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Interaction Between DUSP8 and the Polyglutamine Protein Ataxin-1

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Polyglutamine (polyQ) diseases are a group of inherited neurodegenerative disorders caused by the expansion of polyQ repeats in the disease proteins. Expansion of polyQ repeats presumably leads to misfolding and aggregation of polyQ proteins.[1] Although details of the molecular mechanisms are still elusive, transcriptional dysfunction and oxidative damage are suggested as underlying events responsible for polyQ pathogenesis.[2] Misfolded polyQ proteins are frequently concentrated into intranuclear inclusions, which increase generation of reactive oxygen species (ROS) and activate stress signaling pathways. In cultured cells expressing expanded polyQ proteins, mitogen-activated protein kinases (MAPK), *e.g.*, p38 and JNK, are activated and the cytotoxicity induced by polyQ proteins, partially through sustained activation of MAP kinases, can be reduced by administration of a p38 inhibitor.[3-6] MAPK are activated by MAPK kinase (MEK)-mediated phosphorylation of threonine and tyrosine residues located in the signature sequence (TxY) and MEK is in turn activated by MAPK kinase kinase (MEKK).[7] Since MAPK are involved in a wide variety of cellular processes including cell survival and apoptosis, cells must tightly regulate the magnitude and duration of MAPK activation. The negative regulation of MAPK is often achieved by phosphatase-mediated dephosphorylation of MAPK. A group of protein phosphatases referred as type I cysteine-based protein tyrosine phosphatases including DUSPs (dual-specificity phosphatases) dephosphorylate both tyrosine and serine/threonine residues within the same substrate and as a result counteract MAPK.[8]

Involvement of protein tyrosine phosphatases (PTPs) in polyQ-induced cell death has been reported in previous studies. Several PTP proteins including MKP-1 are up-regulated by over-expression of cytotoxic polyQ-expanded huntingtin (Htt) fragment.[9] Moreover, polyQ-expanded Htt fragment activates JNK pathway by reducing solubility of JNK phosphatase M3/6 (also known as hVH5 or DUSP8).[4] A recent study demonstrated that a DUSP protein laforin can suppress the cytotoxicity caused by misfolded proteins and hinted that phosphatases can be potential targets for pharmacological intervention of neurodegenerative disorders.[10] The polyQ protein ataxin-1 is a soluble protein of about 816 amino acids, which varies depending on the length of polyQ repeats, and located both in the cytoplasm and nucleus. Although the exact functions are not completely understood, ataxin-1 is apparently involved in transcription regulation through its ability to interact with several transcription factors as well as RNA.[11] While it is generally assumed that expansion of polyQ repeats causes misfolding of ataxin-1 and leads to neuronal death in spinocerebellar ataxia 1 (SCA1), contribution of other domains to pathogenesis also becomes increasingly clear. Besides N-terminal polyQ region, AXH domain (570-689; for interaction with transcription factors and RNA), endogenous phosphorylation site (776) and nuclear localization signal (795-798) are present in the C-terminus of ataxin-1.[11] Phosphorylation of ataxin-1 is particularly important since it is implicated in pathogenicity of SCA1. Phosphorylation at Ser776 is critical for the interaction of ataxin-1 with 14-3-3 proteins that regulate its entry into the nucleus.[12]

Previously we demonstrated that ataxin-1, capable of activating JNK by itself, does not directly interact with this MAPK.[5] Such observations led us to suspect that misfolded polyQ proteins, while not directly binding to JNK, could physically associate with PTPs (*e.g.*, DUSP proteins) and thereby regulate JNK activation. To explore this possibility, we first investigated if ataxin-1 actually interacts with MAPK phosphatases *in vivo*. Upon screening for PTPs that physically interact with normal ataxin-1[30Q], we found out that a number of human PTPs - including DUSP2, 8, 13 and 24 - strongly associated with ataxin-1[30Q] (Table 1).

