Regulation of Dyrk1A kinase activity by 14-3-3

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Abstract

Dual specificity tyrosine(Y) regulated kinase 1A (DYRK1A) is a serine/threonine protein kinase implicated in mental retardation resulting from Down syndrome. In this study, we carried out yeast two-hybrid screening to find proteins regulating DYRK1A kinase activity. We identified 14-3-3 as a Dyrk1A interacting protein, which is consistent with the previous finding of the interaction between the yeast orthologues Yak1p and Bmh1/2p. We showed the interaction between Dyrk1A and 14-3-3 in vitro and in vivo. The binding required the N-terminus of Dyrk1A and was independent of the Dyrk1A phosphorylation status. Functionally, 14-3-3 binding increased Dyrk1A kinase activity in a dose dependent manner in vitro. In vivo, a small peptide inhibiting 14-3-3 binding, sc138, decreased Dyrk1A kinase activity in COS7. In summary, these results suggest that DYRK1A kinase activity could be regulated by the interaction of 14-3-3.

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Dual-specificity tyrosine(Y) regulated kinase 1A (DYRK1A) is a member of a novel DYRKs subfamily with dual specificities: autophosphorylation for self-activation on tyrosine residue in the active loop of the catalytic domain, and target protein phosphorylation on serine/threonine residues [1–3]. To date, seven mammalian DYRKs members have been isolated (DYRK1A, DYRK1B, DYRK1C; DYRK2; DYRK3; DYRK4A, and DYRK4B) [2]. Among isoforms the human DYRK1A gene is localized in the Down syndrome (DS) critical region of chromosome 21, which is associated with a variety of anomalies of DS, including mental retardation [4–6]. DYRK1A is expressed in the cerebral cortex, cerebellum, and hippocampus, which are affected regions of the brain in DS [6]. Transgenic mice overexpressing DYRK1A have shown cognitive deficit and loss of learning and memory shown in DS [7,8]. It is also known that Drosophila minibrain (mnb), the human DYRK1A orthologue, is involved in postembryonic neurogenesis, and its mutation causes a reduction of the brain size due to a decrease in the production of neuronal progeny [9]. Taken together, DYRK1A is considered to be a gene that is responsible for at least a portion of mental retardation in relation to DS.

Dyrk1A encodes several structural motifs such as bipartite nuclear localizing signal (NLS), DYRK homology (DH) box, PEST region, serine/threonine rich region, and 13 consecutive histidine repeats [1,3]. Dyrk1A is localized to the nucleus predominantly by its canonical NLS sequence [2,10] and is known to phosphorylate transcription factors including CREB, FHKR, STAT3, and GLI1 in the nucleus [11–14]. For
CREB, its phosphorylation mediates the neuronal differentiation of neural progenitor cells [11]. To date, two different modes of positive regulation for DYRK1A activity have been reported in response to bFGF and E1A [11,15]. However, the detailed upstream signaling that regulates the activity of DYRK1A remains unknown.

14-3-3 is a large family of acidic 28–33 kDa proteins conserved from yeast to mammal that is abundant and widely expressed in all eukaryotic cells [16]. 14-3-3 interacts with over hundreds of various target proteins and the interaction is predominantly dependent on the phosphoserine-containing motif [17,18]. 14-3-3 has attracted substantial attention because of its pleiotropic biological effects [18]. First, binding of 14-3-3 can modulate the localization of several target proteins. For example, 14-3-3 translocates phosphorylated cdc25 from nucleus to cytosol and promotes the retention of phosphorylated FKHRL1 in cytosol [19,20]. Second, binding of 14-3-3 can regulate the intrinsic catalytic activity of target proteins. 14-3-3 increases the activity of serotonin-N-acetyltransferase and Raf-1 kinase, or decreases RSK1 kinase activity upon binding [21–23]. Third, 14-3-3 is also involved in other modes of regulation, including scaffolding, protection of proteolysis, dephosphorylation, and sequestration of target protein [18].

