RESEARCH ARTICLE

Mitochondrial microprotein PIGBOS1 is possibly associated with cellular calcium homeostasis and neuro-protection

Aditya Seemanti, Amal K. Bera*

Abstract

Background: Calcium ion (Ca^{2+}) plays a vital role in many physiological processes like memory formation, cell-cell communication, secretion, neurotransmission, cell survival, and death. The concerted activities of several transporters and ion channels maintain an optimum concentration of Ca^{2+} inside the cell. Disruption of calcium homeostasis may cause abnormal protein aggregation, leading to several neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (PD). *Objective:* Several pieces of evidence indicate that altered calcium homeostasis, oxidative stress, and endoplasmic reticulum (ER) stress are intimately related to the development of PD. PIGBOS1, a recently discovered mitochondrial microprotein, has been shown to play an essential role in ER stress response through its interaction with an ER-resident chloride channel CLCC1. *Materials and Methods:* In the present study, we investigated the role of PIGBOS1 in cellular calcium homeostasis by monitoring Ca^{2+} dynamics in the ER, cytoplasm, and mitochondria using genetically encoded Ca^{2+} indicators. *Results:* In the resting state, PIGBOS1-overexpressed HEK-293T cells exhibited a higher level of basal Ca^{2+} in the ER. Further, cytosolic and mitochondrial Ca^{2+} rise following ionomycin/ histamine treatment was higher in PIGBOS1-overexpressed cells. *Conclusion:* Taken together, it implies that PIGBOS1 is an integral part of the cellular Ca^{2+} signaling network. We have discussed the possible role of PIGBOS1 in neurodegenerative diseases like PD.

Keywords: Calcium homeostasis, ER stress, Microproteins, Neurodegeneration, Parkinson's disease.

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disease (second most prevalent) caused by the loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain.[1,2] Several genetic and environmental factors are known to be involved in the initiation and progression of PD.[3] At the cellular level, mitochondrial dysfunction is one of the hallmarks of PD.[4] Mitochondria function as the powerhouse of cell and are also the site for multiple metabolic processes and reactive oxygen species (ROS) generation. They play a crucial role in cellular calcium homeostasis, senescence, cell survival, and death.[5] Mitochondrial dysfunctions such as the loss of complex I of the electron transport chain and overproduction of ROS are frequently observed in PD.[6] Reports suggest that cells transfected with mitochondrial DNA (mtDNA) taken from PD patients showed reduced complex I activity, indicating the importance of mtDNA in neuronal degeneration.[7] Transplantation of healthy mitochondria may restore the normal physiology of a diseased cell.[8]

In a cell, the endoplasmic reticulum (ER) and mitochondria are interconnected through a specialized microdomain called mitochondria-associated membrane (MAM), which plays a crucial role in calcium (Ca^{2+}) and lipid homeostasis.[9] PD patients often exhibit dysfunction of MAM and altered calcium homeostasis.[10,11] In response to an appropriate signal, the ER releases Ca^{2+}, a major portion of which is readily taken up by the mitochondria. The MAM helps efficiently transfer Ca^{2+} from the ER to mitochondria.[12] Although several mitochondrial enzymes require Ca^{2+} for their activity, uncontrolled Ca^{2+} uptake triggers excessive ROS generation leading to apoptosis and cell death. Altered ROS signaling has also been implicated in PD.[13] The lumen of ER is the hub for protein folding. Accumulation of unfolded/ misfolded proteins in the ER triggers a phenomenon known as ER stress.[14] In order to reduce ER stress, cells activate a response known as unfolded protein response (UPR). Higher the ER stress, more prone the cells are toward apoptotic response. One factor that results in higher ER stress and UPR is the disruption of calcium homeostasis.[15,16] Under normal conditions, ER maintains a higher Ca^{2+} load, constituting the intracellular Ca^{2+} store. If ER fails to maintain a high Ca^{2+} concentration. The chaperone proteins inside the ER cannot function properly as their activity depends on Ca^{2+} binding, Improper functioning of chaperone proteins leads to protein misfolding (Figure 1). It is now established that the disruption of cross-talk between ER and mitochondria contributes to the development of PD.[17] Therefore, targeting ER stress- Ca^{2+}-ROS axis could be an effective way of treating PD.

