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THE DOWN SYNDROME CANDIDATE DUAL-SPECIFICITY TYROSINE PHOSPHORYLATION-REGULATED KINASE 1A PHOSPHORYLATES THE NEURODEGENERATION-RELATED SEPTIN 4

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Abstract—The dual-specific kinase DYRK1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A) is the mammalian orthologue of the *Drosophila* minibrain (MNB) protein kinase and executes diverse roles in neuronal development and adult brain physiology. *DYRK1A* is overexpressed in Down syndrome (DS) and has recently been implicated in several neurodegenerative diseases. In an attempt to elucidate the molecular basis of its involvement in cognitive and neurodegeneration processes, we searched for novel proteins interacting with the kinase domain of DYRK1A in the adult mouse brain and identified septin 4 (SEPT4, also known as Pnutl2/CDCrel-2). SEPT4 is a member of the group III septin family of guanosine triphosphate hydrolases (GTPases), which has previously been found in neurofibrillary tangles of Alzheimer disease brains and in α-synuclein-positive cytoplasmic inclusions in Parkinson disease brains. In transfected mammalian cells, DYRK1A specifically interacts with and phosphorylates SEPT4. Phosphorylation of SEPT4 by DYRK1A was inhibited by harmine, which has recently been identified as the most specific inhibitor of DYRK1A. In support of a physiological relation in the brain, we found that *Dyrk1A* and *Sept4* are co-expressed and co-localized in neocortical neurons. These findings suggest that SEPT4 is a substrate of DYRK1A kinase and thus provide a possible link for the involvement of DYRK1A in neurodegenerative processes and in DS neuropathologies. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: trisomy 21, scaffold, protein interaction, harmine, DYRK1A, SEPT4.

Down syndrome (DS), the most common genetic cause of mental retardation in humans, is characterized by three copies of chromosome 21, particularly of the DS critical region (DSCR) located on its long arm (*Homo sapiens* autosome (HSA) 21q22). The pathophysiological features of DS in the nervous system include a reduction in brain volume and weight in relation to body weight, a reduced number and density of neurons as well as abnormal cytoarchitecture in the cerebral cortex and reduction in cerebellar size (Wisniewski, 1990; Raz et al., 1995). These neuroanatomical alterations may partially explain the cognitive deficits and locomotor impairments of DS individuals. Accordingly, much attention has been paid to HSA21 genes with a known role in brain development. This includes the gene for the protein kinase DYRK1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A), which is the mammalian orthologue of the *minibrain* (*mnb*) gene in *Drosophila* (Tejedor et al., 1995). DYRK1A/minibrain (MNB) is involved in neural progenitor cell proliferation, neurogenesis and neuronal differentiation and thus compellingly linked to several brain developmental alterations observed in DS (reviewed in Hämmerle et al., 2003a). Similar alterations were observed in animal models with a selective loss or gain of DYRK1A/MNB function (Tejedor et al., 1995; Altafaj et al., 2001; Branchi et al., 2004; Fotaki et al., 2004). These observations together with the overexpression of *Dyrk1A* reported in DS brains (Guimera et al., 1999; Ferrer et al., 2005; Dowjat et al., 2007; Wegiel et al., 2008) support the notion of an effect of *Dyrk1A* gene dosage in the etiology of DS and associated cognitive deficits (Kahlem, 2006).

Apart from their developmental roles, it is increasingly recognized that the overexpression of HSA21 genes in the adult may contribute to cognitive deficits in DS (Antonarakis et al., 2004). This is particularly relevant since, in addition to brain developmental alterations and cognitive deficits, most DS individuals develop neuropathological features of Alzheimer disease (AD) (Teipel and Hampel, 2006). Recent insights into the function of DYRK1A suggest that it may contribute to the development of these features (Head et al., 2007), and those of other neurodegenerative diseases, particularly dementia with Lewy bodies (Simard and van Reekum, 2001; Kim et al., 2006) and Parkinson disease (PD (Kim et al., 2006). The DYRK1A kinase may promote the formation of characteristic patho-

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Abbreviations: AD, Alzheimer disease; CC, coiled-coil; DIG, digoxigenin; *dox*, doxycycline; DS, Down syndrome; DYRK1A, dual-specificity tyrosine phosphorylation-regulated kinase 1A; GST, glutathione S-transferase; HSA, *Homo sapiens* autosome; MNB, minibrain; NFT, neurofibrillary tangle; PBR, polybasic region; PD, Parkinson disease; PRD, proline-rich domain; SEPT4, septin 4.