In this study, we identified 14-3-3 as a binding partner of Dyrk1A through yeast two-hybrid screening. We characterized the interaction of 14-3-3 with Dyrk1A and examined the function of the 14-3-3 binding on Dyrk1A kinase activity.

Materials and methods

Plasmids. For bacterial expression, 14-3-3 and Dyrk1A were cloned into pGEX4 and pET25, respectively (Amersham Bioscience, UK). pEBG (GST fusion vector) and pcDNA3.1a (Invitrogen, USA) were used for mammalian expression. Deletion mutants were made by PCR using region specific primers. For single amino acid mutants, site-directed mutagenesis was performed as described in the manual (Quickchange, Stratagene, USA). Dr. Haian Fu kindly provided sc138 and sc174 constructs, encoding small peptides inhibiting 14-3-3 binding [24].

Yeast two hybrid. Yeast two-hybrid assay was performed using a L40 yeast strain, as described previously [25]. Full-length mouse Dyrk1A cloned into pBHA was used for screening human and rat brain cDNA libraries (Clontech, USA).

Purification of recombinant proteins. For Dyrk1A, transformed BL21(DE3) was cultured at 30 °C until OD600 of 0.5, and IPTG (final concentration, 1 mM) was added for induction. Four hours after induction, cells were lysed by sonication in lysis buffer [20 mM Tris–Cl (pH 8.0), 300 mM NaCl, 30 mM imidazole, and 1% protease inhibitor cocktail]. After centrifugation at 20,000 g for 30 min at 4 °C, supernatants were loaded into the Ni–NTA column (Qiagen, Germany), which has affinity to the intrinsic 13 His repeat of Dyrk1A. The column was washed with 5 bed volumes of washing buffer (50 mM imidazole in lysis buffer) and Dyrk1A was eluted with 300 mM imidazole in lysis buffer (>95% purity). Purification of GST fusion protein was performed as recommended in the manual (Amersham Bioscience).

Preparation of cell lysates. COS7 cells were maintained in high glucose DMEM supplemented with 10% FBS (Invitrogen). One day before transfection, cells were seeded at a density of 2 × 10⁵ cells/well in a 6-well plate. Recommended procedures were followed as described in the manual (Invitrogen). After transfection, cells were lysed in RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate]. After centrifugation, supernatants were subjected to immunoblotting, immunoprecipitation, or pull-down assay. For dephosphorylation, supernatants were treated with lambda phosphatase (New England Biolabs, USA) for 2 h at 30 °C.

Immunoprecipitation and pull-down. For Dyrk1A immunoprecipitation, 600 µg of protein lysates was incubated with 2.5 µg affinity purified anti-Dyrk1A antibody (directed against C-terminal fragment of Dyrk1A) with gentle rocking overnight at 4 °C followed by incubation with protein A resin for 1 h. For GST14-3-3 precipitation, lysate was incubated with glutathione–Sepharose resin (Amersham Bioscience). Pull down was carried out by adding 5 µg GST14-3-3 (or its mutant protein) and glutathione–Sepharose resin. Immunoprecipitates or pull-downs were resolved on 10% SDS-PAGE and transferred to PVDF membrane for immunoblotting.

Dyrk1A kinase assay. The kinase reactions were carried out by incubating purified Dyrk1A (50 ng) with dephosphorylated MBP (1 µg. Upstate, USA) in kinase buffer [20 mM Hapes (pH 7.4), 2 mM Na3VO4, 2 mM DTT, 10 mM MgCl2, and 0.5 µCi [γ-32P]ATP (specific activity of 3000 Ci/mmol, Amersham Bioscience)] for 30 min at 30 °C. MBP was separated on 12% SDS-PAGE and its radioactivity was quantified using FLA3000 (Fuji, Japan). For in vivo kinase assay, Dyrk1A immunoprecipitates were used.