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54-amino acid PIGB opposite strand 1 (PIGBOS1), a recently discovered mitochondrial outer membrane protein, has been found to regulate the ER stress response through its interaction with an ER-resident chloride channel CLIC-like1 (CLCC1) protein. When the expression of PIGBOS1 was reduced, it enhanced the sensitivity of cells to ER stress and increased cell death.[18] Based on its strategic location, interaction with ER, and involvement in ER stress, we propose that PIGBOS1 possibly plays a protective role against PD. We explored its role in Ca^{2+} signaling, particularly in transferring Ca^{2+} from ER to mitochondria.

Materials and Methods

Cell Culture

HEK293T cell line was maintained in DMEM with 10% FBS in a cell-culture incubator under standard conditions (37°C, 5% CO_2). Cells were routinely passaged when around 80% confluency was reached.

Live cell Ca^{2+} imaging

Cells were seeded onto glass coverslips. The cells were transfected with 1.5 µg of pcDNA3.1 or PIGBOS1-FLAG and 1.5 µg of sensor plasmids (mtRCaMP, jGCaMP7s, GCEPIAer), using JetPrime transfection reagent (#Polyplus). PIGBOS1 construct was kindly provided by Dr. Alan Saghatelian, The Salk Institute for Biological Studies, USA. Cytoplasmic Ca^{2+} was imaged with jGCaMP7s. pGP-CMV-jGCaMP7s was a gift from Douglas Kim & GENIE Project (Addgene plasmid #104463; http://n2t.net/addgene:addgene:104463; RRID:Addgene_104463).[19] mtRCaMP and GCEPIAer were used for imaging mitochondrial and ER Ca^{2+} imaging, respectively. pCMV G-CEPIA1er was a gift from Masamitsu Iino (Addgene plasmid #58215; http://n2t.net/addgene:58215; RRID:Addgene_58215).[20] Imaging was performed at 44–48 hours post-transfection using a sCMOS camera and appropriate filter set, attached to an Olympus IX83 microscope. Background subtracted images were analyzed using Image J and MATLAB v.R2022a.

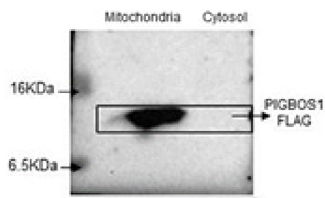

Figure 2: Western blot showing PIGBOS1-FLAG overexpression in mitochondrial fraction of HEK293T cells.

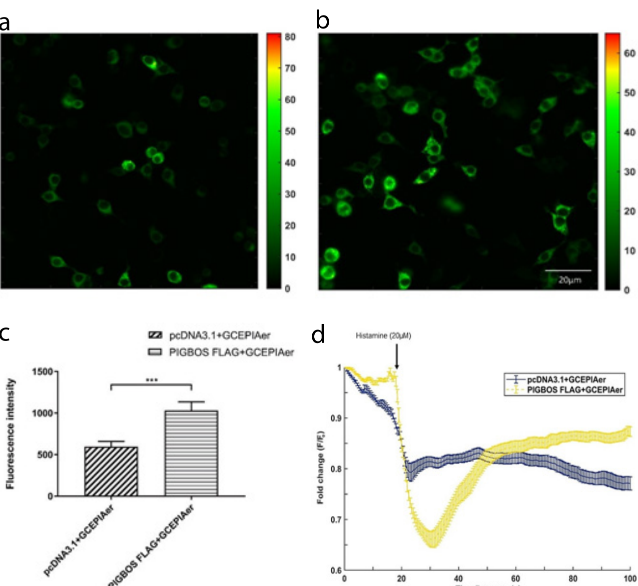

Figure 3: ER Ca^{2+} in control and PIGBOS1 overexpressed cells. ER Ca^{2+} was measured with genetically encoded ER calcium sensor GCEPIAer. (A) pcDNA3.1+GCEPIAer transfected control HEK293T cells. (B) PIGBOS1 overexpressed cells have a higher level of ER Ca^{2+} as reflected by the higher fluorescence intensity. (C) The fluorescence intensity of control and PIGBOS1 overexpressed cells are compared. The basal level of ER Ca^{2+} is significantly higher in PIGBOS1 overexpressed cells (Unpaired t-test; $p \leq 0.001$). (D) Histamine-induced ER Ca^{2+} release, depicted by decrease in fluorescence intensity. PIGBOS1 overexpressed cells exhibited greater release (due to higher level of basal Ca^{2+}).

