Communication

Interaction of Heterotrimeric G Protein Goα with Purkinje Cell Protein-2

EVIDENCE FOR A NOVEL NUCLEOTIDE EXCHANGE FACTOR*

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The heterotrimeric G protein Goα is ubiquitously expressed throughout the central nervous system, but many of its functions remain to be defined. To search for novel proteins that interact with Goα, a mouse brain library was screened using the yeast two-hybrid interaction system. Pcp2 (Purkinje cell protein-2) was identified as a partner for Goα in this system. Pcp2 is expressed in cerebellar Purkinje cells and retinal bipolar neurons, two locations where Goα is also expressed. Pcp2 was first identified as a candidate gene to explain Purkinje cell degeneration in pcd mice (Nordquist, D. T., Kozak, C. A., and Orr, H. T. (1988) J. Neurosci. 8, 4780–4788), but its function remains unknown as Pcp2 knockout mice are normal (Mohn, A. R., Feddersen, R. M., Nguyen, M. S., and Koller, B. H. (1997) Mol. Cell. Neurosci. 9, 63–76). Goα and Pcp2 binding was confirmed in vitro using glutathione S-transferase-Pcp2 fusion proteins and in vitro translated [35S]methionine-labeled Goα. In addition, when Goα and Pcp2 were cotransfected into COS cells, Goα was detected in immunoprecipitates of Pcp2. To determine whether Pcp2 could modulate Goα function, kinetic constants k_on and k_off of bovine brain Goα were determined in the presence and absence of Pcp2. Pcp2 stimulates GDP release from Goα more than 5-fold without affecting k_off. These findings define a novel nucleotide exchange function for Pcp2 and suggest that the interaction between Pcp2 and Goα is important to Purkinje cell function.

Cell surface receptors coupled to G proteins enable cells to respond to a wide range of extracellular signals. G proteins, composed of Go and Gβγ subunits, associate at the plasma membrane in a complex with a serine transmembrane receptor. Agonist-liganded receptors activate Go by inducing a change in conformation that leads to GDP release and GTP-binding. GTP-ligated Go dissociates from Gβγ, and both subunits can interact with a variety of intracellular effectors. Go and Gβγ remain activated until the intrinsic GTPase activity of Go hydrolyzes GTP to GDP. The mechanisms utilized by cells to respond to specific signals in a precise manner is not well understood. In reconstituted systems there is ample evidence that multiple G proteins can couple to the same sets of receptors and effectors (reviewed in Ref. 1), and in transfected cells a single Go subunit can couple to at least three different effector pathways (2). One important mechanism that contributes to regulation of signaling pathways is the existence of proteins that modulate particular points in the pathway. For example, G protein receptor kinases desensitize receptors (such as β-adrenergic receptor kinase, (reviewed in Ref. 3) and RGS (regulators of G protein signaling) proteins turn off effector responses by accelerating the GTPase activity of Ga subunits (reviewed in Ref. 4). Since some G protein family members are predominantly expressed in specific tissues, cell type-specific modulators of G protein signaling are likely to exist.

Goα is a member of the pertussis toxin family of Ga subunits and is predominantly expressed in the central nervous system and heart. Although Goα comprises 0.2–0.5% of brain particulate protein (5), many of its functions are yet to be defined. Goα couples to several well characterized receptors in the brain and can regulate both N-type Ca2+ channels as well as some Kv channels (see Ref. 6 and references therein). In addition, Goα can be regulated by neuromodulin (GAP43 (growth cone-associated protein)) in developing neurites (7). Knockout of neuromodulin in mice causes significant abnormalities in neuronal pathfinding (8), but Goα knockout mice have anatomically normal brains. Despite the normal central nervous system anatomy in the Goα knockout mice, they develop a spectrum of neurologic abnormalities (tremor and impairments of motor control and behavior) and shortened survival (6, 9). We utilized the yeast two-hybrid interaction system to search for unique modulators of Goα that are expressed in the central nervous system. An interaction between Goα and Pcp2 (Purkinje cell protein-2), a protein of unknown function expressed in Purkinje cells and retinal bipolar neurons was identified. Although Goα and Pcp2 have not yet been definitively colocalized in cerebellar Purkinje cells, this interaction was confirmed in vitro by coimmunoprecipitation from transfected cells. Furthermore, Pcp2 can function as a nucleotide exchange factor by stimulating GDP release from Goα.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Goα cDNA in pBS (previously described in Ref. 10) was cloned into EcoRI/SalI sites of PAS2–1 (CLON-TECH), a vector that encodes GAL4 DNA-binding domain. Goα-PAS2–1 was used as a "bait" to screen a mouse brain library in pACT (CLON-TECH). Both Goα-PAS2–1 and the mouse brain cDNA libraries were cotransformed into Y190, a yeast lacZ/HIS3 reporter strain, using standard methods. The transformed mix was screened for growth on plates containing selective medium (synthetic complete medium lacking tryptophan, leucine, and histidine in the presence of 25 mg 3-amino-triazole) and incubated at 30 °C for 8–12 days. His+ colonies were screened for β-galactosidase activity using a filter lift assay, and positive "blue" colonies (β-galactosidase-positive) were further confirmed by a yeast mating assay. Individual plasmids were transformed into Escherichia coli by electroporation and plasmids analyzed by restriction analysis and dyeoxynucleotide sequencing. GenBank™ data bases

