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Identification and characterization of a novel phosphoregulatory site on cyclin-dependent kinase 5

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ABSTRACT

Cyclin-dependent kinase 5 (CDK5) is a serine/threonine kinase essential for embryonic development whose overactivation has been implicated in several pathologies including neurodegeneration, cancer cell metastasis and type II diabetes. Therefore, it is important to investigate molecular mechanism(s) that mediate regulation of CDK5 activity. Here we identify and characterize a novel phosphoregulatory site on CDK5. Our mass spectrometry analysis identified seven putative phosphorylation sites on CDK5. Using phosphomimetic and non-phosphorylatable mutants, we determined that phosphorylation of S47, one of the identified sites, renders the kinase catalytically inactive. The inactivation of the kinase due to the phosphomimetic change at S47 results from inhibition of its interaction with its cognate activator, p35. We connect the effect of this regulatory event to a cellular phenotype by showing that the S47D CDK5 mutant inhibits cell migration and promotes cell proliferation. Together, these results have uncovered a potential physiological mechanism to regulate CDK5 activity. The evolutionary placement of a phosphorylatable residue (S/T) at this position not only in CDK5 but also in the majority of other CDK family members suggests that this phosphosite may represent a shared regulatory mechanism across the CDK family.

1. Introduction

Cyclin dependent kinases (CDKs) are a family of proline-directed serine/threonine kinases with roles in regulation of crucial cellular processes such as cell cycle and transcription [1]. One of the unifying features of this family is its dependence on association with cyclins/cyclin-like regulatory proteins for activation [1,2]. In addition, some CDKs also require phosphorylation of a key threonine residue in the activation loop to stabilize the CDK-Cyclin binding leading to complete activation of the kinase [1–3].

CDK5 is one of the unique members of this family with a primary role in cell migration rather than cell cycle [4,5]. It was initially identified for its essential role in neuronal development including neuronal migration and differentiation; and normal synaptic function post-natally [4,5] but has since been implicated in non-neuronal functions as well [6,7]. Its primary cognate neuronal activator is p35 which has no sequence similarity with classical cyclin proteins, however, structural analysis has revealed that it adopts a cyclin-like fold that mediates interaction with CDK5 [8,9]. Unlike other CDKs, the activation loop phosphorylation is dispensable for activation of CDK5 and it solely depends on binding to its activator to adopt a catalytically active conformation [10]. While CDK5 is a relatively stable protein, p35 has a short half-life owing to its proteasomal degradation which is triggered upon its phosphorylation by CDK5, a feedback mechanism likely to control the duration and level of CDK5 activation [11].

Given its role in important cellular processes, it is not surprising that deregulation of CDK5 activity has been implicated in many pathophysiological conditions including neurodegenerative diseases, cancer and type II diabetes [12–14]. One of the main mechanisms that results in overactivation of CDK5 is calpain mediated cleavage of p35 to generate its 10 kDa shorter counterpart, p25 [15]. This shorter version maintains the CDK5 binding region and has a ~5–10 times longer half-life than p35 resulting in prolonged activation and mislocalization of CDK5 leading to sustained phosphorylation of its substrates [5]. Therefore, it is important to investigate mechanisms that regulate CDK5 activity in

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a manner independent of the degradative clearance of its activator. Here we identify a novel phosphosite and provide the biochemical basis of how phosphorylation of this residue may negatively regulate CDK5 by abolishing its interaction with p35. Together, our study uncovers a phosphoregulatory mechanism that renders CDK5 inactive leading to inhibition of cell migration and promotion of cell proliferation.

2. Materials and methods

Reagents, Antibodies and Plasmid Constructs: All reagents and chemicals were of analytical grade. The purified recombinant CDK5:p35 complex (Catalog#14–477) was purchased from Millipore-Sigma. Recombinant shrimp alkaline phosphatase (rSAP) was from New England Biolabs (NEB). Protease and phosphatase inihibitors were from Fisher Scientific and Sigma, respectively. ProLong Gold Antifade Mountant with DAPI was from ThermoFisher Scientific. Antibodies against CDK5 (Cat#sc-6247) and p35 (Cat#sc-820) were from Santa Cruz Biotechnology. Antibodies against Tubulin (Cat#2128), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat#5174) and phospho-Histone H3 Serine 10 (Cat#53348) were from Cell Signaling. IRDye 680RD and IRDye 800CW secondary antibodies for western blotting were from Li-COR Biosciences and goat anti-rabbit Alexafluor-647 conjugated antibody for immunofluorescence was from Invitrogen. CDK5 (WT and D144N) and p35-HA constructs were gifts from David S. Park (University of Ottawa, Ottawa) and Edward Giniger (NIH/NINDS), respectively. Point mutants were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). The mutations and the integrity of the rest of the insert were confirmed by sequencing.

Cell Culture and Transfection: Cos7 cells (American Type Culture Collection) were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone) supplemented with 10% Fetal Bovine Serum (FBS; HyClone) and Penicillin, Streptomycin, and Glutamine (Corning). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Transfections were performed using TransIT®-LT1 (MirusBio) according to the manufacturer's protocol.

