

**Figure 1.** Accumulation of neurofilament and synaptophysin in the distal end of motor axons. **A**, Immunohistochemistry of skeletal muscle and spinal cord from AR-97Q (4–6), AR-24Q, and wild-type mice (12 weeks) using an antibody for phosphorylated or nonphosphorylated NF-H. **B**, Immunohistochemistry for synaptophysin shows findings parallel to those of neurofilament. **C**, Age-dependent change in antiphosphorylated NF-H immunohistochemistry in skeletal muscle of SBMA mice. **D**, Immunofluorescence of mouse skeletal muscle using  $\alpha$ -bungarotoxin (green) in combination with antiphospho-NF-H antibody (red). Phosphorylated NF-H accumulates in the distal end of motor axons in AR-97 mice (7–8, 12 weeks). **E**, Antiphospho-NF-H immunofluorescence with  $\alpha$ -bungarotoxin staining in skeletal muscle from a human SBMA patient showing similar neurofilament accumulation. **F**, Double-labeling of skeletal muscle from an AR-97Q mouse (4–6, 12 weeks) using antiphospho-NF-H antibody (green) and anti-AR (red) shows that accumulated NF-H does not colocalize with AR. Scale bars, 100  $\mu$ m.

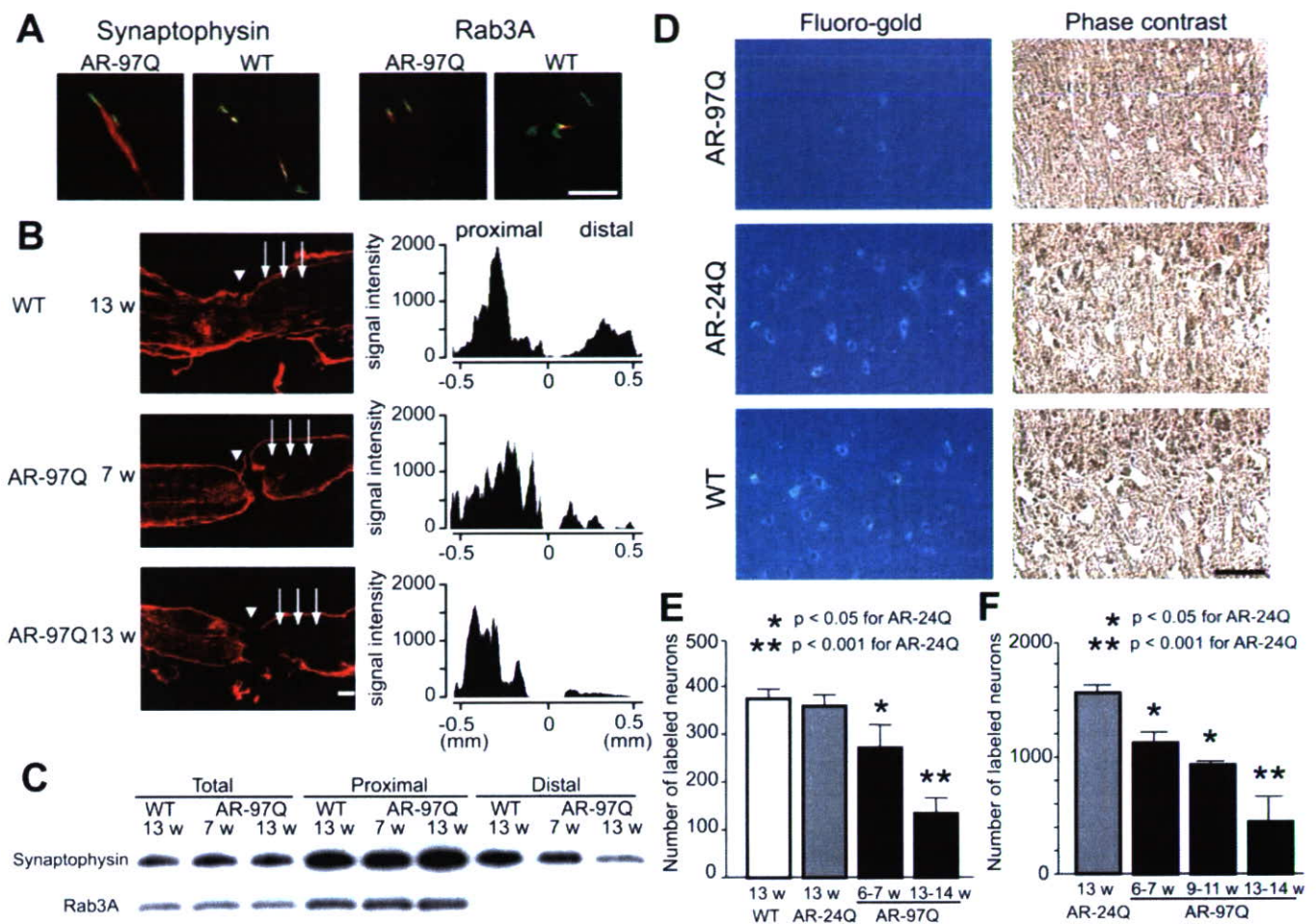
mouse model, and aggravated thereafter (Fig. 1C). These observations suggest that intramuscular accumulation of NF plays a role in the motor neuron dysfunction in this mouse model of SBMA.

To confirm the distribution of NF-H and synaptophysin in skeletal muscle, we examined the localization of these proteins in relation to the neuromuscular junction. Immunohistochemistry using  $\alpha$ -bungarotoxin to mark the junctions, and fluorescent-labeled antibodies showed that both NF-H and synaptophysin accumulated in the most distal motor axon adjacent to neuromuscular junctions (Fig. 1D). A similar intramuscular accumulation of neurofilament was detected in the skeletal muscle of SBMA patients (Fig. 1E). Although

pathogenic AR accumulated in the nuclei of skeletal muscle in the AR-97Q mice, the accumulation of NF-H did not colocalize with AR (Fig. 1F). Moreover, immunoprecipitation demonstrated no interaction between AR and NF-H (data not shown). These findings exclude the possibility that pathogenic AR directly interrupts the axonal trafficking.

#### Retrograde axonal transport is disrupted in SBMA mouse

To elucidate the molecular basis of the abnormal distribution of NF and synaptophysin, we studied axonal transport in this mouse model of SBMA. Axonal components undergo anterograde and/or retrograde axonal transport. Proteins including NF and synaptophysin are bidirectionally transported, whereas some



**Figure 2.** Perturbation of retrograde axonal transport in SBMA mice. **A**, Immunofluorescence of mouse skeletal muscle using  $\alpha$ -bungarotoxin (green) labeling the endplate together with anti-synaptophysin antibody (red) or anti-Rab3A antibody (red). Accumulation of Rab3A is not detected in wild-type or AR-97Q mice (7–8, 12 weeks). **B**, Immunohistochemistry for synaptophysin in the sciatic nerve 8 h after ligation and representative quantification of immunoreactivity. Accumulation of synaptophysin immunoreactivity is decreased on the distal side (arrows) of the ligation site (arrowhead) in preonset (7 weeks) and advanced stage (13 weeks) AR-97Q mice. **C**, Immunoblots of the sciatic nerve segments on both proximal and distal sides of the ligation. The total amount of proteins extracted from the contralateral nonligated sciatic nerve was analyzed as a control. **D, E**, Retrograde labeling of lumbar motor neurons of AR-97Q (7–8), AR-24Q, or wild-type mice (12 weeks) by Fluoro-gold injection into the gastrocnemius muscle (**D**) and the number of labeled neurons (**E**) ( $n = 5$  for each group). **F**, The number of motor neurons labeled by Fluoro-gold using the sciatic nerve stump method ( $n = 5$  for each group). Scale bars: **A, B, D**, 100  $\mu$ m. Error bars indicate SD.

components such as Rab3A, a small GTP binding protein, are transported only anterogradely (Li et al., 1995; Roy et al., 2000). The distribution of Rab3A in skeletal muscle of SBMA mice was equivalent to that of wild-type mice, whereas synaptophysin and neurofilaments accumulated in the most distal motor axons of the SBMA mice only (Figs. 1*D*, 2*A*).

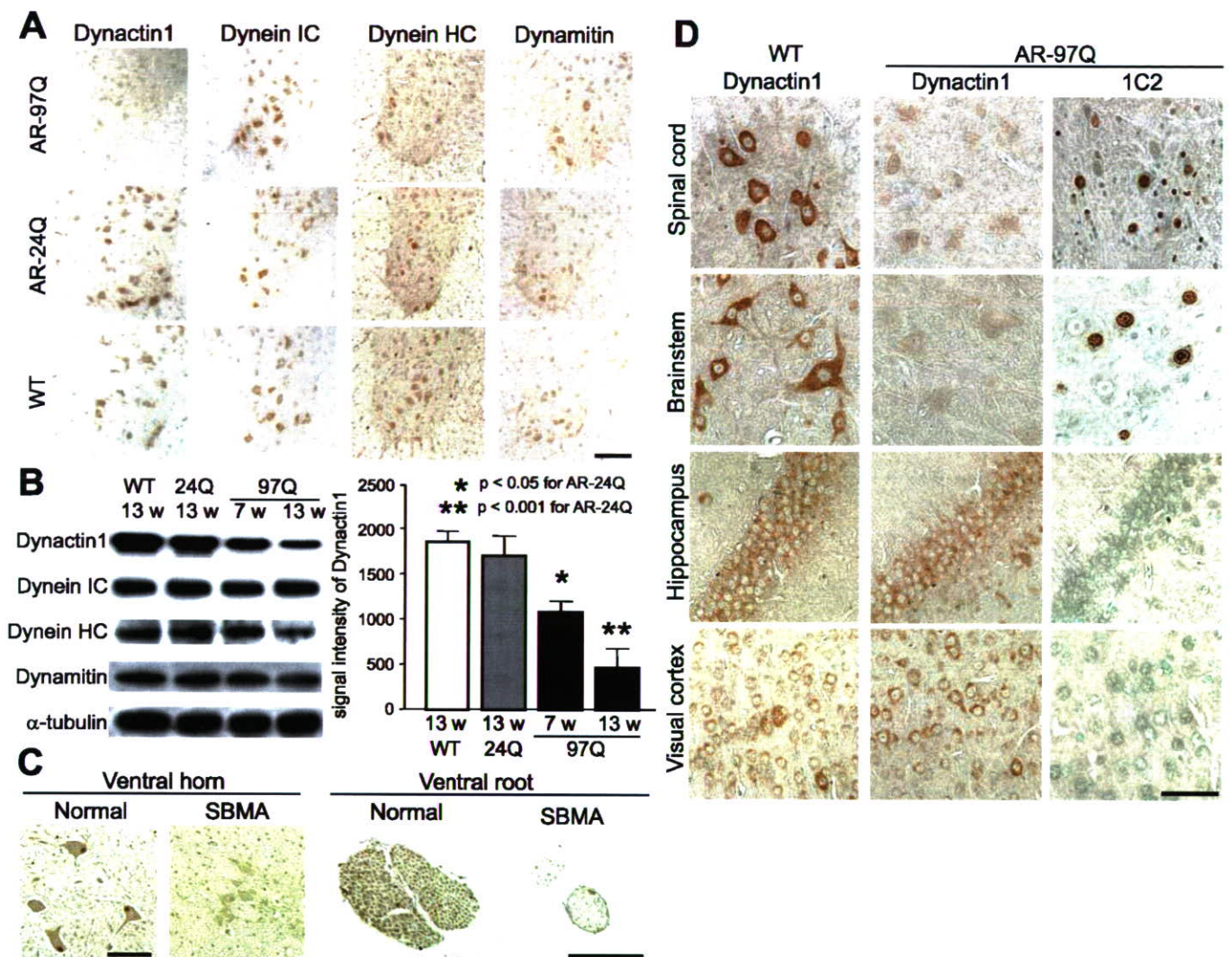
To further examine the nature of the axonal transport anomaly in SBMA mice, the sciatic nerve was ligated at mid-thigh level. Because the transport rate of NF is slower than other axonal components, we analyzed the transport of synaptophysin and Rab3A in this ligation study (Fig. 2*B, C*). In wild-type mice, synaptophysin accumulated predominantly on the proximal side of the ligation, but also on the distal side. Although synaptophysin and Rab3A accumulations proximal to the site of ligation were notable in both preonset and advanced stages of AR-97Q mice, their accumulation on the distal side was decreased before the onset of symptoms and was progressively inhibited. These findings suggest that disrupted retrograde axonal transport gives rise to the accumulation of axonal proteins in the distal motor axon terminals of SBMA mice before the onset of motor impairment.

