Cellular Signalling 23 (2011) 555–565

Contents lists available at ScienceDirect

Cellular Signalling

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

Serine phosphorylation regulates disabled-1 early isoform turnover independently of Reelin

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ARTICLE INFO

Article history:
Received 25 October 2010
Accepted 17 November 2010
Available online 25 November 2010

Keywords:
Disabled-1
Alternative splicing
Phosphorylation
Cyclin dependent kinase
Ubiquitin-mediated degradation
Protein phosphatase

ABSTRACT

The Reelin-Disabled 1 (Dab1) signaling pathway plays an important role in neuronal cell migration during brain development. Dab1, an intracellular adapter protein which is tyrosine phosphorylated upon Reelin stimulation, has been directly implicated in the transmission and termination of Reelin-mediated signaling. Two main forms of Dab1 have been identified in the developing chick retina, an early isoform (Dab1-E) expressed in progenitor cells and a late isoform (Dab1-L, a.k.a. Dab1) expressed in differentiated cells. Dab1-E is missing two Src family kinase (SFK) phosphorylation sites that are critical for Reelin-Dab1 signaling and is not tyrosine phosphorylated. We have recently demonstrated a role for Dab1-E in the maintenance of retinal progenitor cells. Here, we report that Dab1-E is phosphorylated at serine/threonine residues independent of Reelin. Cdk2, highly expressed in retinal progenitor cells, mediates Dab1-E phosphorylation at serine 475 which in turn promotes ubiquitination-triggered proteasome degradation of Dab1-E. Inhibition of protein phosphatase 1 and/or protein phosphatase 2A leads to increased Dab1-E instability. We propose that Dab1 turnover is regulated by both Reelin-independent serine/threonine phosphorylation and Reelin-dependent tyrosine phosphorylation.

1. Introduction

The cytoplasmic adaptor protein, Disabled-1 (Dab1), regulates the proper positioning of migrating neurons in response to Reelin signaling [1,2]. Dab1 contains an N-terminal protein interaction/phosphotyrosine binding (PI/PTB) domain, which associates with the NPxY motifs of the two Reelin receptors, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) [3,4]. The N-terminal domain is followed by a tyrosine-rich region, which consists of five highly-conserved tyrosine (Y) residues (Y185, Y198, Y200, Y220 and Y232) corresponding to two consensus Src family kinase (SFK) recognition sites (Y185 and Y198/Y200) and two consensus Abl recognition sites (Y220 and Y232) [5]. At least three of the four tyrosine phosphorylation sites (Y198, Y220 and Y232) are phosphorylated by SFKs and/or involved in the activation of SFKs in cultured neurons upon Reelin stimulation [6–8]. Tyrosine phosphorylation of Dab1 is essential for Reelin signaling, as mice expressing Dab1 with substitutions at these five tyrosine residues have neuronal cell positioning defects similar to those observed in Reelin-deficient (*reeler*) and *Dab1* −/− mice [9].

Tyrosine-phosphorylated Dab1 transmits the Reelin signal by activating a host of downstream effectors, including SFK, phosphatidylinositol 3 kinase (PI-3 K)/Akt, mTOR, CrkL/C3G/Rap and LIMK1 (LIM kinase 1) [7,10–14]. These events ultimately lead to cytoskeleton remodeling and correct neuronal positioning during development. Importantly, tyrosine-phosphorylated Dab1 also down-regulates Reelin signaling by recruiting SOCS (suppressors of cytokine signaling) proteins, adaptors for cullin-based E3 ligase complexes, thus targeting itself for ubiquitination and degradation [15–17]. This negative feedback mechanism prevents "overmigration" of neurons and ensures precise positioning of migrating neurons during development [17,18].

In addition to tyrosine phosphorylation, Dab1 is phosphorylated by the serine/threonine (S/T) kinase cyclin dependent kinase 5 (Cdk5) [19,20]. In particular, S491 in the C-terminus of Dab1 has been shown to be an important Cdk5 phosphorylation target both *in vitro* and *in vivo* [19]. Like Reelin and Dab1, Cdk5 plays an important role in neuronal cell positioning by phosphorylating substrates involved in cytoskeleton reorganization and cell migration. However, whether Dab1 serves as a convergence point for Reelin and Cdk5 signaling to fine tune neuronal cell migration is not clear at the present time. There is evidence implicating S/T phosphorylation in the modulation of Dab1 tyrosine phosphorylation [16,20]. Moreover, Dab1 levels have been shown to be either elevated or unaltered in *Cdk5* −/− mice

