Gα12 Directly Interacts with PP2A

EVIDENCE FOR Gα12-STIMULATED PP2A PHOSPHATASE ACTIVITY AND DEPHOSPHORYLATION OF MICROTUBULE-ASSOCIATED PROTEIN, Tau

Received for publication, October 26, 2004 Published, JBC Papers in Press, November 3, 2004, DOI 10.1074/jbc.C400508200

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The Gα12/13 family of heterotrimeric G proteins modulate multiple cellular processes including regulation of the actin cytoskeleton. Gα12/13 interact with several cytoskeletal/scaffolding proteins, and in a yeast two-hybrid screen with Gα12, we detected an interaction with the scaffolding subunit (Aα) of the Ser/Thr phosphatase, protein phosphatase 2A (PP2A). PP2A dephosphorylates multiple substrates including tau, a microtubule-associated protein that is hyperphosphorylated in neurofibrillary tangles. The interaction of Aα and Gα12 was confirmed by coimmunoprecipitation studies in transfected COS cells and by glutathione S-transferase (GST)-Gα12 pull-downs from cell lysates of primary neurons. The interaction was specific for Aα and Gα12, and was independent of Gα13. Endogenous Aα and Gα12 colocalized by immunofluorescent microscopy in Caco-2 cells and in neurons. In vitro reconstitution of GST-Gα12 or recombinant Gα12 with PP2A core enzyme resulted in ~300% stimulation of PP2A activity that was not detected with other Ga subunits and was similar with GTPγS- and GDP-ligated Gα12. When tau and active kinase (CdK5 and p25) were cotransfected in to COS cells, there was robust tau phosphorylation. Co-expression of wild type or QLα12 with tau and the active kinase resulted in 60 ± 15% reductions in tau phosphorylation. In primary cortical neurons stimulated with lysophosphatidic acid, a 50% decrease in tau phosphorylation was observed. The Gα12 effect on tau phosphorylation was inhibited by the PP2A inhibitor, okadaic acid (50 μM), in COS cells and neurons. Taken together, these findings reveal novel, direct regulation of PP2A activity by Gα12 and potential in vivo modulation of PP2A target proteins including tau.

* This work was supported by National Institutes of Health Grants GM55223 (to B. M. D.) and CA100869 (to T. E. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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‡‡ The abbreviations used are: G protein, guanine nucleotide-binding protein; CdK, cyclin-dependent kinase; PP, protein phosphatase; FITC, fluorescein isothiocyanate; LPA, lysophosphatidic acid; GSK, glycogen synthase; GST, glutathione S-transferase; GFP, green fluorescent protein; GTPγS, guanosine 5’-O-(thiotriphosphate); HBSS, Hanks’ balanced salt solution.

Heterotrimeric G proteins regulates many fundamental cellular responses, and the canonical receptor-G protein-effector paradigm has become significantly more complex in recent years. G protein signaling is modulated through interactions with families of regulatory proteins that affect nucleotide binding and hydrolysis. These include the RGS (regulators of G protein signaling) protein family that stimulate Ga GTPase activity (reviewed in Ref. 1), and the GPR (G protein regulatory) protein family that share a conserved motif that inhibits GDP release from the Ga/a families of Go subunits (2–4). Gα12/13 have multiple cellular functions including regulation of the actin cytoskeleton (5) and many functions are shared by both Gα12 and Gα13 subunits. Gα12/13 regulation of the actin cytoskeleton and stress fiber formation occurs through direct interactions with Rho regulatory proteins (6, 7), and other aspects of Gα12/13 signaling are regulated through specific interactions with membrane or scaffolding proteins. For example, the binding of Gα12 and Gα13 to the C terminus of E-cadherin displaces β-catenin permitting it to signal (8, 9). We identified direct binding of both wild type and constitutively active QLα12 to ZO-1 and regulation of paracellular permeability of Madin-Darby canine kidney cells (10, 11). In addition, Gα12 interacts with AKAP-lbc, a scaffolding molecule that organizes components of cAMP signaling and Rho signaling machinery (12). Here, we have identified the Aα subunit of Ser/Thr phosphatase PP2A as another “scaffolding” protein that selectively interacts with Gα12.