To confirm this hypothesis, we analyzed retrograde neuronal

labeling with the fluorescent tracer Fluoro-gold after its injection into the mouse calf muscle. The number of Fluoro-gold-labeled spinal motor neurons was significantly less in affected AR-97Q mice compared with AR-24Q or wild-type mice (Fig. 2*D, E*). To exclude the possibility that synaptic pathology contributed to diminished uptake of the tracer, we also examined Fluoro-gold labeling using direct application of the tracer into the sciatic nerve stump (Sagot et al., 1998). Again, AR-97Q mice showed fewer motor neurons labeled by Fluoro-gold applied directly to the proximal stump of the sciatic nerve than did the AR-24Q mice (Fig. 2*F*), suggesting that neither synaptic retraction nor disconnection is the basis for disruption of axonal transport. Furthermore, it should be noted that the decrease in the number of labeled neurons preceded the onset of motor symptoms in both of these experiments. These observations suggest that the disruption of retrograde transport plays an early role in the pathogenesis of motor neuron degeneration in SBMA.

#### Transcriptional dysregulation of dynactin 1 in SBMA

Retrograde axonal transport is microtubule-dependent and is regulated by the axon motor protein dynein and its associated protein complex, dynactin. To elucidate the molecular



**Figure 3.** Decreased levels of dynactin 1 in SBMA. **A**, Immunohistochemistry for motor proteins regulating retrograde axonal transport, dynactin 1, dynein intermediate chain (IC), dynein heavy chain (HC), and dynamitin in the spinal cord from AR-97Q (4–6), AR-24Q, and wild-type mice (12 weeks). Dynactin 1 is markedly diminished in the motor neurons of AR-97Q mice. **B**, Western blot analysis for motor proteins in the ventral spinal root from presymptomatic or advanced AR-97Q mice (4–6) compared with those from AR-24Q and wild-type mice. **C**, Dynactin 1 immunohistochemistry in the anterior horn and the ventral root of an SBMA patient and a normal subject. **D**, Anti-dynactin 1 immunohistochemistry in various affected (spinal cord and brainstem) and nonaffected (hippocampus and visual cortex) tissues from wild-type and AR-97Q mice. Data from AR-97Q mice are compared with immunohistochemistry using the anti-polyglutamine antibody, 1C2. Scale bars: **A**, 100  $\mu$ m; **C**, **D**, 50  $\mu$ m. Error bars indicate SD.

mechanism compromising retrograde axonal transport in SBMA mice, we examined the levels of various dynein and dynactin protein subunits. Immunohistochemistry of spinal cord sections demonstrated that the spinal motor neurons from AR-97Q mice had lower levels of dynactin 1, the largest subunit of dynactin, than did those from either wild-type or AR-24Q mice (Fig. 3A). In the ventral root, significantly decreased levels of dynactin 1 were apparent before the onset of motor symptoms (Fig. 3B). Although the level of dynein heavy chain was diminished in the advanced disease stage in SBMA mice, this phenomenon was not observed before the onset of symptoms (Fig. 3B). No alterations were observed in the levels of dynein intermediate chain or dynamitin, the p50 subunit of dynactin, throughout the disease course (Fig. 3A,B). To confirm the role of dynactin 1 in the pathogenesis of human SBMA, we also examined the protein level in autopsy specimens. As observed in the mouse model, the protein level of dynactin 1 was decreased in the anterior horn cells and in the ventral roots of SBMA patients (Fig. 3C).

To examine the cell specificity of reduced dynactin 1 levels we compared anti-dynactin 1 immunohistochemistry with that of anti-polyglutamine using the 1C2 antibody in various tissues from wild-type and AR-97Q mice (Fig. 3D). The immunoreactivity of dynactin 1 was markedly diminished in 1C2-positive tissues, but not in those lacking nuclear polyglutamine staining. This observation suggests that the reduction in dynactin 1 is relevant to the polyglutamine-mediated neuropathology. In addition, to investigate whether reduced levels of dynactin 1 were correlated with defective retrograde axonal transport, we analyzed anti-dynactin 1 immunohistochemistry in spinal cord sections labeled by Fluoro-gold (supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). The levels of dynactin 1 were decreased in the spinal motor neurons of AR-97Q mice concomitantly with decreased intensities of Fluoro-gold labeling. Together, these data strongly suggest that depletion of dynactin 1 is responsible for the disruption of retrograde axonal transport in SBMA.

To clarify the pathological mechanism responsible for reduc-

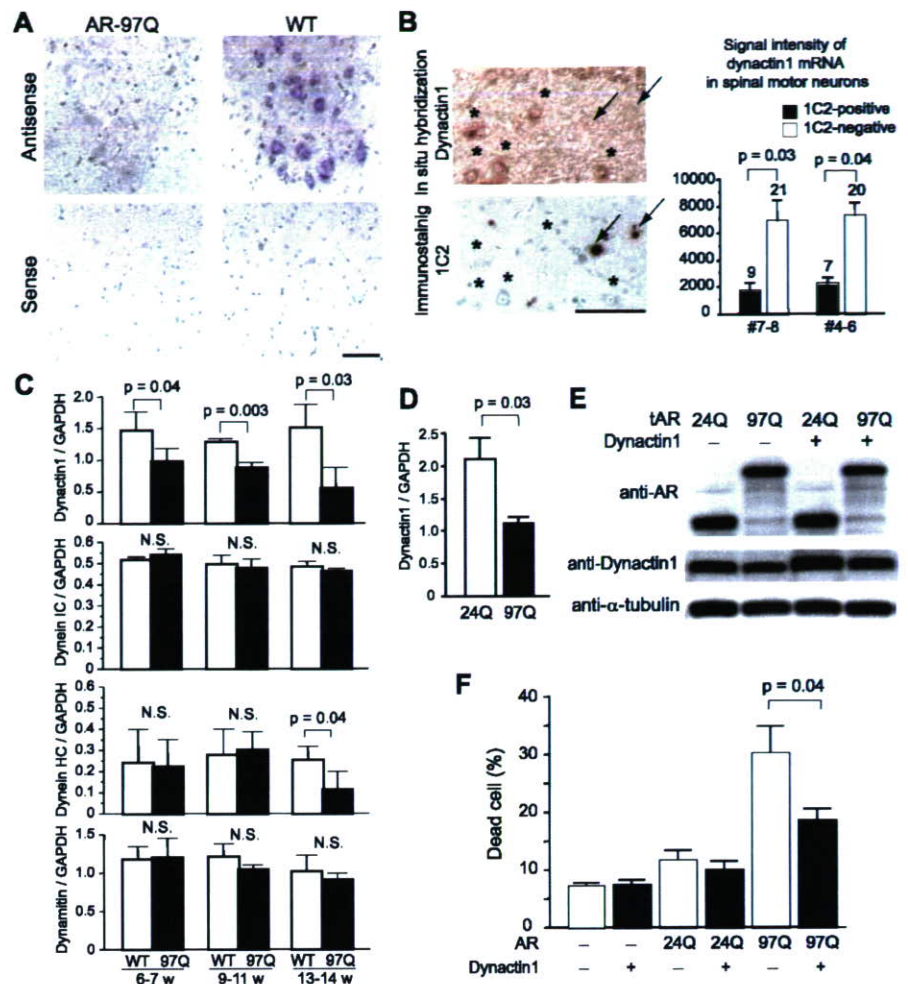
ing the levels of dynactin 1 protein in SBMA, mRNA levels were determined by *in situ* hybridization in AR-97Q and wild-type mice. Although dynactin 1 mRNA was expressed in virtually all motor neurons in the anterior horn, the expression was markedly repressed in AR-97Q mice (Fig. 4A). Moreover, the levels of dynactin 1 mRNA were significantly lower in those motor neurons demonstrating nuclear accumulation of pathogenic AR compared with those without 1C2 nuclear staining (Fig. 4B). Real-time quantitative PCR also demonstrated a significant decrease in dynactin 1 mRNA levels in the spinal cords of AR-97Q mice at all disease stages compared with those of wild-types (Fig. 4C). The level of dynein heavy chain mRNA was decreased in the advanced stage, but not in the preonset period. The levels of dynein intermediate chain mRNA and dynamitin mRNA were not altered either before or after the onset of motor symptoms.

To investigate the role that diminished levels of dynactin 1 play in neurodegeneration in SBMA, we tested whether overexpression of this protein suppressed the cellular toxicity usually observed in the presence of expanded polyglutamine. In SH-SY5Y cells bearing truncated AR containing an expanded polyglutamine, the level of dynactin 1 was decreased both in mRNA and in protein (Fig. 4D,E). In this cellular model of SBMA, overexpression of dynactin 1 alleviated cell death exerted by pathogenic AR (Fig. 4E).

In SBMA mice, the level of dynactin 1 protein in spinal motor neurons was restored by oral administration of sodium butyrate, an HDAC inhibitor that increases the level of histone acetylation leading to promotion of gene transcription (supplemental Fig. 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (Minamiyama et al., 2004). Sodium butyrate-mediated upregulation of dynactin 1 also eventually alleviated the neurofilament accumulation in skeletal muscle (supplemental Fig. 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), although this treatment had no influence on the subcellular distribution of pathogenic AR protein (Minamiyama et al., 2004). These observations indicate that nuclear accumulation of aberrant AR in the nuclei of motor neurons leads to a decrease at the transcription level of dynactin 1, resulting in perturbation of retrograde axonal transport and subsequent motor neuron dysfunction.

