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Dyrk1A-mediated phosphorylation of RCAN1 promotes the formation of insoluble RCAN1 aggregates

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HIGHLIGHTS

- RCAN1 self-associates and forms multimers.
- RCAN1 multimer formation is promoted by the Dyrk1A-mediated phosphorylation.
- Phospho-RCAN1 expression is lower in aged Dyrk1A TG mice than in littermates.
- Dyrk1A plays a role in the formation of insoluble RCAN1 aggregates upon aging.

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ABSTRACT

The mechanisms underlying aggregate formation in age-related neurodegenerative diseases remain not well understood. Here we investigated whether dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (Dyrk1A) is involved in the formation of regulator of calcineurin 1 (RCAN1) aggregates. We show that RCAN1 self-associates and forms multimers, and that this process is promoted by the Dyrk1A-mediated phosphorylation of RCAN1 at the Thr^{192} residue. Transgenic mice that overexpress the Dyrk1A exhibited lower levels of phospho-Thr^{192}-RCAN1 in 10-month-old-group compared to littermate controls, when analyzed with soluble hippocampus lysates. These results suggest that the phosphorylation of RCAN1 by Dyrk1A stimulates the formation of insoluble aggregates upon aging.

1. Introduction

Down syndrome (DS) is the most common genetic disorder and is caused by the presence of an additional copy of human chromosome 21. The genes that encode Dyrk1A and RCAN1 are both located on chromosome 21 and are strong candidate genes for several pathological features that are associated with DS, such as mental retardation and early-onset Alzheimer's disease (AD). Increased Dyrk1A and RCAN1 expression has been reported in human AD brains as well as DS brains [1,2]. Dyrk1A, which is a proline-directed serine/threonine kinase, has multiple functions with diverse substrates such as transcription factors, splicing factors, and synaptic proteins [3]. Transgenic mice that overexpress Dyrk1A (Dyrk1A TG mice) show severe learning and memory defects [4–6]. We have previously shown that the over-expression of Dyrk1A may contribute to AD neuropathology through Tau hyperphosphorylation and increased Aβ production via the phosphorylation of APP and presenilin 1 [7–9].

RCAN1, which is also known as DS critical region 1 (DSCR1), is an endogenous inhibitor of calcineurin, which is a calcium-dependent protein phosphatase that is critical for numerous physiological processes. RCAN1 knockout mice show learning and memory defects [10], and knockdown or overexpression of the *Drosophila melanogaster* RCAN1 homolog leads to severe learning impairment [11]. This suggests that RCAN1 is a candidate protein that is responsible for learning deficit in DS and AD. Recently, we found that Dyrk1A phosphorylates RCAN1 at the Ser^{112} and Thr^{192}

Abbreviations: AD, Alzheimer's disease; DS, Down syndrome; Dyrk1A, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A; GST, glutathione-*S*-transferase; IP, immunoprecipitation; RCAN1, regulator of calcineurin 1; pT192-RCAN1, phospho-Thr^{192}-RCAN1; pT212-Tau, phospho-Thr^{212}-Tau; TG, transgenic; WT, wild-type.

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residues [12]. The phosphorylation of RCAN1 at Ser^{112} primes the protein for GSK3β-mediated phosphorylation of Ser^{108}, while the phosphorylation of RCAN1 at Thr^{192} by Dyrk1A increases its activity as an inhibitor of calcineurin, which results in reduced NFAT transcriptional activity and enhanced Tau phosphorylation [12].

Insoluble protein aggregation is a common feature of many age-related neurodegenerative diseases, including AD [13,14]. It has been reported that the expression of RCAN1 leads to the aggregation of the RCAN1 protein in cultured cells and primary neurons [15]. During aging, multistep processes may cause conformational changes of RCAN1, thereby resulting in the production of diverse oligomeric intermediates and subsequent aggregation. The present study investigated the potential role of Dyrk1A in RCAN1-induced aggregate formation. We found that Dyrk1A-mediated RCAN1 phosphorylation at the Thr^{192} residue stimulates the formation of RCAN1 oligomers, which could result in the formation of insoluble aggregates during aging.

2. Materials and methods

2.1. Plasmids, proteins, and antibodies

Full-length mouse wild-type (WT) Dyrk1A as well as human WT and phosphorylation-defective mutant RCAN1S cDNAs were cloned into pcDNA3.1 as previously described [12]. Dyrk1A as well as WT and mutant RCAN1S proteins were purified with a Ni-NTA resin column or glutathione Sepharose column as previously described [8]. Anti-α-tubulin, anti-β-actin, anti-Myc, and anti-Flag antibodies were obtained from Sigma. Anti-glutathione-*S*-transferase (GST) antibody was obtained from AbFrontier. Antibodies against Tau and phospho-Tau (pT212) were obtained from Biosource. The anti-Dyrk1A antibody was custom-made as previously described [5]. Anti-RCAN1 antibodies were obtained either from Abnova or were custom-made by using synthetic peptides, and a phosphospecific RCAN1 antibody was custom-made as previously described [12].

