Association of ataxin-7 with the proteasome subunit S4 of the 19S regulatory complex

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Spinocerebellar ataxia type 7 (SCA7) is a neurodegenerative disorder characterized by ataxia and selective neuronal cell loss caused by the expansion of a translated CAG repeat encoding a polyglutamine tract in ataxin-7, the SCA7 gene product. To gain insight into ataxin-7 function and to decipher the molecular mechanisms of neurodegeneration in SCA7, a two-hybrid assay was performed to identify ataxin-7 interacting proteins. Herein, we show that ataxin-7 interacts with the ATPase subunit S4 of the proteasomal 19S regulatory complex. The ataxin-7/S4 association is modulated by the length of the polyglutamine tract whereby S4 shows a stronger association with the wild-type allele of ataxin-7. We demonstrate that endogenous ataxin-7 localizes to discrete nuclear foci that also contain additional components of the proteasomal complex. Immunohistochemical analyses suggest alterations either of the distribution or the levels of S4 immunoreactivity in neurons that degenerate in SCA7 brains. Immunoblot analyses demonstrate reduced levels of S4 in SCA7 cerebella without evident alterations in the levels of other proteasome subunits. These results suggest a role for S4 and ubiquitin-mediated proteasomal proteolysis in the molecular pathogenesis of SCA7.

INTRODUCTION

Spinocerebellar ataxia type 7 (SCA7) is a progressive neurodegenerative disorder clinically characterized by cerebellar ataxia and variable signs of dysarthria, ophthalmocelegia, extrapyramidal and pyramidal signs, and macular dystrophy. Post-mortem examination of SCA7 brains reveals variable degrees of neuronal degeneration in the cerebellum, brain stem, globus pallidus, red nucleus, spinal cord and occipital cortex (1,2). Retinas of SCA7 patients also show variable loss of photoreceptors, ganglion and bipolar neurons (1,2). The molecular defect in SCA7 consists of the expansion of a translated CAG repeat encoding a polyglutamine tract in ataxin-7, the SCA7 gene product (3). Thus, SCA7 together with the spinocerebellar ataxia types 1, 2, 3, 6 and 17 (4–10), spinal bulbar muscular atrophy (SBMA or Kennedy’s disease) (11), Huntington’s disease (12) and dentatorubral-pallidoluysian atrophy (13,14) are included within the group of neurodegenerative diseases caused by CAG triplet repeat expansions. Despite phenotypic variations, evidence suggests that most of these diseases share common pathogenetic mechanisms.

Like most CAG triplet repeat disorders (15), SCA7 is characterized by the presence of ubiquitylated neuronal inclusions (NIs) containing an N-terminal fragment of mutant ataxin-7 (16–18). In SCA7 brains, NIs are abundant in retina and brain regions affected by the disease, such as the inferior olives of the brain stem, although they are also detected in other brain regions not considered affected, such as the cerebral cortex (16). Although NIs are observed in cells expressing cDNAs containing long CAG repeats and in animal models of human polyglutamine diseases, the role of the nuclear inclusions in pathogenesis is the subject of controversy (19). NIs are detected in brain areas and cell types not commonly associated with neurodegeneration and some neurons degenerate in the absence of nuclear aggregates (16,20–22). Furthermore, overexpression of HSP70 chaperone suppresses neuropathology and improves motor performance in SCA1 mice without an apparent alteration in NI formation (23). Mutant proteins containing expanded polyglutamine tracts lead to the sequestration of cellular factors into NIs and to alterations in the transcriptional activity of specific genes (24,25). In addition, ubiquitin, components of the proteasome complex and heat-shock proteins are frequently detected in NIs (18,26–30). These data, along with the fact that Ub ligase (E3) activity deficiency, an enzyme implicated in protein ubiquitylation,
results in a more severe pathology in SCA1 transgenic mice (31), suggests that abnormalities in protein folding and degradation by the ubiquitin–proteasome pathway underlie the formation of nuclear inclusions.

Ataxin-7 is a novel protein of unknown function of 892 amino acid residues, an isoelectric point of 9.87 and a predicted molecular mass of 95 kDa (3). The most common allele of the wild-type protein contains 10 glutamines which are flanked by two tracts of alanines and prolines within the N-terminal region. Ataxin-7 is widely distributed in the CNS and peripheral tissues and shows both a nuclear and cytoplasmic localization in neurons (3,32). The presence of a functional phosphoprotein-binding site supports a role for ataxin-7 in signal transduction (33).