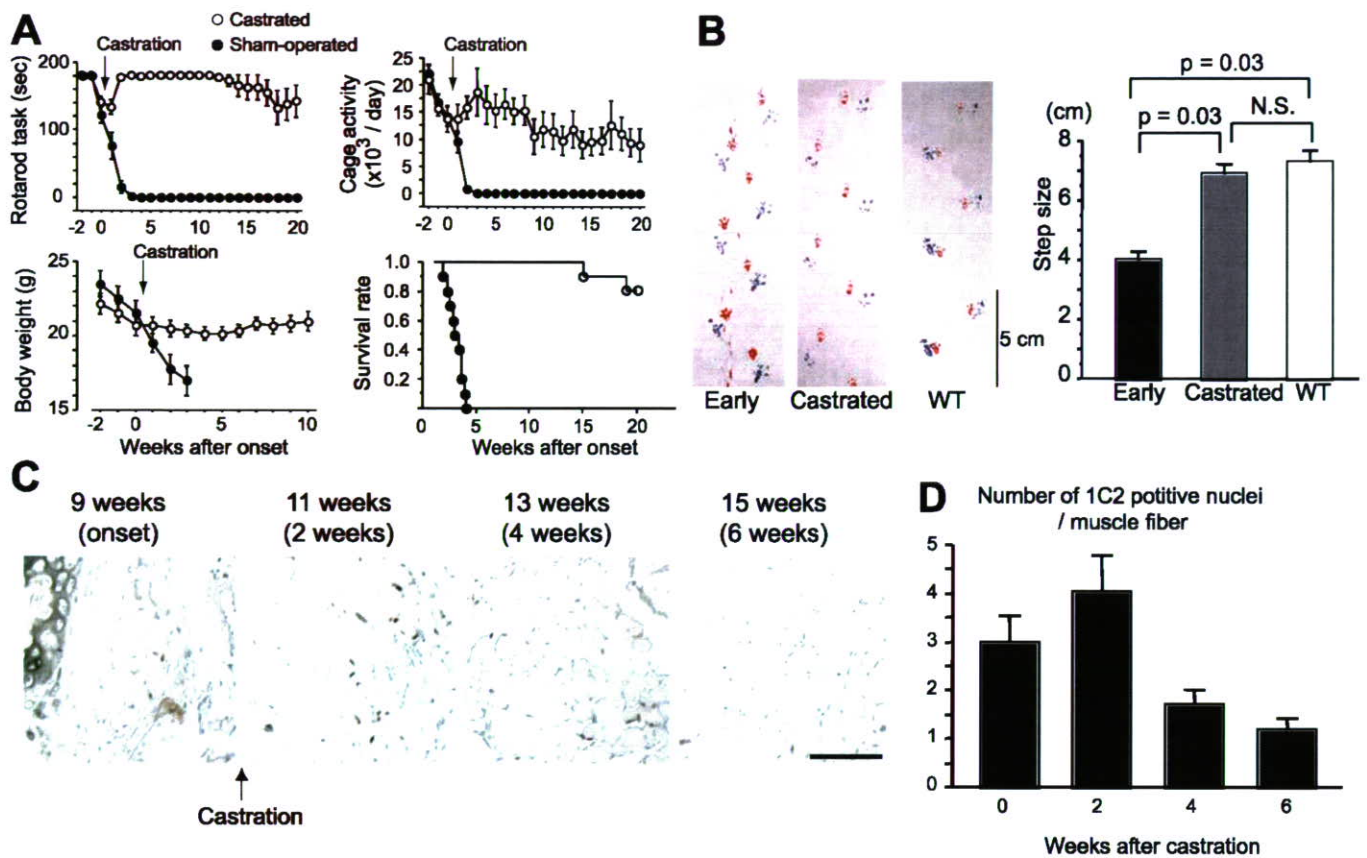
#### Castration reverses symptoms and pathology of SBMA mouse

To examine the reversibility of the phenotypes resulting from polyglutamine-induced neuronal dysfunction, we investigated the effect of castration on early symptomatic SBMA mice. Male AR-97Q mice (7–8 and 4–6) demonstrate a rapid



**Figure 4.** Transcriptional dysregulation of dynactin 1 in spinal motor neurons of SBMA mouse and effects of dynactin 1 overexpression. *A*, *In situ* hybridization of dynactin 1 mRNA in the anterior horn of wild-type and AR-97 (4–6, 9 weeks) transgenic mice. Note the marked decrease in dynactin 1 mRNA levels in the spinal motor neurons of AR-97Q compared with those in wild-type mice. *B*, *In situ* hybridization of dynactin 1 in the anterior horn. The adjacent sections were processed for anti-polyglutamine using the 1C2 antibody and the signals were quantified in representative AR-97Q mice (7–8, 9 weeks; 4–6, 10 weeks). Dynactin 1 mRNA expression is markedly decreased in the motor neurons demonstrating nuclear accumulation of pathogenic AR (arrows), but not in those lacking clear nuclear staining with anti-polyglutamine antibody (asterisks). The number above each bar indicates cell count. *C*, The mRNA levels of dynactin 1 and other motor proteins in the spinal cords of wild-type and AR-97Q mice (7–8, 13 weeks) ( $n = 4$  for each group) demonstrated by real-time, RT-PCR. Data shown are ratios of the various mRNA levels to GAPDH mRNA levels. *D*, The mRNA levels of dynactin 1 in SH-SY5Y cells expressing either AR-24Q or AR-97Q ( $n = 4$ ). *E*, Immunoblots of SH-SY5Y cells expressing either AR-24Q or AR-97Q with or without overexpression of exogenous dynactin 1. *F*, Frequency of cell death detected by propidium iodide staining. Dynactin 1 overexpression significantly reduced cell death in the cells bearing AR with elongated polyglutamine. Scale bars: *A*, *B*, 100  $\mu$ m. Error bars indicate SD ( $n = 6$  for each group). IC, Intermediate chain; HC, heavy chain.

aggravation of neuromuscular phenotypes and usually succumb 3–4 weeks after the onset of motor impairment. The motor-impaired phenotype of the SBMA mouse is dependent on circulating testosterone levels, and we reported previously that castration during the presymptomatic period (4 weeks), to eliminate testosterone, drastically prevents the development of neurological symptoms such as weakness, amyotrophy, and shortened life span (Katsuno et al., 2002). In the present study, we castrated male AR-97Q mice within 1 week after the onset of rotarod task impairment. Castration reversed motor dysfunction in AR-97Q mice, even though it was performed after the onset of symptoms (Fig. 5A). Most mice showed a reduction in daily activity and body weight loss at the onset of rotarod task defect; these symptoms were also reversed by castration. In accordance with these observations,



**Figure 5.** Symptomatic and histopathological reversibility of the SBMA phenotype in AR-97Q mice. **A**, Castration of early symptomatic AR-97Q mice within 1 week after symptomatic manifestation resulted in significant improvement of the symptomatic phenotypes: rotarod task (7–8), cage activity (4–6), body weight (4–6), and survival rate (4–6). There are significant differences in all parameters between the sham-operated ( $n = 10$ ) and castrated ( $n = 10$ ) male AR-97Q mice ( $p < 0.0001$ ,  $p < 0.0001$ ,  $p = 0.0001$ , and  $p = 0.0006$ , respectively). **B**, Representative footprints of an individual AR-97Q mouse (2–6) at the early onset of motor symptoms and after he had been castrated within 1 week after the onset of rotarod impairment, compared with those of a wild-type mouse. Quantification of the gait stride data ( $n = 4$ ). **C**, Nuclear accumulation of pathogenic AR with expanded polyglutamine in the tail muscle of one individual male AR-97Q mouse (4–6). **D**, Castration after motor impairment onset significantly reduced the number of nuclei stained by an anti-polyglutamine antibody, 1C2 ( $n = 4$ ). Scale bar: **C**, 100  $\mu\text{m}$ . Error bars indicate SD.

postonset castration significantly prolonged the life span of the male AR-97Q mice. We confirmed the reversal of motor symptoms by analyzing gait strides in a series of mouse footsteps (Fig. 5B).

To confirm the rescue effects of castration on histopathology, we investigated the nuclear accumulation of pathogenic AR in the skeletal muscle of tail sections sampled over time from the same mouse. Although the number of nuclei positively stained with 1C2 continued to increase for 2 weeks after the castration, at 4 weeks there was a significant decrease in expanded polyglutamine AR-positive nuclei (Fig. 5C,D). This time course corresponds approximately to the that of the symptomatic improvements, suggesting that nuclear accumulation of pathologic AR contributes to neuronal dysfunction and consequent symptomatic manifestation in SBMA mice.

#### Castration reverses dynactin 1 expression and restores retrograde axonal transport

It is important to determine whether disrupted retrograde axonal transport resulting from transcriptional dysregulation of dynactin 1, contributes to the reversible motor neuronal dysfunction in the early disease stage of SBMA mice. We therefore investigated axonal transport and the level of dynactin 1 expression in transgenic mice within 1 week after the onset of rotarod task impairment. In this early stage of the disease, the mice already demonstrated a reduction in the number of spinal motor neurons

labeled by Fluoro-gold (Fig. 6A). Castration of symptomatic AR-97Q mice restored Fluoro-gold staining in the spinal motor neurons to a similar level as seen in wild-types (compare Figs. 2D, 6A). Castration after the onset of muscle weakness reduced the intramuscular accumulation of neurofilaments and synaptophysin in AR-97Q mice (Fig. 6B,C). Immunohistochemistry of spinal cord showed that postsymptomatic castration also eliminated nuclear accumulation of pathogenic AR as detected by the 1C2 antibody, and restored anti-dynactin 1 immunoreactivity in motor neurons (Fig. 6D). Immunoblotting demonstrated that the level of dynactin 1 protein, but not that of dynein heavy chain, was decreased in the ventral root of AR-97Q mice in the early symptomatic stage (Fig. 6E). Castration after the onset of motor impairment restored dynactin 1 to its normal levels in the ventral root, whereas it had no effect on dynactin 1 expression in wild-type mice (Fig. 6E). These observations indicate that the castration-mediated restoration of dynactin 1 expression improves retrograde axonal transport and contributes to the reversal of neuromuscular phenotypes in SBMA mice at an early stage of the disease process.

## Discussion

### Reversibility of neuronal dysfunction in SBMA

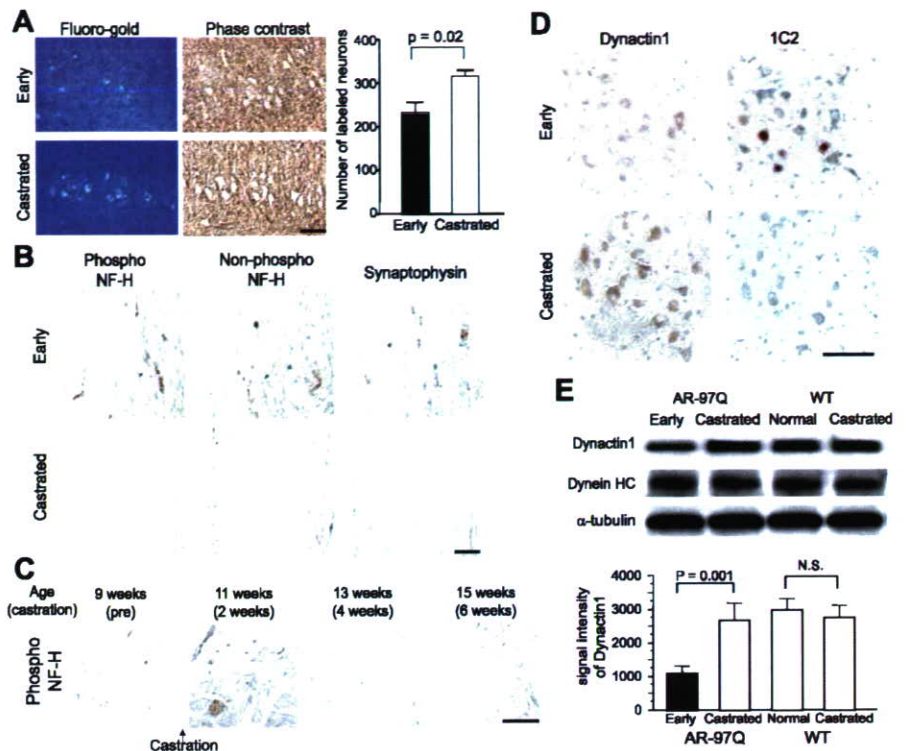
The fundamental pathological feature of polyglutamine diseases is the loss of neurons in selected regions of the CNS. Neuronal cell death, however, is often undetectable in mildly affected HD pa-

tients despite the presence of definite clinical features (Vonsattel et al., 1985). The early HD symptoms may thus result from functional alterations within neurons rather than cell death (Walker et al., 1984). In mouse models of polyglutamine diseases, it has been postulated that neuronal dysfunction, without cell loss, is sufficient to cause neurological symptoms (Mangiarini et al., 1996; Clark et al., 1997). These observations indicate that the pathogenesis of polyglutamine diseases is potentially reversible at an early stage. This hypothesis is supported by the observation that arrest of gene expression after the onset of symptoms reverses behavioral and neuropathological abnormalities in conditional mouse models of polyglutamine diseases (Yamamoto et al., 2000; Zu et al., 2004). The present study supports this hypothesis in that castration after the onset of motor deficit reverses behavioral and histopathological abnormalities by preventing nuclear accumulation of the pathogenic AR protein. These findings imply that cellular protective responses successfully abrogate the toxicity of polyglutamine-containing pathogenic protein, unless it perpetually accumulates in the nucleus.

