·Original Article·

Casein kinase 2 interacts with and phosphorylates ataxin-3

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Abstract: Objective Machado-Joseph disease (MJD)/Spinocerebellar ataxia type 3 (SCA3) is an autosomal dominant neurodegenerative disorder caused by an expansion of polyglutamine tract near the C-terminus of the *MJD1* gene product, ataxin-3. The precise mechanism of the MJD/SCA3 pathogenesis remains unclear. A growing body of evidence demonstrates that phosphorylation plays an important role in the pathogenesis of many neurodegenerative diseases. However, few kinases are known to phosphorylate ataxin-3. The present study is to explore whether ataxin-3 is a substrate of casein kinase 2 (CK2). **Methods** The interaction between ataxin-3 and CK2 was identified by glutathione S-transferase (GST) pull-down assay and co-immunoprecipition assay. The phosphorylation of ataxin-3 by CK2 was measured by *in vitro* phosphorylation assays. **Results** (1) Both wild type and expanded ataxin-3 interacted with CK2α and CK2β *in vitro*. (2) In 293 cells, both wild type and expanded ataxin-3 interacted with CK2β, but not CK2α. (3) CK2 phosphorylated wild type and expanded ataxin-3. **Conclusion** Ataxin-3 is a substrate of protein kinase CK2.

Keywords: Machado-Joseph disease/spinocerebellar ataxia type 3; ataxin-3; casein kinase 2; phosphorylation

1 Introduction

Machado-Joseph disease (MJD)/Spinocerebellar ataxia type 3 (SCA3), one of the most prevalent autosomal dominant cerebellar ataxias, is a neurodegenerative disease caused by an abnormal expansion of the polyglutamine tract near the C-terminus of *MJD1* gene product, ataxin-3[1]. In normal individuals, there are 12-40 glutamines in ataxin-3; whereas in disease states, the repeat expands to a length of 55-84 glutamines[2]. The *MJD1* gene is widely expressed in the brain of normal and affected individuals, preferentially in neurons[3]. Ataxin-3 is expressed ubiquitously in the brain. Expanded polyglutamine tract likely alters the conformation of ataxin-3 and results in its misfolding, thereby conferring a toxic gain of function that is selectively deleterious to neurons[4]. Like many other neurodegenerative diseases, a pathological hallmark of MJD/SCA3 is the aggregate formation due to expanded ataxin-3[5,6]. Phosphorylation, as a common post-translational modification, plays an important role in many neurodegenerative diseases. It has been reported that phosphorylation of some neurodegenerative disease proteins may participate in regulation of the protein functions in association with their pathogenesis[7-14]. In Alzheimer's disease, hyperphosphorylation of tau by kinases leads to the formation of neurofibrillary tangles (NFTs) and accelerates tau-induced neurodegeneration[7,8]. In Huntington's disease, phosphorylations of huntingtin protein at serine421 by Akt and at serine434 by Cdk5 reduce huntingtin toxicity[9-11]. In SCA1, another type of spinocerebellar ataxia caused by the expanded polyglutamine tract in ataxin-1, phosphorylation of ataxin-1 at serine776 by Akt stabilizes ataxin-1 to form nuclear inclusions and influences the pathogenesis of SCA1[12,13]. However, few kinases are known to phosphorylate ataxin-3.

Casein kinase 2 (CK2) is a highly conserved and ubiquitous serine-threonine kinase. It is composed of two catalytic

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subunits (CK2α and/or CK2α'), which are essential for cell viability, and two CK2β regulatory subunits, which deeply affect many properties of CK2[15,16]. The holoenzyme is a tetramer combination of either $\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$. CK2 plays a global role in regulation of cell functions, particular in transcription, protein translation and stability, signal transduction, cell survival, circadian rhythms, and embryonic development[17,18]. In Parkinson's disease (PD), CK2 phosphorylates α-synuclein at serine129[14], which is critical for α-synuclein neurotoxicity and formation of Lewy bodies, thus contributing to the pathogenesis of PD[19,20].

Previously, we reported that phosphorylation of ataxin-3 by glycogen synthase kinase (GSK) 3β at serine256 regulated ataxin-3 aggregation[21]. Here, we identify that ataxin-3 is a new substrate for CK2. We demonstrate that the α- and β-subunits of CK2 interact with normal and expanded ataxin-3 *in vitro*. In 293 cells, both normal and expanded ataxin-3 interacts with CK2β, but not CK2α. Furthermore, we demonstrate that CK2 phosphorylates both normal and expanded ataxin-3.