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Purkinje Cell Protein-2 Interactions with Goα

were screened with each sequence using BLAST analysis. In vitro Binding Assay—Pcp2 was excised from pACT2 using EcoRI/XhoI and cloned into pGEX-4—2 (Amersham Pharmacia Biotech). The resulting Pcp2-gex cDNA and pGEX-4—2 without insert (GST alone) were transformed into E. coli, and the expressed proteins were purified from bacterial pellets after induction with isopropyl-1-thio-β-D-galactopyranoside. Bacterial lysates were incubated with glutathione-agarose beads (2 ml) and rotated at 4 °C for 30 min. After centrifugation, GST-Pcp2 and GST alone were eluted from beads using glutathione elution buffer for 10 min at room temperature (Amersham Pharmacia Biotech). Samples were centrifuged, and the resulting supernatant containing the fusion proteins was analyzed according to the Bradford method to determine protein concentration. Goα, subtypes 1 and 2 in PBS (Goα, plasmid construction described in Ref. 11) and Goα, in pCDNAI (from ATCC, Manassas, VA) were used for in vitro translation. Labeled Goα subunits were made using 1 µc of cdNA, appropriate RNA polymerase in a coupled rabbit reticulocyte translation system (TNT system, Promega, Madison WI) plus [35S]methionine (NEN Life Science Products; 20 µCi/reaction) as described previously (11). [35S]Methionine-labeled Goα subunits were analyzed by SDS-PAGE and autoradiography, and the amounts were normalized by densitometric analysis of translated products (NIH Image 1.61/fat, Wayne Rasband, NIH, Bethesda, MD). Equivalent amounts of fusion proteins (GST or GST-Pcp2) were incubated with glutathione-agarose beads for 30 min at room temperature, followed by incubation with equivalent amounts of in vitro translated [35S]methionine-labeled Goα, Goα, or Goα in PBS with 0.05% Triton X-100 overnight at 4 °C with rocking. Beads were centrifuged, washed with PBS with 0.05% Triton X-100 three times, eluted with SDS sample buffer, and analyzed by SDS-PAGE and autoradiography.

Comunmunoprecipitation from Transiently Transfected COS Cells—To preserve the HA epitope located at the N terminus of Pcp2 in pACT2, the plasmid was cut with BglII and filled with Klenow to generate a blunt end. Following XhoI digest, the fragment was cloned into the EcoRI/XhoI sites of pcDNA3 (Invitrogen). Goα, in pcDNA3 (12) was transfected into COS-7 cells alone or in combination with Pcp2 using LipofectAMINETM (Life Technologies, Inc.) according to the manufacturer’s protocol. At 72 h after the transfection, cells were washed with ice-cold PBS and lysed for 30 min in lysis buffer (50 mM Hepes, pH 7.5, 6 mM MgCl2, 1 mM EDTA, 75 mM sucrose, 2.5 mM benzamidine, 1 mM dithiothreitol, and 1% Triton X-100). Lysates were cleared by low speed centrifugation, and the supernatant was incubated with the HA-specific monoclonal antibody 12CA5 (1:100) overnight at 4 °C. Protein A-Sepharose (Sigma) was added for 1 h, and the samples were rocked at 4 °C. Samples were then centrifuged, and the pellets were washed three times with PBS with 0.05% Triton X-100. The precipitated proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and Western blotting using a rabbit polyclonal anti-Goα antibody (5), and bands were visualized by chemiluminescence (Pierce).