Protein quality control systems, including molecular chaperones, the ubiquitin-proteasome system, and autophagy have been shown to reduce polyglutamine toxicity in various animal models of polyglutamine diseases (Adachi et al., 2003; Ravikumar et al., 2004; Katsuno et al., 2005; Waza et al., 2005). It is thus logical that inhibition of AR translocation into the nucleus restores the protein degradation machinery, such as ubiquitin-proteasome system, leading to the reduction in the amount of aggregates as well as the improvement of neuronal dysfunction in the SBMA mice (Waza et al., 2005).

### Defective retrograde axonal transport in SBMA

The SBMA mice we examined demonstrated impairment of retrograde axonal transport, resulting in the accumulation of neurofilaments and synaptophysin in the distal motor axon. Many proteins required for neuronal survival are synthesized within neuronal perikarya and are transported along the axon toward the synaptic terminals (Shea, 2000). A bidirectional delivery system consisting of anterograde and retrograde transport enables the recycling of cytoskeletons and synaptic vesicle-associated proteins. A histopathological hallmark of amyotrophic lateral sclerosis (ALS) is the accumulation of neurofilaments in cell bodies and proximal axons of affected motor neurons, presumably caused by compromised anterograde axonal transport; nevertheless, this finding has not been observed in SBMA (Sobue et al., 1990; Julien 2001). Transgenic SBMA mice demonstrate marked neurofilament storage in the distal motor axons, but not in the proximal axons or cell bodies. Neurofilament accumulation at motor endplates has also been reported in a transgenic mouse model of spinal muscular atrophy, another lower motor neuron disease (Cifuentes-Diaz et al., 2002). Axonal transport of NF depends on the dynein/dynactin system, disruption of which results



**Figure 6.** Hormonal intervention restores expression level of dynactin 1 and improves axonal transport. **A**, Fluoro-gold labeling of spinal cord from early symptomatic (7–8; 9–11 weeks) and castrated (7–8; 13–16 weeks) male AR-97Q mice ( $n = 5$  for each group). **B**, Immunohistochemistry of skeletal muscle for NF-H and synaptophysin. **C**, Immunohistochemistry for phosphorylated NF-H in the tail muscle of an individual male AR-97Q mouse (4–6). Castration after onset of symptoms depletes NF-H accumulation in the skeletal muscle. **D**, Immunohistochemistry of the spinal cords of early symptomatic (4–6; 11 weeks) and castrated (4–6; 15 weeks) male AR-97Q mice using anti-dynactin 1 and 1C2. Castration eliminated nuclear accumulation of expanded polyglutamine AR. **E**, Immunoblots of ventral roots from early symptomatic (4–6; 11 weeks) and castrated (4–6; 15 weeks) AR-97Q mice together with that from wild-type littermates (15 weeks) using antibodies against dynactin 1, dynein heavy chain (HC), and  $\alpha$ -tubulin. Scale bars: **A–D**, 100  $\mu$ m. Error bars indicate SD ( $n = 3$  for each group).

in accumulation of neurofilaments at the distal axon in both cultured cells and transgenic mice (LaMonte et al., 2002; He et al., 2005). When combined, these findings indicate that the accumulation of axonal components in distal motor axons appears to be a substantial pathology associated with degeneration of lower motor neurons.

In the present study, synaptophysin showed an accumulation pattern similar to that of neurofilaments, whereas the distribution of Rab3A, another synaptic vesicle-associated protein, was not altered in this mouse model. Crush injury experiments have shown that although both proteins are delivered from cell bodies into axons, of the two only synaptophysin undergoes retrograde transport (Li et al., 1995, 2000). In addition, Fluoro-gold labeling experiments clearly demonstrated the disruption of retrograde, but not anterograde axonal transport in the spinal motor neurons of SBMA mice before the onset of muscle weakness. Together, the pathogenesis of motor neuronal dysfunction in SBMA is likely to be based on the perturbation of retrograde axonal transport, and not on an excessive transport of total axonal proteins.

Axonal transport impairment has been implicated in the pathogenesis of HD and SBMA (Gunawardena et al., 2003; Szebenyi et al., 2003). Although axonal inclusion interferes with axonal transport in a cell model of SBMA (Piccioni et al., 2002), AR containing expanded polyglutamine may also inhibit anterograde and/or retrograde axonal transport without visible aggregate formation (Szebenyi et al., 2003; Morfini et al., 2006). Accu-

mulation of neurofilaments at nerve terminals has also been documented in a mouse model of HD (Ribchester et al., 2004). In our SBMA mice, pathogenic AR did not colocalize with accumulated neurofilament, nor did it form axonal inclusions. More intriguingly, sodium butyrate-mediated gene upregulation attenuated the accumulation of neurofilaments, but did not alter the intracellular distribution of AR. These observations suggest that the defective retrograde axonal transport in SBMA mice does not result from the direct interaction between aberrant AR and axonal components, but rather from a secondary mechanism resulting from expanded polyglutamine.

### Dynactin in motor neuron disease

The present study indicates that a decrease in the level of dynactin 1, the p150 subunit of dynactin, in affected neurons is a fundamental early event in the pathogenesis of SBMA. Dynactin is a multiprotein complex regulating dynein, a microtubule-dependent molecular motor for retrograde axonal transport. A mutation in *DCTN1*, the gene encoding dynactin 1, has been identified in a family with an autosomal dominant form of lower motor neuron disease and in another with ALS (Puls et al., 2003; Münch et al., 2005). A gene expression analysis of sporadic ALS patients revealed a significant decrease in dynactin 1 mRNA (Jiang et al., 2005). Overexpression of dynamitin dissociates the dynactin complex, resulting in late-onset motor neuron degeneration in a transgenic mouse model of motor neuron disease (LaMonte et al., 2002). These observations specifically link an impaired dynactin function to the pathogenesis of motor neuron diseases.

The pathological alteration in individual polyglutamine diseases is limited to distinct subsets of neurons, suggesting that the causative protein context influences the distribution of lesions. Motor neurons are selectively affected in SBMA, although pathogenic ARs are expressed in a wide range of neuronal and non-neuronal tissues (Doyu et al., 1994). A decreased level of dynactin 1 may contribute to this pathological selectivity, because a mutation in the *DCTN1* gene causes a lower motor neuron disease resembling SBMA (Puls et al., 2003, 2005).

### Link between altered transcription and neuronal dysfunction

Numerous studies have shown that nuclear accumulation of pathogenic polyglutamine-proteins is essential for neurodegeneration, although cytoplasmic events may also contribute to the pathogenesis (Gatchel and Zoghbi, 2005). Polyglutamine aggregation sequesters a variety of fundamental cellular factors including heat shock proteins and proteasomal components as well as transcriptional factors and coactivators. cAMP response element-binding protein (CBP), a transcriptional coactivator, colocalizes with intranuclear inclusions in SBMA patients as well as in transgenic SBMA mice (McCampbell et al., 2000; Nucifora et al., 2001). In addition to its sequestration in inclusion bodies, the histone acetyltransferase activity of CBP is also inhibited by soluble polyglutamine-protein (Steffan et al., 2001). This theory suggests that HDAC inhibitors, which upregulate transcription through acetylation of nuclear histone, may open new avenues in the development of therapeutics. In a fly model of HD, the HDAC inhibitors, sodium butyrate and suberoylanilide hydroxamic acid, increased histone acetylation, leading to the mitigation of neurodegeneration (Steffan et al., 2001). These compounds also improve motor dysfunction in mouse models of HD and SBMA (Hockly et al., 2003; Minamiyama et al., 2004).

In the present study, a reduction in the level of dynactin 1 protein is ascribed to polyglutamine-mediated transcriptional

dysregulation, because the mRNA level of this protein is decreased in expanded polyglutamine AR-positive spinal motor neurons. It should be noted that this diminution was significant in the neurons demonstrating nuclear accumulation of pathogenic AR, implying that polyglutamine-induced transcriptional perturbation underlies this pathological process. This hypothesis is confirmed by the observation that administration of sodium butyrate, an HDAC inhibitor, restores dynactin 1 expression, resulting in elimination of neurofilament accumulation at distal motor axons. Although, because of the nonspecific nature of sodium butyrate, we cannot at this time rule out the possibility that expression of some other protein was also elevated, leading to the elimination of neurofilament accumulation.

Given that the expression of other axon motor proteins regulating retrograde axonal transport, such as dynein intermediate chain, dynein heavy chain and dynamitin are not altered before the onset of symptoms, the reduction in dynactin 1 appears to instigate the neurodegeneration in SBMA. In addition to our study, the selective perturbation of certain subsets of gene transcription has been demonstrated in other animal models of polyglutamine diseases (Sugars and Rubinstein 2003; Sopher et al., 2004), although the precise mechanism has yet to be elucidated.

In summary, the present study demonstrates that the pathogenesis of SBMA is a reversible dysfunction of motor neurons that occurs in the early stages of the disease. Polyglutamine-induced transcriptional alteration of dynactin 1 appears to disrupt retrograde axonal transport, contributing to the early reversible neuronal dysfunction. These observations suggest that transcriptional alteration and subsequent involvement of retrograde axonal transport are substantial therapeutic targets for SBMA.

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# Archaeal Proteasomes Effectively Degrade Aggregation-prone Proteins and Reduce Cellular Toxicities in Mammalian Cells\*

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Shin-ichi Yamada, Jun-ichi Niwa, Shinsuke Ishigaki, Miho Takahashi, Takashi Ito, Jun Sone, Manabu Doyu, and Gen Sobue<sup>1</sup>

From the Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya-city, Aichi 466-8550, Japan

The 20 S proteasome is a ubiquitous, barrel-shaped protease complex responsible for most of cellular proteolysis, and its reduced activity is thought to be associated with accumulations of aberrant or misfolded proteins, resulting in a number of neurodegenerative diseases, including amyotrophic lateral sclerosis, spinal and bulbar muscular atrophy, Parkinson disease, and Alzheimer disease. The 20 S proteasomes of archaeobacteria (archaea) are structurally simple and proteolytically powerful and thought to be an evolutionary precursor to eukaryotic proteasomes. We successfully reproduced the archaeal proteasome in a functional state in mammalian cells, and here we show that the archaeal proteasome effectively accelerated species-specific degradation of mutant superoxide dismutase-1 and the mutant polyglutamine tract-extended androgen receptor, causative proteins of familial amyotrophic lateral sclerosis and spinal and bulbar muscular atrophy, respectively, and reduced the cellular toxicities of these mutant proteins. Further, we demonstrate that archaeal proteasome can also degrade other neurodegenerative disease-associated proteins such as  $\alpha$ -synuclein and tau. Our study showed that archaeal proteasomes can degrade aggregation-prone proteins whose toxic gain of function causes neurodegradation and reduce protein cellular toxicity.

