was created in the QLα_{12} protein to facilitate the study of Gα_{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 because of 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 Gα_{12} that was selectively uncoupled from a known binding partner, p115RhoGEF (Meigs et al., 2005). It was shown previously that wild type and QLα_{12} interact with PP2A Aα (Zhu et al., 2004). The myc-tagged QLα_{12} was used as starting material so that the subsequently engineered NAAIRS mutants could be distinguished from endogenous Gα_{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* (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 because of 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 under *Materials and Methods*.

Next, an Aα GST fusion protein (GST-Aα) was constructed and purified as described under *Materials and Methods*. GST-Aα migrates at its predicted molecular mass of ~80 kDa during SDS-PAGE (Fig. 5A). GST-Aα was tested for its ability to precipitate myc-tagged QLα_{12} and the full panel of NAAIRS substitution mutants. Seven or eight mutants were screened in each experiment, and myc-tagged QLα_{12} was included as an internal control and was precipitated by Aα in all experiments (Fig. 5B). Whereas 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 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 with the same ratio determined for myc-QLα_{12} within the same experiment. These results are summarized in Fig. 4. The majority of the NAAIRS mutants showed a normalized ratio that was greater than 50% of the control value (no marking in Fig. 4), but several mutants displayed a dramatic loss of

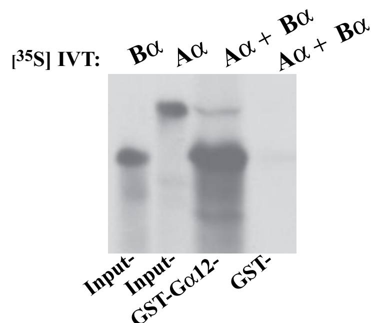

Fig. 3. Gα_{12} binds to labeled Aα and Bα subunits in vitro. Autoradiogram of pull-down with GST-Gα_{12} or GST of [^{35}S]methionine-labeled Aα and Bα that were in vitro-translated (IVT) separately and then mixed together (Aα + Bα) and equilibrated with endogenous proteins in the rabbit reticulocyte lysate for 30 min at 23°C before analysis as described under *Materials and Methods*. Exposure time is 48 h.

N-terminus M S G

a V V R T L S	*b* R C L L P A	*c* E A G A R E	*d* R R A G A A	*e* R D A E R E	*f* A R R R S R
g D I D A L L	*h* A R E R R A	*i* V R R L V K	*j* I L L L G A	*k* G E S G K S	*l* T F L K Q M
m R I I H G R	*n* E F D Q K A	*o* L L E F R D	*p* T I F D N I	*q* L K G S R V	*r* L V D A R D
s K L G I P W	*t* Q H S E N E	*u* K H G M F L	*v* M A F E N K	*w* A G L P V E	*x* P A T F Q L
y Y V P A L S	*z* A L W R D S	*aa* G I R E A F	*bb* S R R S E F	*cc* Q L G E S V	*dd* K Y F L D N
ee L D R I G Q	*ff* L N Y F P S	*gg* K Q D I L L	*hh* A R K A T K	*ii* G I V E H D	*jj* F V I K K I
kk P F K M V D	*ll* V G G Q R S	*mm* Q R Q K W F	*nn* Q C F D G I	*oo* T S I L F M	*pp* V S S S E Y
qq D Q V L M E	*rr* D R R T N R	*ss* L V E S M N	*tt* I F E T I V	*uu* N N K L F F	*vv* N V S I I L
ww F L N K M D	*xx* L L V E K V	*yy* K S V S I K	*zz* K H F P D F	*aaa* K G D P H R	*bbb* L E D V Q R
ccc Y L V Q C F	*ddd* D R K R R N	*eee* R S K P L F	*fff* H H F T T A	*ggg* I D T E N I	*hhh* R F V F H A
iii V K D T I L	*jjj* Q E N L K D	I M L Q — C-terminus			

Fig. 4. Gα_{12} amino acid sequence, NAAIRS mutants, and summary of binding assays. Amino acid sequence of Gα_{12} indicating sites of NAAIRS substitutions. The starting point for the mutants was myc-tagged QLα_{12} cDNA and is described under *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 to 90% decrease in binding to Aα are indicated by open boxes, and those that resulted in a 90 to 100% decrease are indicated by shaded boxes. Mutants with >50% binding were tested twice, and those with <50% binding (open and shaded boxes) were tested three times.

interaction with $A\alpha$. Mutants showing moderate-to-severe impairment of interaction (*t, rr, bbb, eee, fff,* and *jjj*; 10–25% of control) are shown in open boxes in Fig. 4, and those with abolished interactions (<10% of control) are highlighted in the gray boxes in Fig. 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 $G\alpha_{12}$ (Fig. 4). As an additional control, myc-$QL\alpha_{12}$ and all NAAIRS mutants were tested for precipitation by glutathione-Sepharose bound GST; this complex did not precipitate myc-$QL\alpha_{12}$ or any of its mutant variants (Fig. 5B).