Results

To identify Dyrk1A interacting proteins, we carried out yeast two-hybrid screening in rat and human brain cDNA libraries using full-length mouse Dyrk1A as a bait. We found a total of 38 clones including eukaryotic transcriptional initiation factor 2, a known substrate of Dyrk1A. Two independent clones were identified as the exon 1 of 14-3-3η. Since exon 1 of 14-3-3η was composed of 29 amino acids that are highly conserved in the 14-3-3 family, we expected that Dyrk1A could bind other members of the 14-3-3 family. We examined the interaction of Dyrk1A with 14-3-3ε, a brain enriched isotype of the 14-3-3 family.

Dyrk1A binds 14-3-3 in vitro and in vivo

To examine the interaction of Dyrk1A with 14-3-3 in vitro, purified Dyrk1A was pulled down by GST14-3-3. Dyrk1A was co-precipitated with GST14-3-3 in a concentration dependent manner, but not with GST (Fig. 1A). The interaction between Dyrk1A and 14-3-3 was also confirmed in vivo. COS7 cells were transfected with GST or GST14-3-3 and the lysate was incubated with glutathione–Sepharose resin (Fig. 1B, top panel). Dyrk1A was co-precipitated with GST14-3-3 but not with GST (Fig. 1B, middle panel). However, when Dyrk1A was immunoprecipitated, only GST14-3-3 was detected (Fig. 1B, bottom panel). It is known that K(lysine)50 residue in the consensus target binding motif is critical
for 14-3-3 interaction. We tested whether the K50 residue is essential for the interaction between Dyrk1A and 14-3-3. Fig. 1C shows that Dyrk1A in the lysate was pulled down by GST14-3-3 but not by GST14-3-3 (K50E) mutant. In vivo, when lysates transfected with GST14-3-3 or GST14-3-3 (K50E) were precipitated with glutathione–Sepharose resin, co-precipitation was detected only with GST14-3-3, supporting the in vitro result (Fig. 1D).

Since the 14-3-3 generally binds its partner in a phosphorylation dependent manner [17,18], we tested whether phosphorylation of Dyrk1A was required for binding. Cell lysate was treated with lambda phosphatase (Fig. 2, middle panel) and pulled down by GST14-3-3 (Fig. 2, bottom panel). Interaction between 14-3-3 and Dyrk1A was not affected by phosphatase treatment, suggesting that the interaction was phosphorylation independent.

**Binding of 14-3-3 requires N-terminus and K188 residue of Dyrk1A**

To determine the binding domain of Dyrk1A interacting with 14-3-3, we generated deletion mutants of Dyrk1A, as shown in Fig. 3A. COS7 cell lysate expressing myc-tagged deletion mutants of Dyrk1A was pulled down by GST14-3-3 (Fig. 3B). Interaction of C-terminal deletion mutant with GST14-3-3 was comparable to the full-length Dyrk1A. However, interaction was dramatically reduced with N-terminal (Dyrk1A^ΔN) and N- and C-terminal (Dyrk1A^ΔNC) mutants. Amino acid 135–763 and 125–606 fragments include DH box and NLS/ DH box, respectively, suggesting that the N-terminal domain upstream of NLS is important for interaction. We also tested whether kinase activity of Dyrk1A is required for interaction. As shown in Fig. 3C, COS7 lysate expressing kinase mutant of Dyrk1A (K188R) was not pulled down by GST14-3-3. However, the interaction with Dyrk1A (Y321F), another kinase mutant that has lost an autophosphorylation activity, was maintained, indicating that the kinase activity is not necessarily required.