The 20 S proteasome is a ubiquitous, barrel-shaped protease complex responsible for most of cellular proteolysis (1) and is formed by four stacked seven-membered rings (2). The  $\alpha$ -type subunits, which are proteolytically inactive (3), form the outer rings, and the  $\beta$ -type subunits, which contain the active site (4), form the inner rings of the complex (5). The 20 S proteasome of archaeobacteria (archaea) consists of only one type of each of the  $\alpha$ - and  $\beta$ -subunits and is thought to be the evolutionary ancestor of the eukaryotic proteasome (6), which is quite similar in architecture to that of archaea but is composed of seven different  $\alpha$ - and seven different  $\beta$ -subunits (6). Archaea do not have the ubiquitin recognition system for protein degradation and

are thought to have unidentified tags in its degradation pathway (7). Like eukaryotic cells, archaea also have a regulatory complex for the 20 S proteasome, known as proteasome-activating nucleotidase (PAN)<sup>2</sup> (8). PAN is an evolutionary precursor to the 19 S base in eukaryotic cells and thought to be necessary for efficient archaeal 20 S proteasomal protein degradation (8). However *in vitro*, the archaeal 20 S proteasome has been reported to rapidly degrade polyglutamine aggregates without the help of PAN (9). This PAN-independent degradation by the archaeal 20 S proteasome inspired us to introduce and test a novel proteolytic facility in mammalian cells. We have chosen the archaeal *Methanosarcina mazei* (Mm) 20 S proteasome, because its optimal growth temperature is around 37 °C, making it suitable to examine its proteasomal effects in mammalian cells.

The eukaryotic ubiquitin-proteasome system degrades aberrant or misfolded proteins that could otherwise form potentially toxic aggregates (10). These aggregate formations in cells are related to the pathogenesis of several common aging-related neurodegenerative diseases, including Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), polyglutamine diseases (e.g. Huntington disease, some spinocerebellar ataxias, and spinal and bulbar muscular atrophy), and Alzheimer disease (AD), which are thought to be associated with the reduced activities of the proteasome (11–15). However, a critical cause of the accumulation of abnormal proteins remains unclear. Solving this common aspect of many neurodegenerative disorders would be a breakthrough in treating these diseases.

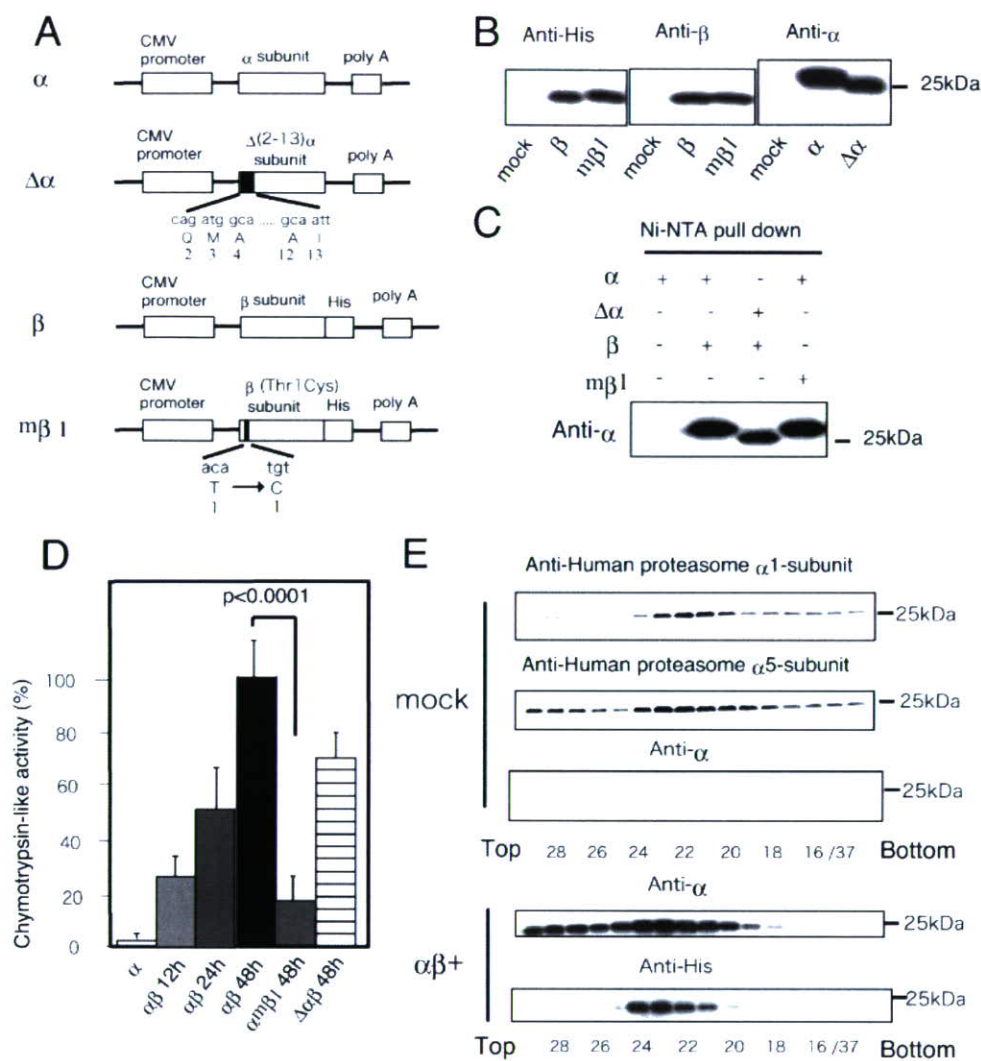
In the present study, we show that the Mm proteasome functions in mammalian cells to accelerate the degradation of the following aggregation-prone proteins: mutant superoxide dismutase-1 (SOD1), a causative protein of familial ALS; mutant androgen receptor (AR) with expanded polyglutamine tract, a causative protein of spinal and bulbar muscular atrophy;  $\alpha$ -synuclein, an accumulated protein in PD; and tau, an accumulated protein in AD.

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<sup>1</sup> To whom correspondence should be addressed. Tel.: 81-52-744-2385; Fax: 81-52-744-2384; E-mail: sobueg@med.nagoya-u.ac.jp.

<sup>2</sup> The abbreviations used are: PAN, proteasome-activating nucleotidase; SOD1, superoxide dismutase-1; Mm, *M. mazei*; ALS, amyotrophic lateral sclerosis; AR, androgen receptor; PD, Parkinson disease; AD, Alzheimer disease; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; WT, wild type; NTA, nitrilotriacetic acid; GFP, green fluorescent protein.

## Archaeal Proteasomes Degrade Aggregation-prone Proteins



**FIGURE 1. Expression of *M. mazei* proteasome in mammalian cells.** **A**, schematic illustration of expression vectors used in this study. The deleted sequences of the  $\Delta\alpha$ -subunit are depicted. The TIC  $\beta$ -subunit ( $m\beta 1$ ) has three mutated base pairs (a to t, c to g, and a to t). **B**, Western blot analysis with anti-proteasome  $\alpha$ -subunit, anti-proteasome  $\beta$ -subunit, and anti-His antibodies. **C**,  $Ni^{2+}$ -NTA pulldown assay. Pulled down proteins run on SDS-PAGE were probed with anti-proteasome  $\alpha$ -subunit. **D**, chymotrypsin-like activity of the  $Ni^{2+}$ -NTA pulled down samples. This protease activity gradually became higher after transfection. Error bars, S.D. ( $n = 3$ ). **E**, glycerol gradient centrifugation experiment: Mm proteasome  $\alpha$ - and  $\beta$ -subunits fractionated into nearly the same fractions as did the human 20 S proteasome subunits  $\alpha 1$  and  $\alpha 5$ ,  $\alpha\beta^-$  and  $\alpha\beta^+$ , indicating that cells were transfected with mock and Mm proteasome  $\alpha\beta$ , respectively.

### EXPERIMENTAL PROCEDURES

**Construction of the Expression Vectors: *M. mazei* Proteasome Subunits  $\alpha$ ,  $\beta$ ,  $\Delta N(2-13)\alpha$ , and Mutant  $\beta$  (TIC)**—The DNA fragment encoding the  $\alpha$ -subunit protein (GenBank™ accession number 1480962) was amplified by PCR from the genomic DNA of *M. mazei* (ATCC) using the following primers:  $\alpha F$  (5'-GCGGGTACCCACCATGCAGATGGCACCACAGATG) and  $\alpha R$  (5'-CGCCTCGAGTTATTCTTTGTTCTCATTTCCCTTTGTG). The  $\Delta(2-13)$   $\alpha$ -subunit ( $\Delta\alpha$ ) was amplified using the following primers:  $\Delta\alpha F$  (5'-GCGGGTACCCACCATGACGGTTTCAGCCCTGACGG) and  $\alpha R$ . The amplified fragments were inserted into the KpnI and XhoI site of the pcDNA 3.1(+) vector (Invitrogen). The  $\beta$ -subunit (GenBank™ accession number 1479036) was amplified by PCR with the following primers:  $\beta F$  (5'-GCCTCTAGACCACCATGGATAATGACAAAATATTTA-AAG) and  $\beta R$  (5'-GCGACCGGTGTTTCCTAAAGCTCTT-