2 Materials and methods

2.1 Plasmid constructs Full-length MJD constructs containing 20 or 80 CAG repeats in pGEX-5X-1, pET-15b or p3×Flag-myc-CMV-24 were cloned as described previously[21]. Full-length human CK2 subunits (CK2α and CK2β) were first amplified from human fetal brain cDNA library (Clontech) by PCR using the primers 5'-CGG GAT CCA CAT GTC GGG ACC CGT G-3', 5'-GCG TCG ACT TAC TGC TGA GCG CCA G-3', and 5'-CGG GAT CCA GAT GAG CAG CTC AG-3', 5'-CGC CTC GAG TCA GCG AAT CGT CTT G-3', respectively. The PCR products were inserted in frame into pGEX-5X-1 (Amersham Biosciences) or pEGFP-C2 (Clontech). The α-synuclein gene was amplified using primers 5'-GAA GAT CTC CAT GGA TGT ATT CAT G-3' and 5'-GCG TCG ACA AGG CTT CAG GTT CGT AG-3' from the previous pCI vector[22] containing α-synuclein gene and inserted in frame into pGEX-5X-1 via *Bam*HI/*Xho*I sites.

2.2 Glutathione S-transferase (GST) pull-down assay The aliquot containing 20 μg of GST or GST-CK2 (CK2α and CK2β) was incubated with 20 μL of glutathione agarose beads (Amersham Biosciences) for 20 min at room temperature. The beads bound with GST or GST-CK2 (CK2α and CK2β) were incubated with 50 μg of recombinant His-ataxin-3-Q20 or His-ataxin-3-Q80 in 0.25 mL HNTG-buffer [20 mmol/L Hepes-KOH (pH 7.5), 100 mmol/L NaCl, 0.1% Triton X-100, and 10% glycerol] for 1 h at 4 ºC. After incubation, the beads were washed with 1 mL HNTG buffer for 4 times. Bound proteins were detected by immunoblotting analysis using anti-ataxin-3 antibody (MJ2-5-3)[23].

2.3 *In vitro* phosphorylation assay Purified GST, GST-α-synuclein or GST-ataxin-3 fusion proteins (Q_{20} and Q_{80}) were incubated with 100 U CK2 (New England Biolabs) in 1× CK2 reaction buffer supplemented with 20 μmol/L ATP and 2.5 μCi of [γ-^{32}P]ATP in total volume of 30 μL for 30 min at 30 ºC. The phosphorylated products were separated by SDS-PAGE and detected by Coomassive brilliant blue staining and autoradiography.

2.4 Cell culture and transfection The 293 cells were cultured overnight in DMEM (Gibco) containing 10% calf serum (Gibco) and transfected with expression vectors using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM without serum. After 48 h, the transfected cells were used for immunoblotting analysis or immunoprecipitation.

2.5 Immunoprecipitation The 293 cells transfected with pEGFP-C2, pEGFP-C2-CK2α or pEGFP-C2-CK2β, and pFlag-ataxin-3-Q_{20} or pFlag-ataxin-3-Q_{80} were collected 48 h after transfection. The cells were sonicated in TSPI buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 μg/mL aprotinin, 10 μg/mL leupeptin, 0.5 μmol/L Pefabloc SC, and 10 μg/mL pepstain] containing 1% NP-40. Cellular debris was removed by centrifugation at 12 000 *g* for 15 min at 4 ºC. The supernatants were incubated with monoclonal anti-GFP antibody (Roche) for 4 h at 4 ºC. After incubation, protein G agarose (Roche) was used for precipitation. Immunoprecipitants were subject to immunoblot analysis.

2.6 Immunoblot analysis Proteins were separated by 12% SDS-PAGE and then transferred onto polyvinylidene difluoride membrane (Millipore). The following primary antibodies were used: MJ2-5-3 antibody[23], monoclonal anti-GFP-antibody (Roche), monaoclonal anti-Flag antibody (Sigma). Sheep anti-mouse IgG-HRP antibody or anti-rabbit IgG-HRP antibody (Amersham Pharmacia Biotech) was used as secondary antibody. The proteins were visualized by using an ECL detection kit (Amersham Pharmacia Biotech).