Under normal conditions

Parkinson's Disease conditions

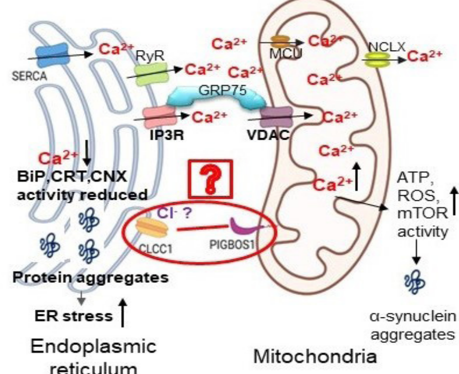

Figure 1: Cartoon diagram of ER-mitochondria cross-talk in normal and PD conditions**.** ER-mitochondria interaction is established by the mitochondria-associated membrane (MAM), made up of IP3R, VDAC, GRP75, and some other proteins. This interaction is known to be hampered in PD. Another such mitochondrial-ER interaction between PIGBOS1 and CLCC1 is possibly affected in PD.

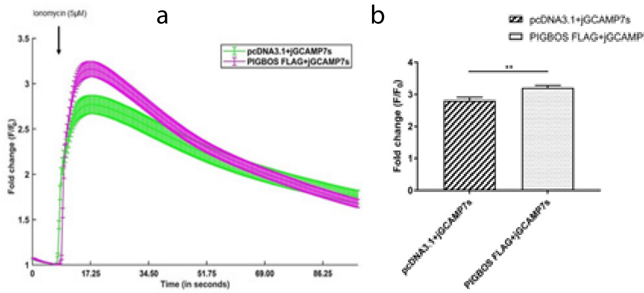

Figure 4: (A) Cytosolic Ca^{2+} rise caused by ER store depletion. Cytosolic Ca^{2+} was measured with jGCaMP7s in HEK293T cells. Ionomycin (5µM) was applied in Ca^{2+}-free buffer to deplete the store. (B) Fold change of cytosolic Ca^{2+} is significantly higher in PIGBOS1-FLAG transfected cells as compared to the control (Unpaired t-test; $p \leq 0.01$).

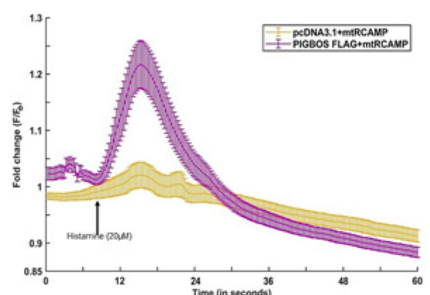

Figure 5: Mitochondrial Ca^{2+} uptake, measured with mtRCaMP. Ca^{2+} uptake is higher in PIGBOS1 overexpressed cells compared to the control. Cells were stimulated with histamine (20µM).

Western Blot

PIGBOS1-FLAG and mock-transfected cells were lysed in RIPA buffer (pH = 8), supplemented with PMSF, and protease inhibitor cocktail. The mitochondria were isolated by differential centrifugation. The mitochondrial pellet was resuspended in RIPA buffer. The proteins were separated on 12% Tricine-SDS PAGE.[21] Resolved proteins were then transferred to a PVDF membrane and probed with anti-FLAG primary antibody (Cell Signaling Technology, #14793S) and an HRP-conjugated secondary antibody. The blot was developed using Bio-Rad clarity western ECL substrate and then imaged with a Bio-Rad gel documentation system.

Results

Overexpressed PIGBOS1 is Localized in Mitochondria

We checked the expression and localization of PIGBOS1-FLAG. As shown in Figure 2, the mitochondrial fraction showed a strong PIGBOS1-FLAG band with an approximate molecular weight of 13 kDa. It is not detected in the cytosolic fraction. It confirms that the FLAG tag does not affect the localization of PIGBOS1; like native protein, the overexpressed protein is also targeted to mitochondria.

