Cellular Signalling 28 (2016) 91–99

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

The breast cancer susceptibility gene product (γ-synuclein) alters cell behavior through it interaction with phospholipase Cβ

V. Siddartha Yerramilli [a], Suzanne Scarlata [a,b,*]

[a] Dept of Physiology & Biophysics, Stony Brook University, Stony Brook, NY, 11794-8661, United States
[b] Dept. of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA, 01609, United States

ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 19 October 2015
Accepted 27 October 2015
Available online 28 October 2015

Keywords:
γ-Synuclein
Phospholipase Cβ2
Calcium signaling
Cell morphology
Cell migration

ABSTRACT

The breast cancer susceptibility gene protein, also known as γ-synuclein, is highly expressed in human breast cancer in a stage-specific manner, with highest expression in late stage cancer. In model systems, γ-synuclein binds phospholipase Cβ2 which is regulated by Gαq to generate intracellular Ca^{2+} signals. PLCβ2, which is also absent in normal tissue but highly expressed in breast cancer, is additionally regulated by Rac to promote migration pathways. We have found that γ-synuclein binds to the same region of PLCβ2 as Gαq. Using cells that mimic stage 4 breast cancer (MDA MB 231), we show that down-regulation of γ-synuclein reduces the protein level of PLCβ but increases the transcript level over 40 fold. γ-Synuclein down-regulation also promotes the interaction between Gαq and PLCβ resulting in a stronger Ca^{2+} response to Gαq agonists. The ability of γ-synuclein to interfere with Gαq–PLCβ interactions allows more PLCβ to colocalize with Rac impacting Rac-mediated pathways that may give rise to cancerous phenotypes.

1. Introduction

Synucleins are a family of small, intrinsically disordered proteins, consisting of three members: α, β and γ (for review see [1]). The synucleins share a conserved N-terminal domain but each member possesses a distinct C-terminal region. Synucleins are predominantly expressed in neuronal tissues, where they have been implicated in neurotransmitter homeostasis and release. However, their exact function remains unknown (for review see [1,2]).

γ-Synuclein is a 127 amino acid protein and possesses a shorter and slightly negative C-terminal domain that distinguishes it from other synucleins [3]. In contrast to the many studies involving α-synuclein which plays a key role in Parkinson's disease, little research has been done on γ-synuclein. However, mouse studies of γ-synuclein have been carried out and these show that knocking-out expression leads to the improvement of working memory, suggesting that γ-synuclein has a role in cognitive function [4].

Aberrant overexpression of γ-synuclein has been observed in various pathological conditions especially in a variety of cancers including prostate, colorectal, pancreatic, ovarian and gall bladder [5,6]. Surprisingly, γ-synuclein was first discovered in 1996 in breast cancers and was named the Breast Cancer Susceptibility Gene Product, but was later identified as being a member of the neuronal synuclein family [7]. In breast cancer, γ-synuclein is overexpressed in later-stage (Stage III and IV) cancer tissues, but not in healthy or early-stage (Stage I and II) cancer tissue [8]. Even though different types of breast cancers are classified by different markers [9], the presence of γ-synuclein been established as a biomarker for later stages of cancer and has been considered to be a prognosis of poor outcome [10].

Studies have shown that knockdown of γ-synuclein expression in prostate [5] and gall bladder [6] cancer cells greatly reduced the occurrence of cancerous phenotypes such as cell proliferation, migration, invasion and cell cycle arrest. Further studies have shown that downregulation of γ-synuclein expression in MCF7 cells (an early-stage breast cancer cell line) resulted in a drastic reduction in cell migration and proliferation [11], as well as the propensity to form tumors when xenografted into mice [12]. A study performed using the triple-negative breast cancer cell line, MDA MB 231 has revealed that knockdown of γ-synuclein results in an inhibition of cell migration and proliferation [13].

