Cellular Turnover of the Polyglutamine Disease Protein Ataxin-3 Is Regulated by Its Catalytic Activity

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Ataxin-3, a deubiquitinating enzyme, is the disease protein in spinocerebellar ataxia type 3, one of many neurodegenerative disorders caused by polyglutamine expansion. Little is known about the cellular regulation of ataxin-3. This is an important issue, since growing evidence links disease protein context to pathogenesis in polyglutamine disorders. Expanded ataxin-3, for example, is more neurotoxic in fruit fly models when its active site cysteine is mutated. We therefore sought to determine the influence of ataxin-3 enzymatic activity on various cellular properties. Here we present evidence that the catalytic activity of ataxin-3 regulates its cellular turnover, ubiquitination, and subcellular distribution. Cellular protein levels of catalytically inactive ataxin-3 were much higher than those of active ataxin-3, in part reflecting slower degradation. In vitro studies revealed that inactive ataxin-3 was more slowly degraded by the proteasome and that this degradation occurred independent of ubiquitination. Slower degradation of inactive ataxin-3 correlated with reduced interaction with the proteasome shuttle protein, VCP/p97. Enzymatically active ataxin-3 also showed a greater tendency to concentrate in the nucleus, where it colocalized with the proteasome in subnuclear foci. Taken together, these and other findings suggest that the catalytic activity of this disease-linked deubiquitinating enzyme regulates several of its cellular properties, which in turn may influence disease pathogenesis.

SCA3/MJD is one of nine known neurodegenerative diseases, including Huntington disease and various spinocerebellar ataxias, that are caused by CAG repeat expansions that encode abnormally long polyglutamine (poly(Q)) tracts in otherwise dissimilar proteins (5–8). All nine are age-related, progressive disorders that typically cause initial symptoms in midlife, leading to death 15–30 years later.

Poly(Q) diseases manifest different clinical and neuropathological features despite their similar poly(Q) expansions, indicating that disease protein context contributes to the range and degree of neurodegeneration in each disease. Indeed, identifying normal functions and interactions of specific poly(Q) disease proteins has helped to define disease pathogenesis. In several poly(Q) disease proteins, domains far removed from the poly(Q) tract contribute to pathogenesis. For example, in SBMA, caused by an expansion in the androgen receptor, testosterone is required for neuronal degeneration, although the hormone-binding domain of the androgen receptor is distant from the poly(Q) region (9–11).

Ataxin-3 contains an N-terminal ubiquitin protease (Josephin) domain, two or three C-terminal ubiquitin interaction motifs (UIMs) depending on splice variant, and a poly(Q) tract that resides between the second and third UIMs. This poly(Q) tract normally contains ~12–40 glutamine residues, which become expanded in disease to ~50–84 repeats. AT3 functions as a DUB, binding ubiquitin chains through its UIMs and cleaving them through the Josephin domain. Its protease activity is lost when the catalytic cysteine at position 14 is mutated to alanine (C14A) (12–14).

Evidence increasingly suggests that AT3 functions in protein quality control. A common pathological feature of poly(Q) diseases is the accumulation of intracellular inclusions containing the disease protein. AT3 is unique among poly(Q) diseases, however, in that the nonpathogenic (i.e. nonexpanded) form of the protein also localizes to aggregates in other disorders (15–17, 40). In addition, individuals homozygous for expanded MJD1 present with more severe symptoms and earlier onset of SCA3/MJD than heterozygous individuals (18, 19), suggesting a dosage effect in SCA3/MJD.

In Drosophila, AT3 serves a DUB-dependent neuroprotective role against expanded poly(Q) proteins (1). Recent evidence also suggests that AT3 regulates endoplasmic reticulum-associated protein degradation by adjusting the rate of extraction of endoplasmic reticulum-associated protein degradation substrates through its interaction with VCP/p97 (20). Together, these reports indicate that AT3 functions in protein quality control.