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logical hallmarks of these diseases by direct phosphorylation of their most abundant components, such as tau in neurofibrillary tangles (NFTs) (Woods et al., 2001; Kimura et al., 2007; Ryoo et al., 2007; Liu et al., 2008), α-synuclein in Lewy bodies (Kim et al., 2006) and Aβ in amyloid plaques (Park et al., 2007; Ryoo et al., 2008). Thus, *Dyrk1A* overexpression may cause the typical hallmarks of AD observed in DS individuals (i.e. tau protein hyperphosphorylation with neurofibrillary degeneration and deposition of fibrillar β-amyloid in plaques). However, the precise contribution of DYRK1A to the pathophysiology of neurodegenerative diseases and the underlying molecular mechanisms remain unclear.

To further understand the molecular mechanisms by which DYRK1A is involved in adult brain function and contributes to the etiology of neurodegenerative diseases, we searched for possible interaction partners of DYRK1A in the adult mouse brain. We identified septin 4 (SEPT4, also known as Pnutl2/CDCrel-2), a neurodegeneration-related septin, as an interacting protein and specific substrate of DYRK1A kinase. We discuss the possible involvement of this interaction in neurodegenerative processes and DS neuropathologies.

EXPERIMENTAL PROCEDURES

Yeast two-hybrid screen

The yeast two-hybrid screen was carried out using the MATCHMAKER GAL4 two-hybrid system 3 (Clontech, Mountain View, CA, USA) according to the manufacturer's protocols and as described previously (Sitz et al., 2004). In brief, yeast cells expressing the kinase domain of DYRK1A, fused to the GAL4 DNA binding domain, were mated with cells pre-transformed with a cDNA library (Clontech MATCHMAKER library from mouse brain). Positive interacting clones were isolated and verified as previously described (Sitz et al., 2004).

Plasmid construction

The construction of the bait plasmids for the yeast two-hybrid screen and the *pEGFP-Dyrk1A* fusion plasmid was previously described (Sitz et al., 2004). To generate the *Sept4_v1* cDNA, the clone from the yeast two-hybrid screen (coding for *Sept4_v7*) was fused to an RT-PCR product from mouse brain (amplified using the primers JS.95, 5′-CATGGACCATTCACTGGGAT-3′, and JS.96, 5′-CCTTGTCATCCTCAGAGGAA-3′), coding for the N-terminus of *Sept4_v1*, and to the IMAGE clone 4166731 (GenBank BF300606), coding for the C-terminus. The complete *Sept4_v1* cDNA was subsequently transferred to pCMV-Tag2A to allow for the expression of *Flag-Sept4*.

A full length *Sept5* cDNA was amplified from mouse brain by RT-PCR (primers JS.93, 5′-GCTCGCAGGAATTCTGAGGT-3′, and JS.81, 5′-CGGCCGTCTATCCTTGTCGA-3′) and also transferred to pCMV-Tag2A. This cDNA corresponds exactly to the rat transcript NM_053931 and represents a previously undescribed splice variant of murine *Sept5.* We termed this isoform *Sept5_v3*.

All sequences amplified by PCR were completely sequenced, and constructs generated by subcloning were verified by sequencing the regions at and adjacent to the cloning sites.

