SUMOylation of the Polyglutamine Repeat Protein, Ataxin-1, Is Dependent on a Functional Nuclear Localization Signal*§

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SUMO (small ubiquitin-like modifier) is a member of the ubiquitin family of proteins. SUMO targets include proteins involved in numerous roles including nuclear transport and transcriptional regulation. The previous finding that mutant ataxin-1[82Q] disrupted promyelocytic leukemia (PML) oncogenic domains prompted us to determine whether ataxin-1 disrupts another component of PML oncogenic domains, Sp100 (100-kDa Speckled protein). Similar to the PML protein, mutant ataxin-1[82Q] redistributed Sp100 to mutant ataxin-1[82Q] nuclear inclusions. Based on the ability of PML and Sp100 to be covalently modified by SUMO, we investigated the ability of ataxin-1 to be SUMOylated. SUMO-1 was found to covalently modify the polyglutamine repeat protein ataxin-1. There was a decrease in ataxin-1 SUMOylation in the presence of the expanded polyglutamine tract, ataxin-1[82Q]. The phospho-mutant, ataxin-1[82Q]/S776A, restored SUMO levels to those of wild-type ataxin-1[30Q]. SUMOylation of ataxin-1 was dependent on a functional nuclear localization signal. Ataxin-1 SUMOylation was mapped to at least five lysine residues. Lys67, Lys194 preceding the polyglutamine tract, Lys610/Lys697 in the C-terminal ataxin high mobility group domain, and Lys746 all contribute to ataxin-1 SUMOylation.

SUMOylation functions as an antagonist to ubiquitylation, generating proteins resistant to degradation (3). SUMO targets include diverse substrates such as promyelocytic leukemia (PML), 100-kDa Speckled protein (Sp100), RanGAP1, histone deacetylase 4, and p53 (4–11). The list of proteins targeted by SUMO is rapidly increasing, and this modification has now been implicated in several neurodegenerative diseases (12, 13).

SUMO was identified in a yeast two-hybrid screen using PML as bait. SUMO has been given numerous other names including PML-interacting protein (PIC1), Sentrin, and GAPP-modifying protein 1 but now is commonly referred to as SUMO (5). In mammalian cells, there are three SUMO genes, SUMO-1, SUMO-2, and SUMO-3. The SUMO-2 and SUMO-3 genes share ~50% identity to SUMO-1. Unlike SUMO-1, SUMO-2 and SUMO-3 contain an internal SUMO consensus sequence and are capable of forming poly-SUMO chains (14). There is some sequence homology between ubiquitin and SUMO (18%), but structurally they adopt nearly identical structures (15). Similar to ubiquitin, SUMO conjugation occurs via covalent modification of target lysine residues (15, 16). Functionally, SUMOylation mimics ubiquitylation in a parallel but non-overlapping E1/E2/E3 enzyme cascade (16). To date, only a single E1 heterodimer complex (Uba2/Aos1) and a single E2-conjugating enzyme (Ubc9) have been described for SUMO (16). Three classes of E3-conjugating enzymes have been reported for SUMOylation: 1) the PIAS (Protein Inhibitors of Activated STATs) of which p53 is a substrate; 2) the nucleoporin RanBP2, a member of the nuclear pore complex; and 3) the polycomb group protein Pc2 (1, 17, 18).

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited progressive neurodegenerative disease that results in atrophy of cerebellar Purkinje cells (19). SCA1 is caused by the expansion of a CAG trinucleotide repeat tract in the SCA1 gene, resulting in an abnormally long polyglutamine tract within the protein (19). Accumulation of mutant ataxin-1[82Q] into nuclear inclusions is a hallmark of disease. Ataxin-1 contains a functional nuclear localization signal (NLS), and mutation of this NLS results in cytoplasmic localization of ataxin-1 and a reduction in disease pathology (20).