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2 The abbreviations used are: AT3, ataxin-3; DUB, deubiquitinating enzyme; SCA3, spinocerebellar ataxia type 3; MJD, Machado-Joseph disease; UIM, ubiquitin interaction motif; HA, hemagglutinin; RIPA, radioimmune precipitation; mRNA, messenger RNA; GST, glutathione S-transferase; mAb, monoclonal antibody; pAb, polyclonal antibody; IP, immunoprecipitation; ATPγS, adenosine 5′-O-(thiotriphosphate).
Deubiquitinating enzymes, through their ubiquitin precursor processing and protein deubiquitinating activities, play central roles in many cellular processes from DNA repair and cell cycle regulation to cell-cell interactions (21, 22). Their importance to cellular homeostasis is highlighted by their involvement in hereditary diseases, including neurodegeneration and certain types of cancer. The significance of DUBs notwithstanding, little is known about their regulation in the cell. Given the importance of protein context in poly(Q) disease pathogenesis and the fact that AT3 functions as a DUB, we investigated whether AT3 catalytic activity alters its cellular properties. Here we provide evidence that the catalytic activity of AT3 influences its steady state levels, ubiquitination pattern, and subcellular localization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Maintenance and Transfections**—Cells were maintained and transfected as described previously (23).

**FLP-in 293 Cell Lines**—Approximately 5 × 10⁶ FLP-in 293 cells were transfected with 5.4 g of pOG44 and 0.6 μg of pcDNA/FRT targeting vector. Selection medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% penicillin/streptomycin, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) was added to the cells 48 h later. Selected cells were then grown in growth medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 50 μg/ml hygromycin).

**Constructs**—Ataxin-3 constructs were maintained in the following vectors: FLAG-AT3Q25(FL), FLAG-AT3Q22(C14A), FLAG-AT3Q80(FL), and FLAG-AT3Q80(C14A) in pVETL-CmcS; FLG-AT3Q22(FL) and FLAG-AT3Q22(SA) in pFLAG; and Myc-AT3Q22(FL) and Myc-AT3Q22(C14A) in pcDNA3. HA-tagged ubiquitin was in pRK5 and was a generous gift from Dr. Ted Dawson.

**Protein Immunoprecipitation from Cells**—Cells were lysed in one of the following ice-cold buffers supplemented with Complete Mini Protease Inhibitor tablets (Roche Applied Science), depending on the experiment: RIPA (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, pH 7.4), Nonidet P-40 buffer (150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1% Triton X-100, pH 7.4), or Buffer A (20 mM HEPES, 120 mM NaCl, 1% Triton X-100, pH 7.4). Lysates were incubated with anti-FLAG M2 affinity beads (Sigma) for 2 h at 4 °C. Beads were washed four times, and protein was eluted with 3× FLAG peptide (Sigma) at 4 °C or with 6% SDS at room temperature. For experiments studying ataxin-3 ubiquitination, cell lysates were denatured with 1% SDS (30 min at room temperature) and then reanimated with 4.5% Triton X-100 (30 min at room temperature) prior to immunoprecipitation in RIPA buffer. For AT3 immunopurification from cells for use in in vitro assays, bead-bound proteins were rinsed five times with RIPA and twice with Buffer B (50 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol, and 0.1 mg/ml ovalbumin, pH 7.5) and eluted with 3× FLAG peptide. For experiments where the proteasome was inhibited before cell lysis, we used MG-132 (Calbiochem) or lactacystin (Boston Biochem) in growth media at a final concentration of 10 μM.

**Quantitative Real Time PCR**—COS-7 cells were transfected with Lipofectamine Plus (Invitrogen) per the manufacturer’s instructions. Messenger RNA (mRNA) was collected 48 h later using TRIZOL reagent (Invitrogen), following the manufacturer’s protocol. cDNA of the extracted mRNA was obtained in a reverse transcription reaction, and it was further used for quantitative real time PCR in an ABI PRISM 7700 sequence detection system (Applied Biosystems), using SYBR Green I (Applied Biosystems) as the reporter dye. c-Myc quantification was used for endogenous control reactions. The primer sequences used were the following: for ataxin-3, forward (5′-TTC TAT ATT TGT CGT TAA GGG TGA TCT G-3′) and reverse (5′-GCA TCT GTT GGA CCC TAA TCA TC-3′); for c-Myc, forward (5′-TCA AGA GGT GCC ACG TCT CC-3′) and reverse (5′-TCT TGG CAG CTG GAT AGT CCT T-3′).

**GST Fusion Protein Purification**—pGEX-6P1 plasmids encoding GST or GST-AT3 were transformed into BL21 E. coli. Individual colonies were grown at 37 °C overnight in LB plus ampicillin, and then 5 ml was used to inoculate 100 ml of LB for additional growth at 37 °C for an additional 3 h. Fusion protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 37 °C. Cells were centrifuged and resuspended in ice-cold phosphate-buffered saline, lysed by sonication, and centrifuged for 10 min to remove debris. Lysates were frozen in liquid nitrogen and stored at −80 °C.

