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# Enhanced SUMOylation in polyglutamine diseases

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**Abstract**

Small ubiquitin-like modifiers (SUMOs) are proteins homologous to ubiquitin that possibly regulate intranuclear protein localization, nuclear transport, and ubiquitination. We examined patients of DRPLA, SCA1, MJD, and Huntington's disease and found that neurons in affected regions of the brain react strongly to SUMO-1, a family member of SUMOs. Western blot with a transgenic mouse expressing mutant ataxin-1 showed the increase of SUMOylated proteins in the cerebellar cortex, which we named ESCA1 and ESCA2. These results indicated activation of SUMO-1 system in polyglutamine diseases and predicted its involvement in the pathology. 

Polyglutamine diseases associated with expansion of the CAG triplet repeat sequence in an exon are the most popular forms of familial spinocerebellar degeneration (see review [1]). Similar molecular pathology was found in Huntington's disease and X chromosome-linked bulbospinal muscular atrophy (Kennedy's disease). Abnormal expansion of the polyglutamine tract leads to nuclear or cytoplasmic aggregates in about 10% of neurons, which have been implicated in neuronal degeneration (see review [2]). Expression of the polyglutamine peptide derived from MJD causes the nuclear aggregate in transgenic mice [3]. However, the aggregate seems to be composed of full-length and even soluble products of the causative gene in human brain of Huntington's disease [4].

A feasible problem in this field of research is the relationship between nuclear aggregates and nuclear functions such as transcription and splicing. Mutant polyglutamine gene products have been shown to affect nuclear localization and function of various transcription-related factors including CBP, $TAF_{II}130$, and PQBP-1 ([5–7]; Okazawa et al., submitted). In some cases, the mutant protein sequester CBP [5] while interaction before sequestration might also play a role in degeneration (Okazawa et al. submitted). Anyway, it is generally believed that interaction between mutant proteins and transcription factors leads to transcriptional suppression and cell death.

On the other hand, nuclear micro-structural change has been implicated in the pathology of polyglutamine diseases. Orr's group first reported co-localization of ataxin-1 and PML [8] that regulates partitioning of transcription and splicing factors into PML nuclear body (see review [9]). They showed that mutant ataxin-1 shifted PML from nucleoplasm to nuclear bodies [8]. As it is known that small ubiquitin-like modifiers (SUMOs) critically regulate transport of PML between nucleoplasm and nuclear body (see review [9]), it suggests the involvement of SUMO in the polyglutamine disease pathology. In addition, SUMOylation is found in various proteins involving RanGAP1, MDM2, c-Jun, and so on (see review [10]). Among them, interaction with RanGAP1, a protein binding to the nuclear membrane pore complex, suggests the role of SUMOylation in nuclear transport [11], which might also be relevant to the pathology of

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polyglutamine diseases. SUMO is covalently linked to lysine within the consensus sequence (ψKxE) of target proteins. As SUMOylation and ubiquitination share the consensus, antagonistic relationship between the two systems is proposed (see review [10]). Recently, SUMO-conjugating enzymes corresponding to E3 ubiquitin ligases were isolated by multiple groups [12–14]. These findings reconfirmed molecular homology of SUMOylation and ubiquitination. Collectively, aforementioned information prompted us to test the activation of the SUMO system in polyglutamine diseases.

