Available online at www.sciencedirect.com
SCIENCE DIRECT
Cellular Signalling 18 (2006) 1439–1446
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Receptor-type PTP-NP inhibition of Dynamin-1 GTPase activity is associated with neuronal depolarization

Shuxian Jiang [a], Hava Karsenty Avraham [a], Tae-Aug Kim [a], Rick A. Rogers [b], Shalom Avraham [a,*]

[a] *Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115, United States*
[b] *Biomedical Imaging Laboratory, Harvard University School of Public Health, Boston, MA 02115, United States*

Received 4 November 2005; received in revised form 16 November 2005; accepted 16 November 2005
Available online 18 January 2006

Abstract

Dynamin-1 is a GTP-hydrolyzing protein and a key element in the clathrin-mediated endocytosis of secretory granules and neurovesicles at the plasma membrane. The unique receptor-like protein tyrosine phosphatase, PTP-NP/Phogrin/IAR/IA-2, is associated with neuroendocrine secretory granules and is highly expressed in the brain. Here, we show by confocal microscopy and biochemical studies that PTP-NP rapidly associates with Dynamin-1 in a depolarization-dependent manner and regulates Dynamin-1 GTPase activity upon KCl depolarization of rat primary hippocampal neurons. Depolarization of primary neurons induced direct association of PTP-NP with Dynamin-1 within 30 s. This association resulted in significant inhibition of Dynamin-1 GTPase activity (~75% inhibition). Mutation within the phosphatase domain of PTP-NP ($PTP\text{-}NP_{D947A}$) abolished the direct interaction of PTP-NP with Dynamin-1 and failed to inhibit Dynamin-1 GTPase activity. To further confirm the endogenous interaction of Dynamin-1 with wild-type PTP-NP, Dynamin-1 was purified biochemically from rat brain and its interaction with purified PTP-NP was analyzed. Highly purified Dynamin-1 specifically associated with wild-type PTP-NP and not with mutated PTP-NP, resulting in significant inhibition (~70%) of Dynamin-1 GTPase activity. This is the first report to suggest a novel function of this unique receptor-type tyrosine phosphatase as a potential regulator of Dynamin-1 GTPase activity upon neuronal depolarization.

Keywords: Tyrosine phosphatases; PTP-NP; Dynamin-1; GTPase activity; KCl depolarization; Neurons

1. Introduction

The receptor-type protein tyrosine phosphatase PTP-NP, also known as phogrin, islet antigen IA-2 and PTP-IAR, belongs to a unique family of receptor-like protein tyrosine phosphatases (PTPs) that is characterized by the lack of PTP enzymatic activity with conventional substrates [1–9]. PTP-NP expression is restricted primarily to the pancreas, pituitary, and brain, with the highest level in the brain [1–9]. Two forms of PTP-NP mRNA were detected: a type 1 with robust expression in pancreatic cells in the early stages of development and a type 2 transcript expressed predominantly in the early stages of neurogenesis. These observations imply a role for PTP-NP in the development of the nervous system and pancreatic endocrine cells [7].

IA-2β (islet cell antigen 512) is the primary target of humoral autoimmunity against type 1 diabetes-associated tyrosine phosphatase autoantigens [3,8,9]. Phogrin is a 60/64-kDa integral membrane protein localized to the dense-core secretory granules of neuroendocrine cells [4]. Phosphorylation of phogrin occurred in response to a variety of secretory stimuli, including glucose. The phosphorylation of phogrin suggests that it may be a regulator of insulin secretion and of secretory-granule mobilization and recruitment to the exocytic site [8].

PTP-NP and its isoform, PTP-NP2, are expressed in secretory granules in the pancreas and are also suggested to be expressed on synaptic boutons in the brain, but their function (s) are unknown [7,9,10]. By analyzing the PTPase activity of wild-type and mutant PTP-NP, we and others showed that a

Abbreviations: (CLSM), confocal laser scanning microscope; (DMEM), Dulbecco's Modified Eagle's Medium; (GFP), green fluorescent protein; (GTPase), guanosine triphosphatase; (IA), islet antigen; (MT), mutant; (PH), pleckstrin homology; (PRD), proline/arginine-rich domain; (PKC), protein kinase C; (PTPases), protein tyrosine phosphatases; (PTPs), receptor-like protein tyrosine phosphatases; (RT), room temperature; (WT), wild type.

* Corresponding author. Tel.: +1 617 667 0063; fax: +1 617 975 6373.
E-mail address: savraham@bidmc.harvard.edu (S. Avraham).

0898-6568/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2005.11.010

single amino acid change (amino acid D947A) [9,10] is responsible for the absence of phosphatase activity in the PTP-NP wild-type protein. Interestingly, an aspartate in the active tyrosine phosphatase consensus sequence is a common feature of the inactive second phosphatase domain of the adhesion type of PTP-like receptors [11], suggesting that PTP-NP may be a common evolutionary ancestor of PTPases with possible functional similarity [11]. The function of this catalytically inactive phosphatase domain in brain is unknown.