**GST Pull-down Experiments**—GST or GST-AT3 (between 100 and 300 nM concentration) was incubated with 50 μl of glutathione-Sepharose beads (GST Microspin columns; GE Healthcare), 26 S proteasomal fractions (between 1 and 10 μM; Boston Biochem), ATPyS (4 μM; Sigma), MgCl₂ (10 μM), and proteasome inhibitors (lactacystin (Boston Biochem) or MG-132 (Calbiochem), used at 100–150 nM) at room temperature for 5–10 min in FLAG lysis buffer, Nonidet P-40 buffer, or Buffer A. Beads were rinsed three or four times with the same buffer. Protein was eluted using 10 μl reduced glutathione in Buffer B (15 min at room temperature).

**Antibodies and Western Blotting**—Proteins were electrophoresed on SDS-PAGE Ready Gels (Bio-Rad) and transferred to polyvinylidene difluoride membrane. Membranes were blocked for 20 min in blocking buffer (TBS-Tween with 5% dry milk powder). Primary antibodies were diluted in blocking buffer and incubated with membranes for 1 h at room temperature or overnight at 4 °C. Secondary antibodies were incubated with membranes for 1 h. The following primary antibodies were used: anti-ataxin-3 mouse monoclonal antibody (mAb) (1:2000; 1H9; Chemicon), anti-ataxin-3 rabbit polyclonal antibody (pAb) (1:20,000; MJD), anti-HA rabbit pAb (1:1000; Y11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ubiquitin mouse mAb (1:10,000; P4D1; Santa Cruz Biotechnology), anti-tubulin mouse mAb (1:50,000; Sigma), anti-FLAG rabbit pAb (1:1000; Sigma), anti-ubiquitin rabbit pAb (1:1000; Dako), anti-glyceraldehyde-3-phosphate dehydrogenase mouse mAb (1:500; Chemicon). Primary antibodies for 26 S proteasomal subunits (from Affiniti unless otherwise noted) were the following: anti-20 S rabbit pAb (1:500; Zymed Laboratories Inc.), anti-20 S rabbit pAb (1:500), anti-RPT2 rabbit pAb (1:500), anti-RPN2 mouse mAb (1:5000), anti-RPT3 mouse mAb
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(1:1000), anti-RPT5 mouse mAb (1:1000), anti-RPT6 mouse mAb (1:1000), anti-RPT4 mouse mAb (1:2500), anti-RPT1 mouse mAb (1:5000), anti-RPN10 mouse mAb (1:500). Goat anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies from Jackson Laboratories were used at 1:15,000.

**Immunofluorescence—**Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 min, rinsed three times with PBT (phosphate-buffered saline plus 0.1% Triton X-100), and blocked for 1 h in 5% normal goat serum in PBT. Cells were stained overnight in primary antibody (anti-20 S rabbit polyclonal (1:500; Affiniti) and anti-FLAG mouse monoclonal (1:500; Sigma)). Fluorescent secondary antibodies were used at 1:1000 (Jackson Laboratories) for 2 h. Fluorescence was visualized with a Bio-Rad krypton/argon mixed gas confocal laser microscope, based on a Nikon Eclipse upright microscope providing excitation wavelengths of 488, 568, and 647 nm. Images were collected at a x100 magnification factor and compiled using ImageJ (National Institutes of Health).

**In Vitro 26 S Degradation and DUB Assay—**GST-AT3 (0.35 μM) was incubated with 10 nM rabbit 26 S fractions (Boston Biochem), 10 mM MgCl₂, and a 1× ATP regeneration system (ERS; Boston Biochem) in Buffer B at 37 °C. At specific time points, aliquots were taken from each tube, and the reaction was stopped by the addition of 6% SDS. For AT3 deubiquitination, ubiquitin-aldehyde (Boston Biochem) was used at a 4 μM concentration. Reaction was stopped by the addition of 6% SDS.