While the exact role of γ-synuclein in the signaling pathways that lead to cancer is currently not known, some studies have shown that γ-synuclein promotes cancerous phenotype by increasing ER-α (Estrogen Receptor) transcription [14], activating MAPK [15], enhancing AKT and ERK signaling [13], and binding and inhibiting BubR1, a mitotic checkpoint protein that prevents the formation of the anaphase promoting complex, thereby allowing the cells to rapidly undergo mitosis [16]. γ-Synuclein is known to regulate signaling pathways by changing its intracellular localization [17] and binding to transcription factors affecting gene expression [18].

* Corresponding author at: Dept. of Chemistry and Biochemistry, Worcester Polytechnic Institute, 100 Institute Rd., Worcester, MA 01602, United States.
E-mail address: sfscarlata@wpi.edu (S. Scarlata).

http://dx.doi.org/10.1016/j.cellsig.2015.10.018
0898-6568/

Our lab has previously shown that both α- and γ-synuclein interact with the signaling enzyme phospholipase Cβ (PLCβ) to alter Ca^{2+} responses [19,20]. There are 4 isoforms of PLCβ (PLCβ1-4) which are all strongly activated by the Gαq family of heterotrimeric G proteins. Receptors that are coupled to Gαq include those that bind acetylcholine dopamine, angiotensin II, bradykinin as well as endothelin I [21]. Both PLCβ2 and β3 can be activated by Gβγ subunits which may be released in response to activation of other G protein families. PLCβ enzymes catalyze the hydrolysis of the signaling lipid phosphatidylinositol 4,5 bisphosphate to generate two second messengers (diacylglyercol and 1,4,5 inositol trisphosphate) which lead to the activation of protein kinase C and release of Ca^{2+} from intracellular stores, respectively (for review see [22,23]). Even though they bind strongly to membranes, PLCβs are soluble and are found both on the plasma membrane and in the cytoplasm. It has been shown that membrane binding of PLCβ2 can be enhanced by Rac which better allows PLCβ to access its $PI(4,5)P_2$ substrate and promote hydrolysis ([24] for review see [25]). It is notable that Rac1 mediates cytoskeletal changes associated with migration and mobility leading to the idea that PLCβ may also be involved in these processes via its association with Rac. Rac1–RhoA signaling plays a role in cell motility, where Rac-mediated signaling is associated with forward movement and Rho-mediated signaling with the contractile movement. The movements associated with Rac or Rho mediated signaling have also been linked to protease-dependent mesenchymal and protease-independent amoeboid modes of invasion [26].

Using purified proteins, it was observed that both α- and γ-synuclein bind strongly to the C-terminal region of PLCβ2 [20]. This binding site of γ-synuclein is also the binding site of Gαq, resulting in the competitive inhibition of $Gα_q$-mediated PLCβ2 activation. Conversely, the levels of γ-synuclein do not affect the binding of Gβγ or Rac to PLCβ2, since they bind to the N-terminal region which is distant from the Gαq binding site. [20]. It is also notable that the binding site of γ-synuclein on PLCβ2 overlaps the calpain cleavage site and therefore, the presence of γ-synuclein prevents PLCβ2 degradation [27].

Like γ-synuclein, PLCβ2 is abnormally overexpressed in late-stage breast cancer cells and tissues [28,29]. Knockdown or overexpression of PLCβ2 affects cell migration only in late stage breast cancer cell lines with no effect on cell proliferation or invasion [29], suggesting that PLCβ2 is part of a signaling pathway that influences transition into the late-stage cancer phenotypes.

From the studies described above, we postulate that γ-synuclein might promote cancer phenotypes through its ability to increase levels of PLCβ2. This idea is supported by observations that in breast cancer cell line MDA MB 231, PLCβ2 and γ-synuclein co-localize with each other and PLCβ2 co-immunoprecipitates with γ-synuclein, suggesting a cellular interaction between the two proteins [20]. Here, we present evidence that γ-synuclein, by its ability to increase PLCβ2 levels, allows for enhanced Rac-mediated signals at the expense of Gαq signals thereby promoting cancerous phenotypes.