3 Results

3.1 Interaction of ataxin-3 with CK2 *in vitro* Few kinases are known to phosphorylate ataxin-3 so far. We analyzed the

primary sequence of ataxin-3 and found that there were some potential CK2 phosphorylation sites in ataxin-3. To investigate if there is a physical interaction between ataxin-3 and CK2, we evaluated the binding of ataxin-3 with CK2 using an *in vitro* GST pull-down assay. CK2α or CK2β, which were expressed by pGEX-5X-1 vectors encoding GST-CK2α or GST-CK2β and coupled to glutathione-agarose beads, interacted with both His-ataxin-3-Q_{20} and His-ataxin-3-Q_{80} from extract of the *Escherichia coli* transformed with pET-15b-MJD-CAG20 or pET-15b-MJD-CAG80 separately, while GST alone did not pull down ataxin-3 (Fig. 1). These data suggest that both normal and expanded ataxin-3 interact with CK2α and CK2β *in vitro*.

3.2 Interaction of ataxin-3 with CK2β, but not CK2α, in 293 cells To further examine the possible interaction between ataxin-3 and CK2, we co-transfected 293 cells with pFlag-ataxin-3-Q_{20} or pFlag-ataxin-3-Q_{80}, and pEGFP-C2, pEGFP-C2-CK2α or pEGFP-C2-CK2β, respectively. Cells were collected at 48 h after transfection and immunoprecipitated with anti-GFP antibody to immunoprecipitate the overexpressed EGFP, EGFP-CK2α or EGFP-CK2β. The immunoprecipitants were subject to immunoblot analysis using anti-GFP antibody or anti-Flag-HRP antibody. As shown in Fig. 2, both Flag-ataxin-3-Q_{20} and Flag-ataxin-3-Q_{80} were not co-immunoprecipitated by anti-GFP antibody in the presence of EGFP or EGFP-CK2α, whereas, they were co-immunoprecipitated by anti-GFP anti-

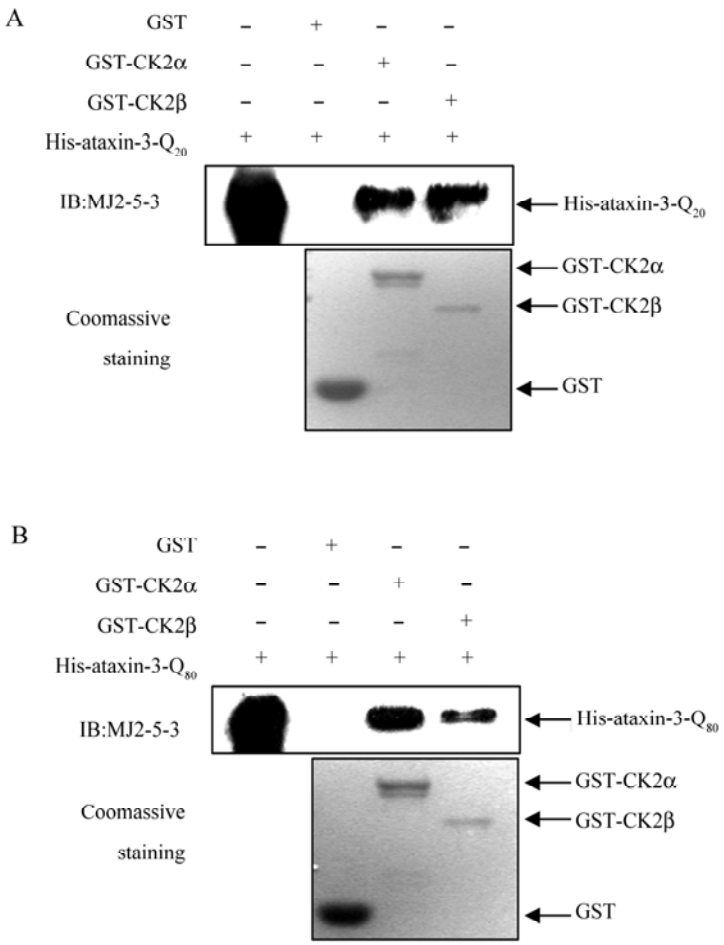

Fig. 1 Ataxin-3 interacted with CK2 *in vitro*. A: Ataxin-3-Q20 interacted with CK2α and CK2β *in vitro*. His-ataxin-3-Q_{20} was incubated with GST, GST-CK2α or GST-CK2β coupled to glutathione-agarose beads. Bound proteins were detected using anti-ataxin-3 antibody (MJ2-5-3) by immunoblotting analysis (IB, upper panel). Purified GST, GST-CK2α and GST-CK2β were detected by Coomassive staining (lower panel). B: Ataxin-3-Q_{80} interacted with CK2α and CK2β *in vitro*. His-ataxin-3-Q_{80} was incubated with GST, GST-CK2α or GST-CK2β coupled to glutathione-agarose beads. Bound proteins were detected using anti-ataxin-3 antibody (MJ2-5-3) by immunoblot analysis (upper panel). Purified GST, GST-CK2α and GST-CK2β were detected by Coomassive staining (lower panel).