To gain insights into ataxin-7 function and the molecular basis of neurodegeneration in SCA7, a yeast two-hybrid assay was performed to identify ataxin-7 interacting proteins. Herein, we report the association between ataxin-7 and the ATPase subunit S4 of the 19S regulatory complex (RC) of the proteasome. The ataxin-7/S4 interaction is inversely correlated with the length of the polyglutamine tract contained in ataxin-7. Immunofluorescence studies demonstrate that endogenous ataxin-7 localizes within nuclear foci that contain additional proteasomal components. Furthermore, immunohistochemical and immunoblot analyses of brain regions from four SCA7 patients show alterations either in the distribution or the levels of S4 in neurons that degenerate in SCA7, whereas the levels of other proteasomal subunits are not altered. These results suggest a role for S4 and ubiquitin-mediated proteasomal degradation in SCA7 neurodegeneration.

RESULTS

Ataxin-7 associates with S4 and the interaction is inversely correlated with the length of the polyglutamine tract

A yeast two-hybrid screen of a human cerebellar cDNA library (Clontech) using wild-type ataxin-7 containing 10 glutamines as a bait was used to identify putative ataxin-7 interacting proteins. The screening of 1.3 × 10^7 cDNA clones resulted in the isolation of 26 clones, two of which corresponded to the full-length cDNA of the ATPase subunit S4 of the 19S proteasomal RC. Yeast two-hybrid analysis showed that S4 does not interact with other polyglutamine-containing proteins including ataxin-1 and huntingtin or with seven additional peripheral tissues and shows both a nuclear and cytoplasmic localization in neurons (3,32). The presence of a functional phosphoprotein-binding site supports a role for ataxin-7 in signal transduction (33).

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These results demonstrate that the association between ataxin-7 and S4 is decreased when a higher number of glutamines is contained within ataxin-7.

We next immunoprecipitated S4 from whole-cell human post-mortem cerebellar extracts with anti-S4 polyclonal antibodies to confirm the ataxin-7/S4 interaction. Examination of the precipitates by western blotting using an affinity-purified polyclonal antibody raised against a synthetic peptide from the N-terminal region of ataxin-7 (amino acids 4–13) demonstrated a physical association of S4 and ataxin-7 (Fig. 3). Specificity of our anti-ataxin-7 antibodies was demonstrated by abrogation of immunoreactivity upon preincubation of the antibody with the synthetic peptide to which the antibody was raised, by immunoblotting detection of a 95 kDa protein (which corresponds to the predicted molecular mass of ataxin-7) in human brain extracts and in COS7 cells transfected with the wild-type SCA7 cDNA, and by detection of a GST–ataxin-7 fusion protein overexpressed in bacteria (34) (data not shown; Fig. 8).

Sedimentation of lymphoblast cellular extracts on a sucrose gradient followed by fractionation and immunoblot analyses demonstrated that ataxin-7 sediments faster than predicted from its molecular mass supporting its association with other cellular components. Furthermore, these studies showed that fractions containing ataxin-7 also contained proteasomal components, including S2, S4, S12 and other ATPases of the 19S RC including S6, S7, S8 and S10b, but not 20S proteasomes (Fig. 4).

Endogenous ataxin-7 localizes to nuclear foci containing other proteasomal subunits

To examine if ataxin-7 and S4 co-localized in mammalian cells, ataxin-7 and a Flag–S4 fusion protein were co-expressed exogenously in COS7 cells and analyzed by immunofluorescence confocal laser microscopy. These studies evidenced co-localization of both proteins in discrete regions of the nuclei (Fig. 5). We next examined the subcellular localization of endogenous ataxin-7 and S4 in COS7 and HeLa cells. These studies demonstrated that ataxin-7 localizes to discrete foci distributed throughout the nucleus (Fig. 6A) and that S4 shows a nuclear bipartite distribution: it is distributed within nuclear foci and also localizes along the perinuclear membrane (Fig. 6B). Immunofluorescent labeling with anti-20S proteasome antibodies showed that endogenous 20S proteasomes are also distributed within nuclear foci (Fig. 6C). We hypothesized that if ataxin-7 is associated with S4 and both proteins co-localize, ataxin-7 may also co-localize with other components of the RC and with 20S proteasomes when 19S RCs and 20S proteasomes are assembled into 26S proteasomes. Using antibodies against ataxin-7 and the subunit S12 of the 19S RC we demonstrated co-localization of both proteins (Fig. 6D–F). Furthermore, co-localization of endogenous ataxin-7 and 20S proteasomes was also observed in some nuclear foci with antibodies against ataxin-7 and 20S proteasomes (Fig. 6G–I). The nuclear foci detected with anti-ataxin-7 antibodies do not contain the promyelocytic leukemia (PML) oncoprotein under basal conditions indicating that they do not correspond to PML bodies (data not shown). These results support a cellular association of ataxin-7 and S4 and are consistent with the fact that antibodies selective for constitutive proteasomes do not react with PML bodies upon basal conditions (35).
Alteration of either the levels or the distribution of S4 in SCA7