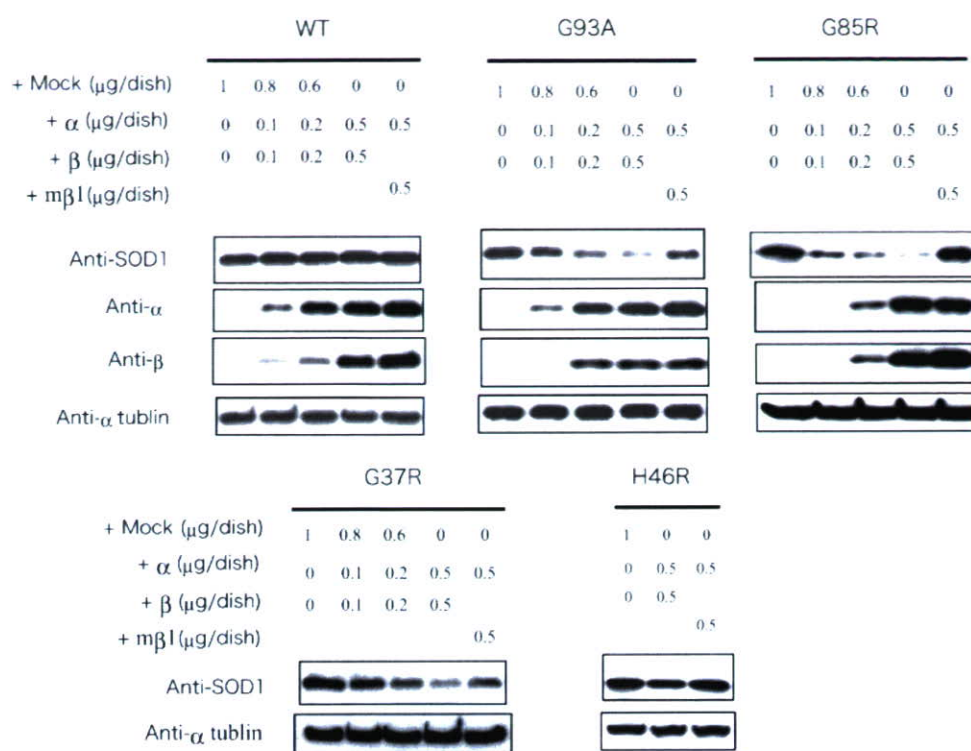
CTG) and inserted into the XbaI and AgeI site of the pcDNA3.1(+)/MycHis vector (Invitrogen) to fuse it to a His<sub>6</sub> tag. The mutated  $m\beta 1$ -subunit (TIC  $\beta$ -subunit) was generated with a site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. Construction of pcDNA3.1/MycHis-SOD1 and pCMV-Tag4-SOD1 vectors (WT, G93A, G85R, H46R, and G37R) (16), pEGFP-N1-SOD1 (WT and G93A) vectors, pCR3.1-AR24Q and pCR3.1-AR97Q vectors, and pcDNA3.1(+)/MycHis- $\alpha$ -synuclein (WT, A53T, and A30P) was described previously (16–18). Six isoforms of tau were amplified by PCR from the pRK172 vectors that were kindly provided by Dr. Michel Goedert and inserted into the KpnI and XbaI site of the pcDNA3.1 vector (Invitrogen).

**Cell Culture, Transfection, and Antibodies**—Neuro2a cells and human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfections were performed using Lipofectamine 2000 (Invitrogen) in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay or Effectene transfection reagent (Qiagen) in other experiments. Antibodies used here were as follows: anti-SOD1 antibody (SOD100; Stressgen Bioreagents), anti-His antibody (Ab-1; Oncogene), anti- $\alpha$ -tubulin antibody (clone B-5-1-1; Sigma), anti-20 S proteasome  $\beta$ -subunit antibody (from *Methanosarcina thermophila*; Cal-

biochem), anti-20 S proteasome  $\alpha$ -subunit antibody (from *M. thermophila*; Calbiochem), anti-AR antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti- $\alpha$ -synuclein antibody (LB509; Zymed Laboratories Inc.), and anti-tau antibody (Mouse Tau-1; Chemicon International).

**Glycerol Density Gradient Centrifugation**—Cells grown on a 10-cm dish were lysed in 1 ml of 0.01 M Tris-EDTA, pH 7.5, by two freeze-thaw cycles, and the lysates were centrifuged for 15 min at  $15,000 \times g$  at 4 °C. The cleared supernatants were loaded on the top of a 36-ml linear gradient of glycerol (10–40%) prepared in 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol and then centrifuged at  $80,000 \times g$  for 22 h at 4 °C in a Beckman SW28 rotor (Beckman Coulter Inc.). Following centrifugation, 37 fractions (1.0 ml each) were collected from the top of the tubes with a liquid layer injector fractionator (model number CHD255AA; Advantech) connected to a fraction col-

## Archaeal Proteasomes Degrade Aggregation-prone Proteins



**FIGURE 2. Reduced expression levels of mutant SOD1 proteins in the presence of *M. mazei* proteasome.** Neuro2a cells grown on 6-cm dishes and co-transfected with 1 μg of SOD1-MycHis vector and increasing doses of *M. mazei* proteasome subunits were harvested and analyzed 48 h after transfection. The levels of mutant SOD1 proteins were gradually reduced as *M. mazei* proteasome αβ increased, whereas no changes in SOD1 proteins were seen with *M. mazei* proteasome αβ1. WT, wild-type SOD1; G93A, SOD1<sup>G93A</sup>; G85R, SOD1<sup>G85R</sup>; G37R, SOD1<sup>G37R</sup>; H46R, SOD1<sup>H46R</sup>.

lector. 200 μl of each fraction was precipitated with acetone; the pellets were lysed with 50 μl of sample buffer and then used for SDS-PAGE followed by Western blotting. The immunostained bands were quantified using ImageGauge software (Fuji Film).

**Ni<sup>2+</sup>-NTA Pulldown**—HEK 293 cells grown on 10-cm dishes, transfected with *M. mazei* proteasome α (as a control), αβ, Δαβ, and αβ1, were lysed by two freeze-thaw cycles in 1 ml of phosphate-buffered saline buffer and centrifuged at 3000 × *g*. Proteasome complexes were pulled down from the supernatants with 200 μl of Ni<sup>2+</sup>-NTA-agarose, washed 4 times in 4 ml of 10 mM imidazole/phosphate-buffered saline buffer, and eluted in 2 ml of 250 mM imidazole/phosphate-buffered saline buffer. Samples were then boiled and subjected to Western blotting.

**Measurement of the Proteasome Activity**—HEK 293 cells grown on 10-cm dishes were transfected with *M. mazei* proteasome α (as a control), αβ, Δαβ, and αβ1. 12, 24, and 48 h after transfection, the cells were lysed and pulled down with Ni<sup>2+</sup>-NTA. The chymotrypsin-like activity of 500 μl of the Ni<sup>2+</sup>-NTA pulled down samples were assayed colorimetrically after 12-h incubations at 37 °C with 100 mM Suc-LLVY-amino-4-methylcoumarin (Sigma) by a multiple-plate reader (PowerscanHT, Dainippon Pharmaceutical). The assay was carried out in triplicate and statistically analyzed by one-way analysis of variance.

**Immunocytochemistry**—Neuro2a cells grown on glass coverslips were co-transfected with pEGFP-N1-SOD1 and *M. mazei* proteasome α- and His-tagged β-subunit. 48 h after transfection, cells were fixed, blocked, and incubated with anti-His antibody

overnight at 4 °C. After washing, samples were incubated with Alexa-546-conjugated anti-mouse antibody (Molecular Probes, Inc.) and visualized with an Olympus BX51 epifluorescence microscope.

**Cycloheximide Chase Analysis**—Neuro2a cells grown on 6-cm dishes were transfected with 1 μg of pcDNA3.1/MycHis-SOD1 with mock (0.6 μg), *M. mazei* proteasome αβ1 (0.3 μg each), or *M. mazei* proteasome αβ (0.3 μg each). 24 h after transfection, cycloheximide (50 μg/ml) was added to the culture medium, and the cells were harvested at the indicated time points. The samples were subjected to SDS-PAGE and analyzed by Western blotting with anti-SOD1 antibody.

**Pulse-chase Analysis**—Neuro2a cells grown on 6-cm dishes were transfected with 1 μg of pCMV-Tag4-SOD1<sup>G93A</sup> with mock (0.6 μg) *M. mazei* proteasome αβ1 (0.3 μg each) or *M. mazei* proteasome αβ (0.3 μg each). 24 h after transfection, cells were pulse-labeled with [<sup>35</sup>S]Cys for 60 min and harvested at the indicated time points. After the immuno-

precipitation by anti-FLAG antibody (M2; Sigma), the samples were subjected to SDS-PAGE, phosphor-imaged (Typhoon 9410; General Electric Co.), and statistically analyzed by one-way analysis of variance.

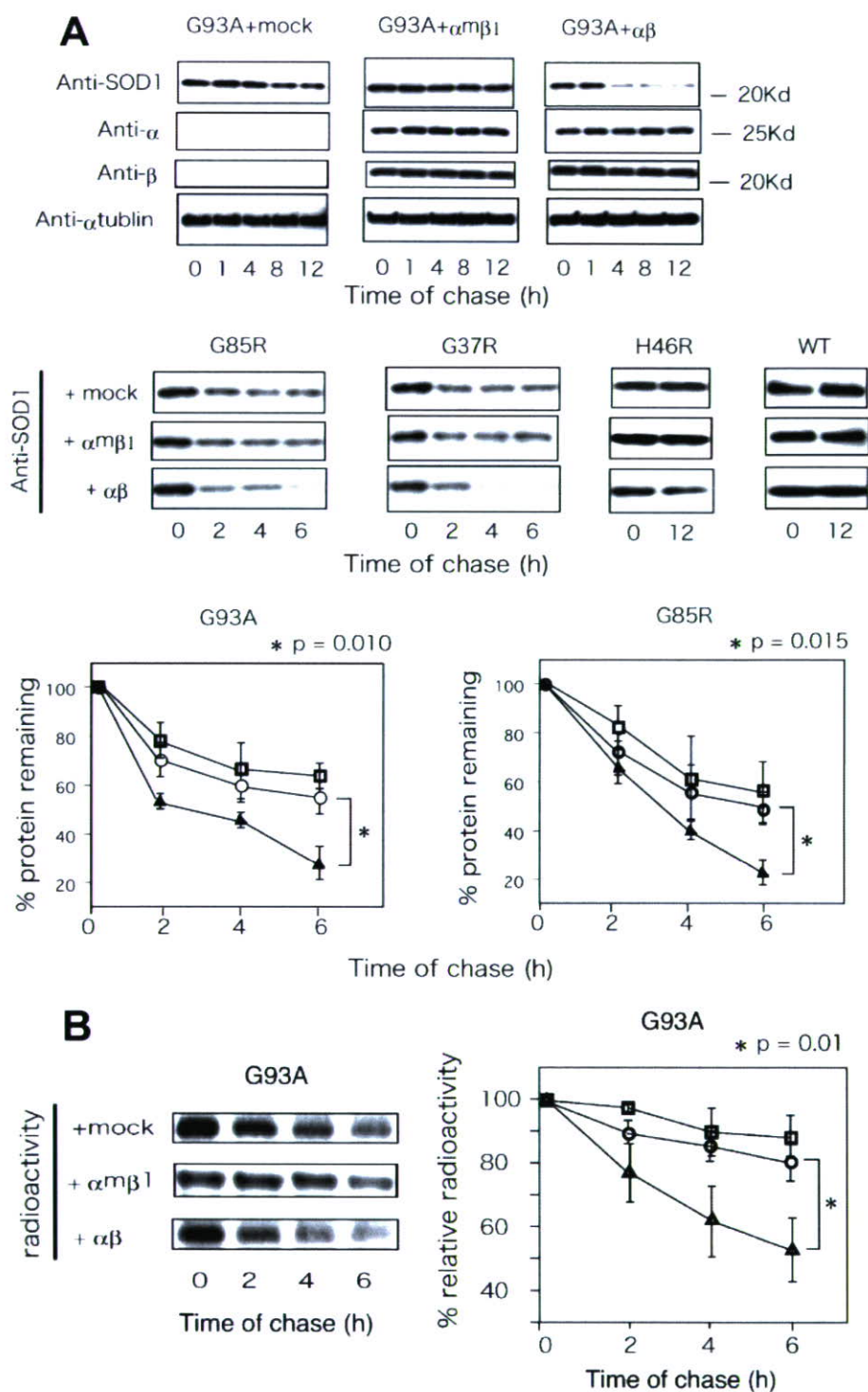
**Cell Viability Analysis**—HEK293 cells were grown on collagen-coated 96-well plates and co-transfected with pcDNA3.1/MycHis-SOD1 (WT, G93A, and G85R) and *M. mazei* proteasome αβ, αβ1, or mock in 12 wells each. The MTS-based cell proliferation assays were performed after 48 h of transfection. Absorbance at 490 nm was measured at 37 °C in a multiple-plate reader (PowerscanHT, Dainippon Pharmaceutical). The assay was carried out in triplicate and statistically analyzed by one-way analysis of variance.

