FEBS Letters 587 (2013) 60–66

journal homepage: www.FEBSLetters.org

Dab1 stabilizes its interaction with Cin85 by suppressing Cin85 phosphorylation at serine 587

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ARTICLE INFO

Article history:
Received 4 September 2012
Revised 30 September 2012
Accepted 29 October 2012
Available online 21 November 2012

Edited by Jesus Avila

Keywords:
Dab1
Cin85
Reelin
Phosphorylation
CrkL
Kinase

ABSTRACT

Crk and CrkL adaptors play essential neuronal positioning roles downstream of Reelin-induced Dab1 tyrosine phosphorylation. Recently we identified Cin85 to be a CrkL-SH3 binding partner from embryonic murine brain while others found Cin85 binds directly to Dab1. Here using mass spectrometry, biochemical and mutational analyses we show that Dab1 suppresses Cin85 phosphorylation at Ser587. Furthermore a Cin85 Ser587 phosphomimetic disrupts the Dab1-Cin85 complex without affecting the Cin85-CapZ complex. These data provide an early glimpse into how Cin85 phosphorylation might alter the composition of its scaffolding partners to regulate its diverse roles including vesicular trafficking, receptor endocytosis and actin remodeling.

1. Introduction

During central nervous system development newly born neurons take fates dependent on instructional cues in their environments. Some instructional cues direct a neuron's ultimate position in the mature tissue. One such master positional cue is the secreted glycoprotein Reelin. Reelin governs neuronal positioning throughout the central nervous system with its function most readily evident in the cerebellum, cerebral cortex and hippocampus. In spite of major advances toward understanding Reelin signaling [1–5], it remains only partially understood.

The canonical Reelin pathway clusters its receptors very low density lipoprotein receptor (VLDLR) and ApoE receptor 2 (ApoER2) found on responsive cells [6]. Bound to Reelin receptors intracellularly is the adaptor protein disabled-1 (Dab1). Reelin receptor clustering leads to Dab1 tyrosine phosphorylation by the Src family of tyrosine kinases (SFKs) [7–10]. At the level of phosphotyrosyl-Dab1 (pY-Dab1) the pathway bifurcates with Tyr185 and Tyr198 responsible for the recruitment and activation of phosphatidylinositol 3-kinase (PI3K)-Akt signaling and Tyr220 and Tyr232 leading to the recruitment of the adaptor molecules Crk and Crk-Like (CrkL) [11,12]. Genetic dissection of this bifurcation indicates that both PI3K-Akt and Crk/CrkL binding are essential in Reelin signaling [11,13]. We have identified several Crk/CrkL binding proteins that could serve as Reelin effectors in both targeted [12] and large-scale proteomic analyses [14]. We hypothesized that Crk/CrkL could recruit effector proteins to the Reelin signaling complex where they could be locally regulated by either PI3K-Akt signaling or by SFKs. Indeed we found that the Crk/CrkL binding partner C3G became tyrosine phosphorylated in response to Reelin and this led to activation of the small G protein Rap1 [12]. Among the proteins from embryonic murine brain extracts that we found bound to the CrkL-SH3 domain was the Cbl-interacting protein of 85 kDa (Cin85) [14]. Intriguingly, Sato et al. found

Abbreviations: Dab1, disabled-1; CIN85, Cbl-interacting protein of 85 kDa; ApoER2, apolipoprotein E receptor 2; Crk, CT10-regulator of kinase; CrkL, Crk-like; SFK, Src family kinase; SH3, Src homology-3 domain; SH2, Src homology-2 domain; PR, proline-rich region; CC, coiled-coil domain; CT, carboxyl terminal region; VLDLR, very low density lipoprotein receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PMA, phorbol 12-myristates 13-acetate; CaMKII, calmodulin-dependent kinase 2; PKC, protein kinase C; CK-59, CamKII inhibitor 59; Cdk-5, cyclin-dependent kinase 5; CapZ, F-actin capping protein; SH3KBP-1, SH3 Kinase binding protein-1; RSK, 90 kDa ribosomal S6 kinase; S6K, 70 kDa ribosomal S6 kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; MSK, mitogen and stress-activated kinase; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WCE, whole cell extract; IP, immunoprecipitation; ECL, enhanced chemiluminescence; FKBP, FK506 binding protein; RXXS, arginine-any two amino acids-serine

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.10.051

Cin85 bound directly to the carboxyl-terminal region of Dab1 and that this interaction was disrupted when Dab1 was phosphorylated by Cyclin-dependent kinase 5 [15], a kinase that plays critical roles in brain development (reviewed in [16]).

Taken together these data suggest that Cin85 might participate in Reelin signaling in a highly regulated way and we therefore asked if Cin85 became phosphorylated at tyrosine residues or in an Akt consensus motif in a setting where Reelin-Dab1 signaling was engaged. To our surprise we found that Dab1 reduced Cin85 phosphorylation in an Akt-like motif. We identified the primary site of this regulated phosphorylation to be Ser587. Furthermore we found that a Ser587 Cin85 phosphomimetic showed dramatically reduced binding to Dab1. The implications of the regulated Cin85-Dab1 complex are discussed.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

The Flag-CIN85 expression construct was a gift of Dr. Ivan Dikic (Goethe University school of Medicine), the FKBP-Dab1-WT and FKBP-Dab1-5F expression constructs were gifts of Dr. Johannes Nimpf (Max Perutz Laboratories), and the Myr-Akt-HA construct was a gift of Phil Tsichlis (Tufts University Medical School). The following constructs were generated using a QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA): Flag-CIN85-ΔCT (Ser587STOP) and Flag-Cin85 Pro492Ala. DNA sequence confirmation was performed by the University of Vermont Advanced Genome Technologies Core. Flag-Cin85 Ser587 Ala and Flag-Cin85 Ser587Asp were generated and sequenced-verified by Bio Basic (Markham, ON).

