Published in final edited form as:

Neurosci Res. 2015 August ; 97: 1–6. doi:10.1016/j.neures.2015.02.008.

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

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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.

Keywords

Disrupted in Schizophrenia 1 (DISC1); Hypoxia; oxygen-glucose deprivation; Protein half-life; Ubiquitin-Proteasome system

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 posttranslational modifications, change in subcellular distribution, and access to proteins involved in the ubiquitin-proteasome cascade (Ciechanover, 2005; Hershko, 2005; Tanaka et al., 1998). In

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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 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 CO₂ 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 2,500g for 2 minutes at 4°C. Cell pellets were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl and 1x protease inhibitor cocktail (Roche)) and sonicated for 3×5 seconds 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 minutes.

2.3. Western Blotting

Standard Western blotting method from our published protocol was used with minor modifications (Kamiya et al., 2005; Kamiya et al., 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 (10µg, Clontech) and mouse monoclonal anti-myc (5µg, SantaCruz) 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 CO₂ incubator (5% CO₂ 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 minutes, followed by a pulse phase with 0.1 mCi [³⁵S] 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-Cl (pH 7.4), 150 mM NaCl, 5mM MgCl₂, 5mM DTT, 1% Triton X-100 and 1X Protease inhibitor cocktail] on ice and triturated through a 26-gage needle. Crude lysates were cleared of insoluble debris by centrifugation at 14,000g 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 minutes. 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, 3×10⁵ 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 CO₂ 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% O₂, 5% CO₂, 94% N₂, 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 2,500g for 2 minutes 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 CO₂ incubator containing 5% CO₂ and were re-oxygenated (ReOxy) for 3 h. Cells were harvested in ice cold PBS and pelleted by centrifugation at 2,500g for 2 minutes 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.

2.7. Quantitative Real Time PCR (qRT-PCR)

qRT-PCR analysis of DISC1 transcripts was performed using our standard protocol, and exon specific primers were carefully designed for amplification specificity (Nakata et al., 2009). Total RNA was extracted from PC12 cells using an RNeasy mini kit (Qiagen). cDNA was synthesized from 1 µg of total RNA using SuperScript III First-Strand Synthesis System with oligo-dT priming (Invitrogen, Grand Island, NY, USA). The qRT-PCR reaction contained 10-fold diluted cDNA from the synthesis reaction, 1 × SYBR GreenER reagent (Invitrogen) and 200 nM specific forward and reverse primers in a 10 µl reaction volume. β-tubulin expression levels were used to normalize the expression data. Primer sequences were

as follows: rat DISC1_ex1-forward, 5'-GACAGTGGTTGTCGGCAAGAAT-3'; reverse, 5'-TGCTCCAAGCTACATCAAGGC-3'; rat DISC1_ex10-forward, 5'-TGAGAGAGTGTGGAAAGCGGA-3'; reverse, 5'-AGGCTCTGCATCAGCAA-3'; β -Tubulin-forward, 5'-TGCAGTGGCAAAGTGGAGATT-3'; reverse, 5'-TTGAATTTGCCGTGAGTGG-3'. Primers were designed using Primer Express 2.0 (Applied Biosystems). The PCR reaction and measurement was carried out with Applied Biosystems PRISM 7900. The PCR reaction conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation curve step was added at the end to verify the presence of a single amplicon in the reaction.

2.8. Statistical analysis

Statistical differences among more than three groups were determined using one-way analysis of variance (ANOVA), or two-way ANOVA with repeated measures followed by the Bonferroni multiple comparison test. A value of $p < 0.05$ was considered statistically significant and data are presented as mean $\pm$ SEM.

3. Results

3.1. Determination of the half-life of DISC1 protein

We first examined the half-life of DISC1 protein in PC12 cells by performing a CHX chase assay and pulse chase assay. Endogenous DISC1 protein expression in the total protein extract was gradually decreased with increased chase time points (from 3 to 24 h) following a characteristic exponential decay in the cells treated with CHX at a concentration of 20 μ g/ml, which inhibits protein synthesis. Using one phase decay equation, the half-life of DISC1 in undifferentiated and differentiated PC12 cells with 3 days NGF treatment was calculated to be 5.8 and 5.2 h, respectively (Fig. 1A and B). These data suggest that the differentiation process induced by NGF treatment has no robust effect on the half-life of DISC1 protein. To test whether the ubiquitin-proteasomal system mediates degradation of DISC1 protein under these conditions, we tested the effect of MG132 (a potent proteasome inhibitor) on DISC1 protein expression during critical CHX chase points, 6 h and 12 h, in undifferentiated P12 cells. Treatment with MG132 (20 μ M) in the presence of 20 μ g/ml CHX induced upregulation in DISC1 protein expression (Fig. 1C), suggesting that the degradation of DISC1 protein is mediated in part by a ubiquitin-proteasomal pathway. The half-life of DISC1 protein overexpressed in undifferentiated PC12 cells was also examined by using a pulse chase assay. We observed that autoradiography signals of [35 S]-L-methionine in immunoprecipitated samples were gradually reduced following an exponential decay curve. The half-life of DISC1 protein was estimated to be 2.7 h (Fig. 2). These results suggest that DISC1 protein in immunoprecipitated samples is less stable than DISC1 assessed in the total protein extract, indicating that turnover rates of DISC1 protein might be affected by its cellular compartmentalization.