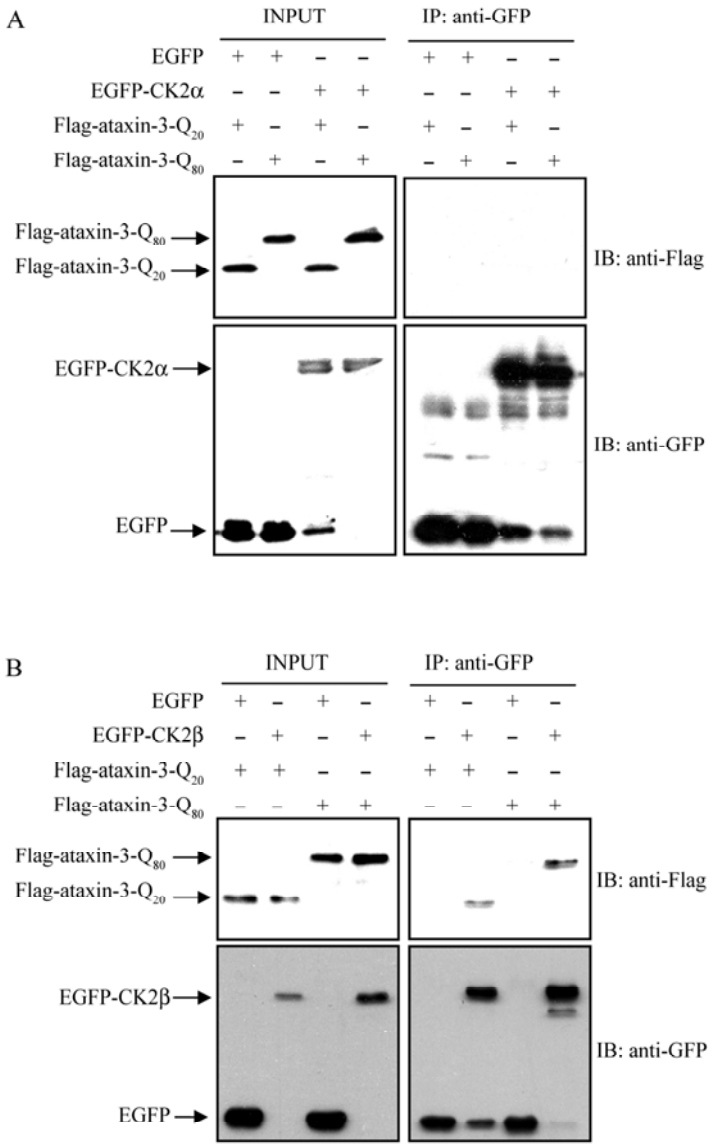

Fig. 2 Ataxin-3 interacted with CK2β, but not CK2α, in 293 cells. A: Ataxin-3 did not interact with CK2α in 293 cells. 293 cells were co-transfected with pFlag-ataxin-3-Q_{20} or pFlag-ataxin-3-Q_{80} and pEGFP-C2 or pEGFP-C2-CK2α, respectively. Cells were collected at 48 h after transfection and immunoprecipitated with anti-GFP antibody to immunoprecipitate (IP) the overexpressed EGFP or EGFP-CK2α. The inputs and immunoprecipitants were subject to immunoblot analysis (IB) using anti-GFP antibody or anti-Flag-HRP antibody. B: Ataxin-3 interacted with CK2β in 293 cells. 293 cells were co-transfected with pFlag-ataxin-3-Q_{20} or pFlag-ataxin-3-Q_{80} and pEGFP-C2, pEGFP-C2-CK2β, respectively. Cells were collected at 48 h after transfection and immunoprecipitated with anti-GFP antibody to immunoprecipitate the overexpressed EGFP or EGFP-CK2β. The inputs and immunoprecipitants were subject to immunoblot analysis using anti-GFP antibody or anti-Flag antibody.

body in the presence of EGFP-CK2β. These results further support that ataxin-3 interacts with CK2 in the transfected cells.