Western blotting: Western blotting was performed following the standard protocol as previously described [16]. All the images were collected using the Odyssey Fc imaging system (Li-COR Biosciences).

***In vitro* Kinase Assay and Mass Spectrometry:** *In vitro* kinase assays were performed on ~1 μg of purified CDK5/p35 complex in 30 μL of kinase buffer (50 mM HEPES pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 5 mM ATP and phosphatase Inhibitors) at 30 °C for 2 h. The samples were electrophoretically separated followed by staining with Instant Blue. The CDK5 band was excised and sent to MS Bioworks (Ann Arbor, MI) to be analyzed using their Post Translational Modification (PTM)-Profiling service.

Co-immunoprecipitation Assay: Cos7 cells co-transfected with CDK5 (WT, D144N, S47A or S47D) and p35 constructs were lysed in the immunoprecipitation buffer (25 mM HEPES pH 7.4, 0.5% Triton X-100, 2 mM DTT, 150 mM NaCl, 10 mM $MgCl_2$, protease and phosphatase Inhibitors) on ice. The cleared cell lysates were incubated with 1 μg of anti-rabbit p35 antibody overnight at 4 °C followed by incubation with 20 μL of Protein A Sepharose beads for 1 h at 4 °C. The beads were then washed, eluted and analyzed by western blotting.

Shrimp Alkaline Phosphatase (SAP) Assay: Lysates of Cos7 cells co-expressing p35 and CDK5 (WT or D144N) were immunoprecipitated as described above except after incubation the Protein A sepharose beads were washed thrice with the immunoprecipitation buffer without the phosphatase inhibitors, resuspended in 300 μL of 1× Cut-Smart Buffer (NEB) and distributed evenly into three microcentrifuge tubes. The beads were incubated at 37 °C for 15 min either without, with SAP (0.5U/μl) or with SAP and EDTA (50 mM). After incubation, the beads were eluted and analyzed by western blotting.

Scratch Wound Healing Assay. Cells expressing p35 and CDK5 (WT, S47A or S47D) were grown to ~90% confluency. Cells were starved in reduced serum medium (DMEM containing 0.2% FBS) overnight before the day of wounding and were maintained in reduced serum medium throughout the remainder of the experiment. The wound was made by scratching the center of the plate with a sterile tip. Images were captured using the Leica DM IRB Inverted Modulation Contrast Microscope (Leica Microsystems). To quantify cell migration, images were analyzed using ImageJ (NIH) to calculate the % wound closure at 40 h post-scratching.

Immunofluorescence Microscopy: Cells expressing p35 and CDK5 (WT, S47A or S47D) were fixed with 4% paraformaldehyde and permeabilized (0.2% TX100 in PBS) for 20 min at room temperature. Cells were then blocked (1% BSA and 0.2% TX100 in PBS) followed by subsequent incubations with the primary (anti-phospho Histone H3; 1:500 dilution in the blocking buffer) and secondary antibody. Cells were mounted on glass slides using the ProLong Gold Antifade Mountant with DAPI. Images were taken using the Olympus IX81 inverted confocal microscope with the 40× oil immersion objective. The images were analyzed using ImageJ.

Statistical Analysis: All graphical data were prepared and statistical analyses were performed using GraphPad Prism. Statistical significance between the differences of means was determined by performing a One-way Analysis of Variance followed by the Bonferroni's *post-hoc* test.

3. Results

CDK5 is phosphorylated in an activation-dependent manner: Reversible phosphorylation is a key regulatory mechanism to control kinase activity. In this study, we wished to identify specific sites and study phosphoregulation of CDK5. Since phosphorylation is known to retard electrophoretic mobility of modified proteins, we first determined if we could detect phosphomodified CDK5 in cell lysates using a mobility shift based western blot. Cos7 cells were transfected with CDK5 - either WT or D144N (catalytically inactive) mutant with or without its cognate activator, p35. Cos7 cells do not have detectable levels of endogenous p35, therefore, they serve as an excellent system to control CDK5 activation by simply including or omitting p35 from the transfections [17]. Cells were lysed 48 h post-transfection and lysates were analyzed by western blotting. As shown in Fig. 1A, when expressed without p35 (lanes 1 and 3), both WT and D144N CDK5 were detected as a single band. D144N CDK5 also showed a single band even in the presence of p35 (lane 4). In contrast, WT CDK5 showed slower migrating bands in addition to the main band when co-expressed with p35 (lane 2) suggesting that CDK5 is post-translationally modified in a p35 *i.e.* activation dependent manner. p35 is also a substrate for CDK5 [17] and it showed a broader/more smeared band (suggestive of its phosphorylation) when co-expressed with WT but not D144N CDK5 further confirming that WT CDK5 was activated in the presence of p35.

To ascertain that the slower migrating bands represented phosphorylated CDK5, we performed a phosphatase assay. Lysates of cells co-expressing p35 and either WT or D144N CDK5 were immunoprecipitated using an anti-p35 antibody. The immunoprecipitates were then dephosphorylated using SAP along with appropriate controls. As shown in Fig. 1B (*Top*), D144N CDK5 samples showed a single band in all three conditions (lanes 1–3) suggesting the absence of any post-translational modification. The WT CDK5 on the other hand, showed the slower migrating bands in

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