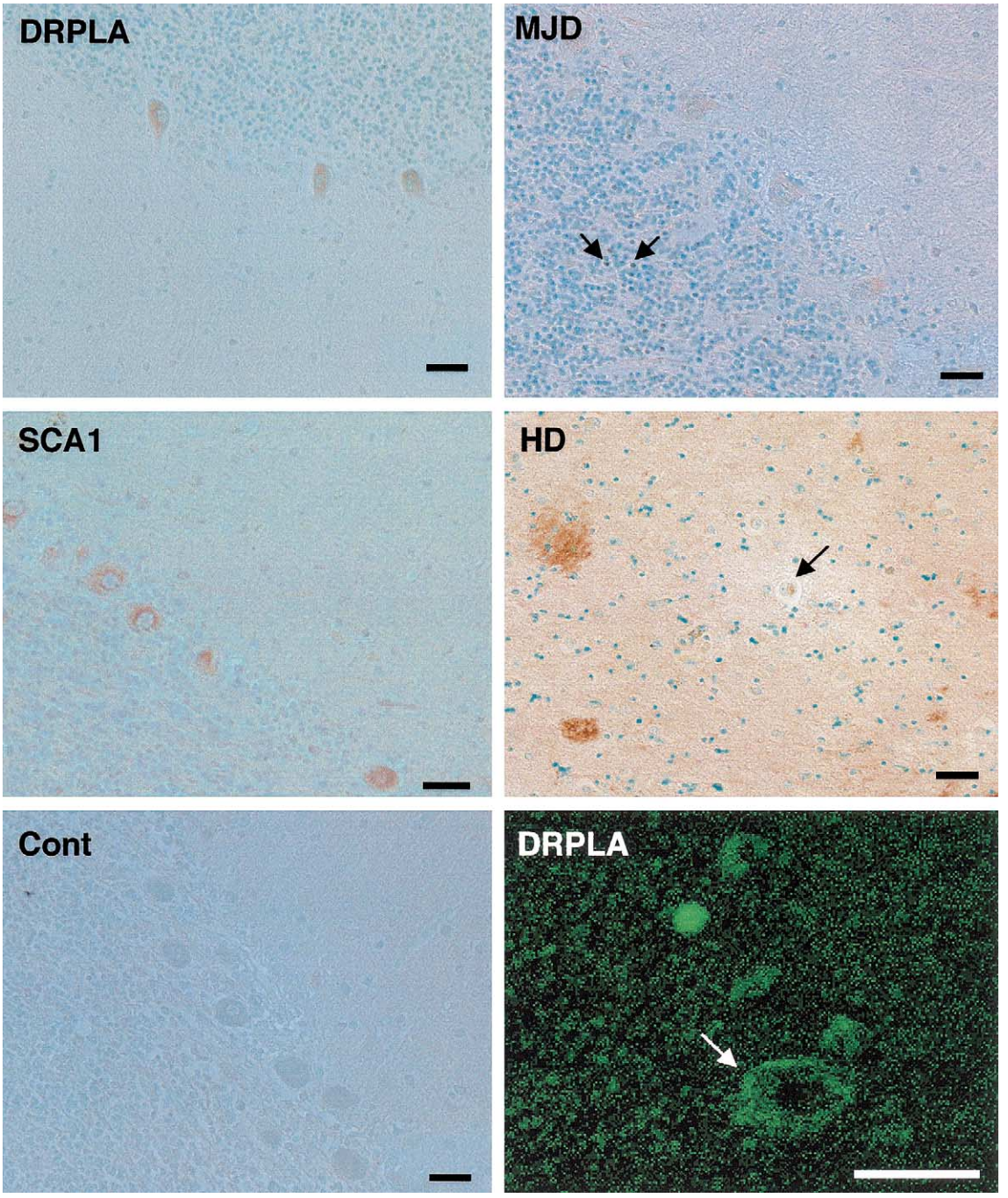


Fig. 1. Immunohistochemistry of SUMO-1 in polyglutamine diseases. DRPLA: dentatorubral pallidoluysian atrophy, MJD: Machado–Joseph disease, SCA1: spinocerebellar atrophy type-1, HD: Huntington's disease. Purkinje's cells were stained in all three spinocerebellar degenerations. In MJD, granule cells that reacted strongly to anti-SUMO-1 antibody are indicated with arrows. In HD, a minor part of striatal neurons were stained. Neural fibers in striatosomes were positively stained. Confocal laser scanning microscopy of a single slice showed a cytoplasm-dominant distribution of SUMO-1 in a Purkinje cell (arrow). Bars indicate 100 μm.

## Materials and methods

*Human materials and SCA1 transgenic mouse*. Human brains were obtained from two patients with DRPLA (69 yr, 62 repeats; 60 yr, 59 repeats), a patient with MJD (33 yr, 78 repeats), a patient with SCA1 (73 yr, 78 repeats), and two controls (31 and 47 yr) and served for the study. For immunoprecipitation, we used a transgenic mouse carrying mutant SCA1 gene (82 repeats) and its litter-mate mouse [15].