To further investigate the implications of the ataxin-7/S4 interaction in SCA7, we analyzed the distribution of ataxin-7 and S4 in control and SCA7 brain sections by immunohistochemical staining. We examined brain areas predominantly affected in SCA7, such as cerebellar Purkinje cells and neurons of the brain stem. We also investigated the localization of ataxin-7 and S4 in neurons of the frontal cortex and the locus ceruleus and in the vestibular nuclei of the medulla. Immunohistochemical analysis of control brains with anti-ataxin-7 antibodies demonstrated strong staining of ataxin-7 in the nucleus of Purkinje cells of the cerebellar cortex (Fig. 7A) (34), and both a nuclear and cytoplasmic localization in neurons of the pons and the inferior olives (34). Ataxin-7 was also detected in the nucleus of neurons of the cerebral cortex, vestibular nuclei and locus ceruleus. Immunostaining with anti-S4 antibodies demonstrated a nuclear distribution of S4 in Purkinje cells, neurons of the inferior olives, pons, nucleus

Figure 1. Representation of constructs used with the two-hybrid system to define the regions of ataxin-7 that interact with S4. Numbers indicate the positions of amino acids in ataxin-7. The N-terminal region spanning the polyglutamine tract and the last 308 C-terminal amino acids of ataxin-7 (585–892) interact with S4. However, the highest level of β-galactosidase activity is detected with the full-length cDNA of ataxin-7 containing 10 glutamines.

Figure 2. Liquid assay of β-galactosidase activity of yeasts containing S4 and ataxin-7 containing either 10, 40 or 60 glutamines. The strongest interaction is observed with wild-type ataxin-7 (10 glutamines). The strength of the interaction significantly decreases with ataxin-7 containing a higher number of glutamines. Error bars indicate the SEM.

Figure 3. ATPase subunit S4 of the 19S proteasome RC associates with wild-type ataxin-7. Co-immunoprecipitation experiments were performed on human post-mortem cerebellar extracts using either pre-immune rabbit serum or rabbit polyclonal anti-S4 antibodies. Protein complexes were separated by 6% SDS–PAGE and assayed by western blot with an affinity-purified polyclonal antibody against ataxin-7.
Interest

gly, we could not detect S4 immunostaining in cerebellar granular cells indicating that in the cerebellar cortex, S4 is primarily distributed in the nucleus of Purkinje cells. These results demonstrate a concomitant distribution of ataxin-7 and S4 in different brain regions.

In sections from three SCA7 post-mortem cerebella, ataxin-7 was mostly detected in the cytoplasm of Purkinje cells, although in a few Purkinje cells ataxin-7 localized to the nucleus (Fig. 7B; data not shown). Interestingly, S4 immunostaining was significantly depleted in most Purkinje cells (Fig. 7D). However, in a few degenerating Purkinje cells, S4 was detected and showed a cytoplasmic localization (data not shown). Immunostaining of SCA7 cerebellar sections with antibodies recognizing the Purkinje cell-specific protein calbindin showed high levels of immunoreactivity in all Purkinje cells (data not shown). We next examined the presence of S4 in other brain regions that degenerate in SCA7, and we observed decreased levels of S4 immunoreactivity in neurons of the inferior olives (Fig. 7E and F) and neurons of the base of the pons (data not shown). By contrast, in brain sections of the vestibular nuclei of the medulla oblongata and neurons of the locus ceruleus of the pons, two regions of the brain stem that are not affected in SCA7, S4 was present in the nucleus and the levels were not altered relative to controls (data not shown). In a few neurons of the inferior olives and in some pyramidal neurons of the cerebral cortex from SCA7 brains, anti-S4 antibodies showed an intense staining of discrete NIs (Fig. 7F and H).

