Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 454 (2006) 160–169
ABB
www.elsevier.com/locate/yabbi

TRP1 interacting PDZ-domain protein GIPC forms oligomers and is localized to intracellular vesicles in human melanocytes

Rajendra H. Kedlaya [a], Kumar M.R. Bhat [a], Julie Mitchell [b], Steven J. Darnell [b], Vijayasaradhi Setaluri [a,*]

[a] *Department of Dermatology, University of Wisconsin, Madison, WI 53706, USA*
[b] *Department of Mathematics and Biochemistry, University of Wisconsin, Madison, WI 53706, USA*

Received 29 June 2006, and in revised form 5 August 2006
Available online 23 August 2006

Abstract

PDZ proteins coordinate assembly of protein complexes that participate in diverse biological processes. GIPC is a multifunctional PDZ protein that interacts with several soluble and membrane proteins. Unlike most PDZ proteins, GIPC contains single PDZ domain and the mechanisms by which GIPC mediates its actions remain unclear. We investigated the possibility that in lieu of multiple PDZ domains, GIPC forms multimers. Here, we demonstrate that GIPC can bind to itself and that the PDZ domain is involved in GIPC–GIPC interaction. Gel filtration, sucrose gradient centrifugation and chemical cross-linking showed that whereas bulk of cytosolic GIPC was present as monomer, oligomers with an estimated molecular mass corresponding to GIPC homotrimer were readily detectable in the membrane fraction. Modeling of GIPC PDZ domain showed feasibility of trimerization. Immunogold electron microscopy showed that GIPC is present in clusters near vesicles. Our data suggest that oligomers of GIPC mediate its functions in melanocytes.

Keywords: PDZ proteins; GIPC; Protein folding; Oligomerization; Chemical cross-linking; Computational analysis

PDZ[1] (Postsynaptic density-95/DISC large/ZO1) domain proteins, play important roles in assembling large signaling complexes involved in diverse biological processes such as phototransduction in *Drosophila*, synaptic transmission, trafficking of receptors and other membrane proteins, and maintenance of epithelial cell morphology and polarity [1–6]. PDZ protein GIPC was originally identified as a protein that interacts with GAIP (RGS 19), a regulator of G protein signaling and thought to play a role in trafficking of clathrin coated vesicles [7]. Subsequently, GIPC was shown to provide a link between signaling by NGF receptor Trk and MAP kinase pathways [8]. GIPC also binds a number of soluble and membrane bound proteins in a variety of cell types and is also known to bind certain viral oncoproteins [9–16]. We showed that in melanocytes GIPC binds C-terminus of newly synthesized melanosomal membrane protein TRP1 (tyrosinase related protein 1) in the Golgi region, and proposed a role for this interaction in trafficking of TRP1[17].

A common and striking feature of PDZ proteins, in general, is the presence of multiple PDZ domains and/or other protein–protein interacting and signaling domains within the same polypeptide. Moreover, PDZ proteins have also been shown to undergo self association to from multimers, thus generating complex signaling scaffolds [18–20]. GIPC, unlike most PDZ proteins, however, has a single PDZ domain and no other recognizable protein interacting domains. Other PDZ proteins that contain a single PDZ domain, PICK1 (protein interacting with C kinase 1) and ERBIN (ERBB2/HER2 receptor interacting protein) also

* Corresponding author. Fax: +1 608 263 5223.
E-mail address: setaluri@wisc.edu (V. Setaluri).

[1] *Abbreviations used:* GFP, green fluorescent protein; GIPC, GAIP-interacting protein C-terminus; GST, glutathione *S*-transferase; PDZ domain, postsynaptic density 95/disc *large*/ZO-1 domain; PAGE, polyacrylamide gel electrophoresis; DTME, dithio-bis-maleimidoethane; CuP, cupric orthophenanthroline; GRIP, glutamate receptor interacting protein.