Previously, it was shown that mutant ataxin-1[82Q] redistributed PML oncogenic domains (PODs) to large mutant ataxin-1[82Q] foci or nuclear inclusions (21). The effect of this redistribution is unknown; however, the cellular distribution of PODs in relationship to nuclear organization is tightly regulated (22–24). The presence of PML is critical for the proper formation of PODs (25). SUMO modification of PML is required for the recruitment of other proteins into the PODs, suggesting a dynamic balance between PML SUMOylation and protein localization to PODs.
SUMOylation of Ataxin-1

Consensus sequence scores (CS) were obtained from SUMOplot. Percent SUMOylation was determined by multiplying the numbers on the y axis of Fig. 4B by 100%. ND, not done.

| Site  | CS  | CS score | % SUMOylation |
|-------|-----|----------|---------------|
| Lys16 | KEKKE | 0.4775 | 39.9 | +++ |
| Lys29 | KEKAP | 0.3944 | ND | ND |
| Lys32 | KKAKE | 0.5167 | 45.8 | ++ |
| Lys39 | KKAEE | 0.4778 | ND | ND |
| Lys47 | KKGKG | 0.5000 | ND | ND |
| Lys48 | KKGKG | 0.3278 | ND | ND |
| Lys49 | KKGKG | 0.5000 | 90.9 | - |
| Lys53 | PKSEE | 0.6056 | 56.5 | + |
| Lys55 | PKVE | 0.4778 | 53.1 | + |
| Lys57 | LKTE | 0.9056 | 68.0 | + |
| Lys60 | LKED | 0.9056 | 43.4 | ++ |
| Lys62 | LKKG | 0.7333 | 100 | - |
| Lys67 | VKKG | 0.7556 | 42.4 | ++ |
| Lys74 | LKFP | 0.8000 | 44.4 | ++ |
| Lys75 | EKKE | 0.3278 | ND | ND |
| Lys78 | EKLE | 0.4444 | ND | ND |
| Lys79 | EKSE | 0.5000 | 61.6 | + |

(16, 26). PML and PODs are thought to have a role in transcription (both as co-activators and co-repressors), translation, DNA repair/DNA replication, mRNA transport/stability, and proteasome-dependent degradation (27).

Although recent studies have focused on PML and its involvement with PODs, the first protein isolated from PODs was Sp100 (5, 28). Sp100 does not interact directly with PML (4). Sp100 is SUMOylated, although SUMOylation is not essential for POD formation or nuclear localization, suggesting that Sp100 SUMOylation is involved in mediating other events. Sp100 contains a high mobility group (HMG-1) domain and has been shown to interact with members of the heterochromatin family (HP1) that function as transcriptional co-repressors. The SUMOylation site of Sp100 overlaps with the HP1 binding domain, and SUMOylation could regulate the interaction of Sp100 with HP1 (4, 16, 29).

In addition to PML and Sp100, SUMOylation has been described for RanGAP1, where SUMOylation targets RanGAP1 to the nuclear pore complex. As a result of a SUMO-1 E3 ligase residing at the nuclear pore, RanBP2, it has been hypothesized that a large number of proteins are SUMOylated at the nuclear pore (1). Besides regulating subnuclear localization, SUMOylation is hypothesized to regulate import/export efficiency.

Here we show that ataxin-1 is SUMOylated and that SUMOylation is dependent on the length of the polyglutamine tract, the ability of ataxin-1 to be phosphorylated at serine 776, and the integrity of the NLS of ataxin-1. The dependence of ataxin-1 on phosphorylation and nuclear localization is reminiscent of other nuclear body proteins, namely PML, Sp100, and histone deacetylase 4, where SUMOylation controls their nucleocytoplasmic trafficking as well as their ability to function as transcriptional regulators.

MATERIALS AND METHODS

Expression Constructs—Plasmids for pCDNA1Amp-ataxin-1[30Q] and [82Q] have been described previously (21). The plasmid for pCDNA-ataxin-1[82Q]-A776 has also been described (30). The plasmids pCDNA1Amp-ataxin-1[82Q]-K772T and ataxin-1[82Q]-A776 were as described previously (20). The pCDNA1Amp-ataxin-1[30Q]-A776 was constructed by digesting pCDNA1Amp-ataxin-1[82Q]-K772T with NsiI and AgeI to cut out the fragment containing 772T. This fragment was then cloned into pCDNA1Amp-ataxin-1[30Q]. The pCDNA1.1Amp-ataxin-1[30Q] expression construct was used as a template for QuickChange II site-directed mutagenesis (Stratagene) to make the lysine to arginine mutations in the SUMO-1 consensus sites. Primer pairs are listed Supplemental Table I. To construct the multiple lysine to arginine mutations in ataxin-1[30Q], the QuickChange multi site-directed mutagenesis kit (Stratagene) was used. The ataxin-1[30Q]-K16R mutant was used as the template. The sequences of the primers used are the same as in Supplemental Table I, but they were 5'-phosphorylated and only the sense strand was used in the mutagenesis reaction. The pCDNA3-HA-SUMO-1 plasmid and pCDNA3-HA plasmid were kindly provided by Dr. Serena Kwek (9).