Dynamins are GTPases required for the budding of clathrin-coated vesicles from the plasma membrane [12,13]. Dynamin is also a high-affinity substrate for calcineurin, and its guanosine triphosphatase activity is determined by analyzing the balance between PKC-mediated phosphorylation and calcineurin-dependent dephosphorylation [14,15]. Moreover, Dynamin-1 serves as a switch for depolarization-evoked synaptic vesicle endocytosis in secretory vessels [16]. The GTPase activity of Dynamin is stimulated 5–10-fold over basal levels by self-assembly [17].

In this study, we present data demonstrating that the receptor type PTP-NP plays a role in the inhibition of Dynamin-1 GTPase activity in primary hippocampal neurons upon KCl depolarization.

2. Experimental procedures

2.1. Materials

Chemical reagents were of analytical grade and were purchased from Sigma (St. Louis, MO). Restriction endonucleases and modifying enzymes were purchased from Roche Applied Sciences (Indianapolis, IN), Stratagene (La Jolla, CA), and Pharmacia Biotech (Piscataway, NJ). Primers for polymerase chain reactions and sequencing were synthesized by Integrated DNA Technologies (Coralville, IA). PCR reagents were purchased from Perkin-Elmer (Branchburg, NJ). Pfu DNA polymerase was obtained from Clontech Inc, and random-primer labeling kits were obtained from Stratagene (La Jolla, CA). Proteinase inhibitors were purchased from Roche Applied Sciences (Indianapolis, IN). pNPP substrate, GTP, Tween 80, and charcoal were purchased from Sigma (St. Louis, MO). Gel electrophoresis reagents and equipment were from Bio-Rad (Hercules, CA). [γ-^{32}P] GTP was from NEN (New England). Dynamin-1 monoclonal antibody, which is highly specific for Dynamin-1 and does not cross-react with Dynamin-2, was obtained from Upstate Biotechnology (Lake Placid, NY) and was used for the immunoprecipitation, Western blotting and confocal analysis. Dynamin-1 constructs were kindly provided by Dr. S. Schmid (Scripps Research Institute, La Jolla, CA). Mutated Dynamin-1 contains a mutation in amino acid residue 44 K→A (K44A). BAPTA/AM was purchased from Calbiochem.

2.2. Cell culture

Primary neurons were prepared from the hippocampus region of Sprague–Dawley rats at gestational day 18. Primary hippocampal neurons were grown for 10 days in neurobasal medium containing B-27 supplement and L-glutamine (1.0 mM) (Gibco BRL). The neurons were then preincubated for 5–6 h in neurobasal medium without any supplements. 293 cells were grown in 6-well plates with DMEM containing 10% FBS and 0.1 mM non-essential amino acids.

2.3. Preparation of PTP-NP constructs and transfection experiments

The PTP-NP aspartate 947 to alanine mutant (PTP-NP$_{D947A}$) was generated using the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer's instructions. Full-length PTP-NP was subcloned into the EcoRI site of pEGFP-C_2, and sequenced. pEGFP-C_2 PTP-NP and pEGFP-C_2 vector alone were used to transfect the 293 cells using Lipofectamine (Life Technologies). After 2 days, the transfected cells were analyzed further for protein expression.

2.4. Purification of dynamin-1 from rat brain

The purification of Dynamin-1 was performed essentially as described previously [15]. Briefly, 20 rat brains (Wistar, 5 weeks old) were homogenized in 30 ml of A buffer (10 mM sodium phosphate, pH 6.9, 70 mM sodium glutamate, 2 mM $MgSO_4$, 2 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml Tosyl arginine methyl ester, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1.0 mM dithiothreitol). The tissue was homogenized by three passes in a 50-ml capacity Teflon homogenizer at 1500 rpm, and the homogenate was centrifuged at 10,000 rpm for 10 min at 2 °C. The supernatant was recentrifuged at 50,000 rpm for 45 min (60 Ti rotor; Beckman, Fullerton, CA). The supernatant (S_1) was applied to an 80-ml column (DE-52; Whatman, Clifton, NJ) pre-equilibrated in A buffer, and the flow-through fraction (S_1) was collected. Ten milligrams of phosphocellulose column-purified porcine brain tubulin polymerized by taxol was added to this S_1 fraction and incubated at room temperature for 15 min. The sample was centrifuged through a 2-ml cushion of 20% sucrose in A buffer at 2 °C for 30 min at 45,000 rpm (Beckman; 60 Ti rotor). The pellet was resuspended in 800 μl of A buffer containing 5 mM AMP-PNP, 2.5 mM GTP and then incubated at 37 °C for 15 min. The sample was centrifuged at 2 °C for 30 min at 55,000 rpm (Beckman; TL-100). The second pellet was resuspended in 800 μl of A buffer containing 10 mM GTP, incubated at 37 °C for 20 min and then centrifuged at 2 °C for 30 min at 55,000 rpm (Beckman; TL-100). The resulting supernatant (S_2) contained dynamin and a small amount of tubulin and other minor bands. To remove the tubulin, S_2 was reapplied to 1.0 ml of Whatman DE-52, and the flow-through fraction was collected (S_2'). S_2' was enriched in dynamin. The final purification step consisted of sucrose gradient centrifugation. S_2' was loaded onto 5–20% linear sucrose gradients containing 12 ml of B buffer (10 mM tris(hydroxymethyl)aminomethane[Tris]–HCl, pH 7.0, 30 mM sodium glutamate, 2 mM EDTA, 2 mM $MgSO_4$). The