Our next goal was to determine whether $G\alpha_{12}$ regulates PP2A activity in cells. To address this question, we used well-characterized MDCK cells with inducible expression of $G\alpha_{12}$ and $QL\alpha_{12}$ (Meyer et al., 2002, 2003). PP2A activity can be assessed by the relative tyrosine phosphorylation on the catalytic subunit (Tyr307), and decreased phosphorylation is associated with increased PP2A activity (Chen et al., 1992). Figure 6 shows that endogenous $G\alpha_{12}$ in MDCK cells is not readily detectable by Western blot with available anti-$G\alpha_{12}$ antibodies. However, after 72 h in the absence of doxycycline, $QL\alpha_{12}$ 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 $QL\alpha_{12}$-expressing MDCK cells (−dox) that was not seen in +dox $QL\alpha_{12}$ cells (no $QL\alpha_{12}$ expression) or in wild-type $G\alpha_{12}$-MDCK cells ± dox (Fig. 6). This finding is consistent with $G\alpha_{12}$ activation of PP2A phosphatase activity in MDCK cells and our prior finding of $G\alpha_{12}$/PP2A-stimulated dephosphorylation of the microtubule-associated protein tau in transfected COS cells (Zhu et al., 2004).

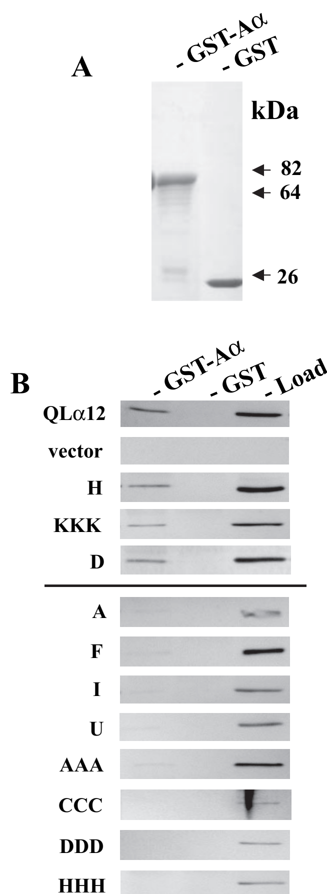

Fig. 5. Representative $G\alpha_{12}$ Western blots of NAAIRS mutants with intact and disrupted $A\alpha$ binding. A, purification and immobilization of GST-fused $A\alpha$. GST-$A\alpha$ and GST, previously expressed in bacteria and immobilized on glutathione-Sepharose (see *Materials and Methods*), were subjected to SDS-PAGE and then stained using Coomassie blue. Molecular mass standards (in kilodaltons) are indicated on the right. B, immunoblot analysis of $G\alpha_{12}$ precipitation. Lysates from HEK293 cells expressing either myc-tagged $QL\alpha_{12}$ or each indicated NAAIRS substitution mutant of this protein (e.g., *I-myc*, *DDD-myc*) were pulled down with GST alone or GST-$A\alpha$ as described under *Materials and Methods*. For each panel, the left lane (GST-$A\alpha$) indicates $G\alpha_{12}$ that was precipitated by GST-$A\alpha$, the middle lane (GST) indicates $G\alpha_{12}$ that was precipitated by GST alone, and the right lane (load) indicates a fraction (5%) of the HEK293 cell lysate that was set aside before precipitation.

Discussion

We reported previously that $G\alpha_{12}$ binds to the $A\alpha$ subunit of PP2A and stimulates its activity (Zhu et al., 2004). We now identify the specific regions of $G\alpha_{12}$ and $A\alpha$ necessary for this interaction and show that $G\alpha_{12}$ binding to PP2A core enzyme stimulates catalytic activity with little effect on substrate affinity. Analysis of well-characterized deletions of individual HEAT repeats in $A\alpha$ identifies repeats 7 to 10 as the critical region for $G\alpha_{12}$ interaction. $G\alpha_{12}$ binding does not require the B subunit to be bound, but in vitro, $G\alpha_{12}$ may interact with the PP2A holoenzyme. Analysis of 61 distinct $G\alpha_{12}$ mutants identifies regions near the N and C termini as important for $G\alpha_{12}$ binding to $A\alpha$. Furthermore, using a cell