Tau is a microtubule-associated protein that is predominantly expressed in neurons and functions to stabilize the cytoskeleton. Tau phosphorylation is necessary for microtubule binding and other protein interactions, but hyperphosphorylated tau is the predominant component of neurofibrillary tangles, a pathologic hallmark finding found in several neurodegenerative disorders including Alzheimer disease and FTDP-17 (frontotemporal dementia and Parkinson’s disease linked to chromosome 17; reviewed in Ref. 13). Several kinases phosphorylate tau including mitogen-activated protein kinase (MAP), glycogen synthase 3β (GSK-3β), tau-tubulin kinase, and cyclin-dependent kinases 2 and 5 (CdK) (reviewed in Ref. 13). Likewise, several protein phosphatases (PP) of the 1, 2A, and 2B families can dephosphorylate tau proteins in vitro, and PP2A directly binds to tau and microtubules (14). To determine the relevance of the yeast two-hybrid interaction between Gα12 and the Aα subunit of PP2A, we demonstrate direct and specific Gα12-dependent stimulation of PP2A activity in vitro. Furthermore, we show that Gα12-mediated signaling in COS cells and primary cultured neurons stimulates PP2A-mediated dephosphorylation of the microtubule-binding protein, tau.

EXPERIMENTAL PROCEDURES
cDNAs, Purified Ga Subunits, and Antibodies—cDNAs for Gα12, Gα13, QLα12 (Q229L), and QL (Q229) were obtained from the Guthrie cDNA Resource Center (www.cDNA.org). GST- and pcDNA3-Gα12, GST, Gα12, and Gα13 were described previously (11). Myc-tagged PP2A Aα subunit was provided by Dr. David Virshup (University of Utah), and CdK5 and p25 plasmids (15) were provided by Li-Huei Tsai (Harvard Medical School). 4R GFP-tau has been described previously (16).
Baculovirus protein Gα12 was provided by Stephen Graber (Wes Virginia University), and Gα12 and Gαq were generously provided by Patrick Casey (Duke University). Polyclonal rabbit anti-Gα12 and Gα13, and GO antibodies were from Santa Cruz Biotechnology, monoclonal anti-Myc mouse antibodies from Invitrogen. Antibodies to tau were from Upstate Biotechnology (Lake Placid, NY), and GFP was from Abcam Inc. (Cambridge, MA). Secondary antibodies were all obtained from Molecular Probes. Polyclonal rabbit anti-Aα was provided by D. Virshup, and tau phosphospecific antibody CP9 was generously provided by Peter Davies (Albert Einstein College of Medicine). Lyso-phosphatic acid (LPA) was from Avanti Polar Lipids (Alabaster, AL).

Yeast Two-Hybrid Screening—The Matchmaker™ (Clontech, Palo Alto, CA) was utilized as described previously (10). Mouse Gα12 cDNA was cloned into the bait vector pAS-2 and a human fetal kidney library, in pACT-2, was screened using standard selection methodology as described previously (10) except that co-transformed yeast were incubated at room temperature for 6–10 days. Clones growing on selective plates at room temperature were regrown and screened for positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones. Two of these were identical and confirmed positive clones.

Immunohistochemistry, Immunoprecipitation, Immunoblot Analysis, and GST Pull-downs—Caco-2 cells and primary cortical neurons were cultured on sterile glass cover slips and costained with rabbit anti-Gα12 and goat anti-Aα both at 1:50 as described previously (10). Donkey anti-rabbit FITC (1:1000) and donkey anti-goat Texas Red (1:1600) were used for staining and images were obtained on a Nikon Labe-phot microscope and Spot Digital camera and software (www.micro-com SpotSoftware). Images were processed in Adobe Photoshop. For immunoprecipitations and Western blots, cells were washed in phosphate-buffered saline, and lysates prepared in modified RIPA buffer (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.02% azide, 1 mM Na3VO4, 25 mM NaF + protease inhibitor mixture). Immunoprecipitation with various antibodies (rabbit Aα, rabbit Gα12, mouse myc, or control antibodies) was performed as described previously (11). SDS-PAGE and Western blot were done as described previously (11). GST pull-downs were performed as described previously (11) from cell lysates prepared from primary cortical neurons.