*Immunohistochemistry*. Human brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and 6 μm paraffin-embedded sections were prepared from the cerebellum or the striatum. The sections were immunostained by the avidin–biotin–peroxidase complex (ABC) method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) with anti-SUMO-1 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100) or anti-ubiquitin rabbit polyclonal antibody (Dakopatts, Glostrup, Denmark; 1:100). For confocal microscopic analysis, SUMO-1 and ubiquitin immunoreactivities were visualized with Fluorescein (excitation: 494 nm, emission 517 nm) and Cyanine 3 (excitation: 550 nm, emission: 667 nm) by using TSA Plus Fluorescence Systems (Perkin–Elmer Life Sciences, Boston, MA, USA). Leica DMRB/E confocal laser scanning microscopy with TCS-NT software was used for the analysis of co-localization of the proteins. Counterstains were performed weakly with methylgreen or hematoxylin–eosin.

*Immunoprecipitation and Western blot analyses*. Immunoprecipitation was performed as described previously [16]. Briefly, 100 mg of the cerebellar cortex tissues was suspended in 1 ml of lysis buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), incubated at 4 °C for 10 min, and disrupted by needle aspiration. Debris was removed by centrifugation and the cell lysates were used directly for Western blot analysis or incubated with anti-ataxin-1 antibody [17] at 1:2000. Immunoprecipitation was performed with protein A–agarose (Oncogene Research Products, Boston, MA, USA). Non-specific binding was excluded by preincubation with protein A–agarose for 1 h at room temperature. After precipitation, the agarose was washed with the lysis buffer five times. The precipitates were solubilized in 50 μl of Laemmli's sample buffer. 10 μl of the cell lysates or the precipitates was subjected to 7.5 and 12% SDS–PAGE and then transferred to NCL membrane (Amersham-Pharmacia Biotechnology, USA). Nitrocellulose membranes were blotted with anti-SUMO-1 monoclonal antibody (Santa Cruz Biotechnology, USA), diluted at 1:1000, and detected with ECL (Amersham-Pharmacia Biotechnology, USA).

## Results

### *Enhanced immunoreactivity of SUMO-1 in neurons of polyglutamine diseases*

First, we performed immunohistochemical analysis of a dentato-rubropallidoluysian atrophy (DRPLA) patient brain with anti-SUMO-1 antibody. In this patient, Purkinje's cells were reduced substantially. Without any enhancing treatment, we found immunoreactivity of SUMO-1 in Purkinje's cells. The signal was stronger in the cytoplasm (Fig. 1). To confirm this, we localized the SUMO-1 immunoreactivity by a confocal laser scanning microscopy and found that signals clearly predominated in the cytoplasm (Fig. 1). SUMO-1-immunohistohemistry was similar in the cerebellum of a spinocerebellar atrophy (SCA1) patient (Fig. 1). In the case of Machado–Joseph disease (MJD), the staining pattern was different in some

