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Dual-specificity phosphatase 18 modulates the SUMOylation and aggregation of Ataxin-1

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ABSTRACT

We previously reported that SUMOylation promotes the aggregation of ataxin-1 and JNK is involved in the process. Here we show that dual-specificity phosphatase 18 (DUSP18), a member of protein tyrosine phosphatases, exerts the opposite effects on ataxin-1. DUSP18 associated with ataxin-1 and suppressed JNK activated by ataxin-1. Interestingly DUSP18, but not the other DUSPs interacting with ataxin-1, caused the mobility shift of ataxin-1. De-phosphorylation by DUSP18 was initially suspected as a cause for such an effect; however, the phosphorylation of ataxin-1 was unchanged. Instead DUSP18 inhibited SUMOylation and reduced ataxin-1 aggregation. The catalytic mutant of DUSP18 failed to reduce the SUMOylation and aggregation of ataxin-1 indicating that the phosphatase activity is indispensable for the effects. Moreover, DUSP18 disrupted the co-localization of ataxin-1 with the PML component Sp100. These results together implicate that JNK and DUSP18 reciprocally modulate the SUMOylation, which plays a regulatory role in the aggregation of ataxin-1.

1. Introduction

Polyglutamine (polyQ) diseases consist of nine inherited neurodegenerative disorders caused by expanded CAG repeats in the coding region of unrelated genes. Expanded CAG repeats result in the abnormally long polyQ tracts, which facilitates the misfolding and formation of aggregates [1]. Although aggregates are invariably observed in the affected neuronal cells and thus considered as the primary culprit for polyQ diseases, their actual roles in the pathogenesis are still controversial. The intracellular inclusions containing polyQ aggregates may be generated by a protective mechanism to sequester potentially harmful misfolded proteins. Alternatively the aberrant protein interaction with the soluble but toxic oligomeric structures is proposed as a cause of toxicity [2].

Spinocerebellar ataxia type 1 (SCA1) is a fatal progressive neurodegenerative disease. Expansion of polyQ repeats in ataxin-1, the SCA1 gene product, results in the neurological dysfunction and the loss of Purkinje cells in cerebellar cortex [3,4]. The length of polyQ repeats, like other polyQ diseases, is inversely correlated to the age of disease onset for SCA1 [5]. Accumulating evidence, however, indicates that the non-polyQ regions are also important for the aggregation and toxicity of polyQ-expanded ataxin-1. AXH domain is crucial for the interaction with a number of partner proteins, e.g., SMRT, CIC and Gfi-1 [6]. The nuclear localization signal (K772) and the major phosphorylation site (S776) are also important for the interaction with partners (e.g., RBM17 and 14-3-3) and the stability of ataxin-1 [3]. Phosphorylation of S776 impacts many functions associated with ataxin-1 including aggregation propensity and the Purkinje cell pathogenesis. Akt, PKA and MSK1 have been suggested as the responsible protein kinase(s) [7].

Ataxin-1 is also modified by ubiquitin and SUMO proteins. Ataxin-1 contains seventeen putative sites for SUMOylation and five of them were revealed as the major sites [7]. We previously showed that SUMO-1 or E2 conjugating enzyme Ubc9 promotes the aggregation of ataxin-1. In addition JNK, which is activated by ataxin-1, is involved in the SUMOylation of ataxin-1 [8]. We lately reported that certain dual-specificity phosphatases (DUSPs), belonging to protein tyrosine phosphatases regulating MAP kinases (MAPKs), interact with ataxin-1 [9]. Despite these findings, the contribution of SUMOylation to SCA1 pathogenesis and the regulatory roles of MAPKs/DUSPs in ataxin-1 functions are largely unknown.

Here we provide evidence that DUSP18, a member of atypical

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DUSPs, associates with ataxin-1 and suppresses JNK activated by ataxin-1. Interestingly, DUSP18 specifically inhibits the SUMOylation of ataxin-1, but not the phosphorylation, and thereby blocks its aggregation and protein interaction. These observations implicate that DUSP18/JNK play regulatory roles in the aggregation of ataxin-1 through their ability to modulate SUMO modification.

2. Materials and methods

2.1. Expression plasmids and transfection

Expression plasmids for ataxin-1 and ataxin-3 were from the previous study [8]. Expression plasmids for DUSPs, SUMO proteins and huntingtin were generously provided by Prof. Sayeon Cho (Chung-Ang University), Prof. Chin Ha Chung (Seoul National University) and Prof. Man Ho Kim (Seoul National University Hospital), respectively. Transfection of the plasmids into BOSC 23 cells, a derivative of HEK 293 T, was carried out using Lipofectamine reagent (Invitrogen).

2.2. RT-PCR

Total RNAs were isolated from cells using Trizol reagent (Thermo Fischer) and reverse transcription was performed using a RT-PCR kit (Thermo Fischer). PCR for DUSP18 was carried out using following primer set (forward, 5′-ACCTCATGAAGTACCACGCC-3’; reverse 5′-GCCAAACAATTGGAACTCA-3′). GAPDH was used as a control (forward, 5′-AAGGTCGGAGTCAACGGATT-3’; reverse, 5′-CTCCTGGAAGATGGTGATGG-3′).