Table 1. Interaction between ataxin-1[30Q] and protein tyrosine phosphatases (PTPs) *in vivo*[a]

PTP	Interaction with Ataxin-1[30Q]	PTP	Interaction with Ataxin-1[30Q]
DUSP1	–	DUSP12	–
DUSP2	++	DUSP13	++
DUSP3	–	DUSP15	–
DUSP4	+	DUSP24	++
DUSP6	+	MTM1	–
DUSP7	+/–	CDC14B	–
DUSP8	++	PTPRH	–
DUSP11	+		

[a]HEK293T cells were transiently co-transfected with FLAG-tagged ataxin-1[30Q] and GST-tagged PTPs. After 48 h of transfection, cells were lysed and then subjected to immunoprecipitation with *anti*-FLAG M2 affinity gel followed by western-blot analysis with *anti*-GST antibody. All data are representative of three independent experiments. ++ = strong interaction; + = moderate interaction; +/– = inconsistent interaction; – = no interaction.

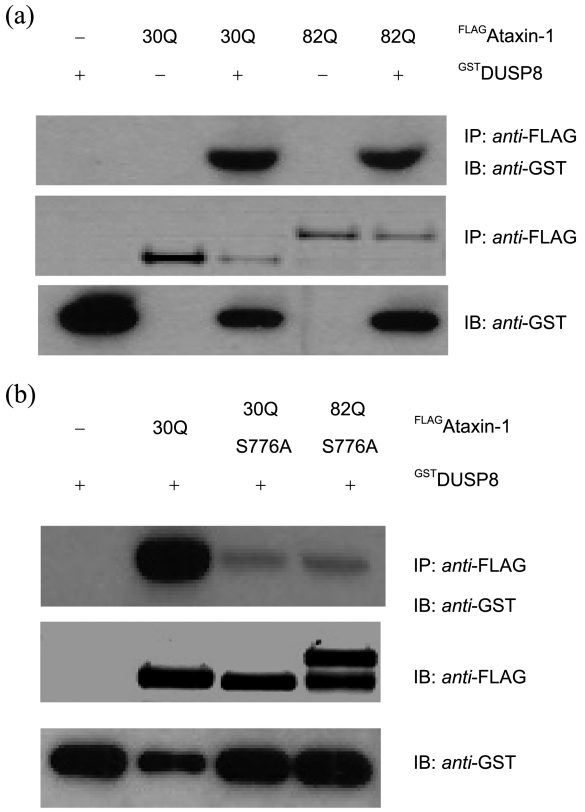

Figure 1. Interaction between DUSP8 and ataxin-1. HEK293T cells were transiently transfected with (a) FLAG-tagged ataxin-1[30Q], ataxin-1[82Q] or (b) ataxin-1 mutant (S776A) together with GST-tagged DUSP8. After 48 h of transfection, cells were collected and lysed with NP40 lysis buffer. Ataxin-1 proteins in cell lysate were isolated using *anti*-FLAG M2 affinity gels and the presence of ataxin-1 and DUSP-8 in the immunoprecipitates was determined by western-blot analysis using *anti*-FLAG antibody and *anti*-GST antibody, respectively.

Among them, we were particularly interested in DUSP8 because it belongs to JNK/p38-selective group of MAPK phosphatases and the solubility of DUSP8 (M3/6) was also reported to be impaired by expanded polyQ proteins, causing JNK activation.[4,7] To further investigate the interaction between DUSP8 and ataxin-1, we then examined if the length of polyQ repeat influences their association. Co-immunoprecipitation experiments were carried out using both normal ataxin-1[30Q] and polyQ-expanded ataxin-1[82Q] together with DUSP8. As shown in Figure 1(a), DUSP8 associated with both ataxin-1[30Q] and ataxin-1[82Q] in a similar extent indicating that polyQ length does not influence the binding and their association is presumably governed by the region(s) outside the polyQ repeats. Indeed interaction analysis using a mutant form of ataxin-1(S776A), in which a major endogenous phosphorylation site (Ser776) is substituted with alanine, revealed that ataxin-1's binding to DUSP8 was impaired by the mutation of this phosphorylation site (Fig. 1(b)).