Abbreviations: ApoER2, apolipoprotein E receptor 2; CsA, cyclosporine A; Cdk, cyclin dependent kinase; Dab1, Disabled-1; CHX, cycloheximide; CPTS, 3,4',4″,4-copper phthalocyanine tetrasulfonic acid, tetrasodium salt; CsA, cyclosporine A; ED, embryonic day; GSK3, glycogen synthase kinase 3; LIMK1, LIM kinase 1; NEM, *N*-ethylmaleimide; OA, okadaic acid; PBD, polo-box domain; P1, post-hatching day 1; PBS, phosphate buffered saline; PI/PTB, protein interaction/phosphotyrosine binding; PI-3 K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PKC, protein kinase C; Plk, polo-like kinase; PPase, phosphatase; RAP, receptor-associated protein; S/T, serine/threonine; SOCS, suppressors of cytokine signaling; SFK, src family kinase; VLDLR, very low density lipoprotein receptor.

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doi:10.1016/j.cellsig.2010.11.007

depending on the study, further confounding the importance of Dab1 S/T phosphorylation in Dab1 function [19–22].

We have identified two alternatively-spliced Dab1 isoforms in the developing chick retina: Dab1-E expressed in undifferentiated retinal progenitor cells, and Dab1-L expressed in differentiated ganglion, horizontal and amacrine cells [23,24]. Dab1-L, commonly referred to as Dab1, contains the five tyrosine residues described above, whereas Dab1-E is missing two SFK tyrosine phosphorylation sites, but retains two Abl tyrosine phosphorylation sites. In addition, Dab1-E has a 19 aa region encoded by alternatively-spliced exon 9a. Dab1-L is tyrosine phosphorylated upon Reelin stimulation and recruits Crk adaptor proteins, whereas Dab1-E is not tyrosine phosphorylated, nor does it associate with Crk proteins. Knockdown of Dab1-E in the developing chick retina results in a decrease in the number of retinal progenitor cells [24]. Here, we demonstrate that there are multiple phosphorylated forms of Dab1-E in the developing chick retina. In contrast to Dab1-L, Dab1-E phosphorylation exclusively involves S/T residues and is independent of Reelin. Unlike Dab1-L which is phosphorylated at S/T residues by Cdk5, Dab1-E appears to be primarily phosphorylated by Cdk2. Dab1-E S475, the counterpart of Dab1-L S491, is a major site of phosphorylation, which in turn destabilizes Dab1-E protein. We also demonstrate that Dab1-E stability is controlled by ubiquitination-mediated proteasome degradation and protein phosphatases 1 and/or 2A.

2. Materials and methods

2.1. DNA constructs

pEGFP-C1-Dab1-E and pEGFP-C1-Dab1-L have been previously described [22]. DNA constructs expressing GST-Dab1-E middle (residues 140–263) and GST-Dab1-E-C-terminus (residues 441–535) were generated by cloning the corresponding PCR fragments from pEGFP-C1-Dab1-E into pGEX-4 T2 at the BamHI and EcoRI sites. Full-length Dab1-E cDNA derived from pEGFPC1-Dab1-E was subcloned into pCMV-Tag4A at the BamHI and XhoI sites to produce the FLAG-tagged Dab1-E. pGEX-4 T2-Dab1-E-S475A, pEGFP-C1-Dab1-E-S475A and other pCMV-Tag4A-Dab1-E mutants were made by quick-change site-directed mutagenesis (Stratagene). Cdk2-HA (plasmid 1884), Cdk2-HA dominant negative (plasmid 1885) and HA-Ubiquitin (plasmid 18712) constructs were obtained from Addgene [25,26]. The construct expressing GST-RAP (receptor-associated protein) was a gift from Dr. Joachim Herz (University of Texas, Southwestern Medical Center).