PIGBOS1 Overexpression Alters Calcium Homeostasis

ER Calcium

Cells were transfected with an equal amount of ER Ca^{2+} sensor plasmid GCEPIAer and pcDNA3.1 or PIGBOS1-FLAG, and the fluorescence intensity was measured. As shown in Figure 3a-c, PIGBOS1 overexpressed cells exhibited higher fluorescence intensity reflecting higher level of basal Ca^{2+} in the ER. The SERCA pump primarily executes the Ca^{2+} entry in the ER. On the other hand, Ca^{2+} release from the ER is mediated by the IP_3/Ryanodine receptors and Ca^{2+} leak channels. We activated the IP_3 receptor by using histamine. Histamine activates its receptor (GPCR) localized in the plasma membrane causing an increase of IP_3 which in turn activates ER-localized IP_3 receptors. Figure 3d depicts the release pattern of Ca^{2+} from the ER in response to histamine. As shown in the figure, the released Ca^{2+} from the ER in PIGBOS1 overexpressed cells is significantly higher compared to the control (Figure 3d).

Cytosolic Calcium

The cytosolic Ca^{2+} levels in control and PIGBOS1 overexpression conditions were measured in Ca^{2+}-free extracellular buffer, using a cytosolic Ca^{2+} sensor jGCaMP7s. In the PIGBOS1 overexpressed cells, Ca^{2+} ionophore ionomycin (5 µM) caused a higher rise of cytosolic Ca^{2+} as compared to the control (Figure 4A, B). In our experimental conditions, the Ca^{2+} entering cytosol comes from the ER, not the extracellular solution.

Mitochondrial Calcium

To check whether the released Ca^{2+} from the ER, is transferred promptly to the mitochondria, the mitochondrial Ca^{2+} was monitored using a mitochondrial Ca^{2+} sensor mtRCaMP. Mitochondrial Ca^{2+} rise by histamine was found to be significantly higher in PIGBOS1 overexpressed cells (Figure 5).

Discussion

Microproteins are small proteins (less than 100 amino acids long) encoded by the small open reading frame. The existence and function of several microproteins have been reported very recently.[22,23] One such microprotein PIGBOS1 is localized in the outer membrane of mitochondria. Although PIGBOS1 is not a part of MAM, it physically interacts with ER-localized chloride channel CLCC1.[18] PIGBOS1 has been associated with ER stress, though the precise mechanism is not known. We looked at the effect of PIGBOS1 overexpression on cellular calcium homeostasis by measuring the concentration and dynamics of Ca^{2+} in different subcellular compartments. Interestingly, PIGBOS1 overexpressed cells exhibited a higher level of basal Ca^{2+} in the ER. When ER Ca^{2+} store was depleted with ionomycin/histamine, PIGBOS1 overexpressed cells showed a higher Ca^{2+} rise in cytoplasm and mitochondria. It is not clear how a mitochondrial protein affects the ER Ca^{2+}, particularly when it is not a part of MAM. Earlier studies showed that CLCC1 has a role to play in determining Cl^- level in the ER.[24] Further, the Cl^- in ER is known to influence ER Ca^{2+} homeostasis.[25] It is possible that PIGBOS1 is not just an interacting partner of CLCC1, but also its functional modulator. The observed changes in ER Ca^{2+} by PIGBOS1 are possibly mediated through CLCC1, which needs further investigation. The increased uptake of Ca^{2+} in mitochondria suggests a positive role of PIGBOS1 in transferring Ca^{2+} from the ER to mitochondria. Alternatively, PIGBOS1 may modulate the mitochondrial Ca^{2+} import-export machinery. The conserved interaction of PIGBOS1-CLCC1 seems to have

immense physiological importance. Knockdown of either protein impacts the cell in the same way, *i.e.* heightened ER stress and cell death,[18,24] which are well-established phenomena associated with PD. Further, the association of PIGBOS1 in Ca^{2+} signaling emphasizes its possible protective role against PD. Although no direct evidence has emerged so far, we anticipate a discovery of the protective role of PIGBOS1 against neurodegenerative diseases like PD.

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Conflict of Interest

None

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