Cell Transfection and Immunoprecipitation—COS-1 cells were plated the day before transfection at 5 × 105 cells/60-mm plate. The following day, the cells were transfected using Lipofectamine Plus (Invitrogen). 48 h post-transfection, cells were lysed in SDS-containing buffer (one part SDS sample buffer (5% SDS, 0.15 M Tris, pH 7.5, 30% glycerol) plus three parts of lysate buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton-X-100) containing 1× protease inhibitors (Roche Applied Science), phosphatase inhibitors cocktails I and II (Sigma) (3, 9). After scraping in a 60-mm plate, lysis buffer plus cells were diluted 10 times with phosphate-buffered saline containing 0.5% Nonidet P-40 plus protease/phosphatase inhibitors. Cell lysates were collected into 1.5-ml tubes, incubated on ice for 15 min, and passed 10 times with a 21-gauge needle followed by passage through a 25-gauge needle four times. Extracts were centrifuged at 14,000 rpm for 10 min at 4 °C, and supernatants were quantified by Bio-Rad D500 protein assay (Bio-Rad). For immunoprecipitations, 500 μg of cell lysate, 50 μl of protein A (Amersham Biosciences), and 2 μl of 11NQ (residues 164–197) or 269 residues of ataxin-1 were incubated with rocking overnight at 4 °C (21).

Alternately, cells were washed as described above and then harvested using denaturing buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, and 0.1% SDS containing protease/phosphatase inhibitors as described above) directly in a 60-mm plate. After the addition of denaturing buffer, the cells were lysed by rocking at 4 °C for 15 min (31). Extracts were centrifuged as described above. Immunoprecipitations were performed with 500 μg of cell lysate, 50 μl of protein A, and 2 μl of anti-ataxin-1 antibody 11750 with rocking overnight at 4 °C (4, 5). The following day, the beads were pelleted by centrifugation for 20 s at 12,000 rpm and washed four times with denaturing buffer. The beads were heated for 10 min at 70 °C in SDS-loading dye (4 times, 30 μl, Invitrogen) plus sample reduction reagent (3 μl, Invitrogen). Immunoprecipitations were then electroblotted through 4–12% Bis-Tris gels using MOPS buffer (Invitrogen) and transferred to nitrocellulose membranes (Protran, Schleicher & Schuell). Membranes were probed overnight at 4 °C with anti-SUMO-1 antibody (1:1000, Zymed Laboratories Inc.) or with anti-HP1 antibody (1:1000, Santa Cruz Biotechnology). Protein was visualized with enhanced chemiluminescence (ECL) (Amersham Biosciences). Total ataxin-1 protein levels (10–15 μg) were electrophoreosed as described above; however, membranes were probed for 1 h with anti-ataxin-1 antibody (11750, 1:2500) at room temperature followed by detection with Western lightening chemiluminescence (PerkinElmer Life Sciences).

Immunofluorescence—COS-1 cells were plated onto coverslips at 2 × 105 density in a 6-well plate. The following day, they were transfected using lipofectamine Plus. 48 h post-transfection, cells were immunostained as previously described (21). To visualize endogenous ataxin-1, rabbit anti-Sp100 antibody (1:200) (provided by Jacob Seeler, Pasteur Institut) (29) followed by rabbit anti-Cy3 (1:200) (Jackson Immunoresearch) was used. Endogenous SUMO-1 was visualized with anti-SUMO-1 antibody (10 μg/ml, Zymed Laboratories Inc.). The different forms of ataxin-1 were visualized with anti-ataxin-1 (antibody 11750, 1:200) followed by rabbit Alexa 488 (1:200, Molecular Probes) or mouse FLAG M2 (Sigma) and 4′,6-diamidino-2-phenylindole (100 ng/ml, Sigma) to visualize the nucleus for 1 h at 37 °C. Coverslips were then washed once, mounted on slides, and analysis was performed using confocal microscopy (Bio-Rad).