0003-9861/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2006.08.010

have, respectively, a coiled–coiled domain and leucine-rich repeats, which are thought to mediate their functions [21,22]. The mechanisms that mediate the cell biological actions of GIPC however, remain to be understood. We hypothesized that, in addition to binding to its target protein TRP1 by PDZ domain, GIPC in melanocytes, also binds to other signaling proteins and to itself to form oligomers. In this study, we show that GIPC can bind to itself and occurs as trimers *in vivo*. GIPC–GIPC interaction occurs through surface interactions between PDZ domains. Immunogold electron microscopy showed clustering of GIPC molecules near intracellular membranes. Our data suggest a functional role for GIPC oligomers in trafficking of TRP1.

Materials and methods

GIPC expression plasmids

Cloning of full-length and ΔNH_2-GIPC into pFLAG-CMV2 vector were described earlier [17]. N- and C-terminal deletion mutants ΔPDZ and ΔPDZ2 were generated using specific primers that incorporated a termination codon and an initiation codon at 126 and 225, respectively, and cloned into pFLAG-CMV2 vector (Sigma Aldrich Corp. St. Louis, MO). The C-terminal deletion mutant ΔACP-GIPC, lacking amino acid residues 248–333 was generated by digesting the pFLAG CMV2-GIPC plasmid with restriction enzyme *Sma*1 (at nucleotide 742 in the open reading frame of GIPC and the pFLAG-CMV2 vector at 1012) and the large plasmid fragment was religated generating a truncated GIPC protein with 1–247 amino acids. Expression plasmid for the fusion protein GIPC-EGFP was generated by cloning full-length GIPC into pEGFP-N3 vector (Clontech, Mountain View, CA). Mutations of cysteine residues at 100 and 189 positions to alanines were produced using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using specific primers according to the manufacturer's instructions.

Transfection, cell lysis and subcellular fractionation

Semi-confluent SK-MEL-23cl.22a (clone 22a) melanoma cells in 100 mm dishes were transfected with a total of 3–5μg of indicated plasmids using Lipofectamine Plus reagent (Invitrogen Life Technologies Inc., Carlsbad, CA) according to manufacturer's instructions. Forty hours after transfection, cells were harvested, lysed in 50 mM phosphate buffer, pH 7.4, containing 1% Triton-X-100 and a mixture of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Detergent lysates were cleared by centrifugation at 15,000*g* for 20 min. For preparation of cytosolic and membrane-bound proteins, clone 22a cells in semi-confluent 100 mm dishes were washed with ice-cold phosphate–buffered saline (PBS), harvested by scrapping, suspended in 50 mM phosphate buffer, pH 7.4 containing mixture of protease inhibitors and homogenized in Dounce homogenizer (20 strokes). Post nuclear supernatants (PNS) were centrifuged for 2 h at 100,000*g* in a Beckman TLA-100.1 rotor at 4 °C and supernatants were collected. The membrane pellet was solubilized in lysis buffer containing 1% Triton X-100 and cleared as described above. For sucrose gradient fractionation, the membrane fraction was washed with buffer containing 0.5 M NaCl for 1 h and clarified by centrifugation for 2 h at 100,000*g* [23]. The supernatant was collected and subjected to fractionation. The heavy membrane and light vesicle fractions were prepared by centrifuging the PNS at 10,000*g* for 30 min, and the supernatant (light vesicle fraction) was collected. The pellet (heavy membrane fraction) was then resuspended in SDS sample buffer. For cross-linking of GIPC with cupric orthophenanthroline (CuP), 48 h after transfection, cells were washed twice with PBS and once with 5 ml of lysis buffer (10 mM Tris–HCl, pH 7.4, and containing mixture of protease inhibitors). The cells were lysed with the lysis buffer and homogenized in Dounce homogenizer (20 strokes). The PNS was centrifuged at 100,000*g* for 2 h in a Beckman TLA-100.1 rotor. The resulting pellet was resuspended in buffer containing 20 mM Tris–HCl, pH 8.0, 1 mM MgCl2, 5 mM CaCl2 and 100 mM NaCl.