Immunoblot analyses with anti-S4 antibodies confirmed the immunohistochemical observations in three SCA7 cerebella. Soluble fractions of cerebellar extracts from post-mortem tissue from three SCA7 patients were analyzed by western blot analysis with anti-S4 antibodies and compared to protein extracts from control tissues. In the SCA7 patients examined, the levels of S4 were depleted relative to the control extract (Fig. 8; data not shown). However, it is important to note that
two smaller products of 40 and 45 kDa immunoreactive with anti-S4 antibodies were detected in one SCA7 cerebellum (Fig. 8). These products have not been identified in five control cerebellar extracts. Immunoblot analysis with antibodies recognizing the ATPase subunit S10b of the RC base complex did not reveal significant differences in the levels of this protein in SCA7 cerebellar extracts when compared to controls (Fig. 8). Similarly, the levels of the subunits S9, S10a and S12 of the RC lid complex, and 20S proteasomal subunit HC2 were equivalent in control and SCA7 extracts (data not shown). Lastly, immunoblot analyses of four lymphoblast cell lines from SCA7 patients with anti-S4 antibodies showed similar levels of S4 when compared to the levels in control extracts. Altogether, these results suggest that alterations in the levels or in the distribution of S4 immunoreactivity occurs in cell types that degenerate in SCA7.

**DISCUSSION**

In this report, we show that ataxin-7, the spinocerebellar ataxia type 7 (SCA7) gene product, associates with the ATPase proteasome subunit S4. The ataxin-7/S4 interaction is modulated by the number of glutamines contained in ataxin-7 indicating that a short polyglutamine tract is required for the stability of the interaction. The association of ataxin-7 with S4 is supported by two-hybrid analyses, immunoprecipitation, sucrose gradient sedimentation and co-localization studies.

S4 is one of the six ATPase subunits of the base of the 19S RC (or PA700) of the 26S proteasome. The 26S proteasome, a
large multisubunit complex composed of two distinct particles, the 20S catalytic core and the 19S RC, is implicated in ubiquitin-mediated degradation, the major non-lysosomal proteolytic cellular pathway (36–38). The 19S regulatory component of the 26S proteasome participates in the initial recognition and binding of substrates, in addition to unfolding and translocation of ubiquitylated targets to the 20S core for degradation.

Our sucrose gradient sedimentation data demonstrates that ataxin-7 sediments to an ∼380 kDa fraction that also contains S4 and other subunits of the 19S RC. Immunofluorescence studies demonstrate that ataxin-7 localizes within discrete foci distributed within the nucleoplasm in mammalian cells and that it co-localizes with S4 and other components of the proteasomal RC. In addition, these studies show a nuclear bipartite distribution of S4 in COS7 and HeLa cells, whereby S4 is detected within nuclear foci in the nucleoplasm and along the perinuclear membrane. Immunofluorescence studies in fission yeast cells with antibodies recognizing the 19S regulator

**Figure 7.** Immunohistochemical analysis of ataxin-7 (A and B) and S4 (C–H) in control and SCA7 brain sections. In control cerebella, ataxin-7 (A) and S4 (C) are abundantly detected in the nucleus of Purkinje cells. In SCA7 cerebellar Purkinje cells, ataxin-7 shows either a nuclear (B) or cytoplasmic localization (data not shown) and S4 immunoreactivity is depleted (D). Depletion of S4 immunoreactivity and nuclear inclusions immunoreactive with anti-S4 antibodies in inferior olives neurons (F) and pyramidal neurons of cerebral cortex (H) from SCA7 brains when compared to controls (E and G). Magnification: 400× (A and B); 200× (C–H).
subunit Mts4, the yeast counterpart of the regulatory subunit S2, demonstrated that the protein is found predominantly around the nucleus in a punctate manner (39). Since Mts4 interacts specifically with the yeast S4 homolog Mts2 (40), this data is consistent with the localization of S4 to the perinuclear membrane in mammalian cells. In higher eukaryotes, proteasomes are found dispersed in the cytoplasm and/or in the nucleus, associated with the centrosome, or localized with intermediate filaments and with the membrane of the endoplasmic reticulum (41). The subcellular distribution of the proteasome changes under overexpression of structurally abnormal proteins or upon pharmacological inhibition of proteasome activity (41). Altogether, these data indicate that the subcellular localization of the proteasome is dynamically regulated. Our studies also showed that the PML myeloid protein does not co-localize with ataxin-7 under basal conditions. However, it is important to note that since the subcellular localization of PML is altered in acute promyelocytic leukemia and in response to trans retinoic acid or interferon γ (35,42), we cannot rule out co-localization of ataxin-7 and PML in non-basal conditions.