**In Vitro Deubiquitination Assay—**FLAG-AT3Q22(C14A) that was coexpressed in COS-7 cells with HA-ubiquitin constructs was immunopurified using anti-FLAG antibody beads (Sigma) with an additional, stringent, denature/renature step (see “Protein Immunoprecipitation from Cells”). FLAG-AT3Q22(C14A) (ranging between 0.1 and 1 μM concentration) was eluted in Buffer B using 3× FLAG peptide (Sigma) and incubated with GST-AT3Q22(FL) or GSTQ22(C14A) (used between 4 and 8 μM) in Buffer B and protease inhibitor mixture P8340 (Sigma) at 37 °C. Aliquots were taken at specific time points, and the reaction was stopped by the addition of 6% SDS. As a positive control, we used Lys-63 linked hexaubiquitin chains (Boston Biochem) at 250 nM concentration.

**Pulse-Chase Analysis—**COS-7 cells were transfected with FLAG-AT3Q25(FL) or FLAG-AT3Q22(C14A) DNA to yield comparable protein levels. 48 h later, cells were starved for 30 min with methionine/cysteine-free Dulbecco’s modified Eagle’s medium plus 5% dialyzed fetal bovine serum (Invitro). Eagle’s medium plus 5% dialyzed fetal bovine serum (Invitro). Methionine/cysteine-free Dulbecco’s modified Eagle’s medium for 0–24 h. RIPA buffer cell lysates were immediately frozen in liquid nitrogen and maintained at −80 °C until time of immunopurification. FLAG-AT3 was immunoprecipitated as described above.

**Densitometry and Statistical Analysis—**Immunoblots were scanned using a Canon LiDE 60 flatbed scanner. Images were collected in Adobe Photoshop 7.0. Densitometry was measured using identically sized regions with ImageJ, and data were analyzed using Microsoft Excel. Student’s t test was used to determine statistical differences between experimental or control groups. Prior to analysis, recorded intensities were corrected for background intensity and normalized to loading control lanes.

**RESULTS**

**Ataxin-3 Catalytic Activity Affects Its Steady State Levels in Cells—**Ataxin-3 can be rendered catalytically inactive by mutating its active site cysteine at position 14 (cysteine to alanine; C14A). In transfected cells, we noticed that AT3(C14A) is consistently expressed at much higher levels than fully functional AT3. As shown in Fig. 1A, expression of equal amounts of plasmids encoding epitope-tagged, normal ataxin-3 (FLAG-AT3Q25(FL)) or catalytically inactive ataxin-3 (FLAG-AT3Q22(C14A)) led to markedly different levels of AT3 protein. The higher levels of catalytically inactive AT3 were observed whether AT3(C14A) had a normal repeat (Gln-22) or a pathogenic, expanded repeat (Gln-80; Fig. 1A, left and middle panels; supplemental Figs. 1 and 2). This phenomenon was observed both in COS-7 cells (Fig. 1A) and in HEK293 cells (data not shown). Moreover, AT3 that had been mutated elsewhere in the protein did not show this same effect. For example, AT3 with mutations in each UIM (AT3Q22(5A)), which does not bind polyubiquitin chains but retains catalytic activity, did not display increased steady state levels (Fig. 1A, right; supplemental Fig. 2). Parallel experiments employing enhanced green fluorescent protein and AT3 cotransfection confirmed that the difference in protein levels between active and inactive AT3 was not due to variable transfection efficiency (data not shown). These results suggest that the much higher AT3(C14A) protein levels are functionally linked to loss of protease activity.

Intriguingly, co-expression of wild type AT3 noticeably reduced the levels of inactive AT3 (Fig. 1B). Normal (Gln-25) and expanded (Gln-80) AT3 were able to suppress the levels of coexpressed, inactive AT3 whether it had an expanded (Gln-80) or normal (Gln-22) repeat (Fig. 1B). These results suggest both that the catalytic site of AT3 modulates steady state levels and that AT3 may act in trans to regulate the cellular fate of other AT3 proteins.

We confirmed the modulatory effect of functional AT3 on catalytically inactive AT3 protein levels in stably transfected cell lines. The FLP-in 293 cell line was used to generate pooled, transfected cells that stably express various forms of ataxin-3. Levels of catalytically inactive, expanded FLAG-AT3Q80(C14A) were lowered only in the presence of stably expressed, active FLAG-AT3Q22(FL) (Fig. 1C).