GST pull-down assay

GST and GST–GIPC fusion proteins were produced in *Escherichia coli* BL21 after induction with 0.1 mM isopropyl β-D-thiogalactopyranoside for 2 h. Cells were pelleted and resuspended in 300 μl B-PER, (Bacterial Protein Extraction Reagent, Pierce Biotechnology, Rockford, IL). Supernatants were incubated with glutathione (GSH)-Sepharose beads (Amersham Biosciences Corp., Piscataway, NJ) for 30 min and washed three times with 10 ml of PBS and resuspended in PBS. Lysates from clone 22a cells transfected with FLAG-GIPC and its deletion mutants, were prepared as described earlier. Five hundred microliter aliquots of cell lysates were incubated with 25 μg of GST protein immobilized on 50 μl of GSH–Sepharose beads for 1 h at 4 °C followed by incubation with GST-fusion proteins immobilized on GSH–Sepharose beads. After extensive washing with lysis buffer and PBS, bound proteins were eluted by thrombin (Amersham) digestion for 16 h at 22 °C. The Sepharose beads were then centrifuged and the supernatants were resolved by 9% or 15% SDS–PAGE, transferred to PVDF membrane (PerkinElmer Life and Analytical Sciences, Boston, MA), and probed with anti-GIPC and/or anti FLAG mAb M2 (Sigma).

Gel filtration

Gel filtration chromatography was performed with Sepharose 6B column (20 × 400 mm, 72 ml) (Amersham). The column was calibrated with ribonuclease A (13.7 kDa ±15%), chymotrypsinogen A (25 kDa ±25%), ovalbumin (43.0 kDa ±15%) and albumin (67 kDa ±10%) (Amersham). Each standard protein (2–5 mg) was dissolved in 1 ml of equilibration buffer (50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl) and loaded on to the column in a 500 μl volume at a flow rate of 12 ml/h and eluted with the same buffer and flow rate and elution was monitored by measuring absorbance at 280 nm. Soluble protein fraction (1.5 ml) obtained from clone 22a cells was subjected to gel filtration, 3 ml fractions were collected and analyzed for GIPC protein by SDS–PAGE followed by immunoblotting with anti-GIPC antibody.

Sucrose density gradient centrifugation

Clone 22a cells were harvested, lysed and cytosolic and membrane fractions were prepared as described earlier. Two hundred microliters of cytosolic and membrane fractions were layered on top of 4.5 ml of 8–40% (w/v) discontinuous sucrose gradient and centrifuged at 100,000 *g* for 24 h at 4 °C in a Beckman SW 55Ti rotor for 24 h [24]. After centrifugation, 500 μl fractions were collected from top of the gradient and analyzed by Western blotting with anti-GIPC antibody. The protein markers (200 μl of 5 mg/ml) described above in gel filtration section were loaded on to the gradient and fractionated in a parallel tube and the fractions were analyzed by spectrophotometry at 280 nm.

Chemical cross-linking

For chemical cross-linking with dithio-bis-maleimidoethane (DTME, Pierce) melanoma cells transfected with 3 μg of FLAG-GIPC plasmid, in culture, were resuspended at approximately 1×10^6/ml in cross-linking buffer (HBSS containing 10 mM HEPES, pH 7.4). Membrane permeable thiol cross-linker DTME (Pierce) was dissolved in DMSO and diluted 100 fold with the cross-linking buffer immediately prior to use. The cells were then incubated with varying concentrations of cross-linker for 1 h at 4 °C with occasional gentle shaking. Reactions were stopped by two 5 min washes with HBSS containing 5 mM cysteine and lysing the cells in buffer containing 2 mM cysteine. The cells were centrifuged at 1200*g* for 10 min, washed in PBS and lysed in 50 mM phosphate buffer, pH 7.4, containing