**Caspase-3/7 Assay**—HEK293 cells were grown on black 96-well plates and co-transfected with pcDNA3.1/MycHis-SOD1 (WT, G93A, and G85R) and *M. mazei* proteasome αβ, αβ1, or mock. 24 h after transfection, the medium was replaced with serum-free medium (Dulbecco's modified Eagle's medium). After 24 h, activated caspase-3/7 activity was analyzed by the Apo-ONE homogeneous caspase-3/7 assay (Promega) following the manufacturer's instructions.

## RESULTS

**Cloning and Expression of *M. mazei* Proteasome**—We cloned the *M. mazei* proteasome α-subunit (GenBank<sup>TM</sup> accession number 1480962) and β-subunit (GenBank<sup>TM</sup> accession number 1479036) from genomic DNA of *M. mazei* (Fig. 1A) and generated a mutant α-subunit lacking amino acids 2–13, Δ(2–13) α-subunit (Δα) (Fig. 1A). These amino acids (positions 2–13) nor-

## Archaeal Proteasomes Degrade Aggregation-prone Proteins



**FIGURE 3. *M. mazei* proteasome-accelerated degradation of mutant SOD1 proteins.** A, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of various mutant SOD1 proteins were reduced in the presence of Mm 20 S proteasome  $\alpha\beta$ . The graphs represent the percentage of degraded SOD1<sup>G93A</sup> and SOD1<sup>G85R</sup> proteins in three independent experiments. The error bars indicate S.D. B, pulse-chase analysis (see "Experimental Procedures") showing that the degradation of SOD1<sup>G93A</sup> was accelerated in the presence of Mm 20 S proteasome  $\alpha\beta$ . Circle, mock; triangle,  $\alpha\beta$ ; square,  $\alpha$ m $\beta$ 1. Error bars, S.D. (n = 3).

mally form a gated channel in the  $\alpha$ -ring that regulates substrate entry into the 20 S proteasome (19). We also generated a mutant  $\beta$ -subunit with T1C (m $\beta$ 1) (Fig. 1A). Thr-1 in the  $\beta$ -subunit of the archaeal proteasome is essential for proteolysis, and Thr-1 mutants lose their proteolytic activities (20). The

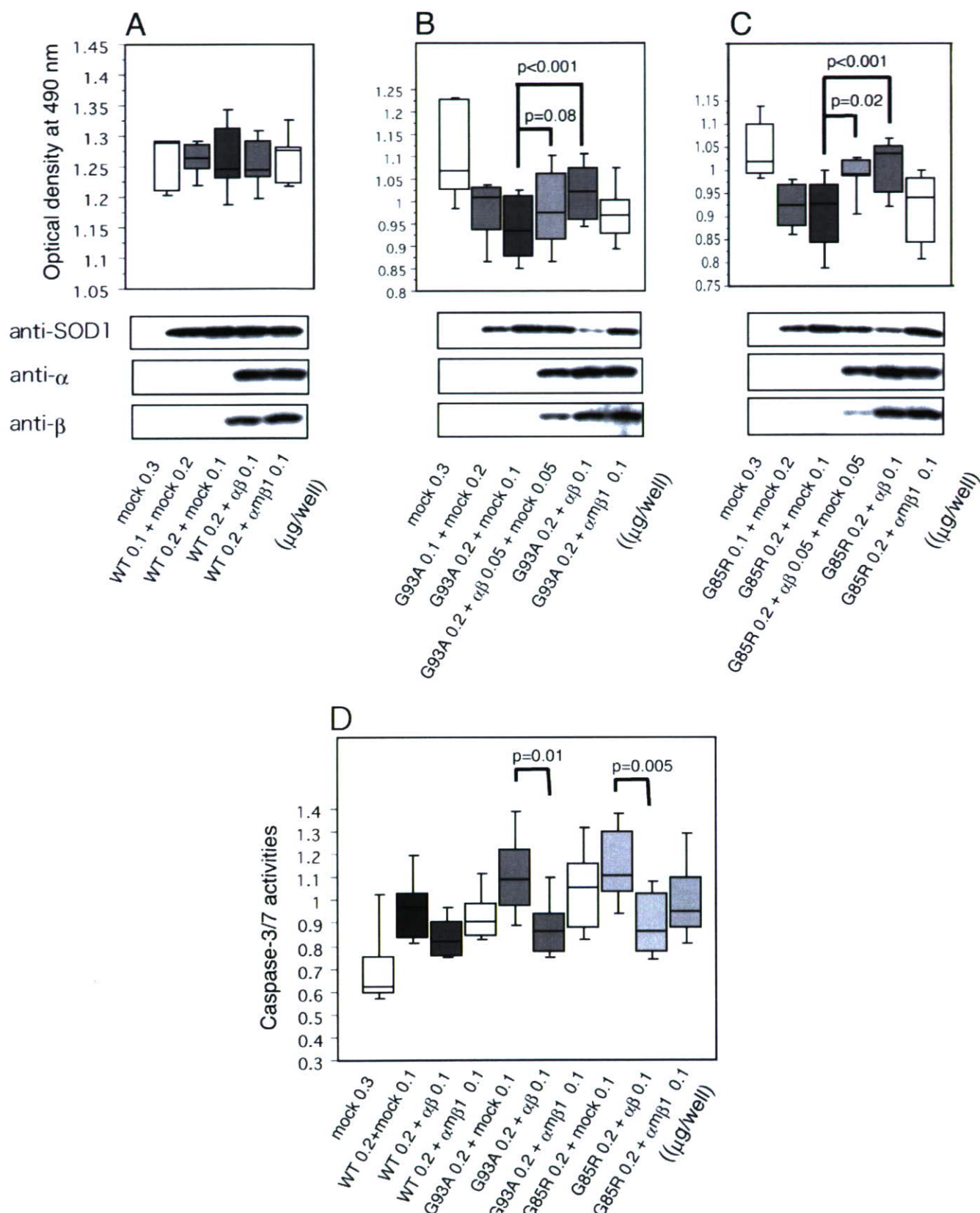
m $\beta$ 1-subunits could properly assemble to form four stacked seven-membered rings and that an active Mm proteasome could be reproduced in mammalian cells. The cells expressing Mm proteasome  $\Delta\alpha\beta$  displayed cellular toxicity, whereas the cells expressing Mm proteasome  $\alpha\beta$  showed little toxicity

following experiments were performed in both HEK293 and Neuro2a cells with similar results in both cell lines.

To confirm protein expression of the Mm subunits, HEK293 cells transfected with mock,  $\alpha$ ,  $\Delta\alpha$ ,  $\beta$ , or m $\beta$ 1 were lysed, subjected to SDS-PAGE, and immunoblotted with anti-proteasome  $\alpha$ -subunit, anti-proteasome  $\beta$ -subunit, and anti-His antibodies. Fig. 1B demonstrates that the  $\alpha$ - and  $\beta$ -subunit antibodies detected the Mm proteasome  $\alpha$ -subunit at 26 kDa, the  $\Delta\alpha$ -subunit around 25 kDa, and the  $\beta$ -subunit at 22 kDa, respectively, and faintly recognized endogenous human proteasome subunits. A Ni<sup>2+</sup>-NTA pull-down assay showed that the Mm proteasome  $\alpha$ - and  $\Delta\alpha$ -subunits cosedimented with the Mm proteasome  $\beta$ - and m $\beta$ 1-subunits but not with mock (Fig. 1C), and protease activity of the pulled down samples of the cells lysed 48 h after transfection showed significantly higher chymotrypsin-like protease activity in the Mm proteasome  $\alpha\beta$  than in the  $\alpha$ m $\beta$ 1 or mock-transfected samples (Fig. 1D). This protease activity was confirmed to become gradually higher after transfection (Fig. 1D).

Glycerol density gradient centrifugation fractionated the  $\alpha\beta$ ,  $\Delta\alpha\beta$ , and  $\alpha$ m $\beta$ 1 complexes of the Mm proteasome into nearly the same fractions as those of the human 20 S proteasome subunits  $\alpha$ 1 and  $\alpha$ 5 (Fig. 1E, data not shown for  $\Delta\alpha\beta$  and  $\alpha$ m $\beta$ 1). Moreover, of the anti-His-immunoblotted bands (Fig. 1E), the density of staining in fractions 20–25 accounts for about 80–90% of the total anti-His staining. That these fractions constitute the majority of the anti- $\alpha$  staining as well suggests that about 80–90% of the  $\beta$ -subunit expression is incorporated into the Mm proteasome. These results suggested that the Mm proteasome  $\alpha$ -,  $\Delta\alpha$ -,  $\beta$ -, and

## Archaeal Proteasomes Degrade Aggregation-prone Proteins



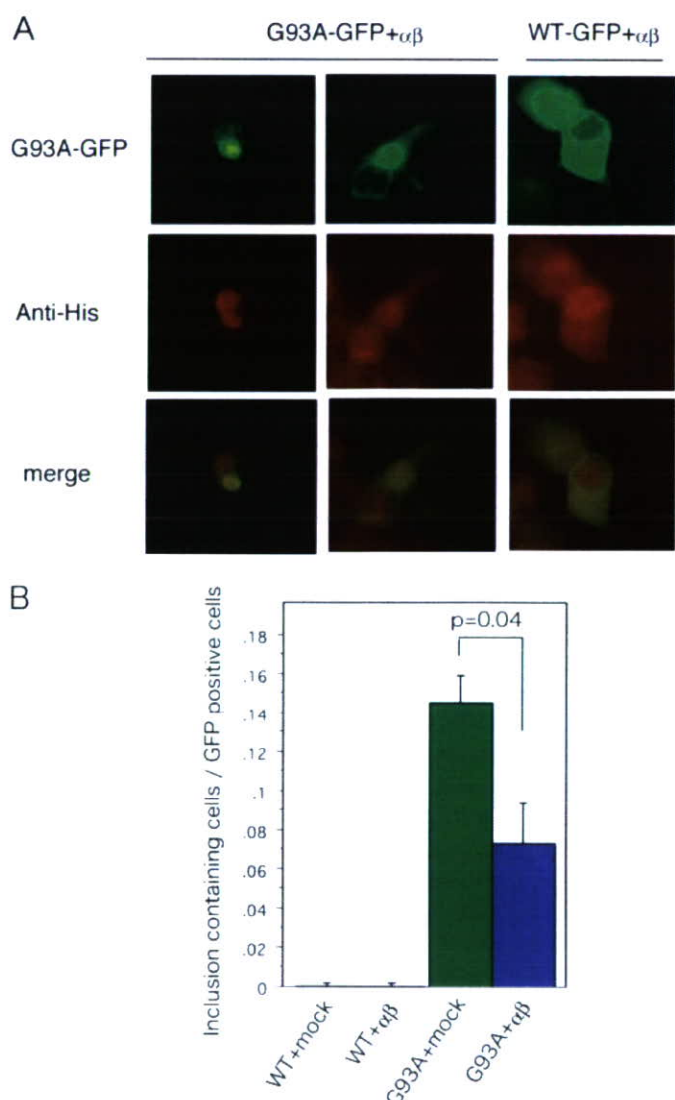
**FIGURE 4. *M. mazei* proteasome reduces the cellular toxicity of mutant SOD1.** The dose-dependent rescue effect of *Mm* proteasome  $\alpha\beta$  expression on cell viability in SOD1<sup>WT</sup>- (A), SOD1<sup>G93A</sup>- (B), and SOD1<sup>G85R</sup>-transfected HEK293 cells (C) as shown in MTS-based cell proliferation assays. The box plots show the median values (center line of box), the 25th (lower line of box), 75th (upper line of box), 10th (lower T bar), and 90th (upper T bar) percentiles in each group ( $n = 3 \times 6$  wells). The numbers indicate the dose of DNA transfected in each well of a 96-well plate ( $\alpha\beta$ , 0.1  $\mu\text{g}$ ;  $\alpha$ , 0.05  $\mu\text{g}$ ;  $\beta$ , 0.05  $\mu\text{g}$ ). The expression levels of SOD1,  $\alpha$ -subunit, and  $\beta$ -subunit at the analyzed points are shown. D, relative activities of cleaved caspase-3/7 were analyzed with the fluorescent caspase substrate, benzyloxycarbonyl-DEVD-R110. Production of *Mm* proteasome  $\alpha\beta$  prevents activation of caspase-3/7. Positive control value was  $3.2 \pm 0.2$  (S.D.) ( $n = 3 \times 4$  wells) (1  $\mu\text{M}$  staurosporin, 24 h).

