Neuroscience Research xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

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

Half-life of DISC1 protein and its pathological significance under hypoxia stress

Sandeep Kumar Barodia[a], Sang Ki Park[a,b], Koko Ishizuka[a], Akira Sawa[a,c,*], Atsushi Kamiya[a,**]

[a] Molecular Psychiatry Program, Department of Psychiatry, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21287, USA
[b] Department of Life Sciences, Pohang University of Science and Technology, Pohang, Republic of Korea
[c] Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form 21 February 2015
Accepted 23 February 2015
Available online xxx

Keywords:
Disrupted in schizophrenia 1 (DISC1)
Hypoxia
Oxygen–glucose deprivation
Protein half-life
Ubiquitin-proteasome system

ABSTRACT

DISC1 (disrupted in schizophrenia 1) is an intracellular scaffolding molecule which regulates multiple signaling pathways for neural cell differentiation and function. Many biological studies utilizing animal models of DISC1 have indicated that loss of DISC1 functions are associated with pathological psychiatric conditions. Thus, DISC1 protein stability is a prerequisite to its goal in governing neural function, and modulating the protein stability of DISC1 may be a key target for understanding underlying pathology, as well promising drug discovery strategies. Nonetheless, a half-life of DISC1 protein has remained unexplored. Here, we determine for the first time the half-life of DISC1, which are regulated by ubiquitin-proteasome cascade. Overexpression of PDE4B2, a binding partner of DISC1, prolonged the half-life of DISC1, whereas NDEL1 does not alter DISC1 protein stability. Notably, the half-life of DISC1 is diminished under hypoxia stress by increasing protein degradation of DISC1, suggesting that alteration of DISC1 stability may be involved in hypoxia stress-mediated pathological conditions, such as ischemic stroke.

1. Introduction

Regulation of protein stability via multiple steps of protein synthesis and proteasome-dependent degradation is critical for maintaining cellular function (Fonseca et al., 2006). The half-life of a protein is affected by multiple factors, which include post-translational modifications, change in subcellular distribution, and access to proteins involved in the ubiquitin-proteasome cascade (Ciechanover, 2005; Hershko, 2005; Tanaka et al., 1998). In particular, protein ubiquitination and subsequent proteasome-dependent degradation are major mechanisms underlying degradation or transient sequestration from the available functional pool, allowing the cell to fine-tune protein availability (Patrick et al., 2003).

The gene for disrupted in schizophrenia 1 (DISC1) was originally discovered as a transcript disrupted by a hereditary chromosomal translocation co-segregated with major mental illnesses in a Scottish pedigree (Millar et al., 2000). After this unique finding in psychiatric genetics, many biological studies have taken place: in summary, cellular and animal models have indicated that the process involving DISC1 can underlie biology relevant to major mental illnesses (Brandon and Sawa, 2011; Kamiya et al., 2012; Narayan et al., 2013). Multiple studies have indicated that loss of DISC1 functions is associated with pathological conditions, whereas the effects of gain of DISC1 functions are milder. Loss of DISC1 function impairs multiple developmental processes during brain development (Ishizuka et al., 2011). Suppression of DISC1 protein expression during brain development causes altered interneuron and dopamine neuron maturation as well as behavioral abnormalities after puberty relevant to major mental conditions, such as schizophrenia (Niwa et al., 2010). Nonetheless, we should note that there is no "genetic" support on the *DISC1* gene and mental illness after initial discovery of the Scottish pedigree. The discrepancy between biological significance and little genetic support is reminiscent of Tau in Alzheimer's disease: there is no clear evidence that the *Tau* gene is involved in the disease at the genetic levels (Davies and Koppel, 2009), whereas Tau protein plays a crucial role in neurobiology of the disease (Buee et al., 2000). The biological process involving DISC1 includes many interacting proteins, such as phosphodiesterase-4 (PDE4) family proteins and nuclear distribution element-like (NDEL1), critical molecules for