Our immunohistochemical analysis using anti-ataxin-7 antibodies confirmed a wide neuronal distribution of ataxin-7 throughout the brain as reported previously (17,32,43), but also showed high levels of ataxin-7 in the nuclei of Purkinje cells, one of the major sites of SCA7 pathology. These studies also demonstrated a concomitant spatial distribution of S4 and ataxin-7 in the brain and suggest that alterations in the levels of S4 occur in the main brain regions that degenerate in SCA7. We also demonstrated localization of S4 within NIs in neurons of the inferior olives of the medulla oblongata and pyramidal cells of the cortex, two brain areas in which we detected numerous NIs with anti-ataxin-7 antibodies. Although available SCA7 brain tissues are limited in number, selective depletion of S4 is supported by the fact that the levels of S9, S10a, S10b, S12 and HC2 subunits of the proteasome are not significantly altered in SCA7 when compared to controls. The decreased S4 immunoreactivity observed in SCA7 cerebella may be caused by either reduced S4 synthesis, epitope masking or by increased degradation of this proteasomal subunit. Since ataxin-7 does not have intrinsic transactivation activity in yeast (data not shown) and does not contain evident DNA- or RNA-binding motifs, alterations of the levels of S4 by ataxin-7 directly at the transcriptional or translational level are unlikely. Several observations argue against epitope masking as a cause for reduced S4 immunoreactivity in SCA7 brains. First, immunohistochemical analysis with anti-S4 antibodies showed a cytoplasmic localization of S4 in a few surviving cerebellar Purkinje cells and in a few basal pontine neurons. Staining with anti-S4 antibodies also demonstrated accumulation of the protein in nuclear aggregates in most of the pyramidal neurons of the cerebral cortex and in some neurons of the inferior olives. Secondly, in SCA7 brains S4 can be detected in neurons in which neurodegeneration is not observed including those of the vestibular nuclei of the medulla oblongata and the locus ceruleus of the pons. Lastly, immunoblot studies demonstrated the presence of some S4 in two SCA7 cerebellar extracts, albeit at significant lower levels when compared to controls, and in a third SCA7 cerebellar extract, two smaller products immunoreactive with anti-S4 antibodies were also detected. Taken together, these data support that S4 can be detected in SCA7 brains and that S4 is depleted in SCA7 cerebella.

Alterations in the distribution of ataxin-7 and some proteasomal subunits, and in particular the S4 subunit of the 19S RC, have recently been shown in SCA7 transgenic mice (18). These studies demonstrated that in diseased neurons, mutant ataxin-7 is abnormally processed and that N-terminal fragments are either accumulated in NIs or localize to the cytoplasm as a result of changes in their subcellular distribution during the progression of the disease. These studies also showed that most of the NIs were immunoreactive with anti-S4 antibodies despite the fact that they did not show immunostaining with S6′ and S10b of the 19S RC. These data are consistent with the altered distribution of S4 in affected neurons observed in SCA7 brains. Our data, together with the presence of S4 and ubiquitin immunoreactivity in NIs of SCA7 neurons, suggest that abnormalities in either recognition or degradation of ubiquitin–protein conjugates by the 26S proteasome complex may contribute to SCA7 pathogenesis. One hypothesis is that alterations in the levels or distribution of S4 may affect the assembly and function of the 19S RC of the 26S proteasome. This would in turn diminish the ability of the RC to translocate ubiquitylated substrates to the proteasomal core for degradation, thus producing accumulation of ubiquitin–protein conjugates in NIs. Proteasome inhibition induces rapid cell death in numerous cancer cell types and occurs in neuroblastoma cells lines under neurotoxic oxidative stress (44–47). However, proteasome inhibition prevents apoptosis under different apoptotic stimuli and prevents brain damage caused by ischemia (46,48). It also protects sympathetic neurons from undergoing programmed cell death upon deprivation of nerve growth factor (49). Interestingly, differentiated PC12 cell lines expressing mutant α-synuclein, the Parkinson’s disease gene product, show decreased activity of proteasomes and increased