2.2. Mammalian cell culture, transfections, inhibitors, stimuli, and lysis

E1 A-transformed Human embryonic kidney (HEK 293E) cells were grown in DMEM (Mediatech, Manassas, VA) supplemented with 5% Fetal Bovine Serum (FBS), 5% Cosmic Calf Serum (sera were from Hyclone, Logan, UT), 50 units/ml of penicillin and 50 μg/ml of streptomycin. The cells were transfected by calcium phosphate precipitation when at 75% of confluence and between 6 and 16 h after plating. Cells were washed with warm PBS 4 h after transfection and returned to full growth media for 72 h. In the case of stimulations, cells were washed once with PBS and returned to media without serum for 12 h prior to treatments as indicated in the figure legends. Stimuli and inhibitors were used at the following concentrations and were from the following sources: Calyculin A (100 nM), epidermal growth factor (EGF, 100 nM), Phorbol 12-myristate 13-acetate (PMA, 100 nM) were from Cell Signaling Technology (Danvers, MA). AP20187 (200 nM) was from ARIAD Pharmaceuticals (Cambridge, MA). The CaMKII inhibitor (CK-59, 500 nM) was from Calbiochem (San Diego, CA). After treatments cells were washed once in ice-cold PBS and lysed in ice-cold lysis buffer (25 mM Tris pH 7.2, 137 mM NaCl, 10% glycerol, 1% Igepal, 25 mM NaF, 10 mM $Na4P_2O_7$, 1 mM Na_3VO_4, 1 mMP MSF, 10 μg/mL leupeptin, and 10 μg/mL pepstatin). The insoluble material was cleared using a tabletop micro-centrifuge at 13,000 rpm for 30 min at 4 °C and the clarified supernatant was kept for biochemical analyses.

2.3. Antibodies, immunoprecipitations and immunoblotting

The anti-CIN85 (H-300), anti-Dab1 (H-103) and anti-CapZ (130309) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Flag M2 antibody and anti-Flag M2 affinity

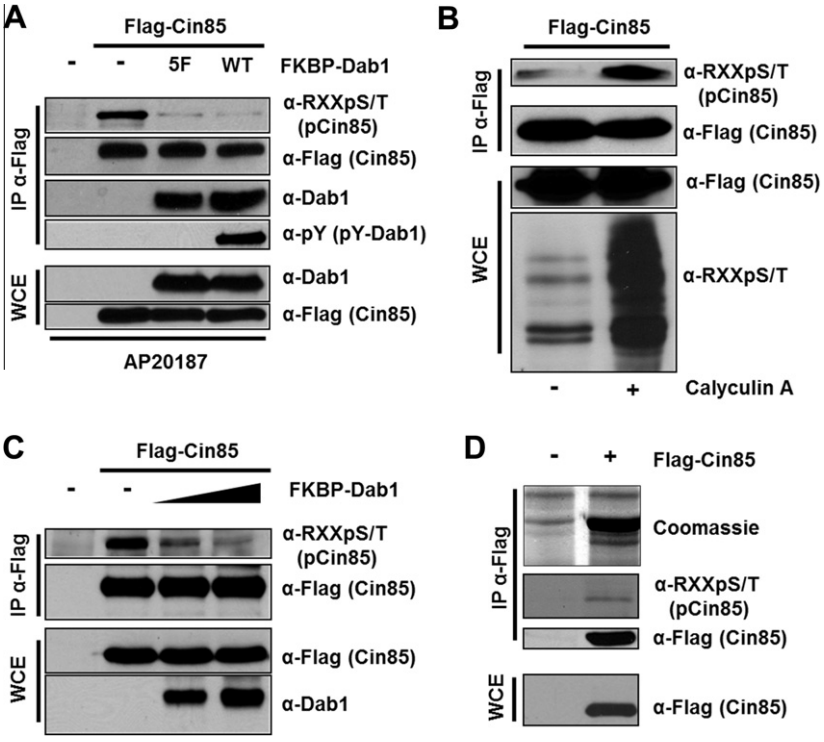

Fig. 1. Dab1 negatively regulates Cin85 phosphorylation in a RXXS/T motif. (A) The negative regulation of Cin85 RXXS/T phosphorylation by Dab1 is independent of Dab1 tyrosine phosphorylation. HEK 293 cells were transfected with the indicated constructs, starved of serum and treated with AP20187 (200 nM) for 30 min prior to lysis. Clarified extracts were either directly subjected to SDS–PAGE and immunoblotting with the indicated antibodies or first to anti-Flag immunopreciptation as indicated. (B) HEK 293 cells were transfected with Flag-Cin85, starved of serum and treated with or without 100 nM Calyculin A for 30 min prior to lysis as indicated. Clarified extracts were treated as in A. (C) HEK 293 cells were transfected with the indicated constructs. Co-transfected 10 cm dishes received 15 μg of Flag-Cin85 and either zero, 5 μg or 10 μg (as in A) of FKBP-Dab1. (D) Flag-Cin85 was immunoprecipitated from transfected cells, subjected to SDS–PAGE and stained with coomassie blue (upper panel). Whole cell extract or 10% of the immune complexes were subjected to immunoblotting with the indicated antibodies.