3.3 CK2 phosphorylated ataxin-3 Given the fact that ataxin-3 interacted with CK2 (Fig. 1, 2), we further determined whether CK2 phosphorylates ataxin-3. We performed phosphorylation assays *in vitro* to clarify the ability of CK2 to phosphorylate ataxin-3 using GST-ataxin-3-Q20 and GST-ataxin-3-Q80 as the substrates. The phosphorylations of ataxin-3-Q20 and Q80 were detected by autoradiography. GST-α-synuclein, a known substrate of CK2, served as a positive control, whereas GST alone served as a negative control. As shown in Fig. 3A, ataxin-3-Q_{20} and Q_{80} were indeed phosphorylated by CK2 in the presence of the kinase CK2 (Fig. 3B). These results suggest that ataxin-3 is a substrate for CK2.

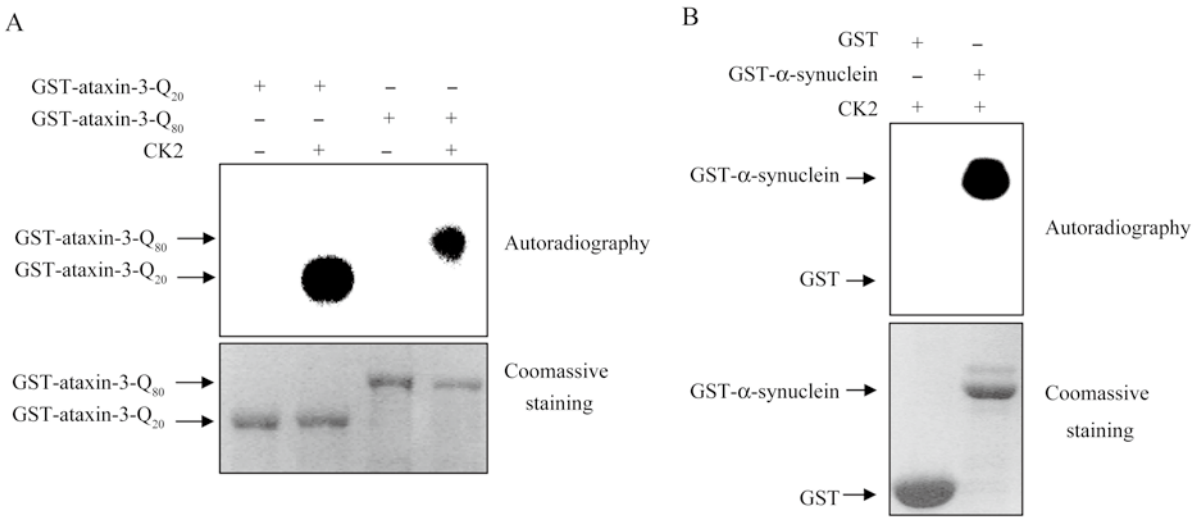

Fig. 3 CK2 phosphorylated ataxin-3 *in vitro*. A: GST-ataxin-3-Q_{20} or Q_{80} coupled to glutathione-agarose beads was incubated with or without kinase CK2, in the presence of [γ-^{32}P]ATP. The phosphorylation of ataxin-3-Q_{20} and Q_{80} were detected by autoradiography (upper panel). Purified GST-ataxin-3 (Q_{20} and Q_{80}) were detected by Coomassive staining (lower panel). B: GST-α-synuclein, a known substrate of CK2, is a positive control, whereas GST alone is a negative control (upper panel). Purified GST and GST-α-synuclein were detected by Coomassive staining (lower panel).

4 Discussion

Phosphorylation is a ubiquitous cellular regulatory mechanism. It is a reversible, covalent modification of a protein and plays important roles in regulating protein functions and in the pathogenesis of neurodegenerative diseases. In our present study, we demonstrated that CK2 interacted with and phosphorylated both normal and expanded ataxin-3. CK2 holoenzyme is composed of two catalytic α (or α') subunits and two regulatory β subunits. Interestingly, we showed that both catalytic α and regulatory β subunits of CK2 interacted with ataxin-3 *in vitro*. But in 293 cells, only regulatory β subunit interacted with ataxin-3. We did not observe the interaction between the catalytic α subunit and ataxin-3-Q_{20} or ataxin-3-Q_{80} in 293 cells. The α and β subunits of CK2 play different roles in cellular processes. The α (or α') subunit has the catalytic kinase domain and is catalytically activate by itself[24]; while the β subunit is inactive, but it increases the enzyme activity, stabilizes the holoenzyme and determines the substrate specificity[25]. Therefore the catalytic α (or α') subunit and regulatory β subunit of CK2 fulfill different tasks in the cell. We speculate that in 293 cells, the β subunit may be responsible for interaction with ataxin-3, and the α subunit may be responsible for phosphorylation of ataxin-3. It is also possible that the interaction between ataxin-3 and CK2α is dynamic, so that we could not detect their interactions in certain low protein amount in 293 cells; but they could be detected if we incubated these two proteins in high concentrations when they were expressed by *E. coli*.