3.2. PDE4B2, but not NDEL1 stabilizes DISC1 protein against proteasome-dependent degradation

The protein stability of DISC1 may be modulated by its binding partners. To test this idea, PDE4B2 and NDEL1, direct protein interactors of DISC1 (Brandon and Sawa, 2011), were

overexpressed with DISC1 in undifferentiated PC12 cells and a half-life of DISC1 protein was assessed using pulse chase assays. When PDE4B2 was co-expressed with DISC1, we found that the protein stability of DISC1 was increased, whereas no effect was observed of either wild type NDEL1 or mutant NDEL1 lacking DISC1 binding affinity (mutNDEL1) on the half-life of DISC1 (Fig. 3A and B). These data suggest that DISC1 protein stability may be specifically regulated by interaction with PDE4B2.

3.3. In vitro hypoxia suppresses the DISC1 protein synthesis

Deficits in brain development caused by hypoxic stress maybe partly mediated by the disruption of protein stability in certain molecules, which are critical for regulating multiple cellular processes in neuronal development (Hu et al., 2006). To investigate whether DISC1 protein stability is altered under hypoxic conditions, we measured DISC1 expression after cells were exposed to oxygen-glucose deprivation (OGD). We found a severe reduction in DISC1 protein expression under hypoxic conditions, which was reversed by re-oxygenation of hypoxic cells (Fig. 4A). To examine whether reduction of DISC1 protein expression is mediated by posttranslational events, DISC1 expression at the mRNA levels was measured by qRT-PCR experiments. Of note, DISC1 mRNA expression was up-regulated under hypoxic conditions, compared with normoxic and reoxygenated conditions (Fig. 4B). Thus, the reduction in DISC1 protein expression caused by hypoxic stress may be mediated by alteration in posttranslational mechanisms, such as protein degradation processes.

4. Discussion

To the best of our knowledge, this is the first study determining the half-life of DISC1 protein. Interestingly, DISC1 protein shows a shorter half-life in the pulse chase assay, compared to its half-life measured by the CHX chase assay. Note that in the CHX assay, half-life of DISC1 is assessed in total protein extract from crude cell lysate, which yields predominantly insoluble DISC1 protein fraction, whereas its half-life was analyzed in immunoprecipitates that would contain only the soluble DISC1 protein in the pulse chase assay. Protein stability is reportedly affected by its subcellular locations: for example, multiple proteins in the mitochondria, endoplasmic reticulum, and nucleus have longer half-lives on average than proteins in other cellular compartments (Price et al., 2010; Toyama and Hetzer, 2013). p53 protein is another example of varying turnover rates depending on subcellular compartmentalization (O'Brate and Giannakakou, Drug Resist Updat 2003). Given that DISC1 is expressed in multiple subcellular locations (Brandon and Sawa, 2011; Kamiya et al., 2012; Narayan et al., 2013), varied cellular compartmentalization of DISC1 may affect the results. DISC1 is an intracellular anchoring protein that has multiple protein binding partners (Brandon and Sawa, 2011). Thus, cellular compartmentalization of DISC1 may in part be regulated by its protein interactors, which may affect the half-life of DISC1. In fact, we also found that PDE4B2, but not NDEL1 specifically prolonged DISC1 protein turn over. Hypoxia causes an up-regulation of PDE4B2 expression leading to an increase of intracellular cAMP levels (Millen et al., 2006). Up-regulation of PDE4B2 caused by hypoxia stress might act as a compensatory mechanism for impaired DISC1 protein stability.

We report that DISC1 expression is significantly diminished at the posttranslational level under *in vitro* hypoxia stress (OGD). Interestingly, we observed an increase in DISC1 mRNA expression under these conditions as compared to normoxia. Up-regulation of DISC1 expression at the mRNA level may occur as a compensatory mechanism for aberrant translational machinery of DISC1 protein. Reoxygenation after hypoxia increases levels of reactive oxygen species (Fernandez-Checa et al., 1997). Oxidative stress induces various post-translational modifications, including carbonylation and glutathionylation (Gianazza et al., 2007). Thus, posttranslational modifications of DISC1 protein after re-oxygenation may protect against its proteolysis. Further investigation of underlying mechanisms is important in addressing how the protein stability of DISC1 is involved in hypoxic conditions.