RESULTS

Ataxin-1 Disrupts Sp100 Localization—Previously, it was shown that mutant ataxin-1[82Q] redistributed PODs to large mutant ataxin-1[82Q] nuclear inclusions (21). PODs are a large arsenal of SUMOylated proteins involved in diverse functions in the cell (16). Besides PML, Sp100 is another important member of PODs, although it does not interact with PML (25). Because mutant ataxin-1[82Q] redistributed PML, we sought to determine whether ataxin-1 could also redistribute Sp100 (4). To address this possibility, we overexpressed wild-type ataxin-1[30Q], mutant ataxin-1[82Q], the phospho-mutant, ataxin-1[82Q]-A776 (30, 32), and the NLS mutant, ataxin-1[82Q]-K772T (20). Immunostaining 48 h post-transfection...
demonstrated that, similar to PML, mutant ataxin-1-[82Q] redistributed endogenous Sp100 to mutant ataxin-1 inclusions (Fig. 1, g–i). Endogenous Sp100 exhibited diffuse nuclear staining with additional dot-like staining in COS-1 cells (Fig. 1, red fluorescence). Wild-type ataxin-1-[30Q], either as diffuse nuclear staining or nuclear inclusions, showed very little, if any, co-localization with Sp100 (Fig. 1, a–f). Ataxin-1-[82Q]-A776 (Fig. 1, m–o) also did not show localization with Sp100, even in the presence of ataxin-1 inclusion bodies (m–o). However, Sp100 was redistributed to the nucleolus in the presence of wild-type ataxin-1-[30Q] and ataxin-1-[82Q]-A776, as demonstrated by staining with 4′,6-diamidino-2-phenylindole (data not shown). Strikingly, expanded ataxin-1 with a mutation rendering the NLS non-functional sequestered Sp100 to cytoplasmic ataxin-1 inclusions (Fig. 1, j–l).

**Ataxin-1 Co-localization with SUMO-1**—Nuclear localization of PML and Sp100 is integral for covalent modification by SUMO. As a first step in assessing the SUMOylation status of ataxin-1, ataxin-1 co-localization with SUMO-1 was determined. Immunofluorescence studies using cells overexpressing wild-type ataxin-1-[30Q] or mutant ataxin-1-[82Q] were carried out using anti-ataxin-1 antibody 11750 and anti-SUMO-1 antibody. In COS-1 cells, endogenous SUMO-1 showed diffuse nuclear staining (data not shown). In the presence of wild-type ataxin-1-[30Q], SUMO-1 co-localized with ataxin-1 (Fig. 2, a–c). In COS-1 cells overexpressing mutant ataxin-1-[82Q], SUMO-1 staining was at the periphery or juxtaposed on the outside of mutant ataxin-1 inclusions (Fig. 2, e–g). It is possible that this pattern reflects insufficient SUMO-1 antibody penetration within the mutant ataxin-1-[82Q] nuclear inclusions. SUMO-1 conjugation is regulated by phosphorylation, and ataxin-1 is phosphorylated. Therefore, the ataxin-1 construct that cannot be phosphorylated at serine 776, ataxin-1-[82Q]-A776, was utilized (7, 33). In the presence of ataxin-1-[82Q]-A776, there was SUMO-1 co-localization (Fig. 2, i–k). To date, the majority of SUMO-1 targets described have been nuclear proteins and, as such, SUMO-1 modification has been suggested to be a predominantly nuclear process. Ataxin-1 has two possible nuclear localization signals, Lys16 and Lys772, the latter of which has.

**FIG. 1.** Redistribution of Sp100 by mutant ataxin-1-[82Q] and the NLS mutant ataxin-1-[82Q]-K772T. COS-1 cells were transfected with wild-type ataxin-1-[30Q], mutant ataxin-1-[82Q], ataxin-1-[82Q]-K772T, and ataxin-1-[82Q]-A776 (Lipofectamine Plus). Endogenous Sp100 (shown in red in a, d, g, j, and m) was visualized with rabbit anti-Sp100 antibody followed by Cy3. Ataxin-1 constructs are shown in green labeled with mouse anti-FLAG antibody followed by Alexa 488 (b, e, h, k, and n). Co-localization is shown in yellow in the merged images (c, f, i, l, and o). Images were captured with a confocal microscope.