Determination of kcat and koff—Bovine brain Goα was kindly provided by E. Neer (Harvard Medical School) and used for kinetic analysis in the presence and absence of Pcp2. Pcp2 was prepared from GST fusion proteins by cleavage with thrombin at 25 units/mg protein for 1 h at room temperature and separated from GST by incubation with glutathione-agarose beads. Samples were concentrated and protein concentration determined (Bradford). Single turnover GTP hydrolysis (kcat) was determined by incubating Goα (50 nM) with 1 µM [γ-32P]GTP (5500 cpm/pmol) for 20 min in the presence (1:1 molar ratio) or absence of Pcp2 in buffer A (50 mM Tris, pH 7.6, 5 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100). The hydrolysis reaction was started by addition of MgCl2 (final concentration, 10 mM) and 100 µM GTP. Aliquots were diluted into 1 ml of 5% (w/v) trichloroacetic acid in 5% charcoal and counted as described previously (13). The amount of [γ-32P]GTP released at each time point was fit to an exponential function using GraphPad Prism. For determining koff, Goα (2 pmol) in the presence and absence of Pcp2 or GST (1:1 ratio with Goα) was incubated in buffer A with 10 mM MgCl2, 1 µM GTP [α-32P]GTP (specific activity 1.1 × 106 cpm/pmol), and 10 µg/ml bovine serum albumin for 20 min at room temperature. The stoichiometry of binding was approximately 60%, and the amount bound prior to initiating nucleotide exchange was set at 100%. GDP dissociation from Goα was initiated by the addition of 1 mM GDP, and aliquots were filtered onto nitrocellulose, washed, and counted. The data were fit to a one phase exponential decay using GraphPad Prism (San Diego, CA).

The yeast two-hybrid interaction system detects low affinity protein interactions and has been successfully used for finding novel proteins that interact with G protein α subunits (14). The initial screen yielded 34 β-galactosidase-positive clones, and this number was reduced to six positive clones after yeast mating controls. These six clones were sequenced and searched in GenBankTM (BLAST) data bases. Three of these cDNAs had no sequence homology to known genes, and one was identified as lactate dehydrogenase. One of the remaining genes was a previously identified human mosaic protein, LGN, that was discovered in a yeast two-hybrid screen using Goα as bait (15). The other remaining gene was a full-length clone of PCD5 (now called Pcp2) (16) that had been initially identified as a candidate gene important for Purkinje cell development. In pcd mice, Purkinje cells develop normally but then begin to degenerate at 15–18 days after birth leading to the development of ataxia (17).

To determine whether Goα could interact with Pcp2 in an independent assay, GST pull-down experiments were performed. Goα subunits were translated and [35S]methylabeled in vitro and then incubated with equivalent amounts of GST or GST-Pcp2 fusion proteins. Fig. 1 shows that in vitro translated Goα binds to GST-Pcp2 (lane 2), whereas no significant interaction was seen with the GST control protein (black arrow, lane 1). We consistently detected approximately 10% of in vitro translated Goα coprecipitating with GST-Pcp2. Nonspecific interactions of Goα with GST were less than 1%. We next asked whether the conformation of Goα affected the interaction with GST-Pcp2. Goα was preincubated with GTPγS (nonhydrolyzable GTP analogue) or GDP prior to binding. As shown in Fig. 1, the amount of Goα that associates with Pcp2 is similar irrespective of the nucleotide bound to Goα. To address the issue of which Goα subunits could interact with Pcp2, two other Goα subunits expressed in the central nervous system were studied. Goα2 (pertussis toxin family member; ~70% amino acid identity to Goα) and Goα5 (cholera toxin family member; ~40% amino acid identity to Goα) were characterized in pull-down experiments with GST-Pcp2. Equal amounts of [35S]-labeled Goα subunits were translated in vitro and used for binding to GST proteins, and the amount of nonspecific binding between Goα2 or Goα5 with GST was similar to that seen with Goα (not shown). Fig. 1 shows that in vitro translated Goα2 interacts with GST-Pcp2, but Goα5 is barely detectable in GST-Pcp2 precipitates (lane 6, 52 kDa, open arrow). Taken together, these results suggest that the related pertussis toxin family
members (Goα and Gαo2) interact in vitro with Pcp2 but that Goα subunits do not.