**Binding of 14-3-3 increases Dyrk1A kinase activity in vitro and in vivo**

To examine the regulatory function of 14-3-3 on the Dyrk1A activity in vitro, we carried out a Dyrk1A kinase assay using MBP as a substrate. Phosphorylation of MBP by purified Dyrk1A was increased 2.5-fold in the presence of GST14-3-3 (Fig. 4A). The kinase activity
was increased in a dose-dependent manner, showing a maximum activity at 5 μg GST14-3-3 (data not shown). To confirm the activation of Dyrk1A by 14-3-3 in vivo, the kinase activity was measured for the immunoprecipitate of COS7 lysate transfected with GST14-3-3 (top panel) and sc138 or sc174. Lysate was precipitated with glutathione-Sepharose resin (bottom panel). (C) COS7 cells were transfected with sc138 or sc174, and Dyrk1A kinase activity was measured as (A). The results are represented as means ± SE (n = 4, three independent experiments). *p < 0.05.

Discussion

The binding of yeast Yak1p (DYRK family) and Bmh1p or Bmh2p (14-3-3 family) was previously reported, suggesting an interaction between mammalian counterparts [26]. In the present report, we showed the interaction of 14-3-3 with Dyrk1A, and the positive regulation of Dyrk1A kinase activity by 14-3-3 binding. Bmh1/2p binds Yak1p in a phosphorylation-dependent manner in response to the glucose supply and Dyrk1A has several consensus candidate sites (RSXpSXP or RXXpSXP) for 14-3-3 binding. However, the interaction of Dyrk1A and 14-3-3 was maintained regardless of Dyrk1A phosphorylation status (Fig. 2), similar to a few reports on 14-3-3 binding [27,28]. In support of this result, no serine phosphorylation was reported in the phosphopeptide mapping study of Dyrk1A [29] and phosphoserine residue was not detected by immunoblotting for the immunoprecipitated Dyrk1A (data not shown). Furthermore, four candidate serine residue mutants (S20A, S47A, S54A, and S97A) at the N-terminus...
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did not affect the interaction (data not shown). We also found that the N-terminus and K188 of Dyk1A is a required domain and a critical residue for the interaction, but Y321 does not play a role in binding (Fig. 3). We reasoned that the failure of interaction between K188R mutant and 14-3-3 might be due to the tertiary structural changes of the N-terminal domain. In fact, K188 residue constitutes an ATP binding pocket located near the tertiary structure formed by a N-terminal domain different from Y321 [29].

Dyk1A kinase activity was increased by 14-3-3 up to 2.5-fold in vitro, and was significantly decreased by an interaction disrupting peptide, sc138 (Fig. 4). It is known that 14-3-3 increased the activity of serotonin N-acetyltransferase by stabilizing the region involved in the substrate binding and Raf-1 by stabilizing active conformation as a cofactor [21,30]. These findings indicate that Dyk1A activity regulation may be mediated by increasing or decreasing the stability of the active structure by 14-3-3. However, an increase of Dyk1A activity in vivo was not observed, possibly due to the abundance of endogenous 14-3-3 in COS7 and HEK 293 cell lines used. Similar to our result, overexpression of 14-3-3 did not increase the specific activity of Raf-1 kinase in NIH3T3 cell line [31], whereas microinjection of 14-3-3 into Xenopus oocytes could increase the kinase activity of Raf-1 [22].

Since 14-3-3 is known to translocate the target protein from the nucleus to cytosol, we tested whether the presence of 14-3-3 changes the localization of Dyk1A. However, translocation of Dyk1A from the nucleus to cytosol was not observed by overexpression of 14-3-3 (data not shown). Instead, 14-3-3 was co-localized in the nucleus with Dyk1A in some cells highly overexpressing Dyk1A (data not shown). Failure of Dyk1A translocation by 14-3-3 from the nucleus to cytosol is supported by a report that Dyk1A constitutes a complex subnuclear splicing speckle, composed of sc35 and cyclins [32,33].

Considering implication of DYRK1A in the brain pathology of DS, it is very important to investigate the regulation mode of DYRK1A kinase activity. We found that binding of 14-3-3 could positively modulate the Dyk1A kinase activity. Understanding the precise mechanism of DYRK1A kinase activity regulation in a structural base would contribute to the development of therapeutics to alleviate some forms of DS pathology, especially mental retardation.

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