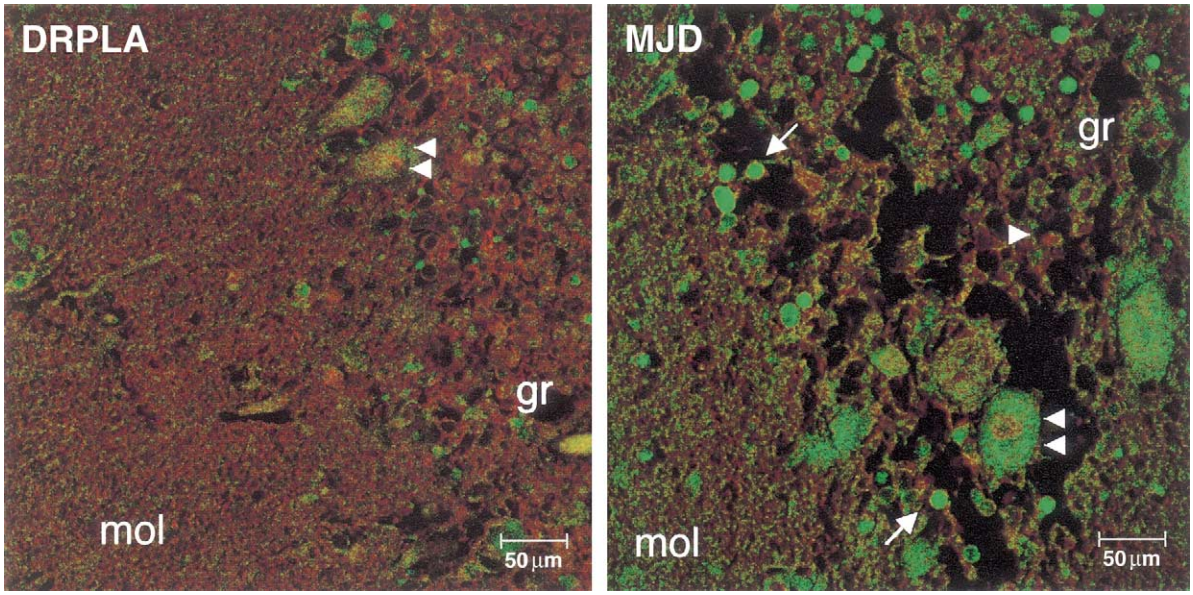


Fig. 2. Confocal laser scanning microscopic analysis showed the relationship between SUMO-1 and ubiquitin immunoreactivities in the cerebellar cortex of DRPLA and MJD patients. Molecular and granular cell layers are indicated as mol and gr, respectively. Representative Pukinje cells are indicated with double arrowheads. SUMO-1 was visualized with Fluorescein (green) and ubiquitin was visualized with Cyanine-3 (red). 16 images of a single section were accumulated with each fluorescence and then merged. In MJD, Pukinje cells showed granule-like signals in the cytoplasm while granule cells showed homogenous signals in the nucleus. A representative granule cell with high ubiquitin signals is indicated with single arrowhead (MJD). Although SUMO-1-positive Purkinje's cells showed strong signals in the nucleus, no definite co-localization of the two substances was observed in any cell type. Nuclear ubiquitin signals in Purkinje's cells of DRPLA were lower than those of MJD.

points from that of DRPLA and SCA1. Granule cells clearly showed higher signals than those of DRPLA whereas the DAB stains of Purkinje's cells looked weaker (Fig. 1). The nuclear inclusions were very rare in Purkinje's cells of all the patients as reported [18]. We could not find definitely positive neurons of the normal controls (Fig. 1).

Moreover, we tested whether anti-SUMO-1 antibody stains striatal neurons of Huntington's disease. Also in this case, we found clearly positive neurons in the striatum of a patient (Fig. 1). However, the percentage of SUMO-1-positive neurons was very small (less than 1% of total striatal neurons). Interestingly, the antibody stained neural fibers in striatosomes.

*Relationship between SUMO-1 and ubiquitin in the cerebellum*

To analyze the relationship between SUMOylation and ubiquitination in neurons, we double-stained the cerebellar tissues of DRPLA and MJD patients with anti-SUMO-1 and -ubiquitin antibodies and observed the samples with a confocal laser scanning microscopy. We could not find definite aggregates stained by anti-ubiquitin antibody in Purkinje's and granule cells, although we found intracellular auto-fluorescence of lipofuscin. However, some nuclei of Purkinje's cells reacted strongly with anti-ubiquitin antibody in MJD (Fig. 2). Localization of the SUMO-1 immunoreactivity did not overlap with that of ubiquitin in Purkinje's cells (Fig. 2), indicating that two systems function in different cellular compartments. Interestingly, neurons possessing higher signals of SUMO-1 showed a stronger nuclear stain of ubiquitin (Fig. 3). About 60–70% of Purkinje's cells showed a similar staining pattern in MJD. Neurons of lower SUMO-1 signals showed diffuse but weak stains of ubiquitin (Fig. 3).

Meanwhile, granule cells showed the SUMO-1 signal clearly in the nuclei. The signal was stronger in MJD than in DRPLA (Fig. 2). Anti-ubiquitin antibody stained the cytoplasm but not the nucleus in granule cells. The relationship between SUMO-1 and ubiquitin in granule cells was opposite to that in Purkinje's cells. However, segregation between the two systems was also clear in granule cells. These findings suggest distinct and cell-specific relationships between SUMO-1 and ubiquitin systems.