To investigate whether the observed differences in AT3 protein levels merely reflect dissimilar mRNA levels for the expression constructs, we measured transcription levels by quantitative real time PCR analysis. Plasmids encoding FLAG-AT3Q25(FL) or FLAG-AT3Q22(C14A) were transiently transfected in COS-7 cells, and AT3 mRNA levels were standardized to an endogenous control mRNA (c-Myc). As shown

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**A**

Ataxin-3 catalytic activity affects its steady state levels in cells. A, COS-7 cells were transiently transfected with the indicated constructs, harvested 48 h later, and probed on Western blot with the indicated antibodies. Left, transfection of equal amounts of FLAG-AT3Q25(FL) or FLAG-AT3Q22(C14A) plasmid DNA results in markedly higher levels of catalytically inactive AT3 protein compared with normal AT3. These lanes are from a larger Western blot, shown in full in supplemental Fig. 1. Middle, similar increased levels are observed for poly(Q)-expanded, catalytically inactive AT3. In fact, comparing the first two lanes, note that about one-tenth the DNA amount of inactive AT3 needs to be transfected compared with active AT3 to yield similar protein levels. Right, mutating the ubiquitin-binding motifs of AT3 (FLAG-AT3Q22(5A)) does not lead to increased levels of AT3. Loading controls were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin. Each group of cells received the same total amount of DNA by using a combination of empty vector and AT3 DNA. B, steady state levels of FLAG-AT3Q80(C14A) or FLAG-AT3Q22(C14A) are reduced by co-expression of catalytically active AT3 containing repeats of 25 or 80 poly(Q). AT3 with 25Q migrates at 75 kDa (gray arrow), AT3 with 80Q migrates at 50 kDa (black arrow). Loading control was glyceraldehyde-3-phosphate dehydrogenase. Each group of COS-7 cells received the same total amount of DNA by using a combination of empty vector and AT3 DNA. C, transient expression of AT3Q80(C14A) and AT3Q22(C14A) is reduced by co-expression of catalytically active AT3 containing repeats of 25 or 80 poly(Q). AT3 with 25Q migrates at 75 kDa (gray arrow), AT3 with 80Q migrates at 50 kDa (black arrow). Loading control was glyceraldehyde-3-phosphate dehydrogenase. Each group of COS-7 cells received the same total amount of DNA by using a combination of empty vector and AT3 DNA. D, quantitative real time PCR analysis of AT3 mRNA levels in COS-7 cells transiently transfected with the indicated amounts of FLAG-AT3Q25(FL) or FLAG-AT3Q22(C14A) DNA to yield comparable steady state AT3 levels. Each group of cells received the same total amount of DNA by using a combination of empty vector and AT3 DNA.

**B**

In transfected COS-7 cells (Fig. 3) or HEK293 (data not shown), we expressed active or inactive AT3 together with HA-tagged ubiquitin, with plasmid DNA levels adjusted to yield similar AT3 protein levels. Cells were harvested 48 h after transfection, and the lysates were subjected to a stringent denaturation/renaturation step before immunopurification with anti-FLAG antibody to isolate FLAG-AT3 protein. Immunoprecipitated AT3 was then probed with anti-HA antibody to detect ubiquitin. As shown in Fig. 3A, AT3 becomes ubiquitinated in cells in the presence of excess ubiquitin, resulting in a ladder of ubiquitinated species, confirming previously published results (23). However, we observed that the ubiquitination pattern of AT3Q22(C14A) differs from that of AT3Q25(FL), with catalytically inactive AT3 being more heavily ubiquitinated (Fig. 3A). This greater ubiquitination of catalytically inactive AT3 was always observed, although the degree of increased ubiquitination of
inactive AT3 varied somewhat from experiment to experiment (e.g. see Fig. 3B for a marked difference in ubiquitination of active versus inactive AT3). Thus, the catalytic activity of AT3 influences not only the AT3 turnover rate but also its post-translational modification by ubiquitin.

Ataxin-3 Does Not Deubiquitinate Other AT3 Proteins in Trans—Considering that inactive AT3 is more heavily ubiquitinated (Fig. 3) and that wild type AT3 can destabilize inactive AT3 (Fig. 1B), we investigated whether wild type AT3 can deubiquitinate inactive AT3 and thus affect its turnover rate. FLAG-AT3Q22(C14A) and wild type HA-ubiquitin were coexpressed in cells, and FLAG-AT3 was then immunopurified from lysates with anti-FLAG antibody. Immunopurified FLAG-AT3Q22(C14A) was then incubated with recombinant GST-AT3Q22(FL) or with GST-AT3Q22(C14A) as a negative control for 0–24 h. As shown in Fig. 4A, recombinant AT3 could not deubiquitinate AT3Q22(C14A), since there was no change in the ladder of ubiquitinated AT3 species (Fig. 4A, top). As a positive control, GST-AT3Q22(FL) readily cleaved hexaubiquitin chains (Fig. 4A, bottom), indicating that the recombinant AT3 used here displays catalytic activity against ubiquitin chains.