2.2. In vitro RCAN1 phosphorylation by Dyrk1A

Purified RCAN1 protein was incubated with or without Dyrk1A for 1 h at 37 °C in a kinase buffer (20 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, and 20 μM sodium orthovanadate) that contained 25 μM ATP, as described previously [12]. The reaction mixtures were separated on SDS-polyacrylamide gels and were analyzed by carrying out immunoblotting with anti-RCAN1 antibodies.

2.3. Preparation of lysates from cell cultures and brains

HEK293T cells were transfected with the indicated plasmids by using the calcium phosphate precipitation method. One day later, the cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholic acid) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), a protease-inhibitor cocktail, and a phosphatase-inhibitor cocktail from GenDEPOT. Subsequently, they were subjected to western blot analysis. Dyrk1A TG mice were generated and maintained as previously described [5]. Experiments were performed in accordance with guidelines set forth by the Inje University Council Directive for the proper care and use of laboratory animals. The mice were sacrificed by performing cervical dislocation. Their brains were dissected, snap-frozen in liquid nitrogen, Dounce homogenized in RIPA buffer that contained 1 mM PMSF and 0.5 mM sodium orthovanadate, and centrifuged. The supernatants (Nonidet P-40-soluble fraction) were used for western blotting and the pellet (Nonidet P-40-insoluble fraction) was solubilized in 4% SDS sample buffer (0.5 M Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 10% mercaptoethanol) as described by Lee et al. [16]. Protein concentrations were determined using the Bradford method (Bio-Rad). Typically, 25–50 μg of the brain lysates was used for western blotting. Densitometric quantification was carried out by using the ImageJ 1.42 software (NIH, USA).

2.4. Coimmunoprecipitation and GST pull-down assay

For coimmunoprecipitation (co-IP), HEK293T cell lysates (500 μg) that had been transfected with the indicated plasmids were incubated with control IgG (R&D systems), anti-Myc or anti-Flag antibodies overnight at 4 °C in RIPA buffer with protease inhibitors and 1 mM PMSF. The next day, the bead mixture was gently washed with 1% Triton X-100 in RIPA buffer after 1 h of incubation with protein A beads (Pierce), and the bound proteins were subjected to immunoblot analysis with the indicated antibodies. For the GST pull-down assay, purified GST-RCAN1 WT or the T192A mutant was incubated with HEK293T cell lysates (500 μg) that had been transfected with the plasmid that encoded Myc-RCAN1, for 1 h at 4 °C in binding buffer (50 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 0.01% Nonidet P-40, 0.5 mM EDTA, and 1 mM PMSF). The beads were then washed 3 times with binding buffer with 300 mM NaCl, and the bound proteins were subjected to immunoblot analysis with the indicated antibodies.

3. Results

3.1. RCAN1 self-associates and this process is promoted by Dyrk1A-mediated phosphorylation

RCAN1 aggregation potentially involves a multistep process of protein conformational change. To understand this process, we first assessed whether RCAN1 self-interacts to form homodimers. Differentially tagged full-length RCAN1 were generated for co-IP and GST pull-down experiments. IP was performed using the cell lysates of HEK293T cells that had been transfected with expression vectors that encoded Myc- and Flag-tagged RCAN1. Flag-tagged RCAN1 was co-immunoprecipitated with Myc-tagged RCAN1 (Fig. 1A). Furthermore, reverse co-IP experiments with the anti-Myc antibody also revealed an interaction with Flag-RCAN1, which suggests that transfected RCAN1 self-interacts and is capable of forming dimers (Fig. 1B). To further confirm this observation, a GST pull-down assay was performed. Myc-tagged RCAN1 that was overexpressed in HEK293T cell lysates was pulled down by GST-RCAN1, but not by GST alone, which showed the self-association of RCAN1 (Fig. 1C). Taken together, these results indicate the existence of an interaction between full-length RCAN1 proteins, which suggests that RCAN1 can occur as a homodimer within cells.

We have previously shown that Dyrk1A phosphorylates RCAN1 on residues Ser^{112} and Thr^{192} [12]. Phosphorylation of RCAN1 at Ser^{112} by Dyrk1A primes the protein for subsequent phosphorylation by GSK3β at Ser^{108} [12]. There was little difference between the RCAN1(S112A) mutant and the RCAN1 WT in terms of their effect on calcineurin binding and activity as well as Tau phosphorylation [12]. On the other hand, the phosphorylation of RCAN1 at Thr^{192} by Dyrk1A activates its function as a calcineurin inhibitor, which leads to reduced NFAT transcriptional activity and enhanced Tau phosphorylation [12]. We investigated the role of Dyrk1A-mediated phosphorylation of RCAN1 at Thr^{192} on the self-association of RCAN1. The phospho-Thr^{192}-RCAN1 (pT192-RCAN1) was detected after co-IP with the anti-Flag antibody, but not with the anti-Myc antibody (Fig. 1A and B), perhaps because of technical issues such as varying efficiencies of the 2 antibodies for immunoprecipitation of RCAN1. To further examine the role of Dyrk1A in the RCAN1 self-association, the interaction between Myc-RCAN1 and