2. Materials and methods

2.1. Cell culture

MDA MB 231 cells were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 units/mL of penicillin and 50 μg/mL of streptomycin at 37 °C and 5% CO2. To knockdown protein expression levels, cells were transfected using Dharmafect reagent with 40 pg of γ-synuclein (Sigma), PLCβ2 (Sigma) or non-specific control (Ambien) siRNAs and were incubated for 96 h before performing an experiment. For migration, invasion and viability assays, cells were transfected with 40 pg each of γ-synuclein and PLCβ2 siRNAs or with 40 pg each of γ-synuclein and control siRNAs, or with 80 pg of the control siRNA.

2.2. Quantitative real time reverse transcriptase PCR (qRT-PCR)

Total RNA was extracted using RNAeasy kit (Qiagen) according to the manufacturer's instructions. 2 μg of total RNA was used for cDNA synthesis with random hexamers using Reverse Transcriptase kit (Qiagen). Using TaqMan® Gene Expression Assays (ThermoFisher) for SNCG (γ-synuclein), PLCB2 (PLCβ2) and ACTB (β Actin) as primers and using DNA Engine Opticon® 2 System (BioRad), real-time PCR was carried out with a denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing at 60 °C for 15 s and extension at 72 °C for 30 s. Upon completion of the cycling steps, the reaction was stored at 4 °C. Real-time PCR was carried out. Reactions were run in triplicate in three independent experiments. The geometric mean of housekeeping gene ACTB was used as an internal control to normalize the variability in expression levels. Expression data were normalized to the geometric mean of housekeeping gene ACTB to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method [30].

2.3. In vitro protein association studies

Binding between purified coumarin-PLCβ2 and purified C3PO in the presence and absence of purified γ-synuclein was carried out using the materials and procedure described in [20]. Briefly, purified proteins were labeled with the thiol-reactive probe7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) at a probe:protein ratio of 4:1 on ice. The reaction was stopped after 60 min by adding 10 mM DTT and the protein was purified either by extensive dialysis. Protein solutions were diluted into 20 mM Hepes (pH 7.2), 160 mM NaCl, and 1 mM DTT.

Spectral measurements were performed on an ISS spectrofluorometer (Champaign, IL) using 3 mm quartz cuvettes. Peptide and the emission spectrum of CPM-labeled protein were measured from 400 to 550 nm ($\lambda_{ex} = 380$ nm). The background spectra of unlabeled protein or peptide were subtracted from each spectrum along the titration curve, which was also corrected for dilution.

2.4. Immunofluorescence

Cells were fixed using 3.7% formaldehyde and permeabilized with 0.2% nonyl phenoxypolyethoxylethanol (NP40) and incubated with 0.2% NP40 in phosphate buffered saline (PBS) for 5 min and then blocked in PBS containing 4% goat serum for 1 h. Cells were then incubated with the primary antibody (anti-PLCβ2, anti-Rac1 or anti-Gαq (Santa Cruz Biochemicals, Inc.) diluted to 1:1000 for 1.5 h at 37 °C, followed by incubation with Alexa-labeled secondary antibody for 0.5 h at the same temperature. Cells were washed with Tris buffered saline (TBS) buffer after the incubations. Images of the cells were obtained using Olympus Fluoview FV1000 laser scanning confocal microscope, and were analyzed using Olympus (Fluoview) software and Image J (NIH).

2.5. Förster resonance energy transfer (FRET)

FRET measurements were performed using an Olympus Fluoview1000 instrument on MDA MB 231 cells co-expressing eCFP-tagged Rac1, eCFP-tagged Gαq and/or eYFP-tagged PLCβ2 proteins as described [31]. eCFP and eYFP were excited using 458 and 515 nm argon ion laser lines, respectively, and 480–495 and 535–565 nm bandpass filters to collect emission images, respectively. FRET efficiencies were calculated using the Olympus Fluoview software whose algorithm calculates FRET by sensitized emission after correcting for spectral bleed-