Figure 8. Depletion of the ATPase subunit S4 of the 19S RC in SCA7 cerebella. Cerebellar protein extracts from a control and two SCA7 individuals with ataxin-7 containing 41 and 42 glutamines were assayed by western blot analyses with the antibodies indicated on the left. Immunodetection with anti-ataxin-7 antibodies in SCA7 cerebellar extracts demonstrate lower levels of ataxin-7 caused by Purkinje cell loss. Cerebellar extracts from SCA7 individuals demonstrate significant depletion of S4 when compared to a control cerebellum, whereas the levels of the ATPase subunit S10b of the 19S RC and calbindin in SCA7 cerebella are not altered when compared to the control.
sensitivity to apoptotic cell death with sub-toxic concentrations of the proteasome inhibitor lactacystin (50). In cellular models of polyglutamine diseases, proteasome inhibition causes accumulation of ubiquitin–protein conjugates and enhancement of protein aggregation and formation of nuclear inclusions (28,51). In gad mice, defects in the ubiquitin pathway caused by intragenic deletions in the gene encoding ubiquitin C-terminal hydrolase produce ataxia and neurodegeneration (52). Furthermore, loss of function mutations in a novel ATPas of the AAA family cause neurodegeneration in autosomal dominant hereditary spastic paraplegia (53). Moreover, yeast S4 mutants undergo cell cycle arrest and show accumulation of polyubiquitin-conjugated proteins and progressive cell death (54,55). Accumulation of S4 and 19S RCs in nuclear inclusions is observed when the androgen receptor protein containing a long polyglutamine tract accumulates in NIs (29).

The association between ataxin-7 and S4 and their localization in nuclear foci containing additional proteasomal components suggest a role of ataxin-7 in 26S proteasome function. Ataxin-7 could transport S4 to the 19S RC or exert a protective role on S4. Decreased interaction between both proteins, because of the presence of a long polyglutamine tract contained in ataxin-7, may render S4 susceptible to cleavage and degradation or abnormal distribution in nuclear inclusions with a concomitant decrease in proteasome activity. Normal S4/ataxin-7 interactions may be important for proper incorporation of S4 into the 19S RC or, alternatively, ataxin-7 may serve to localize S4 within the nuclei of neurons to confer non-proteolytic functions to the 19S RC. The regulatory subcomplex of the 26S proteasome is able to recognize misfolded proteins and inhibit aggregation of misfolded proteins (56). S4 along with five additional ATPas present in the base of the RC form a subfamily within the AAA superfamily of ATPas that has been associated with the assembly and disassembly of protein complexes by exhibiting chaperone-like activity (57,58). Therefore, association of S4 with ataxin-7 may confer chaperone-like functions to the RC to mediate assembly or disassembly of protein complexes. This is consistent with the presence of protein aggregation in polyglutamine expansion disorders and with findings that protein aggregation is diminished by overexpression of chaperonins (27,29).

In conclusion, we demonstrate that ataxin-7 associates with the proteasomal subunit S4 and that both proteins co-localize to discrete nuclear foci in mammalian cells. Our data suggest that alterations in the levels or distribution of S4 in SCA7 may underlie proteasomal or chaperonin dysfunction resulting in progressive accumulation of nuclear ubiquitin–protein conjugates. Further investigation of the ataxin-7/S4 association should provide additional insights into ataxin-7 function and the roles of S4 and the ubiquitin-mediated proteasomal degradatory pathway in SCA7 pathogenesis.

**MATERIALS AND METHODS**

**Plasmids and yeast strains**

The full-length wild-type human SCA7 cDNA containing 10 CAG repeats was isolated by screening a brain cDNA library (Stratagene, La Jolla, CA) and assembled by RT–PCR using a Marathon cDNA brain library (Clontech, Palo Alto, CA). The SCA7 cDNAs containing 40 and 60 CAG repeats were obtained by genomic DNA PCR amplification with Pfu DNA polymerase (Gibco BRL, Rockville, MD) of the 5′ coding region of the SCA7 gene containing the CAG repeat tract from two SCA7 individuals carrying 40 and 60 CAG repeats within the SCA7 gene. The region flanking the CAG repeats from the wild-type SCA7 cDNA was replaced with the corresponding PCR fragments containing 40 and 60 CAG repeats by restriction enzyme digestion and subcloning. Subsequently, the full-length SCA7 cDNAs containing either 10, 40 or 60 CAG repeats were subcloned into the pAS2-CYH2 vector next to the GAL4 DNA-binding domain (Matchmaker GAL4 system; Clontech). An H7c yeast clone expressing ataxin-7[10Q] was transformed with a human cerebellar cDNA library cloned in the pACT2 vector which includes the GAL4 activation domain (Clontech). cDNA clones from yeast colonies auxotrophic for histidine, leucine and tryptophan expressing the lacZ reporter gene were isolated and sequenced. To assess the specificity of the interaction between ataxin-7 and S4, Y187 yeast containing the full-length S4 cDNA were mated with Y190 yeast containing either ataxin-7[10Q], ataxin-7[40Q], ataxin-7[60Q], ataxin-1[30Q], ataxin-1[82Q], huntingtin[16Q], huntingtin[30Q], huntingtin[64Q], LNP, Lamin, p53, p53BP2, SV40-T, SNF or CDK2. Diploid yeast were tested for β-galactosidase activity. The bait constructs containing partial truncations of the wild-type SCA7 cDNA were generated by PCR with Platinum Pfu DNA polymerase (Gibco BRL).