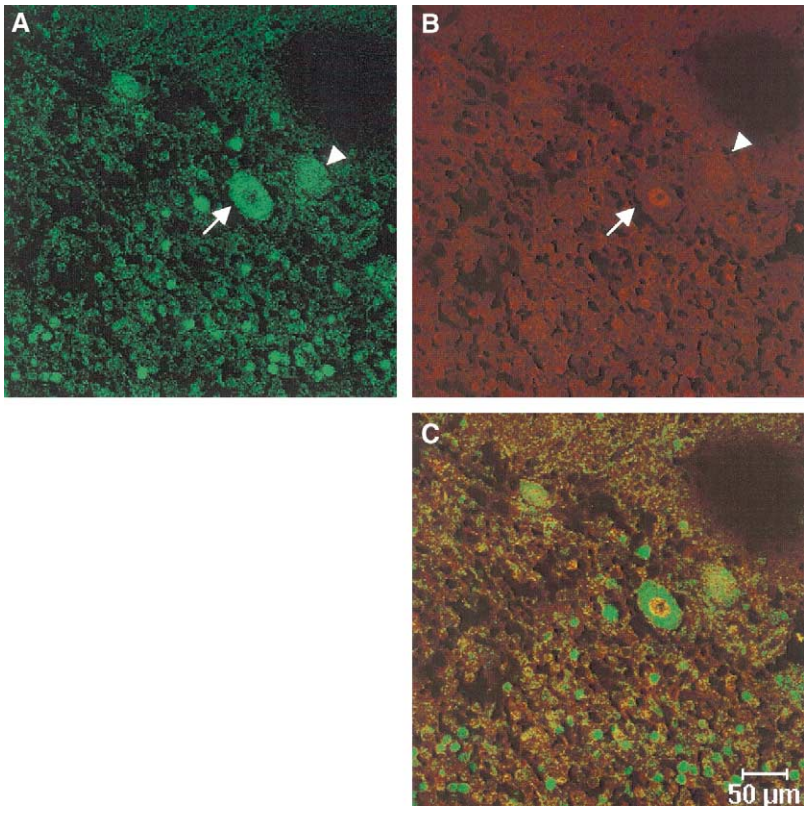


Fig. 3. SUMO-1 immunoreactivity was positively correlated with the nuclear ubiquitin signal in MJD. In a high-SUMO-1-signal Pukinje cell (a, arrow), ubiquitin stain in the nucleus was remarkable (b, arrow). On the other hand, a neighboring Pukinje cell with a low signal of SUMO-1 showed a relatively homogeneous stain of ubiquitin in the cytoplasm (arrowhead). Signals of SUMO-1 and ubiquitin were obtained from a single section 16 times and accumulated on a single picture. Merge picture (c) clearly demonstrated the relationship.

*Enhanced SUMOylated proteins in the SCA1-transgenic mouse brain*

We have shown enhanced immunoreactivity to SUMO-1 in neurons of polyglutamine diseases. To examine whether it means activation of the SUMO-1 system, we observed SUMOylated (strictly, SUMO-1ylated) proteins immunoprecipitated with ataxin-1. A transgenic mouse carrying a mutant ataxin-1 (B05:82 polyglutamine repeats) was sacrificed and the cerebellar cortex was prepared for immunoprecipitation with anti-ataxin-1 antibody (diluted at 1:2000). As control, a litter-mate non-transgenic mouse and a normal mouse were used. Immunoprecipitates were separated with 7.5% and 12% SDS-PAGE and then blotted with anti-SUMO-1 antibody.