* Corresponding author at: Molecular Psychiatry Program, Department of Psychiatry, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21287, USA. Tel.: +1 410 955 4726; fax: +1 410 614 1792.
** Corresponding author at: Molecular Psychiatry Program, Department of Psychiatry, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21287, USA. Tel.: +1 410 502 0060; fax: +1 410 614 1792.
E-mail addresses: asawa1@jhmi.edu (A. Sawa), akamiya1@jhmi.edu (A. Kamiya).

http://dx.doi.org/10.1016/j.neures.2015.02.008
0168-0102/

regulating neuronal development and function (Brandon and Sawa, 2011; Kamiya et al., 2012; Narayan et al., 2013).

Here, we determine the half-life of DISC1 protein under the perspective that this information is essential for basic understanding of DISC1 and is also important for future drug discovery. Given that loss of, but not gain of, function of DISC1 is likely to be associated with pathological conditions in most cases, modulating the protein stability (in most cases, to make it more stable in a reasonable range) may be a promising drug discovery strategy. We also explore another pathological condition where DISC1 stability may be changed: examining the half-life of DISC1 in PC12 cells exposed to oxygen–glucose deprivation (OGD).

2. Materials and methods

2.1. Cell culture and maintenance

PC12 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 5% horse serum (HS, Invitrogen). Differentiation was initiated by treatment with nerve growth factor (NGF, Sigma–Aldrich) at a concentration of 50 ng/ml for 3 days, according to our published protocols (Kamiya et al., 2005).

2.2. CHX chase assay

The half-life of DISC1 protein was evaluated by CHX chase assay using published methods with some modifications (Patrick et al., 1998). Briefly, PC12 cells were seeded into 6-well plates at a density of 3×10^5 cells/well in DMEM media containing 10%FBS and 5%HS, and incubated overnight at 37 °C in a CO_2 incubator. Cells were treated with 20 μg/ml of cycloheximide (CHX) dissolved in absolute ethanol, and harvested in ice cold phosphate buffered saline (PBS, pH 7.4) at varying chase points by centrifugation at 2500 × *g* for 2 min at 4 °C. Cell pellets were lysed in a lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl and 1× protease inhibitor cocktail (Roche)) and sonicated for 3 × 5 s on ice. Total protein from the crude cell lysate was quantitated using the Lowry method. For each sample, protein concentration was normalized to 2 μg/μl and diluted (1:2) using an equal volume of SDS loading buffer with 5% β-mercaptoethanol. Samples were heated at 95 °C for 10 min.

2.3. Western blotting

Standard Western blotting method from our published protocol was used with minor modifications (Kamiya et al., 2005, 2006). Protein samples were analyzed by SDS-PAGE and Western blotting with the following antibodies: rabbit anti-DISC1 polyclonal antibody (1:500, ECM Biosciences), mouse anti-GAPDH monoclonal antibody (1:10,000, Santa Cruz), mouse anti-β-tubulin monoclonal antibody (1:5000, Sigma–Aldrich). For co-immunoprecipitation, we used rabbit polyclonal anti-HA (10 μg, Clontech) and mouse monoclonal anti-myc (5 μg, Santa Cruz) antibodies. Species-appropriate secondary antibodies were conjugated to HRP (Pierce) and detected by enhanced chemiluminescence (Thermo Scientific) using ImageQuant LAS4000 mini (GE Healthcare). Quantification of immunoblot was performed with ImageJ64 (National Institutes of Health). Optical density of immunoreactivity in Western blotting was acquired with Image J software (http://rsb.info.nih.gov/ij/). Protein levels were normalized to GAPDH and expressed as relative % integrated intensity.

2.4. Treatment with proteasome inhibitors

Treatment with a potent proteasome inhibitor, MG 132 was performed following modified published protocol (Lee et al., 2005). PC12 cells (3×10^5/well) were pretreated overnight with MG-132 (20 μM, Sigma–Aldrich) in a CO_2 incubator (5% CO_2 at 37 °C). CHX was directly added to the media and cells were harvested at 0, 6 and 12 h. Western blot was performed for the quantitative analysis of DISC1 protein levels as described above.