**Liquid assay of β-galactosidase activity**

A liquid assay of eight independent Y190 yeast clones containing S4 and ataxin-7 with either 10, 40 or 60 glutamines, was performed in duplicate as described by Matilla et al. (59). Statistical significance of the averaged data was determined by non-parametric Kruskal–Wallis one-way ANOVA analysis.

**Antibody reagents**

Polyclonal antibodies recognizing ataxin-7 were generated by immunizing New Zealand rabbits with a synthetic peptide corresponding to amino acids 4–13 of ataxin-7 (RAAD-DVRGEP) conjugated to KLH (34). The peptide was generated by conventional FMOc chemistry with an ABI 431A peptide synthesizer. Antisera were affinity purified against the synthetic peptide coupled to activated CH-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). Antibodies recognizing calbindin (Chemicon International, Temecula, CA), ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA) and different subunits of the 19S RC and 20S proteasome, including S4, S10a, S10b and H2C (Affiniti, Exeter, UK) were used according to the manufacturers’ instructions. Polyclonal antibodies recognizing proteasome subunits S2, S4, S6, S7, S8, S9, S10b, S12 and 20S proteasomes were a generous gift from Martin Rechsteiner.

**Co-immunoprecipitation, SDS–PAGE and immunoblot analyses**

Post-mortem cerebellar human tissue from a control individual and two SCA7 patients were frozen immediately after extraction and homogenized in a hand-held Dounce homogenizer in immunoprecipitation buffer (20 mM Tris–HCl pH 7.5,
phoresis, blots were transferred onto nitrocellulose membranes with ATP, 10 mM MgCl₂, 1 mM EDTA and protease inhibitors.

Proteins from lymphoblastoid cell lines were extracted with 0.5% Triton X-100, 1 mM DTT, 10 mM Tris pH 7.6, 2 mM Proteins were incubated on ice for 30 min and the soluble and insoluble fractions were collected by centrifugation at 17 500 g for 30 min at 4°C. Protein concentration was determined by the Bradford method (Pierce, Rockford, IL). Five hundred micrograms of protein extracts from wild-type human cerebellum were first precleared with 20 μl of 50% Protein A/G Agarose (Santa Cruz Biotechnology) in immunoprecipitation buffer containing protease inhibitors for 1 h at 4°C with continuous mixing. Rabbit polyclonal antibodies anti-S4 (Affiniti) were then added to the sample and incubated overnight at 4°C. A negative control was performed by incubating the cerebellar extract with the corresponding amount of pre-immune rabbit antiserum. Immune complexes were precipitated by incubating the samples with 40 μl of 50% protein A/G Sepharose in immunoprecipitation buffer for 1 h at 4°C with continuous mixing followed by centrifugation at 500 g for 5 min at 4°C. The supernatant fractions were collected and the sedimented beads were washed three times with immunoprecipitation buffer, mixed with SDS sample buffer (60 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% Bromphenol blue), boiled for 5 min and resolved by 6% SDS–PAGE in Tris–glycine electrophoresis buffer. After electrophoresis, blots were transferred onto nitrocellulose membranes (Protran 0.2 μm pore size; Schleicher & Schuell, Keene, NH) overnight at 4°C, and subsequently blocked for 1 h at room temperature (RT) with 5% blocking grade blocker (Bio-Rad, Hercules, CA) in TBST (150 mM NaCl, 50 mM Tris pH 7.4, 0.001% Tween-20). Membranes were incubated for 1 h at RT with the primary antibody diluted in 5% blocking grade blocker in TBST, and subsequently washed five times, 5 min each with TBST. Secondary horseradish peroxidase-conjugated antibodies (Sigma, St Louis, MO) diluted 1:2000 in 5% blocking grade blocker in TBST were added for 45 min at RT and membranes were washed five times with TBST, for 5 min each. Immunoblots were detected with the ECL Plus system (Amersham Pharmacia Biotech).