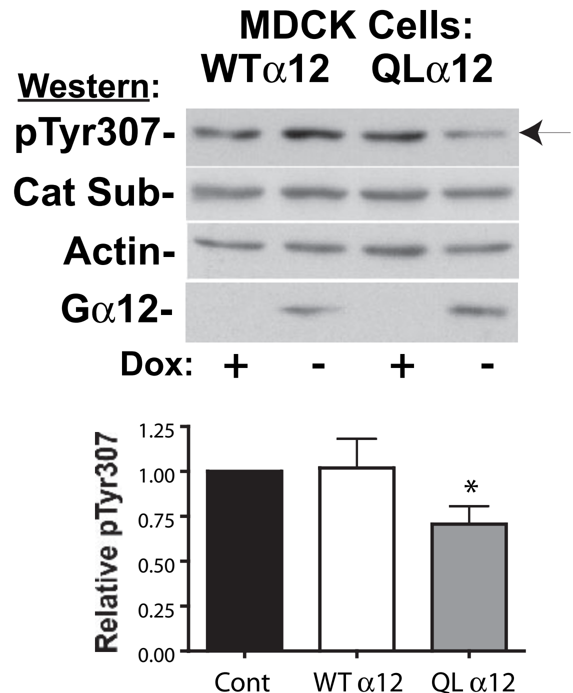

Fig. 6. $QL\alpha_{12}$ expression in MDCK cells reduces Tyr307 phosphorylation. Previously characterized, stably transfected MDCK II cells with inducible expression of $G\alpha_{12}$ (WT) or a constitutively active mutant of $G\alpha_{12}$ (QL) were cultured for 72 h ± doxycycline and analyzed by Western blotting as described under *Materials and Methods*. Equivalent amounts of total protein (50 μg) 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 with +dox control for WT and $QL\alpha_{12}$-MDCK cells. Results are the mean of four separate experiments analyzed with GraphPad Prism software. Two-tailed t test indicated significant reduction; *, $p = 0.03$.

culture model we provide evidence for Gα_{12}-dependent activation of PP2A. Based on 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 (Janssens and Goris, 2001). SV40 small-T antigen requires Aα repeats 3 to 6, and Polyoma small T and middle T antigens require repeats 2 to 8 (Ruediger et al., 1992, 1994). The results of the binding experiments with Aα deletion mutants shown in Fig. 2 reveal a unique pattern of Gα_{12} interaction. Gα_{12} binds to repeats 7 to 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 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. Gα_{12} 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 Gα_{12} interaction. Gα_{12} binding does not overlap on Aα with C subunit binding (repeats 11–15), a finding consistent with simultaneous binding of C subunit and Gα_{12} 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, 2007). In an analogous manner, the binding of Gα_{12} to the site immediately adjacent to the C subunit may permit direct interaction(s) of Gα_{12} with the C subunit.

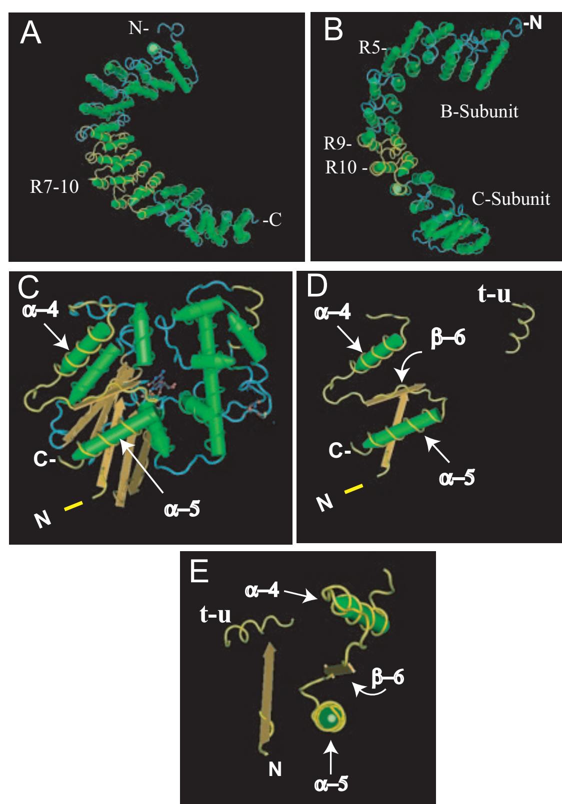

Fig. 7. Crystal structures of Aα and Gα_{12} binding domains. A and B, the PP2A-Aα crystal structure was analyzed using Cn3d as described under *Materials and Methods*. The N and C termini are noted. In A, the Gα_{12} binding region (repeats 7–10) is colored yellow within this hook-shaped structure. In B, the structure has been rotated to highlight an intrarepeat surface (the inside region where B and C subunits bind) and the inter-repeat loops on the outside. Repeats 9 (R9) and 10 (R10) are shown in yellow. C, crystal structure of Gα_{12} (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 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 (α5 helix) is projecting directly out of the image.