**FIG. 2.** Co-localization of overexpressed ataxin-1 and endogenous SUMO-1 in COS-1 cells. Endogenous SUMO-1 staining (shown in red) was visualized using a monoclonal anti-SUMO-1 antibody followed by mouse anti-Cy3 (a, e, i, m, and q). The different forms of ataxin-1 are visualized using rabbit anti-FLAG antibody followed by Alexa 488 (b, f, j, n, and r). Co-localization is shown in yellow as merged images (c, g, l, o, and s). 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei is shown in d, h, l, p, and t. Dtype merged images (c, g, l, o, and s). 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei is shown in d, h, l, p, and t.
SUMOylation of Ataxin-1

**SUMOylation of Ataxin-1 in Vivo**—Sequence analyses of SUMO-1 target proteins led to identification of a loose consensus sequence for SUMOylation in which a target lysine is modified, namely Ψ-K-X-D/E, where Ψ is a hydrophobic residue and D/E represent an acidic residue (35). The examination of the sequence of ataxin-1 revealed seventeen possible SUMOylation sites (Fig. 3A). The putative SUMO-1 consensus sequences are depicted in relation to previously characterized regions within ataxin-1 (Fig. 3A). Twelve of the 17 sites are clustered near the C terminus of ataxin-1. Intriguingly one of the predicted SUMOylation sites in the N terminus is at the start of the polyglutamine stretch. Within the C terminus of ataxin-1, there is a region with high homology to the HMG-box transcription factor HBPI (heterochromatin-binding protein 1) and Sp100-HMG that is designated the AXH domain (ataxin HMG domain) (Fig. 3A) (36). This domain in HBP1 and Sp100 is important for chromatin remodeling and also functions as a transcriptional repressor domain (29, 37). In ataxin-1, this domain has been shown to be involved in RNA binding (38) and self-association (20, 39), interaction with the nuclear protein p80 coilin (40), and the ubiquitin protease, USP7 (41).

SUMO-1 has a calculated mass of 11.5 kDa and migrates in SDS-PAGE at 17–22 kDa (6, 10). SUMO-1-conjugated proteins can be identified in SDS-containing cell lysates (9, 10, 33), presumably because of inhibition of rampant SUMO hydrolases and the necessity to solubilize subnuclear structures such as the nuclear matrix (3, 9, 33). Ataxin-1 resides in the nuclear matrix and interacts with chromatin (21, 42). SUMO modification occurs only on a small fraction of total substrate, and as a result, SUMO conjugates can be difficult to detect in vivo (16). To enhance the ability of detecting SUMOylated ataxin-1, HA-tagged SUMO-1 modification occurs in vivo (16). The cells were lysed in the presence of SDS to denature the cells were lysed in the presence of SDS to denature sumolytes overexpressing ataxin-1 and HA-SUMO-1 using anti-ataxin-1 antibody 11NQ. Western blots (WB) were probed with monoclonal SUMO-1 antibody to determine SUMO-1 conjugation. To visualize total ataxin-1, Western blots were probed with monoclonal anti-FLAG antibody. Lane 1, wild-type ataxin-1[30Q]; lane 2, mutant ataxin-1[82Q]; lane 3, ataxin-1[82Q]-A776; lane 4, ataxin-1[82Q]-A777.

**Requirements for SUMO-1 Conjugation**—By immunofluorescence, only the NLS mutation K772T in either ataxin-1[30Q] or ataxin-1[82Q] affected the ataxin-1 co-localized with SUMO-1. To assess ataxin-1 SUMOylation quantitatively at a biochemical level, only the NLS mutation K772T in either ataxin-1[30Q] or ataxin-1[82Q] was predominantly cytoplasmic in COS-1 cells (Fig. 3A). The putative SUMOylation sites (Fig. 3A) were divided by wild-type ataxin-1[30Q] to normalize the data.
ical level, immunoprecipitations using anti-ataxin-1 antibody (11750) were prepared from COS-1 cells overexpressing different forms of ataxin-1 and HA-tagged SUMO-1. Ataxin-1 immunoprecipitations were performed and subjected to Western blotting with antibodies to HA or SUMO-1 (Fig. 4A and data not shown). A comparison of the ladder/smear of bands detected with wild-type ataxin-1[30Q] and mutant ataxin-1[82Q] showed a substantial decrease in the intensity of SUMO-1 staining for ataxin-1[82Q] (Fig. 4A, lane 2 versus 3). These data suggest that increasing polyglutamine length decreases ataxin-1 SUMOylation. Inspection of the possible lysine residues within SUMO-1 consensus sequences revealed a lysine at residue 194 directly preceding the polyglutamine tract (Fig. 3A and Table I). The level of SUMOylation of ataxin-1[82Q]-A776 was similar to that of wild-type ataxin-1[30Q], suggesting that phosphorylation of ataxin-1 at serine 776 affects SUMO-1 conjugation of ataxin-1 (Fig. 4A, lane 4 versus 3). Consistent with the immunofluorescence staining, there was an absence of SUMO-1-reactive bands when ataxin-1 with a mutated NLS was screened, ataxin-1[82Q]-K772T (Fig. 4A, lane 6 versus 3).