CK2 consensus phosphorylation motif is (S/T)XX(D/E), where S/T is the phosphorylated serine or threonine, X is any amino acid, and D/E is an acidic residue[24]. Within ataxin-3, there are two potential CK2 phosphorylation sites upstream of the polygluatamine sequence. One is serine235 in SRQE, and the other is threonine277 in TSEE. A putative nuclear localization signal (NLS) follows the second CK2 potential phosphorylation sites TSEE in ataxin-3[26,27]. It has been reported that the CK2 phosphorylation site near the NLS may determine the rate of protein transport into nucleus[28]. Ataxin-3 has been reported to transport to the nucleus, and its nuclear localization is required for the manifestation of symptoms in MJD/SCA3[26,29]. So the phosphorylation of ataxin-3 by CK2 may regulate the rate of ataxin-3 transport into nucleus and contribute to the pathogenesis of MJD/SCA3. Our further study will focus on identification of ataxin-3 phosphorylation sites and explore the physilogical changes of phosphorylated ataxin-3.

Usually, the substrate phosphorylated at different sites by different kinases may be regulated differentially. For

example, OMI/HtrA2 (a PD-related protein) is a serine protease[30-32], and its protease activity is attenuated through phosphorylation at serine212 by Akt[33]; however, the protease activity is enhanced when OMI/HtrA2 is phosphorylated at serine142 through activation of the p38 pathway in a phosphatase and tensin homologue-induced putative kinase 1 (PINK1)-dependent manner[34]. Ataxin-3 is a protein of 360 amino acids. There are some potential phosphorylation motifs of various kinases within it. In previous study, we demonstrated that ataxin-3 was phosphorylated by GSK 3β at serine256[21]. Therefore, phosphorylation of ataxin-3 by these two different kinases may regulate properties of ataxin-3. It has been reported that phosphorylation of ataxin-3 by GSK 3β regulates ataxin-3 aggregation[21], whereas phosphorlation of ataxin-3 by CK2 may regulate the rate of ataxin-3 transport into nucleus. Both aggregation and translocation of ataxin-3 play important roles in MJD/SCA3 pathogenesis. Thus, phosphorylations of ataxin-3 by CK2 and GSK 3β may participate in the pathological process of MJD/SCA3.

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酪蛋白激酶 2 结合并磷酸化 ataxin-3

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摘要：目的 马查多 - 约瑟夫病 / 脊髓小脑共济失调 3 型，是由 *MJD1* 基因产物 ataxin-3 的 C- 末端的多聚谷氨酰胺发生重复扩展突变而引起的一种常染色体显性遗传的神经退行性疾病，目前它的发病机制还不清楚。很多研究表明磷酸化修饰作用在很多神经退行性疾病的发病过程中起到重要作用，然而已知可以磷酸化ataxin-3的激酶仍然很少。本研究的目的是探讨酪蛋白激酶 2(Casein kinase 2, CK2)对于 ataxin-3 的磷酸化作用。**方法** 通过 GST pull-down 和免疫共沉淀技术鉴定 ataxin-3 和 CK2 的相互作用。通过体外磷酸化技术检测 CK2 对 ataxin-3 的磷酸化。**结果** (1) 正常和扩展突变型 ataxin-3 在体外与 CK2α、β 亚单位均发生相互作用；(2) 在 293 细胞中正常和扩展突变型 ataxin-3 只和 CK2β 亚单位相互作用，而与 α 亚单位没有结合；(3) 正常和扩展突变型 ataxin-3 都可以被 CK2 磷酸化。**结论** Ataxin-3 是蛋白激酶 CK2 的底物。

关键词： 马查多 - 约瑟夫病 / 脊髓小脑共济失调 3 型；ataxin-3; 酪蛋白激酶 2；磷酸化