The results shown in Fig. 3 suggest that a tetrameric complex of Gα_{12} 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 to 7 (Cho and Xu, 2007), indicating that for this specific holoenzyme, the Gα_{12} binding sites at repeats 8 to 10 should be accessible to Gα_{12} for binding. However, we have not excluded the possibility that Gα_{12} binds to Bα in the absence of Aα or C subunits. Furthermore, our analysis is unable to distinguish Gα_{12} binding to the intrarepeat 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 Gα_{12} may depend on the specific B subunit. A more refined mutational analysis using purified proteins will be necessary to determine whether Gα_{12} binds to Bα in the absence of Aα and C subunits and whether Gα_{12} interacts with PP2A holoenzymes.

The mutational analysis of Gα_{12} 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) Gα_{12} protein. The crystal structure of the Gα_{12} N terminus is not known, and the unique N-terminal structure of Gα_{12} is believed to inhibit Gα_{12} protein expression and crystallization (Kreutz et al., 2006). To crystallize Gα_{12}, it was necessary to generate a chimeric Gα_{12} missing the first 46 amino acids and containing the first 28 amino acids of Gα_{i1} (Kreutz et al., 2006). The N-terminal Gα_{12} sequence missing from the crystal structure contains 2 of 8 regions in which NAAIRS mutants (*a* and *f*; Fig. 4) resulted in nearly complete loss of binding to Aα. The N terminus of Gα subunits contributes to protein binding and contains lipid modifications important for membrane attachment (Busconi and Denker, 1997; Busconi et al., 1997); G$\alpha_{12/13}$ are palmitoylated within this region (Jones and Gutkind, 1998). The N terminus of Gα subunits also binds to G$\beta\gamma$ (Denker et al., 1992). However, QLα_{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 Gα_{12} 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

$G\alpha_{12}$ N terminus is unknown, it is predicted to lie in close proximity to the C terminus (Fig. 7). The C terminus of $G\alpha_{12}$ contained the largest region important for binding to $A\alpha$. Most of the region necessary for $A\alpha$ binding comprises the $G\alpha_{12}$ $\alpha4$ helix, $\beta6$ sheet, and $\alpha5$ helix that ends at the C terminus. This region forms an exposed surface (Fig. 7, C–E) available for potential interaction with repeats 7 to 10 of $A\alpha$. NAAIRS mutants *t* and *u* are located apart from the N- and C-terminal binding regions (Fig. 7, C–E) and are not likely to directly contribute to interactions with $A\alpha$ but may induce secondary conformational changes in the $\alpha4$-$\beta6$-$\alpha5$ 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, 2003) through an interaction with the 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. Although $G\alpha_{12/13}$ stimulation of protein phosphatases may be a broad regulatory mechanism, the modes of activation of PP5 versus PP2A by $G\alpha_{12}$ seem to be distinct. PP5 is a single subunit phosphatase stimulated by both $G\alpha_{12}$ and $G\alpha_{13}$. In contrast, we did not detect $G\alpha_{13}$ stimulation of PP2A (Zhu et al., 2004), although preliminary results indicate that $G\alpha_{13}$ can bind to the GST-$A\alpha$ fusion protein (data not shown). In addition, there is no sequence homology between the tetratricopeptide repeat domain of PP5 and the HEAT repeats 7 to 10 of $A\alpha$.

The numerous functions of PP2A require sophisticated 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 $G\alpha_{12}$ 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 $G\alpha_{12}$ 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 on the specific B subunit in the PP2A trimer. The question of whether there are differences in PP2A stimulation in cells by active and inactive $G\alpha_{12}$ requires additional study. The findings in this report provide the basis for future studies to dissect aberrant signaling seen in some pathological conditions. For example, the $G\alpha_{12}$-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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Address correspondence to: Dr. Bradley M. Denker, Brigham and Women's Hospital, Harvard Institutes of Medicine, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail: bdenker@rics.bwh.harvard.edu

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