2.3. Immunoprecipitation and immunoblot

Forty-eight hours after transfection, cells were collected and lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP-40) supplemented with protease inhibitors (Roche). The lysate was clarified and the supernatants were subjected to the immunoprecipitation. For HA-tagged proteins, the lysate was mixed with anti-HA agarose gels (Sigma) at 4 °C for 2 h with gentle agitation. For FLAG-tagged proteins and Xpress-tagged proteins, anti-FLAG M2 affinity gels and anti-Xpress antibodies (and then protein G agarose) were used, respectively. The immunoprecipitates were collected and boiled in 1X SDS sampling buffer. Proteins separated by SDS-PAGE were transferred to PVDF membrane, probed with the appropriate antibodies and then visualized by ECL reagents (Pierce). Antibodies were obtained as follows; anti HA-agarose gel, anti-FLAG M2 affinity gel, anti-tubulin, anti-SUMO-1, anti-SUMO-2/3 (Sigma), anti-HA (Santa Cruz), anti-Xpress (Invitrogen), anti-JNK, anti-phospho-JNK (Cell Signaling).

2.4. SUMOylation assay

For SUMOylation assay, HA-SUMO-1 together with FLAG-ataxin-1 or Xpress-ataxin-1 were transfected into BOSC 23 cells. After 48 h of transfection, cells were collected and lysed as described above except that 10 mM NEM was added to inhibit SUMO proteases. Immunoprecipitation and immunoblot analysis were carried out as described above.

2.5. Fractionation of cell lysate

Cell lysate was clarified at 1000xg for 10 min and then subjected to the second round of centrifugation at 20,000xg for 30 min. The supernatant was collected and mixed with 4X SDS sampling buffer. The pellets were washed with lysis buffer and then solubilized with 1X SDS sampling buffer.

2.6. Filter retardation assay

To assess the aggregation of ataxin-1, filter retardation assay was performed. After cells were lysed, the insoluble pellets were collected by centrifugation at 20,000 x g for 20 min. Pellets were re-suspended in 100 μl of DNase buffer (20 mM Tris-HCl, pH 8.0; 15 mM $MgCl_2$) plus DNase I and heat-denatured for 5 min. Heat-denatured proteins (30 μg) were filtered through cellulose acetate filters using a slot-blot apparatus. Ataxin-1 in the retained aggregates was detected by immunoblotting.

2.7. In vivo labeling with [^{32}P] and autoradiography

After 48 h of transfection, cells were washed with phosphate-free DMEM and then incubated for 4 h with 700 μCi/ml [^{32}P]-orthophosphate (GE Healthcare). The cell lysate was subjected to immunoprecipitation with anti-FLAG M2 affinity gels. For calf intestinal alkaline phosphatase (CIP) treatment, the immunoprecipitates were washed with the phosphatase buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT) and then incubated with 15 units of CIP (Sigma) for 1 h at 37 °C. [^{32}P]-labeled ataxin-1 was visualized by immunoprecipitation and autoradiography.

2.8. Confocal immunofluorescence analysis

Cells seeded onto coverslips were grown to semi-confluence and then transfected with expression plasmids. After 12 h, the transfected cells were washed with PBS and then fixed/permeabilized with the cytotoxic solution (BD Bioscience) for 30 min at room temperature. Incubation with primary antibody was carried out at room temperature over-night. After PBS washing, coverslips were incubated with secondary antibodies conjugated with Alexa Fluor® dyes for 1 h. For nuclear staining, cells were incubated with 1X DAPI in PBS for 15 min at room temperature. The cells were observed under a laser confocal fluorescence microscope (Carl Zeiss).

3. Results

3.1. DUSP18 interacts with ataxin-1 and suppresses the JNK activation

MAPKs are most prominent DUSP substrates and many DUSPs are thus classified as MAPK phosphatases [10]. Individual DUSP, however, exhibits different specificity toward MAPK and some DUSPs also have non-MAPK targets [11]. For example, laforin (EPM2A) binds to misfolded polyQ proteins and promotes their degradation [12]. Previously, we showed that a number of DUSPs interact with ataxin-1 by co-immunoprecipitation [9]. Especially DUSP2, 4, 6, 11, 13, 18, 24 and EPM2A showed strong interaction (Fig. 1A). Among them, we were interested in DUSP18 because it shows specificity against JNK [13] and also interacts with ataxin-3 and huntingtin (Fig. 1A; data for huntingtin not shown). Immunofluorescence analysis revealed that DUSP18 was detected mostly in cytosol without ataxin-1. In the presence of ataxin-1, some DUSP18 were re-located to the nucleus and co-localized with diffusely distributed ataxin-1. However, they were not recruited to large aggregates containing ataxin-1 (see nuclear foci in Fig. 1B). These findings implicated that DUSPs associate preferentially with oligomeric forms of ataxin-1, which are not included in large aggregates.

Studies on the tissue specificity showed that DUSP18 was expressed highly in testis, liver, brain and ovary while it was barely detected in kidney and thymus [14]. We also observed that DUSP18