We extended these in vitro results with cell-based studies in which AT3Q80(C14A) was coexpressed with AT3Q25(FL) or empty vector.
Ataxin-3 Catalytic Activity Affects Its Proteasomal Degradation Rate in Vitro—To investigate whether the observed difference in degradation of AT3Q25(FL) versus AT3Q22(C14) (Fig. 2) depended on AT3 ubiquitination, we incubated recombinant GST-AT3Q22(FL) or GST-AT3Q22(C14A) with 26 S proteasomal fractions and an ATP regeneration system and then assessed degradation over time. As shown in Fig. 5A, AT3Q22(C14A) was degraded more slowly than AT3Q22(FL). This indicates that the rate of proteasomal degradation of AT3 depends, in part, on the catalytic site of AT3 and suggests that AT3 does not need to be ubiquitinated to be degraded by the proteasome in vitro.

Next, we sought to determine how ubiquitinated AT3 was handled by 26 S proteasomal fractions in vitro. For this experiment, we used ubiquitinated AT3(FL) or AT3(C14A) that had been immunopurified from transfected cells. As shown in Fig. 5B, both active and inactive AT3 were quickly deubiquitinated by the proteasomal fraction (Fig. 5B, compare 0 with 20 min); ubiquitinated AT3 bands (Fig. 5B, triangle) were rapidly converted to nonubiquitinated AT3 species. This deubiquitination of AT3 was prevented by the inhibitor ubiquitin-aldehyde, which blocks most proteasome-associated DUB activity (Fig. 5C). These data indicate that one or more DUBs present in 26 S proteasomal fractions can efficiently deubiquitinate AT3.

Ataxin-3 Catalytic Activity Affects Both Its Interaction with VCP/p97 and Its Subcellular Distribution—AT3 has previously been reported to interact with the proteasome (24). Therefore, we decided to investigate whether interaction of AT3 with proteasomal subunits and proteasome-associated proteins was influenced by its catalytic site. Recombinant GST-AT3Q22(FL) or GST-AT3Q22(C14A) was incubated with 26 S proteasomal fractions that had been pretreated with proteasome inhibitors to inhibit proteolysis. We tested the ability of AT3 to interact with various proteasomal subunits, including 19 S non-AAA ATPases RP2 and RP10; AAA ATPases RPT1, RPT2, RPT3, RPT4, RPT5, and RPT6; and various 20 S core subunits. These tests for interaction employing GST pull-downs, together with IPs from cell lysates, or nuclear extracts using mild buffers (see “Experimental Procedures”) did not identify specific interactions of AT3 with any of the 19 or 20 S subunits tested (data not shown).

However, when AT3 was expressed in COS-7 cells, we did notice colocalization of AT3 with the endogenous proteasome in the nucleus (Fig. 6A). Together, these results suggest that exogenous AT3 does not directly interact with the proteasome or does so only transiently or with low affinity.

In the GST pull-down assays, we noticed that catalytically inactive AT3 associated less avidly with a known AT3-interacting protein, the AAA protein VCP/p97, which is present in the proteasomal fractions (Fig. 6, B and C). VCP/p97 participates in diverse cellular processes, including the shuttling of substrates to the proteasome (25–32). This prompted us to test whether the ability of AT3 to co-precipitate endogenous VCP/p97 from cells is also affected by its catalytic site. Although there was a trend toward reduced interaction of catalytically inactive AT3 with VCP/p97 in cells, this did not reach significant statistical difference (Fig. 6D). Based on these data, one reason for reduced AT3Q22(C14A) degradation by the 26 S proteasome in vitro may be reduced shuttling or presentation of AT3 to the proteasome by VCP/p97.

Differences in steady state levels of active versus inactive AT3 could also reflect differences in subcellular distribution. When conducting confocal microscopy analyses, we observed a difference in the subcellular distribution between nonexpanded, catalytically active and inactive AT3. Catalytically active AT3 pref-
erentially localized to the nucleus, whereas inactive AT3 often distributed similarly in the cytoplasm and nucleus or preferentially in the cytoplasm (Fig. 7A). In these experiments, we used AT3Q25(FL) and AT3Q22(C14A) plasmid DNA amounts that had been determined to yield similar protein levels on Western blots (for an example, see Fig. 1D). These data suggest that AT3 catalytic activity modulates its distribution in the cell.