Previous studies have reported that the dimerization of a substrate can be important for its SUMOylation (4, 8). Ataxin-1 contains a functional self-association domain (Δ77) (Fig. 3A) (20, 39). Deletion of this region results in a protein that does not form inclusion bodies and does not alter PML distribution but still retains the ability to bind the nuclear matrix (20). Consistent with oligomerization having a role in SUMOylation, ataxin-1[82Q]-Δ77 showed a reduction in SUMOylation (Fig. 4A, lane 5 versus 3).

As a first step toward characterizing the biological functions of SUMOylation of ataxin-1, a series of experiments were performed to identify SUMO-1-modified lysine residues in ataxin-1. Thus, a number of lysine to arginine mutations were made in the context of full-length wild-type ataxin-1[30Q] and their effect on SUMOylation was determined (Fig. 4A and Table I). SUMOylation of each ataxin-1 variant was normalized to wild-type ataxin-1[30Q] (Fig. 4B). The mutation of Lys621 and Lys692 to arginine had no effect on SUMOylation. In contrast, the mutation of Lys16 abrogated ataxin-1 modification by SUMO-1 to the greatest extent. The mutation of Lys194, Lys610, Lys697, and Lys746 to arginine all decreased the level of SUMOylation. Table I compares the level of SUMOylation and percentage homology to the SUMO-1 consensus sequence. Consistent with the multiple bands of SUMOylated ataxin-1 seen in Fig. 3B, these data indicate there are 5–7 possible sites of SUMO-1 SUMOylation in ataxin-1.

To confirm that SUMOylation of Lys16, Lys194, Lys610, Lys697, and Lys746 represents the major SUMOylation sites on ataxin-1, constructs were made with multiple lysine to arginine mutations (Fig. 5A). As shown in Fig. 5B, the mutants generated were expressed at similar levels. Moreover, treatment with proteasome inhibitor did not result in an increase in total levels of ataxin-1 or the ataxin-1 SUMOylation mutants (data not shown). Thus, ataxin-1 lacking these SUMOylation sites did not appear to be less stable. The mutation of all five SUMOylation sites did not completely abrogate ataxin-1 SUMOylation, suggesting the presence of more than five SUMOylation sites (Fig. 5B, lane 2 versus 4). As shown in Fig. 5B, lane 3, the NLS mutant, ataxin-1[30Q]-K772T, completely eliminated ataxin-1 SUMOylation.

Mutation of the SUMOylation sites in PML resulted in the complete loss of POD formation, whereas mutation of the SUMOylation site of Sp100 does not affect nuclear localization or POD targeting (4, 43). To determine whether SUMOylation of ataxin-1 had an affect on ataxin-1 localization, the subcellular localization of ataxin-1 SUMO mutants was examined by immunofluorescence (Fig. 5C). There was a decrease in the ability of ataxin-1 to co-localize with SUMO-1 as the number of putative ataxin-1 SUMOylation sites were increasingly mutated (Fig. 5C, a–t). Similar to Sp100, the mutation of ataxin-1 SUMOylation sites did not affect ataxin-1 nuclear import or inclusion formation (Fig. 5C, a–t).

**DISCUSSION**

Ataxin-1 overexpressed in transfected cells was found to be SUMOylated at multiple lysine residues. SUMOylation of ataxin-1 was dependent on its nuclear localization, phosphorylation at Ser776, self-association region, and polyglutamine length, all of which have a role in the subcellular distribution of ataxin-1. Thus, an important factor controlling ataxin-1 SUMOylation seems to be its subcellular distribution. Besides PML (43) and GRIP1 (44), which have three SUMOylation sites, the majority of substrates reported to date are SUMOylated at a single lysine residue. Consequently, SUMOylated targets can be divided into two groups, those that are conjugated to one SUMO moiety and those that are SUMOylated at multiple residues. Ataxin-1 is SUMOylated on at least five residues and is therefore a member of the latter group.