Since the functional relationship of oxidative stress in polyQ pathogenesis and JNK activation was already well-documented, we then examined if oxidative stress affects the

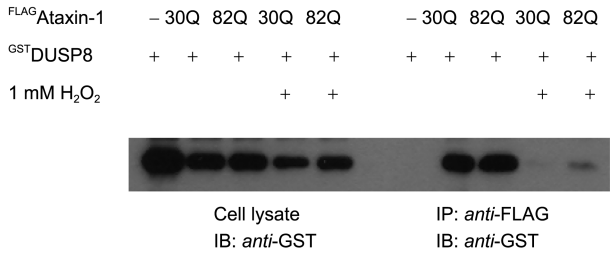

Figure 2. Effects of oxidative stress on the interaction between DUSP8 and ataxin-1. After transfection with ataxin-1 and DUSP8, cells were treated with or without 1 mM H_2O_2 for 24 h. Co-immunoprecipitation using *anti*-FLAG M2 affinity gels was carried out as in Fig. 1.

interaction between DUSP8 and ataxin-1. As expected, the treatment of cells with 1 mM H_2O_2 nearly abolished the interaction of ataxin-1 and DUSP8 (Fig. 2). Above findings together suggest that the segment near C-terminus of ataxin-1 which contains a major phosphorylation site, but not polyQ repeats, is important for the interaction with MAPK phosphatase and their interaction is strongly influenced by the cellular state of oxidative stress.

It was previously demonstrated that DUSP8, even though its solubility was impaired by polyQ proteins, was not directly recruited into nuclear inclusions.[4] Analysis of domain structure has revealed that DUSP8 contains nuclear export signal (NES) but not nuclear localization signal (NLS).[7] These results led us to predict that the interaction between DUSP8 and ataxin-1 takes place mainly in cytoplasm but not likely in the nucleus. Confocal microscopic analysis indeed

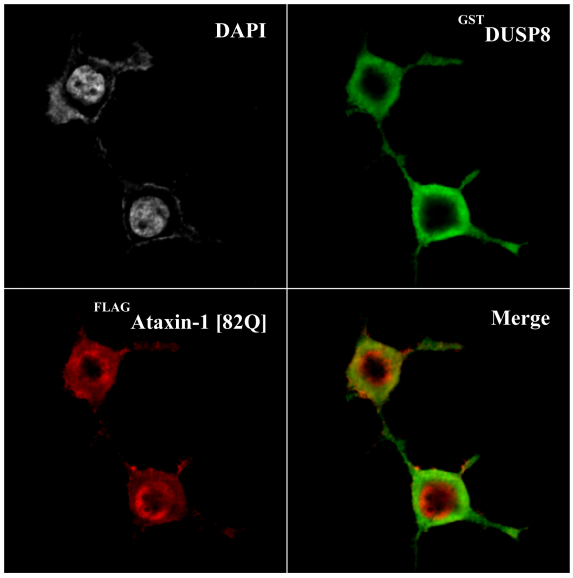

Figure 3. Confocal analysis of interaction between ataxin-1 and DUSP8. For immunofluorescence analysis, HEK293T cells were seeded onto glass slide and then transfected with FLAG-tagged ataxin-1 and GST-tagged DUSP8. After 48 h of transfection, cells were fixed and then incubated with *anti*-FLAG antibody and *anti*-GST antibody followed by incubation with Alexa Fluor-conjugated secondary antibodies. DAPI was used for nuclei staining. Magnification = 100X.

confirmed that DUSP8 and ataxin-1[82Q] were co-localized primarily in the cytoplasm and revealed that DUSP8 was not recruited into the nuclear inclusions containing ataxin-1[82Q] (Fig. 3).

Under our experimental conditions, the majority of ataxin-1 is phosphorylated *in vivo* since the phosphorylated form of ataxin-1 is readily detected in cultured cells even in the absence of external stimuli.[13] Our findings that the phosphorylation state of ataxin-1 influences interaction between ataxin-1 and DUSP8 and the association takes place predominantly in cytoplasm suggest a role of DUSP8 in nuclear transport. Binding of ataxin-1 to certain subtypes of 14-3-3 proteins, which recognize and associate with many phosphoproteins inside cells, is reported to prevent de-phosphorylation and thereby stabilize ataxin-1.[12] Whether DUSP8 actually promotes de-phosphorylation of ataxin-1 *in vivo* is yet to be determined; however, it can be speculated that 14-3-3 proteins and DUSP8 competitively bind to the phosphorylated form of ataxin-1.