Unexpectedly, we only found a small number of bands in Western blot using the cerebellar cortex tissues (Fig. 4). Notably, a band of about 65 kDa was stronger in the transgenic mouse than in the control. Considering the dilution effect by non-stained neurons and glial cells, we judged the increase of the band intensity to be significant. We could not find this 65 kDa band in the co-precipitates with ataxin-1. Instead, we found a slight

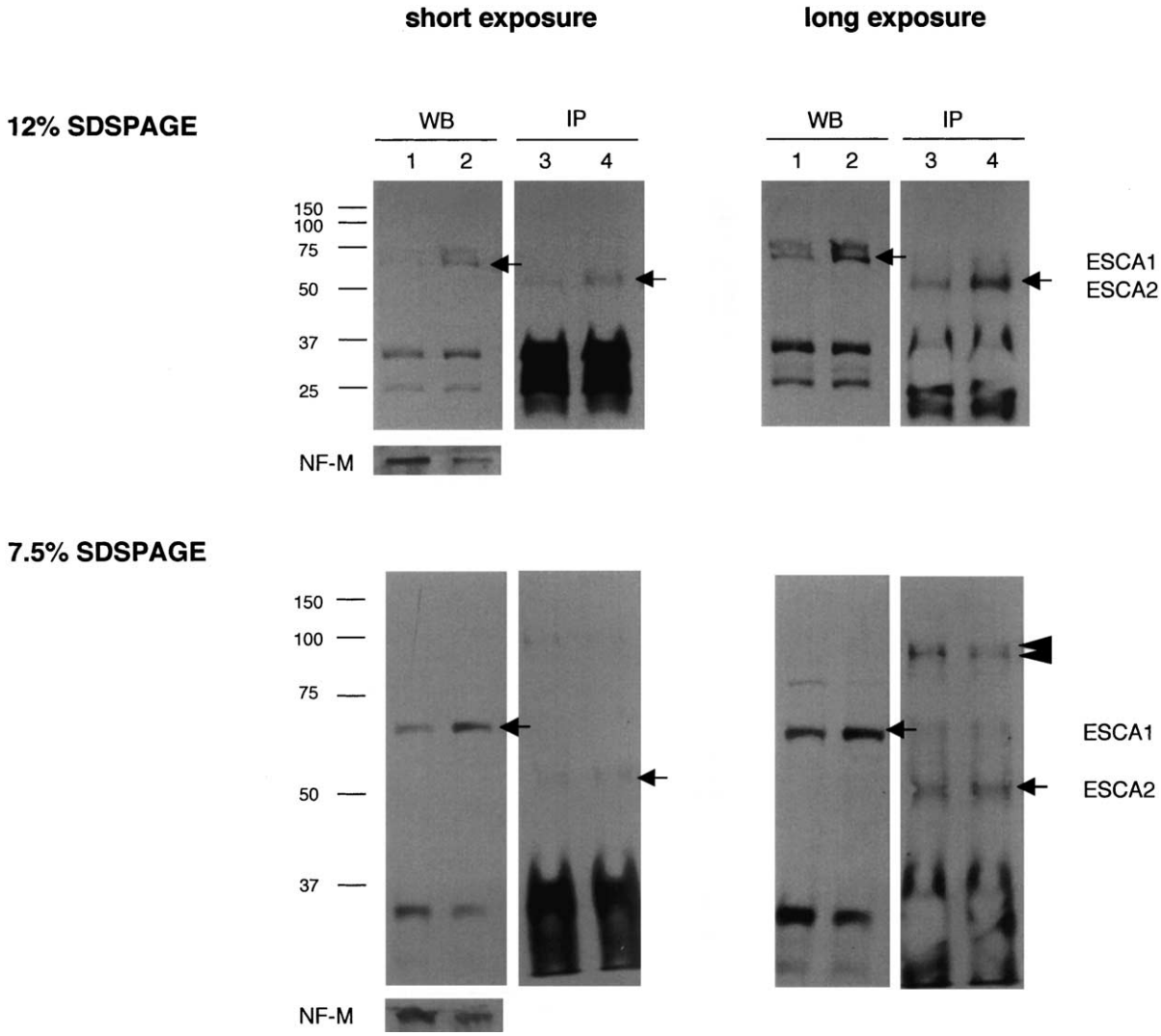


Fig. 4. SUMOylated proteins were increased in the cerebellar cortex of a transgenic mouse carrying the mutant ataxin-1 gene of 82 polyglutamine repeats [15]. Samples from the Tg mouse were used in lanes 2 and 4, while samples from a non-Tg litter-mate mouse were used in lanes 1 and 3. In lanes 1 and 2, the cerebellar cortex tissues were solubilized with TNE buffer, subjected to SDS–PAGE, and then blotted with anti-SUMO-1 monoclonal antibody (diluted at 1:2000). In lanes 3 and 4, a half of each lysate was immunoprecipitated with anti-ataxin-1 antibody and then blotted with anti-SUMO-1 antibody. In both cases, anti-SUMO-1 antibody was diluted at 1:1000. Western blots (WB) showed an increase of a ~65 kDa band (arrow) in the cerebellar cortex of the transgenic mouse. Immunoprecipitations (IP) showed the increase of a ~50 kDa band (arrow). We tentatively named these proteins as enhanced SUMOylated proteins in cerebellar ataxia 1 and 2 (ESCA1 and 2). Around the size of ataxin-1, we found two weak bands in IP (arrow heads), whereas these bands were not increased in the transgenic mouse. The same filters were blotted with anti-neurofilament 160 kDa (NF-M) antibody for the control.

increase of another band of about 50 kDa (Fig. 4). We named these proteins as ESCA (enhanced SUMOylated protein in cerebellar ataxia) 1 and 2, respectively. We found faint bands around 90 kDa. As ataxin-1 itself possesses several consensus sequences for SUMOylation, these bands might correspond to ataxin-1 though they were not increased in the transgenic mouse. Anti-SUMO-1 antibody reacts strongly to RanGAP1 in Hela cells (data not shown). Thus, one of the ~90 kDa bands might correspond to RanGAP1. In this regard, the increase of 50 kDa SUMOylated protein co-precipitated with ataxin-1 in the transgenic mouse is also of interest. It is because RanBP3 of about 54 kDa, which interacts with RanGAP1 to form nuclear pore complex (NPC), is sequestered into polyglutamine-induced aggregates [19].