PP2A Phosphatase Activity—Purified PP2A core enzyme (Aα and catalytic unit) was obtained from Upstate Biotechnology and kinetic analysis of phosphatase activity determined by Malachite Green phosphatase assay (Upstate Biotechnology). The phospho-peptide (K-R-pT-I-R-R) was used as substrate to determine PP2A activity alone and in combination with GST, and GST-Gα12, GST-Gα13, and GST-Gαq, at 1.5 molar ratio. A similar comparison was done with recombinant Gα12, Gα13, and Gαq, at 1:1 molar ratio. G proteins were incubated with GDP or GTPγS (50 µM) or AlF4− (3 mM NaF + 50 µM AlCl3) for 15 min at 30 °C. After incubation with 200 U/ml PP2A for 30 min at 23 °C, the reaction was initiated by addition of substrate at t = 0, and after 30 min the reaction was terminated, absorbance measured at 624 nm, and enzyme activity determined (nmol of phosphate/min/units). Cell Culture and COS Cell Transfections—COS cells were cultured and transfected as described previously (18) using Lipofectamine™ (Invitrogen) according to the manufacturer’s protocol. Total cDNA was constructed by amplification of Gα, Cdk5, p25, and wild-type Cdk5 with various antibodies (rabbit Aα, rabbit Gα12, mouse myc, or control antibodies) was performed as described previously (11). SDS-PAGE and Western blot were done as described previously (11). GST pull-downs were performed as described previously (11) from cell lysates prepared from primary cortical neurons.

Primary Neuronal Cultures and Agonist Stimulation—The cortex of E18 Sprague-Dawley rats was separated in 2.5% trypsin (Invitrogen) and 0.5–1 ml 0.16% (w/v) DNease I (Sigma) dissolved in HBSS. Cells were plated at ~1 × 10⁶ cells/cm² on poly-l-lysine-coated plates or coverslips. HBSS was replaced with Neurobasal media (Invitrogen) supplemented with glutamine and B-27 and penicillin/streptomycin antibiotic solution. Experiments were done after 10–14 days in culture by adding vehicle, isoproterenol (1 µM), or LPA (10 µM) for 30–60 min followed by washing, scraping in modified RIPA buffer, and Western analysis on identical amounts of total protein using CP-9 antibody. Blots were stripped and reprobed with tau antibodies.

Statistics and Quantization—Western blots with exposures in the linear range were scanned using a desktop scanner and the images analyzed in NIH Image 1.63 (Wayne Rasband). Statistics were compiled with GraphPad Prism (GraphPad Software, San Diego, CA). Results are expressed as the mean ± standard deviation. Statistical significance was determined using two-tailed t test.

RESULTS AND DISCUSSION

During our search for novel binding partners for Gα12, we identified an interaction with the Aα subunit of PP2A. This interaction was confirmed in several assays and we found direct Gα12-dependent stimulation of PP2A activity in vitro. Furthermore, studies in COS cells and primary neurons reveal Gα12-dependent stimulation of PP2A activity resulting in reduced phosphorylation of a target protein, tau.

Yeast two-hybrid screening of a human embryonic kidney library with mouse wild type Gα12 subunit resulted in five confirmed positive clones. Two of these were identical and encoded a 1.1-kb fragment of the regulatory subunit A (PR 65) of PP2Aα (GenBank™ accession number NM_014225). The coding sequence in this fragment included amino acids 225 to the C terminus (590) encompassing repeats 7–15. PP2A is one of eight classes of serine/threonine phosphatases, is ubiquitously expressed, and is a major regulator of many fundamental cellular processes (reviewed in Ref. 19). PP2A is composed of a catalytic subunit (C), a scaffolding subunit (A), and a regulatory subunit (B). The catalytic and scaffolding subunits bind tightly to form a core dimer (AC) that is a functional unit. The regulatory subunits are numerous and diverse and provide specificity and localization for the many functions of PP2A. Many proteins interact with PP2A including signaling proteins and transcription factors, membrane receptors and transporters, protein kinases, cytoskeletal proteins, and others (reviewed in Ref. 20).