Possibly prolonged interaction with DUSP8, which contains nuclear export signal, not only blocks the nuclear translocation but also causes de-stabilization and the eventual degradation of ataxin-1. DUSP8 (M3/6) belongs to JNK/p38-selective group of MAPK phosphatases and is inactivated by protein damaging stresses including oxidative stress and heat shock.[7] Upon heat shock, DUSP8 dissociates from JNK and then becomes inactivated as its solubility is reduced. In addition, DUSP8 is relatively unstable and has an apparent half-life of 2 h in cultured cells.[14,15] Reportedly arsenite-induced oxidative stress modulates interaction of DUSP8 with JNK isoforms differentially, which is mediated by modification of DUSP8 (*e.g.*, inactivation by ROS) rather than that of JNK proteins.[16] Our finding that oxidative stress negatively regulates the association between ataxin-1 and DUSP8 suggests a similar possibility of DUSP8 modification. In fact, we also observed that the presence of ataxin-1 and oxidative stress reduced the relative amount of GST-DUSP8 substantially (Fig. 1 and 2; see *anti*-GST blot). In polyQ diseases, persistent oxidative stress may perturb physical interaction between polyQ proteins and DUSP8, which under normal circumstances prevents unnecessary entry of misfolded polyQ proteins into the nucleus and subsequent aggregation.

Members of DUSP subfamily, through their ability to negatively regulate MAP kinase signaling cascades, are now recognized as important controllers of many cellular processes. Hence controlling of DUSP activity could provide tools to intervene certain human diseases including neurodegenerative disorders. Recently, efforts to identify selective inhibitors of DUSP have been attempted. Structural analysis of several DUSP proteins will allow us better understanding of molecular architecture and help design more specific inhibitors for DUSP proteins.[17] However, the physiological roles of individual DUSP proteins are still poorly understood and some of DUSP proteins may have additional functions, such as regulation of protein-protein interaction. Further studies on molecular details of how certain PTPs recognize misfolded proteins and affect their normal functions or localization probably will open up opportunities to utilize this class of PTPs as therapeutic targets for inherited neurodegenerative diseases including Huntington's disease and spinocerebellar ataxia and to develop small molecule inhibitors specific for PTPs.

Experimental

Cell Culture and DNA Transfection. Human embryonic kidney (HEK) 293T cells were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin in the presence of 5% CO_2. FLAG-tagged ataxin-1 expression plasmid was constructed in pFLAG/CMV-2 mammalian expression vector and GST-tagged DUSP8 was constructed in pEBG expression vector, respectively. For transient transfection, 1.4×10^6 cells were plated in 60-mm cell culture plates, grown overnight, and transfected with various plasmid DNA using Lipofectamine reagent (Invitrogen).

Immunoprecipitation and Western-blot Analysis. After 48 h of expression of FLAG-tagged ataxin-1 with GST-tagged PTP constructs (for oxidative stress experiments, cells were treated with 1 mM hydrogen peroxide for 24 h), HEK293T cells were collected and then lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with Complete-MINI™ protease inhibitor mixture (Roche) for 20 min at 4 °C, followed by centrifugation at 13,000 × *g* for 20 min. For co-immunoprecipitation, the cell lysates were incubated with 20 μL of *anti*-FLAG M2 agarose (Sigma) at 4 °C for overnight with rotation. After washing with phosphate buffered saline (PBS), the beads were resuspended in 1x SDS sample buffer and boiled for 10 min. Western-blot analysis using *anti*-GST antibody (1:1,000; Santa Cruz) or *anti*-FLAG antibody (1:1,000; Sigma) was performed to detect individual proteins. The protein bands were visualized using ECL detection system (PIERCE).

Confocal Microscopy Analysis. HEK293T cells were cultured to confluence and then seeded onto glass slide (1-2 × 10^5 cells). After 48 h of transfection with FLAG-tagged ataxin-1 and GST-tagged DUSP8, the cells were washed in 1 × PBS/3.7% formaldehyde for 15 min and then twice in 1 × PBS/0.1% triton X-100 for 15 min at room temperature. After Image-iT™ FX signal enhance (Molecular Probe) treatment, the slides were incubated with primary antibodies (1:100-1:200) for 1-2 h. After washing twice in 1 × PBS, slides were incubated with Alexa Fluor® 488 goat *anti*-rabbit IgG (for DUSP8) or Alexa Fluor® 546 goat *anti*-mouse IgG (for ataxin-1) for 1 h. The stained cells were observed under a laser confocal fluorescence microscope (Carl Zeiss). For nuclei staining, cells were incubated with 1 × DAPI/PBS for 10 min at room temperature.

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