Ataxin-1 with a mutated NLS showed a dramatic decrease in its ability to be SUMOylated. However, ataxin-1 nuclear localization and nuclear inclusions were indistinguishable between wild-type ataxin-1 and the ataxin-1 SUMOylation site mutants. Thus, ataxin-1 SUMOylation does not appear necessary for nuclear import. However, ataxin-1 SUMOylation could contribute to nuclear import/export efficiency of ataxin-1. Nuclear-cytoplasmic trafficking and subcellular localization are important themes for SUMOylated target proteins. The SUMO E3 ligase, RanBP2, and the SUMO isopeptidase Senp2 localize to the nuclear pore, indicating a role for SUMOylation in the dynamic import/export process (45). However, the question remains as follows. Does SUMOylation depend on nuclear import, or does import depend on SUMOylation (1)? Mutation of the NLS in PML and Sp100 disrupted their SUMOylation (4, 43). Restoring nuclear import of Sp100 by fusing the large T-antigen NLS to Sp100 NLS mutants restored Sp100 SUMOylation (4). The same was true for PML but complicated by the presence of three SUMOylation sites on PML. In both cases, *in vitro* SUMOylation assays were used with NLS mutants and all of the constructs were SUMOylated, even though *in vivo* modification was only detected in the presence of an intact NLS (8, 43, 46). This finding suggests that SUMOylation does not always regulate specific subnuclear targeting but could regulate import/export efficiency. SUMOylation could also have a role in regulating the trafficking of ataxin-1 within the nucleus. Using fluorescence recovery after photobleaching, trafficking of wild-type ataxin-1[30Q] within the nucleus and between the nucleus and cytoplasm has been demonstrated (47, 48). In contrast, once in the nucleus, mutant ataxin-1-[82] no longer traffics and does not shuttle between the nucleus and cytoplasm (47, 48). The decrease in mutant ataxin-1-[82] nucleocytoplasmic shuttling could be due to the decreased SUMOylation of mutant ataxin-1-[82] compared with wild-type ataxin-1-[30Q].

The role of PODs as either “nuclear closets” or “catalytic surfaces” remains controversial (27). Despite this controversy, an overall theme of PML and Sp100 is their involvement with events at the chromatin level (2). Specifically, Sp100 functions as a co-repressor when bound to DNA (29). Further, multiple isoforms of Sp100 have been described, including Sp100 with a C-terminal HMG (29). Sp100 interacts with HP1, a non-histone chromosomal protein (29), and SUMOylation of Sp100 increases interaction with HP1 (49). This is true for other SUMO targets. SUMOylation of PML increases interaction with Daxx,
and SUMOylation of RanGAP increases interaction with RanBP2. Because ataxin-1 may also function as a co-repressor when tethered to DNA (42) and has a C-terminal AXH domain with sequence similarity to HMG proteins (36), it is reasonable to suggest that ataxin-1 has a significant function within PODs. Perhaps one such function could be regulating the SUMOylation of other POD components. Sp100 SUMOylation regulates its ability to interact with HP1 (49). An alteration in Sp100 SUMOylation could control the ability of Sp100 ability to interact with HP1. In this way ataxin-1 could be involved in trafficking within PODs.

SUMO substrates include proteins implicated in gene transcription, including both co-activators and co-repressors (50). Recent reports (42, 51, 52) suggest a defect in transcription underpins polyglutamine repeat diseases. Moreover, ataxin-1 was shown to interact with the transcriptional co-repressor SMRT (Silencing Mediator of Retinoid and Thyroid hormone receptors), histone deacetylase 3, and function as a transcriptional co-repressor when tethered to DNA (42). Thus, it is intriguing to speculate that functional roles of the multiple SUMOylation events on ataxin-1 are to: 1) facilitate/stabilize macromolecular complexes with promoter specific proteins and 2) regulate trafficking at gene specific promoters. As a result of a decrease in SUMOylation of mutant ataxin-1[82Q], the cooperative interactions between ataxin-1 and other proteins would be disrupted, leading to a decrease in transcription at target genes.

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