Collectively, these results support the idea that altered DISC1 protein stability induced by hypoxic stress may have detrimental effects on cellular function, which may be involved in the pathophysiological basis behind neurological conditions, such as ischemia (Ohira et al., 2010; Schmidt-Kastner et al., 2006). Thus, stabilization of the DISC1 protein might have a beneficial effect on neuronal abnormalities caused by hypoxic stress. PC12 cells can only differentiated into sympathetic neuron-like cells by treatment with NGF (Bibel et al., 2007), and we cautiously state this interpretation without superficially generalize the idea to all differentiated neurons. Given that multiple studies report that the suppression of DISC1 protein expression leads to diverse structural and functional brain abnormalities during neurodevelopment (Ishizuka et al., 2011; Kamiya et al., 2005; Kubo et al., 2010; Kvalo et al., 2008; Mao et al., 2009; Taya et al., 2007), stabilization of the DISC1 protein may also be beneficial for proper brain development, the idea which is presently tested in our group (Hikida et al., 2012).

Acknowledgements

We thank Drs. Peter Espenshade and Wei Shao for sharing their lab equipments towards the successful completion of this work. We thank Dr. Miles Houslay for the kind gift of PDE4B2 expression construct and critical discussions. We thank Ms. Yukiko Lema, Mrs. Maxwell Towe, and Michael Ballinger for organizing the manuscript. This work was supported by Janssens Pharmaceuticals Inc. through a collaborative drug discovery project involving the Brain Science Institute at Johns Hopkins University School of Medicine (A.S., A.K.), MH-091230 (A.K.), AT-008547 (A.K.), MH-069853 (A.S.), MH-092443 (A.S.), MH-105660 (K.I., A.S.), and MH-094268 (A.S., A.K.).

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Fig. 1.

Measurement of DISC1 protein half-life by Cycloheximide (CHX) chase assay in PC12 cells. **(A)** Undifferentiated PC12 cells treated with CHX (20 µg/ml) yield a smooth exponential decay curve. The half-life of endogenous DISC1 was measured to be 5.8 h. **(B)** Differentiated PC12 cells upon treatment with CHX (20 µg/ml) for the indicated chase points yield a smooth exponential decay curve. The half-life of endogenous DISC1 was measured to be 5.2 h. **(C)** Selective proteasome inhibitor MG132 (20 µM) significantly upregulated DISC1 protein level at 6 h of CHX (20 µg/ml). Quantitative analysis of Western

blots was performed using integrated intensity of DISC1 protein bands normalized with GAPDH for each individual chase points. One phase exponential decay curve was obtained from the average of three independent experiments (**A, B**). $**P < 0.01$ was determined by two-way ANOVA with post hoc Bonferroni test (**C**).

Fig. 2.

Evaluation of the half-life of DISC1 protein by [^{35}S] pulse-chase assay. Undifferentiated PC12 cells expressing DISC1 protein showed a considerable short half-life of 2.7 h. Quantitative analysis of [^{35}S]-L-methionine labeled DISC1 protein band signals was performed using integrated intensity of each individual chase points. One phase exponential decay curve was obtained from the average of three independent experiments.

A

B

Fig. 3.

Binding partner PDE4B2 selectively ameliorates the half-life of DISC1 protein. **(A)** Co-expression of DISC1-myc with PDE4B2-HA significantly ameliorates the half-life of DISC1 protein i.e. half-life of protein increased to >6 h. **(B)** Either co-expression with NDEL1-HA or mutNDEL1-HA did not enhance the DISC1 protein level. Densitometric analysis was done for autoradiography signals from 21 ³⁵ [S]-L-methionine labeled DISC1 protein and data was obtained from the average of three independent experiments **(A, B)**. ** $P < 0.01$ was determined by two-way ANOVA with post hoc Bonferroni test **(A)**.

A

B

Fig. 4.

Hypoxia downregulates DISC1 protein expression in PC12 cells. **(A)** Hypoxic stress for 6 h robustly reduced the DISC1 protein expression in PC12 cells as compared to the normoxic condition. Re-oxygenation (ReOxy) of hypoxic PC12 cells for 3 h recovered the DISC1 protein expression. **(B)** Quantitative real time PCR (qRT-PCR) analysis showed significant increase in DISC1 mRNA levels in hypoxic PC12 cells, suggesting that DISC1 turnover is changed at the posttranslational level under hypoxia stress. Change in DISC1 mRNA expression induced by hypoxia is restored to normal levels by re-oxygenation of hypoxic

cells. Western blot analysis data are represented as bar graphs showing integrated intensity of DISC1 protein bands normalized with β -tubulin. Data from qRT-PCR is presented as relative quantification (RQ) values normalized with β -tubulin. Data were obtained from the average of three independent experiments (**A, B**). * $P < 0.01$ and ** $P < 0.001$ determined by one-way ANOVA with post hoc Bonferroni test (**A, B**).