2.5. Pulse chase assay

Transfection of Myc-tagged mouse DISC1 expression constructs together with PDE4B2 and NDEL1 expression constructs was carried out with Lipofectamine 2000 reagent (Invitrogen) for PC12 cells according to our published protocols (Kamiya et al., 2005). The molar ratio of expression constructs was 1:1 for transfection. Pulse chase assays were carried out using previously published methods (Hara et al., 2005). At 48 h post-transfection, cells were starved in methionine-free DMEM and 1% dialysed bovine serum for 30 min, followed by a pulse phase with 0.1 mCi [^{35}S] methionine (Perkin-Elmer) in methionine-free DMEM with 1% dialysed bovine serum for 1 h. The media was then changed to methionine-free DMEM containing 1% dialysed bovine serum and 1 mM L-methionine. Proteins were extracted with the lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1% Triton X-100 and 1× Protease inhibitor cocktail) on ice and triturated through a 26-gauge needle. Crude lysates were cleared of insoluble debris by centrifugation at 14,000 × *g* for 10 min. 800 μl of supernatant from each sample was used for immunoprecipitation experiments by incubating with mouse anti-Myc antibody (Santa Cruz) overnight at 4 °C. Protein samples were then incubated with 30 μl of protein Asepharose 4B beads (50% slurry, Sigma–Aldrich) for 1 h on a rotator at 4 °C. Beads were washed three times with lysis buffer and bound proteins were extracted with 30 μl of SDS sample buffer (containing 5% β-mercaptoethanol) by boiling at 95 °C for 10 min. Immunoprecipitates were analyzed on SDS–PAGE and the gel was dried using a gel drier (Biorad). Dried gel was exposed to Kodak Biomax MR Film (Sigma–Aldrich) in a cassette at −80 °C for 1–3 days. Film was developed using Hope Micro-Max processor (Omni Imaging) and the autoradiography signals corresponding to DISC1 (~100 kDa) were quantified using ImageJ software. Protein levels were expressed as relative % integrated intensity.

2.6. Cell culture in hypoxic conditions

In vitro ischemia was induced in PC12 cells using a published method with some modifications (Agrawal et al., 2013). In brief, 3×10^5 cells/well of 6-well plates were sub-cultured in DMEM containing 10% FBS and 5% HS and incubated overnight at 37 °C in a CO_2 incubator. Hypoxia stress was induced to PC12 cells by transferring the culture plates to an airtight chamber [Invivo2 400 Hypoxia Workstation (Biotrace, Inc.)] with inflow and outflow valves that were infused with a mixture of 1% O_2, 5% CO_2, 94% N_2, in glucose deprived culture media (Leibovitz L-15 media with 1% dialysed bovine serum) to attain an OGD anaerobic atmosphere. Culture media was deoxygenated by incubating in a hypoxia chamber overnight before exposing cells to the hypoxic environment. For acute hypoxia, cells were incubated in a hypoxia chamber for 6 h and harvested in ice cold PBS and pelleted by centrifugation at 2500 × *g* for 2 min at 4 °C. To assess the recovery from hypoxia injury, cells were quickly transferred from the hypoxia chamber after 6 h of acute hypoxia to a humidified CO_2 incubator containing 5% CO_2 and were re-oxygenated (ReOxy) for 3 h. Cells were harvested in ice cold PBS and pelleted by centrifugation at 2500 × *g* for 2 min at 4 °C. Protein extracts were analyzed by Western blotting as described above. The integrated intensity of DISC1 protein bands was normalized with that of β-tubulin in the case of each protein sample.

Please cite this article in press as: Barodia, S.K., et al., Half-life of DISC1 protein and its pathological significance under hypoxia stress. Neurosci. Res. (2015), http://dx.doi.org/10.1016/j.neures.2015.02.008