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Cyclin-dependent kinase 5 phosphorylates and induces the degradation of ataxin-2

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HIGHLIGHTS

- Ataxin-2 is phosphorylated at multiple sites by Cdk5.
- Cdk5-mediated phosphorylation induces the proteasomal degradation of ataxin-2.
- Ataxin-2-41Q is also degraded after phosphorylation by Cdk5.
- Cdk5 activity may be a therapeutic approach for SCA2.

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ABSTRACT

The expansion of a polyQ repeat within the ataxin-2 protein causes spinocerebellar ataxia type 2 (SCA2). However, neither the precise pathological mechanism nor the physiological functions of ataxin-2 are known. Ataxin-2 contains 47 (S/T)P sequences, which are targeted by proline-directed protein kinases such as the cyclin-dependent kinase 5 (Cdk5). We hypothesized that ataxin-2 is phosphorylated by Cdk5. In fact, phosphorylation of ataxin-2 by Cdk5–p25 was shown using two methods: *in vitro* ^{32}P labeling and electrophoretic mobility shift on Phos-tag SDS-PAGE. The fractionation of ataxin-2 into three portions, the N-terminal fragment (NF, amino acids 1–507), the middle fragment (MF, amino acids 508–905), and the C-terminal fragment (CF, amino acids 906–1313) showed that NF and MF were phosphorylated slightly and highly, respectively, by Cdk5–p25 when expressed in COS-7 cells. Cdk5-mediated phosphorylation induced the degradation of NF remarkably and MF moderately. Furthermore, toxic ataxin-2-41Q underwent proteasomal degradation after phosphorylation by Cdk5. These results suggest that Cdk5 controls the abundance of both normal and polyQ-expanded ataxin-2 protein in neurons, which implies that Cdk5 activity is a therapeutic approach for SCA2.

1. Introduction

Spinocerebellar ataxia type 2 (SCA2) is a polyglutamine (polyQ) neurodegenerative disease that is caused by the expansion of a CAG trinucleotide repeat, usually with CAA interruptions, in exon 1 of ataxin-2 [16,25,28]. The normal ataxin-2 contains 22 repeats, whereas an expansion of the repeated sequence (>34 repeats) is found in SCA2. Repeats with an intermediate length were recently shown to be associated with an increased genetic risk for amyotrophic lateral sclerosis (ALS) [9]. The ataxin-2 protein is expressed ubiquitously in the cytoplasm of most cells and has been suggested to be involved in RNA translation and splicing, endocytosis, and actin cytoskeletal organization [20,22]. However, neither the toxic mechanism nor the physiological functions of ataxin-2 are known.

Cyclin-dependent kinase 5 (Cdk5) is a member of the Cdk family, which is activated mainly in postmitotic neurons [8]. Cdk5 requires its activator p35 or p39 for activation and exhibits a proline-directed kinase activity that targets (S/T)P sequences. Cdk5 plays a role in neurite formation, neuronal migration, membrane trafficking, and neuronal survival [8,29]. Conversely, it is well documented that the overactivation of Cdk5 via the cleavage of p35 to p25 by calpain is associated with Alzheimer's and Parkinson's diseases [7,10,12,24]. p25 is more stable than p35 and mislocalizes

Abbreviations: ALS, amyotrophic lateral sclerosis; Cdk5, cyclin-dependent kinase 5; HD, Huntington's disease; htt, huntingtin; SCA, spinocerebellar ataxia; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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into cytosolic compartment from the membrane bound form of p35 [24], resulting in hyperactivation of Cdk5. In contrast, Cdk5 shows protective activity against Huntington's disease (HD), which is one of the polyQ neurodegenerative disorders [1,17,21]. It will be interesting to determine whether Cdk5 has protective activity against the cytotoxicity of other polyQ proteins.

The examination of the amino acid sequence of ataxin-2 revealed the presence of 47 (S/T)P possible Cdk5 phosphorylation sites, with two (S/T)PX(K/R) best consensus sequences. We hypothesized that ataxin-2 is a substrate of Cdk5 and examined its phosphorylation by Cdk5 using a cultured-cell overexpression system.

2. Methods

2.1. Antibodies and chemicals

The anti-p35/p25 C19 and anti-Cdk5 C8 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-myc 4A6 antibody was obtained from Millipore (Billerica, MA). The anti-GFP antibody was purchased from Roche Diagnostics (Basel, Switzerland). The anti-actin antibody was purchased from Sigma (St Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, alkaline phosphatase (AP)-conjugated goat anti-mouse IgG, and HRP-conjugated swine anti-rabbit IgG were purchased from Dako (Glostrup, Denmark). Phos-tag acrylamide was obtained from Wako (Osaka, Japan). Benzyloxycarnonyl-leucyl-leucyl-leucinal (MG132) was obtained from the Peptide Institute (Osaka, Japan).