Generation of a stable doxycycline (*dox*)-inducible cell line expressing *EGFP-Dyrk1A*

The Flp-In T-REx™ system from Invitrogen (Carlsbad, CA, USA) was used to generate a stable, *dox*-inducible mammalian cell line for controlled overexpression of *Dyrk1A*. *EGFP-Dyrk1A* was subcloned from *pEGFP-Dyrk1A* (Becker et al., 1998) between the *Hin*dIII and the *Not*I site of pcDNA5/FRT/TO. The resulting expression plasmid was introduced into the HEK293 Flip-In T-REx (HEK293 F-T) host cell line (Invitrogen) by Flp recombinase-mediated integration according to the supplier's instructions. The cells were selected for resistance to 50 μg/ml hygromycin (PAA Laboratories, Pasching, Austria). After 5 days, the remaining cells were selected for five additional days in the presence of 50 μg/ml hygromycin and 15 μg/ml blasticidine before the stable cell line was fully established and stored in liquid nitrogen. Expression of *EGFP-Dyrk1A* was induced with 2 μg/ml *dox*.

Immunoprecipitation

For co-immunoprecipitation experiments, the *EGFP-Dyrk1A* overexpressing HEK293 cells (see above) were transfected in 9-cm culture dishes with 6 μg of the expression plasmid for *Flag-Sept4* or *Flag-Sept5*, and subsequently treated with *dox* to induce expression of *EGFP-Dyrk1A*. Three days after transfection, the cells were lysed for 30 min on ice in 1 ml of lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 μg/ml aprotinine, 1 mM PMSF, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 1% NP-40). The lysate was cleared by centrifugation at 4 °C for 10 min at 20,000$\times$*g* and the supernatants were pre-absorbed to protein A agarose (without antibody) for 2 h. FLAG-tagged proteins were then immunoprecipitated with 40 μl of anti-FLAG M2 affinity matrix (Sigma, Taufkirchen, Germany) for 3 h at 4 °C. The matrix was subsequently washed twice with 1 ml of wash buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% NP-40) and once with wash buffer without NP-40. Bound proteins were eluted with 3$\times$ FLAG peptide (Sigma) for 30 min at room temperature and subjected to Western blot analysis with a polyclonal goat anti-GFP antibody (1:1000, Rockland Inc., Gilbertsville, PA, USA) and mouse monoclonal anti-FLAG M2 antibody (4 μg/ml, Sigma). Signals were revealed by enhanced chemiluminescence using horseradish peroxidase–coupled secondary antibodies. Blots were reused after stripping off the primary antibody by incubation in 2% SDS, 50 mM Tris–HCl, 150 mM NaCl, pH 7.4 in the presence of 100 mM 2-mercaptoethanol.

In vitro kinase assays

To generate the substrate for the kinase assay, FLAG-tagged SEPT4 was expressed in transiently transfected HEK293 cells and purified by immunoprecipitation using anti-FLAG M2 affinity resin (Sigma). The recombinant FLAG-tagged protein was eluted with an excess of 3$\times$ FLAG-peptide (Sigma; 100 ng/μl in 50 mM Hepes pH 7.4; 150 mM NaCl) for 30 min at 4 °C, and 25% of the eluate from one near-confluent 9-cm plate was used as a substrate in each kinase reaction. $MgCl_2$ was added to a final concentration of 5 mM, and the eluate was incubated for 30 min at 30 °C with 0.1 units glutathione S-transferase– (GST-) DYRK1A-ΔC in the presence of 50 μM [γ-^{32}P]ATP (100 μCi/ml). One unit of kinase activity was defined as the amount that catalyzed the phosphorylation of 1 nmol DYRKtide per min at 30 °C (de Graaf et al., 2004). Preparation of bacterially expressed GST-DYRK1A-ΔC was described previously (Himpel et al., 2000); the C-terminally deleted mutant was used because of its higher specific activity. The samples were separated by SDS-PAGE, proteins were blotted onto nitrocellulose, and phosphorylated proteins were detected with the help of phosphor storage screens.

For the immunocomplex kinase assays, NP-40 in the lysis buffer was substituted by 1% BRIJ-97 (Sigma) as a milder detergent, and the lysate was incubated with the anti-FLAG affinity matrix for 4 h at 4 °C. The resin was washed twice with wash buffer supplemented with 1% BRIJ-97, once with wash buffer without detergent, and once with kinase buffer (25 mM Hepes pH

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