Sucrose density gradient sedimentation

Proteins from lymphoblastoid cell lines were extracted with 0.5% Triton X-100, 1 mM DTT, 10 mM Tris pH 7.6, 2 mM ATP, 10 mM MgCl₂, 1 mM EDTA and protease inhibitors (Complete), by alternating cycles of 30 s vortexing at 4°C and 30 s incubation on ice for a total of 30 min. The soluble fraction was collected by centrifugation at 17 500 g for 30 min at 4°C and layered atop 4.9 ml 5–20% sucrose gradients in sedimentation buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) and centrifuged at 39 000 r.p.m. in a Beckman SW 50.1 rotor for 24 h at 4°C as described previously (60). The gradients were fractionated from the bottom (125 μl fractions) and 30 μl aliquots were applied to SDS–PAGE. Sedimentation of protein standards (thyroglobulin, 669 kDa; catalase, 232 kDa; aldolase, 158 kDa; bovine serum albumin (BSA), 67 kDa; Amersham Pharmacia Biotech) at a concentration of 2.5 mg/ml in sedimentation buffer and detection by Coomassie brilliant blue staining were used to estimate the approximate size of protein complexes. The distribution of each subunit across the sucrose gradient was determined by densitometry of the X-ray films using the NIH Image v1.61 software package.

Immunofluorescence

COS7 and HeLa cells (ATCC, Manassas, VA) were grown at 37°C and 5% CO₂ in 35 mm dishes with DMEM (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM L-glutamine until 70% confluency. Cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 15 min, washed three times in PBS at RT and permeabilized with 0.5% Triton X-100 in PBS for 30 min at RT. Cells were washed three times in PBS, rinsed in TBST, incubated with blocking solution (10% blocking grade blocker in TBST) for 1 h at RT and incubated with the primary antibody diluted 1:50 in blocking solution for 1 h at 37°C. Cells were washed six times in TBST at RT and then incubated with anti-rabbit FITC (Vector, Burlingame, CA) and either anti-mouse-Texas Red (Vector) or anti-sheep-TRITC (Jackson Immunoresearch, West Grove, PA) diluted 1:50 in blocking solution for 1 h at 37°C. When counterstained, cells were incubated with DAPI (1 mg/ml) for 1 min and rinsed in H₂O. Slides were mounted in Vectashield antifade medium (Vector) and cells were analyzed by laser confocal microscopy (Bio-Rad 1024). Transient expression of ataxin-7 and S4 in COS-7 cells was performed according to published protocols (59). Full-length S4 cDNA was subcloned in frame with the Flag tag of pFLAG-CMV-2 vector (Kodak) and ataxin-7 cDNA was subcloned in the pGFP-N1 vector (Clontech). Transient expressed proteins were detected with mouse anti-Flag antibody (Sigma) and anti-mouse-TRITC (Vector) and rabbit anti-ataxin-7 and anti-rabbit-FITC (Vector) as described previously (59).

Immunohistochemistry

Post-mortem human brain necropsies were obtained from the Department of Pathology at the University of Utah Medical Center. Brain tissues were formalin fixed, embedded in paraffin and standard 8 μm thick sections were obtained. Sections were dewaxed in xylene, rehydrated by passing through a standard series of graded alcohols and rinsed in deionized distilled water. High temperature antigen retrieval was performed in a 1100 W microwave for 9 min using 10 mM sodium citrate buffer, pH 6.0, and rinsed in potassium-free PBS (PBS-K⁺) (PBS-K⁺: 0.01 M Na₂HPO₄, 0.01 M NaH₂PO₄, 0.15 M NaCl) at RT. Sections were immersed in 3% H₂O₂ for 5 min at RT to quench endogenous peroxidase activity. Blocking of non-specific sites was carried out by incubating the sections in 10% normal human serum (Scantibodies, Santee, CA), 5% normal goat serum (Biosesign International, Kennebunk, ME), and 1% BSA, fraction V (Roche Molecular Biochemicals) in PBS-K⁺ for 30 min at RT. Sections were incubated with the primary antibody diluted 1:1000 in blocking solution overnight at RT. Sections were washed three times in PBS-K⁺ and incubated with a biotinylated goat anti-rabbit antibody (Jackson Immunoresearch), diluted 1:1000 in 5% normal goat serum, 1% BSA in PBS-K⁺ for 30 min at RT. The signal was amplified using the TSA™-Indirect kit (NEN Life Science Products, Boston, MA) following the manufacturer’s protocol. The labeled complex was visualized with diaminobenzidine (Vector) for 2 min at RT and rinsed with deionized distilled water. When counterstained, sections were...
incubated with hematoxylin (Vector) and bluing solution (1.5% NH\textsubscript{4}OH in 70% ethanol) for 1 min at RT. Slides were dehydrated in a series of alcohols to xylenes, mounted with CytoSeal 60 (VWR, West Chester, PA) and visualized with an Olympus AX70 photomicroscope.

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