2.2. Mammalian cell expression vectors

Mouse p25-myc, mouse HA-Cdk5, the kinase-negative (kn) K33T mutant of Cdk5 (knCdk5) were reported previously [3]. GFP-tau was constructed by inserting human tau cDNA (1N4R) [11] at the BglII/SalI site of pEGFP-C1 vector (Invitrogen). Human ataxin-2 was amplified from a human fetal cDNA library by polymerase chain reaction (PCR) using 5′-AGATCTATGCGCTCAGCGGCCGCAGCTCCT-3′ and 5′-GCTGACCAACTGCTGTTGGTGGTGGGCTTG-3′ as forward and reverse primers, and was inserted at the BglII/SalI site of pGEM-T easy (Promega, Madison, WI). The clone obtained was ataxin-2-22Q. GFP-fusion was constructed by insertion of ataxin-2-22Q into a pEGFP-C1 vector. The CAG repeat tract was expanded as reported previously [6]. GFP-tagged ataxin-2 fragments were constructed by PCR using pEGFP-C1-ataxin-2 as a template. The GFP-tagged N-terminal fragment (NF, amino acids 1–507) of ataxin-2 was constructed by PCR using pEGFP-human ataxin-2 as a template and 5′-TAAGTCGACGGTACCGCG-3′ and 5′-CGGGATCCTTATCCCCAGGATATGACTTCTCT-3′ as forward and reverse primers. The GFP-tagged C-terminal fragment (CF, amino acids 906–1313) was constructed similarly using 5′-GCTGAGCAAGTTAGGAAA-3′ and 5′-AGATCTGAGTCCGGACTT-3′ as forward and reverse primers. The GFP-tagged middle fragment (MF, amino acids 508–905) was constructed from the GFP-NF-MF fragment, which was once constructed by PCR using 5′-CGGGATCCTTATCCCCAGGATATGACTTCTCT-3′ and the reverse primer for NF, by secondary PCR using 5′-AGTGGGAGACAGAATT-3′ and 5′-AGATCTGAGTCCGGACTT-3′ as forward and reverse primers. All plasmid vectors were verified by DNA sequencing.

2.3. Cell culture, transfection, immunoblotting, quantification and statistical analysis

COS-7 cell culture and transfection were performed as described previously [3]. COS-7 cells were lysed by sonication in Laemmli sample buffer. Laemmli sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 6% or 12.5% polyacrylamide gels [4], and Phos-tag SDS-PAGE was performed using 5% polyacrylamide gels containing 50 μM Phos-tag and 100 μM $MnCl_2$ [13,18]. Immunodetection after blotting was performed using a Millipore Immobilon chemiluminescent HRP substrate (Millipore) and an ECL system (GE Healthcare, Piscataway, NJ) or BCIP/NBT phosphatase substrate kit (KPL, Gaithersburg, MD). Quantification was performed with image-J software, and the statistical significance of data was determined by one-way analysis of variance with Tukey's post hoc test.

2.4. Immunoprecipitation and in vitro phosphorylation by Cdk5–p25

GFP-tagged proteins, ataxin-2, its fragments or tau, were expressed in COS-7 cells and immunopurified using an anti-GFP antibody [4]. These proteins were subjected to *in vitro* phosphorylation by Cdk5–p25 purified from Sf9 cells [27]. Phosphorylation was detected on a FLA7000 Image Analyzer (GE Healthcare) after SDS-PAGE.

3. Results

3.1. Ataxin-2 is phosphorylated by Cdk5 in COS-7 cells

Ataxin-2 contains 47 (S/T)P Cdk5 minimum consensus sequences, with two preferred sequences (S/T)PX(K/R) at Ser132 and Thr580 (Genbank accession number; NP_002964) (Fig. 1A). We examined if ataxin-2 was phosphorylated by Cdk5 by co-expressing GFP-ataxin-2 with Cdk5–p25 in COS-7 cells. We used p25 C-terminal fragment for activation of Cdk5 in this study because of its high and constant activation of Cdk5 activity. The expression of ataxin-2 alone led to its detection at 160 kDa via Laemmli SDS-PAGE (Fig. 1B). Coexpression with Cdk5–p25, but not with kinase negative (kn) Cdk5 and p25, shifted the electrophoretic mobility slightly, to a higher position (Fig. 1B, arrows), which suggests phosphorylation by Cdk5. An *in vitro* kinase assay showed the direct phosphorylation of ataxin-2 by Cdk5 (Fig. 1C). These results indicate that ataxin-2 is a substrate of Cdk5.

3.2. Phosphorylation of the N-terminal and middle regions of ataxin-2 by Cdk5

Ataxin-2 is a large protein composed of 1313 amino acids (Fig. 1A). To determine which parts of ataxin-2 are phosphorylated, we divided ataxin-2 into three portions: the N-terminal fragment (NF, amino acids 1–507), the middle fragment (MF, amino acids 508–905), and the C-terminal fragment (CF, amino acids 906–1313), each of which contained 8, 22, and 17 (S/T)P sequences, respectively (Fig. 1A). These fragments were constructed as the GFP fusion as described in Section 2.

We coexpressed each of these fragments with Cdk5–p25 in COS-7 cells (Fig. 2A, wt). MF exhibited an upward shift in electrophoretic mobility when coexpressed with Cdk5–p25, whereas no changes were observed for NF and CF in Laemmli SDS-PAGE (Fig. 2A). Nevertheless, the expression levels of NF were greatly reduced to about 30% by coexpression with Cdk5–p25 (right panel of Fig 2A). MF was also reduced moderately to about 70% by Cdk5–p25. To determine if NF is phosphorylated by Cdk5–p25, we used the Phos-tag SDS-PAGE method, which enhances the phosphorylation-dependent mobility shift [13,18]. About half of NF proteins were shifted upward by coexpression with Cdk5–p25, but not by coexpression with knCdk5 (Fig. 2B, NF). MF exhibited an upward shift in the absence of Cdk5–p25, suggesting that MF is phosphorylated by endogenous kinase(s). The Cdk5-induced upward shift of MF was remarkable