Finally, we sought to understand how functional AT3 modifies the protein levels of catalytically inactive AT3, as shown earlier (Fig. 1). We conducted IP experiments in which separately epitope-tagged active and inactive AT3 were coexpressed in cells, and AT3 was then immunoprecipitated from the lysate using bead-bound antibody. We noticed that ataxin-3 can interact with other ataxin-3 molecules (Fig. 7B). Thus, wild type AT3 may reduce levels of catalytically inactive AT3 through a mechanism involving physical interaction between AT3 molecules (Fig. 8).

DISCUSSION

Here we have presented evidence that the catalytic activity of the deubiquitinating enzyme ataxin-3 affects its own protein levels, ubiquitination pattern, and subcellular localization. AT3 has been implicated to function in protein quality control pathways (1, 13, 15–17, 20, 40). Regulation of AT3 by its catalytic activity may be an important component to the normal biological function of this protein; determining the precise catalytic activity of AT3, including its possible specificity for cleavage of certain types of ubiquitin linkages, is an ongoing point of investigation. Our findings may also provide insight into SCA3/MJD pathogenesis, since evidence links poly(Q) toxicity to various non-poly(Q) domains in disease proteins. Nuclear localization of the disease poly(Q) protein is required for pathogenesis in some poly(Q) diseases (33–36), and in SCA3/MJD patients, expanded AT3 accumulates in neuronal nuclei (37, 38). Additionally, recent data provide strong evidence that nuclear localization of poly(Q)-expanded AT3 enhances SCA3 phenotype manifestation in mouse models (39). Therefore, our finding that AT3 subcellular localization is activity-dependent deserves further attention, since it may help lead to a better understanding of SCA3/MJD pathogenesis.

Considering the slower turnover rate of catalytically inactive, expanded ataxin-3 in cells (AT3Q80(C14A); Fig. 1), it will be interesting to compare the pathogenicity of this protein in the mouse brain to that of the expanded, catalytically active AT3. Our data would suggest that the catalytically inactive form of expanded AT3 may accumulate more readily in neurons and therefore prove more neurotoxic. Expression of expanded, catalytically inactive AT3 is indeed more toxic in Drosophila (1); thus, extending this analysis to a mammalian nervous system will be important.

AT3 cleaves polyubiquitin chains via its N-terminal Josephin domain (12–14). In our studies employing AT3 mutated in its active site cysteine residue (C14A), the catalytically inactive protein was consistently expressed at higher steady state levels (Fig. 1; supplemental Figs. 1 and 2) and was more heavily ubiquitinated than its active counterpart (Fig. 3). The higher levels of inactive AT3 could be reduced by co-expression of active AT3 in transiently or stably transfected cells (Fig. 1, B and C), without altering the ubiquitination pattern of inactive AT3 (Fig. 4). The mechanism behind our observation that active AT3 reduces levels of inactive AT3 may rely on the ability of AT3 molecules to interact together (Fig. 7B) and with the proteasome shuttle protein VCP/P97 (Fig. 6). In this view, active AT3 can aid in bringing catalytically inactive AT3 proteins in closer...
proximity to the proteasome, where degradation then can occur (Fig. 8).

Ataxin-3 has many lysine residues interspersed throughout the protein. The presence of multiple lysine residues in AT3, the increased ubiquitination of AT3 when it is catalytically inactive, and the fact that AT3 is itself a DUB together suggest that AT3 could be a substrate for itself. Although we cannot rule out this possibility, in our deubiquitination experiments

FIGURE 6. Ataxin-3 subcellular distribution and interaction with VCP/p97 are modulated by catalytic activity. A, AT3 colocalizes with the 20 S proteasome. COS-7 cells were transiently transfected with FLAG-AT3Q25(FL), probed with anti-FLAG (red) and anti-20S (green) antibodies, and imaged using confocal microscopy. Inset, magnification of the boxed area showing colocalization of AT3 and 20 S to subnuclear foci. B, GST pull-downs of GST-AT3Q22(FL), GST-AT3Q22(C14A), or GST, incubated with 26 S proteasomal fractions in the presence of proteasome inhibitors (lactacystin or MG-132), and ATP-γS. VCP/p97 co-purifying with AT3 was detected by anti-VCP antibody. Less VCP/p97 co-purified with AT3Q22(C14A) than with AT3Q22(FL). C, quantification of results from B. Mean ± S.D. (n = 13), p < 0.01. D, quantification of VCP/p97 subjected to co-IP with AT3 from COS-7 cell lysates (mean ± S.D.; n = 9; p = 0.11).
employing immunopurified AT3, we have not noticed a change in the levels or migration pattern of functional or catalytically inactive AT3, even after overnight incubations.4