To confirm this interaction, we cotransfected myc-tagged PP2A Aα and wild type Gα12, or constitutively active Q229Lα12 in COS cells. Endogenous Gα12 protein is not detectable nor can any be immunoprecipitated (last lane in Fig. 1A). Immunoprecipitation of myc-tagged Aα subunits precipitated the co-expressed Gα12 subunits (wild type and QL, Fig. 1A). Control immunoprecipitations with Gα12 antibody and myc antibody in vector-transfected cells did not detect any Gα12. Stripping and reprobing the blot with Aα rabbit polyclonal antibody revealed the myc-tagged Aα subunit migrating at ~80 kDa. Fig. 1B shows that immunoprecipitating the endogenous Aα subunit coprecipitated a fraction of the transfected wild type and QLα12 subunits. Controls with serum and beads alone were negative. Parallel experiments with Gα12, Gα13, and Gαq failed to detect any interaction with Aα (results not shown). To document this interaction in non-transfected cells, GST pull-downs from primary cortical neurons were performed. Fig. 1C shows interaction of endogenous Aα from neurons with GST-Gα12 but not GST, GST-Gα13, or GST-Gαq. These findings suggested that the interaction of Gα12 with Aα subunits did not depend upon Gα12 conformation. To confirm this, wild type Gα12 and myc-Aα were cotransfected into COS cells and divided into three equal fractions. Lysates were incubated at 23 °C for 20 min in the presence of GDP, GTPγS, or AlF4−. Following immunoprecipitation of myc-Aα subunits and analysis by Gα12 Western blot, there were no significant differences in the amount precipitated (Fig. 1D). It was previously shown in separate studies that Gα12 and PP2A are localized in epithelial cell tight junctions (21, 22). Endogenous Gα12 and Aα were colocalized by immunofluorescent microscopy using antibodies to Gα12 and Aα in Caco-2 cells and primary cultured neurons (Fig. 1E). The two proteins co-localize in the lateral membrane of Caco-2 cells (Fig. 1E) and in the cell body and processes of primary neurons (Fig. 1E).

Although Gα12 and Aα interact, there is no known regulation of PP2A by G proteins. To determine whether Gα12 affects PP2A activity, phosphatase activity of purified core enzyme (Aα and catalytic subunit) was measured in the presence and absence of several Gα subunits. Fig. 2A shows that GST-Gα12 significantly stimulates PP2A phosphatase activity by nearly 300% above the activity of the enzyme alone. Parallel assays done with GST alone or GST-Gα13 failed to demonstrate any significant effect on PP2A activity. Consistent with binding results in Fig. 1, preincubation of GST-α subunits with GDP or
Gα12 Regulates PP2A

**Fig. 1.** Gα12 and Aα coimmunoprecipitate in COS cells and colocalize in Caco-2 cells and primary cortical neurons. A, Western blot of COS cells cotransfected with myc-tagged Aα and wild type or activated (QL) Gα12. Cotransfections and immunoprecipitations (IP) were done as described under “Experimental Procedures.” Plasmids used for cotransfections are listed below and immunoprecipitations (IP) done with Gα12 rabbit polyclonal antibody or mouse anti-myc monoclonal antibody is shown above. The blot was probed with Gα12 antibody (top lane) and then reprobed with rabbit Aα antibody (lower lane). B, Gα12 Western blot of COS cells transfected with wild type or QLα12 and immunoprecipitated with endogenous Aα subunit antibody, serum, or beads alone as described under “Experimental Procedures.” The arrow denotes Gα12. C, GST pull-downs from primary neuron lysate (500 μg). Neuron lysate (20 μg) is shown in first lane and represents 4% of input. GST, GST-Gα12i, and GST-Gα12i (1 μg) were incubated with neuronal lysates, washed, and eluted. Samples were analyzed by SDS-PAGE followed by Western blot analysis with Aα antibody. D, Western blots of myc-Aα and wild type Gα12-transfected COS cells incubated with guanine nucleotides. COS cells were transfected in a single plate and divided into 3 equivalent aliquots followed by incubation with the nucleotide. Aα was immunoprecipitated using myc antibody and samples analyzed by Western for Gα12. Similar results were seen in three experiments. The blot was stripped and reprobed myc antibody (top row). The lysate lane represents 5% of the input used for immunoprecipitation. E, Caco-2 cells (A–C) and primary cultured neurons (D–F) were double stained with Aα and Gα12 as described under “Experimental Procedures.” Scale bar = 10 μm.