To look for evidence that Goα and Pcp2 could interact in cells, cotransfection studies were performed in COS cells. COS cells were transfected with Goαo, Pcp2, empty expression vector pcDNA3 (PC) alone or in combination. Cell lysates were immunoprecipitated using the 12CA5 antibody directed toward the hemagglutinin epitope on the N terminus of Pcp2 and then analyzed by Western with anti-Gαo antibody (Fig. 2). COS cells do not normally express Gαo and in cells transfected with vector (PC), Pcp2, or Goαo, and then immunoprecipitated with 12CA5 there is no detectable Gαo immunoreactivity (Fig. 2, arrow). However, when both Goαo and Pcp2 are cotransfected into the same cells, a fraction of Goαo is found in association with Pcp2 (Fig. 2, lane 4). We were unable to detect Pcp2 in immunoprecipitates of Goαo presumably due to disruption of the association with Goαo by the stringent detergent conditions necessary to immunoprecipitate Goαo (18). Attempts to detect Goαo in Pcp2 immunoprecipitates were unsuccessful, although the level of Gαo2 expression was much lower than for Goαo. The observation that Goαo and Pcp2 interact in an intact cell raises the possibility that Pcp2 could be a modulator of Goαo.

There are several possible mechanisms for regulation of Go subunits by accessory proteins, including effects on nucleotide binding or GTP hydrolysis. To address the possibility that Pcp2 modulates the enzymatic properties of Goαo, we measured kcat and koff of bovine brain purified Goαo (5) in the presence and absence of Pcp2. Fig. 3 shows a representative experiment, and the results are summarized in Table I. The kcat and koff values obtained for Goαo are similar to literature values (19), and there was no significant difference in kcat of Goαo in presence of Pcp2 (Fig. 3a and Table I). However, as seen in Fig. 3b and Table I, there is significant stimulation of koff in the presence of Pcp2. In experiments simultaneously comparing koff of Goαo in the presence and absence of Pcp2 or GST, there was 5.2 ± 0.5-fold stimulation of koff in the presence of Pcp2 (n = 7). Incubating Goαo with GST had no effect on koff (Fig. 3b and Table I), and including bovine serum albumin in the binding buffer also had no effect. The increase in koff of Goαo in the presence of Pcp2 is similar to the observed increases seen with Goα subunits reconstituted with receptors plus agonists (4–6-fold increases in koff) (20). This finding suggests that Pcp2 could mimic a receptor by stimulating GDP release. A family of proteins that promote nucleotide exchange have been described for monomeric G proteins such as Ras (21), but only neuromodulin has been shown to affect nucleotide binding to Goαo. This mechanism of activation is likely to be distinct from Pcp2 because neuromodulin stimulates GTP·S binding through its N-terminal domain, which is homologous to the cytoplasmic tail of G protein-coupled receptors (7).

The gene for Pcp2 is located on mouse chromosome 8 and encodes a cytosolic 99-amino acid protein without significant homology to other proteins (including G protein-coupled recep-
tors). There is some amino acid sequence similarity to the c-sis/PDGFR2 gene, but the implications of this are unknown (22). The expression profile of Pcp2 is consistent with a role in Purkinje cell development (23), but in mice without Pcp2 expression, Purkinje cells develop normally (presumably through other compensatory mechanisms) (24, 25). The function of Pcp2 (26). Several G protein subunits trigger apoptosis (27), and Go may be involved in apoptosis triggered by a mutated amyloid precursor protein in Alzheimer’s disease (28). The results described in these studies suggest that Pcp2 may be involved in a signal transduction pathway involving Go, but it remains to be determined whether this pathway relates to apoptosis. In addition, our results do not exclude important interactions of Pcp2 with other Ga subunits, particularly Go11, which is highly expressed in the brain. Increasingly, novel functions (and locations) for G proteins are being identified (such as in the Golgi, in the endoplasmic reticulum, and on intracellular vesicles) where they regulate protein processing and vesicular targeting (29–31). Because G protein-coupled transmembrane receptors have not been identified in many of these locations, Go subunits may be activated by nucleotide exchange factors. Pcp2 may be such a factor for Go in cerebellar Purkinje cells, and future studies will define the functional consequences of this interaction.

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