AT3Q22(C14A) is ubiquitinated more heavily than its fully functioning counterpart (Fig. 3) yet is less rapidly degraded (Figs. 2 and 5A). This may seem surprising, since ubiquitination is often associated with proteasomal targeting of proteins. However, increased ubiquitination does not necessarily specify a higher proteasomal degradation rate. Indeed, we presented in vitro evidence that AT3 can be degraded by the proteasome independently of ubiquitin (Fig. 5A) and observed a marked temporal discrepancy between the rapid deubiquitination of AT3 by 26S proteasomal fractions and its subsequent, slower degradation (Fig. 5, B and C). Other proteins are targeted for proteasomal degradation independent of ubiquitination by virtue of their association with proteasome-interacting proteins (41, 42). We suggest that AT3, as an enzyme directly involved in ubiquitin pathways, may be “handled” by the proteasome differently than typical substrates.

Our immunoprecipitation studies suggest that the influence of the catalytic activity on AT3 degradation includes activity-modulated interaction of AT3 with the proteasomal shuttling factor, VCP/p97 (Figs. 6 and 8). VCP/p97 has been linked to a diverse array of cellular processes, including endoplasmic reticulum-associated protein degradation, proteasomal degradation, and organelle formation (25–32). VCP/p97 interaction with AT3 has been previously reported in vitro and in vivo (20, 24, 43). An earlier report indicated that the VCP-AT3 interaction in cells was not affected by the catalytic site of AT3 (20). The discrepancy between this earlier study and our current findings may reflect the different conditions used. In our hands, we observe a modest, but statistically significant, decrease in VCP/p97 interaction with AT3(C14A) in vitro and a similar trend in cells (Fig. 6) using a variety of buffers and incubation times (see “Experimental Procedures”).

Another report described AT3 interaction with the proteasome (24). Despite extensive testing using mild buffer conditions, we did not confirm a physical interaction between AT3

4 S. V. Todi and H. L. Paulson, unpublished observations.
and various proteasomal subunits (data not shown). We did, however, observe colocalization of AT3 with the proteasome in subnuclear foci, consistent with a functional interaction (Fig. 6A). It may be that AT3–proteasome interactions are transient, highly dynamic, and observable by immunopurification only under certain circumstances.

Interestingly, we did not observe a difference in steady state AT3 protein levels in stably transfected cell lines expressing AT3Q22(FL) versus AT3Q22(C14A) (Fig. 1, gray arrow). There are several possible reasons for this. FLP-in cells integrate a single plasmid copy, leading to nearly physiological expression of the transfected gene product. As such, endogenous (fully active) AT3 may be able to modulate the levels of stably expressed AT3Q22(C14A) to lower levels than when the construct is overexpressed transiently. Alternatively, feedback mechanisms that come into play in stable cell lines could alter the half-life of AT3 protein in a manner different from the behavior observed in transiently transfected cells. Another possibility is that the AT3Q22(FL) cell line expresses more RNA from its integrated plasmid than does the AT3Q22(C14A) line, leading to similar protein levels.

Finally, it deserves mentioning that although our pulse-chase analysis (Fig. 2) and in vitro experiments (Fig. 5A) demonstrated a difference between the half-lives of active and inactive AT3, they do not fully account for the markedly dissimilar protein levels observed in transiently transfected cells. Another possibility is that the AT3Q22(C14A) construct is overexpressed transiently. Alternatively, feedback mechanisms that come into play in stable cell lines could alter the half-life of AT3 protein in a manner apparently independent of its ubiquitination. This finding suggests that other DUBs should be investigated for activity-dependent actions on DUB cellular fate.

In conclusion, we have presented evidence that ataxin-3 regulates its own levels, ubiquitination pattern, and subcellular localization in a catalytic activity-dependent manner. These findings provide clues to the cellular fate of AT3 in cells and provide insights into the cellular function of AT3, both as a DUB and as a pathogenic protein when its poly(Q) domain is expanded.

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