## Discussion

SUMO-1 is a member of the ubiquitin-like modifiers (UBLs), including Rub1/Nedd8, Apg8, and Apg12. In contrast to ubiquitin-domain proteins (UDPs) containing ubiquitin-homologous domains, such as Parkin, RAD23, and DSK, UBLs are conjugated to the other substrate proteins to signify them for degradation or transport (see review [10]). SUMO-1 composes of a family with SUMO-2 and SUMO-3 that share ~50% sequence identity with SUMO-1. Their conjugation pathway to substrates is similar to that of ubiquitin and mediated by three types of enzymes, activating enzyme, conjugating enzyme, and ligating enzymes. In contrast to ubiquitin, SUMO conjugation does not seem to lead to poly-SUMO chains. Biological functions of SUMO have been analyzed through identification of target proteins. It is noteworthy that a number of targets belong to transcription-related factors including c-Jun, androgen receptor, IκB, p53, and p73 (see review [10]). In analogy to PML, SUMOylation might regulate intranuclear distribution of these transcription factors and thereby affect transcriptional activity of cells.

Another important example of SUMO substrates is a component of nuclear pore complex (NPC), RanGAP1 [11]. SUMOylation promotes recruitment of RanGAP1 into NPC thereby modulates nuclear transport. Because components of NPC are sequestered into aggregates induced by expression of huntingtin [19] and because nuclear transport of the polyglutamine disease gene products is an essential process for cell death [20,21], SUMO-1 activation might be involved in the pathology of polyglutamine diseases. In MJD, SUMO-1-positive Purkinje's cells showed higher ubiquitin signals in the nucleus (Fig. 3). Cytoplasmic stain of SUMO-1 in such cells would prefer this hypothesis rather than the former one of relocalization of proteins in the nucleus. SUMOylation and ubiquitination were segregated and their relationship was cell-specific (Figs. 2 and 3). In granule cells, the antagonistic action of SUMOylation against ubiquitination (reviewed by [10]) might affect the inclusion body formation by repressing ubiquitination.

Collectively, our study proposed a novel protein modification pathway in the polyglutamine disease pathology. Further studies on SUMOylation would provide a novel viewpoint for investigating polyglutamine diseases.

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