2.2. Antibodies and pharmacological reagents

The rabbit anti-Dab1-E and rabbit anti-DDX1 antibodies have been described previously [24,27]. The following antibodies were used for western blot analysis: rabbit anti-Dab1-E (1:400), rabbit anti-Dab1 (100-4101-225, Rockland, 1:5000), mouse anti-actin (A5441, Sigma, 1:200,000), rabbit anti-Cdk2 (sc-163, Santa Cruz, 1:400), mouse anti-Cdc2/Cdk1 (CC16, Calbiochem, 1:100), mouse anti-Cdc2 p34 (sc-54, Santa Cruz, 1:200), rabbit anti-Cdk4 (sc-260, Santa Cruz, 1:200), rabbit anti-Cdk4 (06-139, Millipore), rabbit anti-Cdk5 (sc-173, Santa Cruz, 1:400), rabbit anti-p35 (sc-820, Santa Cruz, 1:200), mouse anti-Cyclin A (ab39, Abcam, 1:500), rabbit anti-Cyclin B1(sc-752, Santa Cruz, 1:200), mouse anti-Cyclin D1/2 (05-362, Upstate, 1:1000), mouse anti-Cyclin E (554182, BD Bioscience, 1:500), rabbit anti-pS491 Dab1 (ab5776, Abcam, 1:1000), mouse anti-phosphotyrosine antibody (pY-100 9411, Cell Signaling Technology, 1:1000) and rabbit anti-phosphoserine CDK substrate antibody (2324, Cell Signaling Technology, 1:1000). The following antibodies were used for immunohistochemical analysis: rabbit anti-pS491 Dab1 (ab5776, Abcam, 1: 200), rabbit anti-Cdk2 (sc-163, Santa Cruz, 1:400) and rabbit anti-Dab1-E (1:400).

MG132, okadaic acid (OA), roscovitine [2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine], purvalanol A [2-(1R-Isopropyl-2-hydroxyethylamino)-6-(3-chloroanilino)-9-isopropylpurine], glycogen synthase kinase 3 (GSK3) inhibitor and the serine/threonine kinase inhibitor set were obtained from Calbiochem. Cycloheximide (CHX), cyclosporine A (CsA) and *N*-ethylmaleimide (NEM) were obtained from Sigma.

2.3. Cell culture, drug treatment and DNA transfection

Hela and HEK293T cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Primary retinal cultures were prepared from embryonic day (ED) 5 or ED10 chick retinas dissociated with trypsin as previously described [8]. The treatments and transfections were carried out 24 h after plating. For drug treatment, cells were treated with different kinase inhibitors, CHX, MG132, Reelin, or RAP, as indicated. For transfection, the DNA was introduced into cells by calcium phosphate-mediated DNA precipitation and removed after 16–18 h. Cells were lysed in RIPA buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4 and 1 × Complete protease inhibitor cocktail (Roche)] followed by immunoprecipitation or western blot analysis.

2.4. Purification of GST fusion protein

pGEX constructs encoding the GST-fused middle (140–263 aa) or C-terminus (441–535 aa) of chicken Dab1-E, and RAP, were transformed into the *E. coli* strain BL21. Expression of the fusion protein was induced with 1 mM IPTG for 4 h at 30 °C. Cells were resuspended in phosphate buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and 2 mM DTT and lysed by sonication (40% output for 10 bursts). Triton X-100 was added to a final concentration of 1% to increase protein solubility. Cleared lysates were incubated with glutathione-Sepharose beads (GE Healthcare) and bound proteins were eluted in 10 mM reduced glutathione (Sigma). The eluants were concentrated using Centricon-30 (Millipore) with three buffer exchanges in PBS.

2.5. Western blot analysis, immunoprecipitation and phosphatase treatment

Chick retinal tissue and cultures were lysed in RIPA buffer. For western blotting, lysates were either used fresh, or stored at −80 °C before use. For immunoprecipitation, cell lysates were precleared with protein A (for primary antibodies raised in rabbit) or protein G (for primary antibodies raised in mouse) Sepharose beads (GE Healthcare) for 1 h at 4 °C, incubated with primary antibodies or IgG control overnight at 4 °C. The immunocomplexes were then collected with protein A or protein G Sepharose beads. Immunoprecipitates or cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose or PVDF membranes and immunostained with antibodies as indicated. For phosphatase treatment, Dab1 immunoprecipitates bound to protein A Sepharose beads were washed in lysis buffer three times and incubated in phosphatase buffer [50 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM dithiothreitol (DTT), 0.1 mM EGTA, 0.01% Brij-35 and 20 μM $MnCl_2$] containing 400 U protein phosphatase (λ PPase, New England Biolabs) at 30 °C for 1 h.

2.6. In vitro kinase assay

ED5 chick retinas were lysed in ELB buffer (50 mM HEPES pH 7.2, 250 mM NaCl, 0.5% NP-40, 5 mM NaF, 0.5 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4 and 1 × Complete protease inhibitor cocktail). Endogenous Cdk1, Cdk2, Cdk4 and Cdk5 proteins were immunoprecipitated from precleared retinal lysates as described above. The immunocomplexes were washed three times in lysis buffer and twice in kinase buffer (50 mM HEPES pH 7.2, 10 mM $MgCl_2$, 1 mM DTT). The immunoprecipitates were incubated with 2 μg of GST-fused Dab1-E fragments in 30 μl kinase buffer supplemented with 10 μM cold ATP and 5 μCi [γ-^{32}P]-ATP at 30 °C for