GTPγS did not significantly affect the Gα12i-mediated stimulation of PP2A activity. AIF1 modestly decreased base-line PP2A activity (by ~25%), but there was still nearly 3-fold stimulation of PP2A activity (data not shown). These results suggest that the effect on PP2A activity was specific for Gα12 and independent of bound nucleotide. However, GST-Gα proteins may not appropriately exchange guanine nucleotides. To address this, PP2A phosphatase activity was measured in the presence and absence of purified Gα subunits. Fig. 2B shows that recombinant Gα12 stimulates PP2A phosphatase activity over 300%, whereas recombinant Gαi and Gα11 had no demonstrable effect. Similar to the results with GST-Gα12, and consistent with the binding data (Fig. 1D), the Gα12-stimulated phosphatase activity was similar for GDP- and GTPγS-ligated Gα12.

Based upon these findings, we hypothesized that Gα12-stimulated PP2A activity would result in less phosphorylation of a target substrate. To test this in cells, we utilized the microtubule associated protein, tau (GFP-tagged) and determined the effect of Gα12 expression on its phosphorylation in COS cells. We have previously demonstrated that the function of GTP-tau is indistinguishable from wild type tau (16). Fig. 3A shows a Western blot of COS cells transfected with GFP-tau (4R), Cdk5+–p25 (the active kinase), and either wild type or QLα12. As expected for COS cells, expression of tau (4R, GFP-tagged) with cotransfected wild type and QLα12 along with tau and the active kinase results in ~60% reduction in Thr-231 phosphorylation (as detected with CP9 antibody; Fig. 3A, lane 1). When GFP-tau is coexpressed with the active kinase (4R+kinase) a robust signal is apparent. Cotransfection of either wild type or QLα12 along with tau and the active kinase results in ~60% reduction in Thr-231 phosphorylation. The total tau in each lane was similar, and the bar graph (Fig. 3A) summarizes the relative effect of Gα12 expression on Thr-231 phosphorylation. Consistent with the effects of Gα12 occurring through regulation of PP2A, the PP2A phosphatase inhibitor, okadaic acid (50 nM), nearly completely inhibited the Gα12 mediated stimulation of PP2A activity (+O.A., Fig. 3). As expected from the lack of PP2A stimulation by other Gα subunits (Fig. 2), parallel experiments with cotransfected wild type and QLα12 in COS cells showed no change in tau phosphorylation (results not shown). Finally, we determined the extent of tau phosphorylation in primary cortical neurons stimulated with the Gα12 agonist, LPA. Fig. 3B shows a 50% reduction in endogenous tau phosphorylation when the cells were stimulated with LPA for 60 min. There was no effect of iso-proterenol on tau phosphorylation, and the effect of LPA was inhibited by preincubation with okadaic acid (Fig. 3B).

Taken together, these results provide direct evidence for stimulation of PP2A phosphatase activity by Gα12 but not the related Gα13 subunit. In addition, Gα12-stimulated PP2A ac-
The stimulation of PP2A phosphatase activity by Gα12 independent of nucleotide bound suggests a novel mechanism of regulation. The Gα12-stimulated PP2A phosphatase activity in vitro reveals that core enzyme (Aα and catalytic subunit) and Gα12 are sufficient for the stimulation. As a result, Gβγ and PP2A regulatory B subunits are not necessary for this effect, but future studies are needed to determine any potential regulatory role of Gβγ and/or B subunits on this activity. Our findings are consistent with a direct interaction of Aα and Gα12, although we cannot exclude the possibility that Gα12 also interacts with the catalytic subunit of PP2A. Additional studies are needed to address this question and whether the mechanism of stimulation by Gα12 is direct or through a secondary conformational change in Aα. The observation that Gα12 binds to the scaffolding Aα subunit of PP2A and regulates phosphatase activity is another example of signaling modulation through multiprotein complexes. This novel pathway of Gα12-stimulated PP2A phosphatase activity was not seen with other related Gα subunits and raises the possibility for specific modulation of some PP2A target proteins in vivo. This may have major implications for treating specific diseases such as neurodegenerative processes mediated by tau hyperphosphorylation.

Acknowledgment—We thank David Virshup for helpful suggestions.

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