(data not shown); thus, further experiments were carried out with Mm proteasomes  $\alpha\beta$  and  $\alpha m\beta 1$ .

**M. mazei Proteasome Degrades Specifically Mutant Superoxide Dismutase-1**—We then assessed whether the Mm proteasome actually affects mutant SOD1 protein (SOD1<sup>G85R</sup>, SOD1<sup>G37R</sup>, SOD1<sup>G93A</sup>, and SOD1<sup>H46R</sup>) expression. In cultured cells, mutant SOD1<sup>G85R</sup>, SOD1<sup>G37R</sup>, and SOD1<sup>G93A</sup> are more likely to form aggregates than is SOD1<sup>H46R</sup> (16), and cases of familial ALS expressing these mutant forms are also more severe than those expressing SOD1<sup>H46R</sup>. Western blot analyses demonstrated that the levels of mutant SOD1 were markedly reduced as the expression of Mm proteasome  $\alpha\beta$  increased (Fig. 2). However, wild-type SOD1 levels were not affected by the expression of Mm proteasome  $\alpha\beta$ . Furthermore, mutant SOD1 levels were not affected by the expression of Mm proteasome containing the  $m\beta 1$ -subunit in all mutant species, indicating that Mm proteasomal activity was important to reduce the levels of mutant SOD1 proteins. That the expression level of SOD1<sup>H46R</sup> was less affected by Mm proteasomal expression than other mutant SOD1 species may be associated with the lower toxicity of SOD1<sup>H46R</sup>.

To determine whether the reduced levels of mutant SOD1 protein were due to accelerated degradation of mutant SOD1 or to the reduction of mutant SOD1 expression, we examined the stability of mutant SOD1 proteins expressed in Neuro2a cells co-expressed with Mm proteasome  $\alpha\beta$ ,  $\alpha m\beta 1$ , or mock (Fig. 3, A and B). Chase experiments with cycloheximide, which halts all cellular protein synthesis, demonstrated mutant species-dependent acceleration in SOD1 protein degradation, whereas the expression levels of Mm proteasome  $\alpha$ - and  $\beta$ -subunits did not change (Fig. 3A). The degree of wild-type SOD1 degradation was not affected by the expression of Mm proteasome  $\alpha\beta$ . Pulse-chase experiments further confirmed that <sup>35</sup>S-labeled SOD1<sup>G93A</sup> degradation was significantly accelerated when co-expressed with Mm proteasome  $\alpha\beta$  but not with Mm proteasome  $\alpha m\beta 1$  or mock (Fig. 3B). These facts strongly suggest that the catalytic center in the Mm proteasome  $\beta$ -subunit is important to accelerate the degradation of mutant SOD1 proteins.

**M. mazei Proteasome Reduces Cellular Toxicities of Mutant Superoxide Dismutase-1**—Next, we investigated the viability of HEK293 cells evoked by SOD1 (wild-type, SOD1<sup>G93A</sup>, and SOD1<sup>G85R</sup>) when co-expressed with Mm proteasome  $\alpha\beta$ ,  $\alpha m\beta 1$ , or mock by the MTS-based cell proliferation assay (Fig. 4). We confirmed a linear response between cell number and optical density at 490 nm between 0.85 and 1.30 (data not shown). The viability of cells expressing wild-type SOD1 with Mm proteasome  $\alpha\beta$  did not change as the transfected DNA doses of SOD1 and Mm proteasome  $\alpha\beta$  increased (Fig. 4A). However, the viability of cells expressing mutant SOD1 was reduced as the transfected DNA dose of SOD1 increased (Fig. 4, B and C), and this reduction was prevented by the co-transfection with Mm proteasome  $\alpha\beta$  but not with Mm proteasome  $\alpha m\beta 1$ . Toxicities of mutant SOD1 proteins are associated with the activation of caspase family proteins, especially caspase-3 (21). Using fluorescent substrates of activated caspase-3/7 as markers, we analyzed caspase-3/7 activities in the cells co-transfected with SOD1 proteins and with mock, Mm proteasome  $\alpha\beta$ , and  $\alpha m\beta 1$ . Mm proteasome  $\alpha\beta$  suppressed the

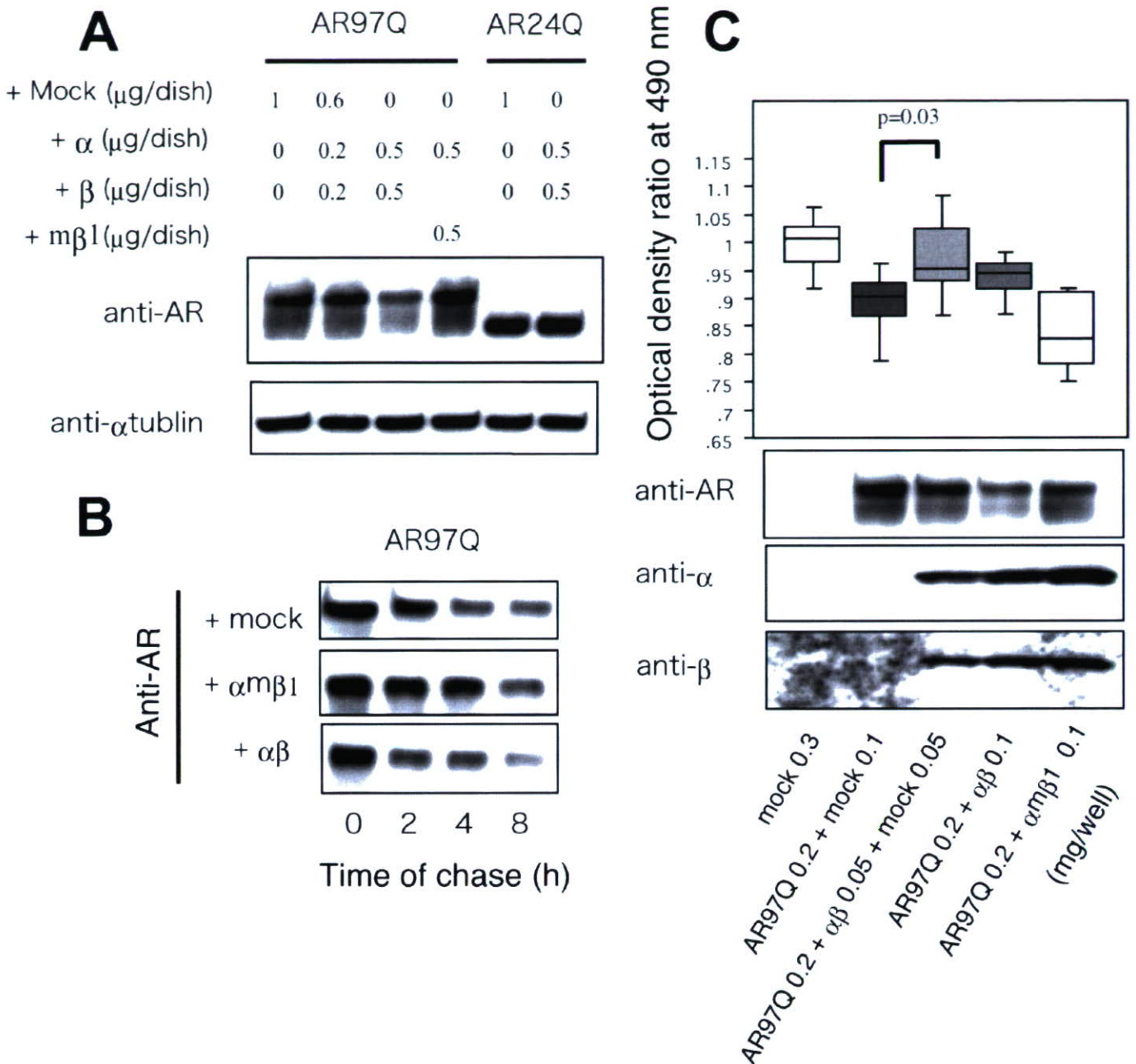


**FIGURE 5. Co-localization of mutant SOD1 and *M. mazei* proteasomes.** A, Neuro2a cells grown on glass coverslips were co-transfected with SOD1<sup>WT</sup>-GFP or SOD1<sup>G93A</sup>-GFP and Mm proteasome  $\alpha$ - and His-tagged  $\beta$ -subunit. 48 h after transfection, cells were fixed, blocked, and incubated with anti-His antibody for 24 h. After washing, samples were incubated with Alexa-546-conjugated anti-mouse antibody. SOD1<sup>G93A</sup> and the Mm proteasome co-localized and formed aggregates together. WT, wild-type SOD1; G93A, SOD1<sup>G93A</sup>. B, the percentages of aggregate-positive cells among the GFP-positive cells were determined. SOD1<sup>G93A</sup> aggregates were significantly reduced when co-expressed with Mm proteasome  $\alpha\beta$ . Error bars, S.D. ( $n = 3$ ). Statistical analyses were carried out by Mann-Whitney's *U* test.

activation of caspase-3/7, resulting in reductions of cellular toxicities of SOD1 proteins (Fig. 4D). These results show that Mm proteasome  $\alpha\beta$  has a protective effect against the decrease in cellular viability evoked by mutant SOD1.

**M. mazei Proteasome Co-localizes with Aggregates Formed by Mutant SOD1**—In the assembly process of the archaeal proteasome,  $\alpha$ -subunit assembly is required for  $\beta$ -subunit incorporation into the proteasome (20), and since the anti-His-stained  $\beta$ -subunit is restricted largely to that incorporated into the Mm proteasome (Fig. 1E), we used anti-His staining to localize the transfected proteasome in Neuro2a cells. GFP-tagged wild-type and G93A mutant SOD1 vectors were transfected along with Mm proteasome  $\alpha\beta$  into Neuro2a cells, which were then fixed and immunostained with anti-His antibody. Fig. 5A shows that

## Archaeal Proteasomes Degrade Aggregation-prone Proteins



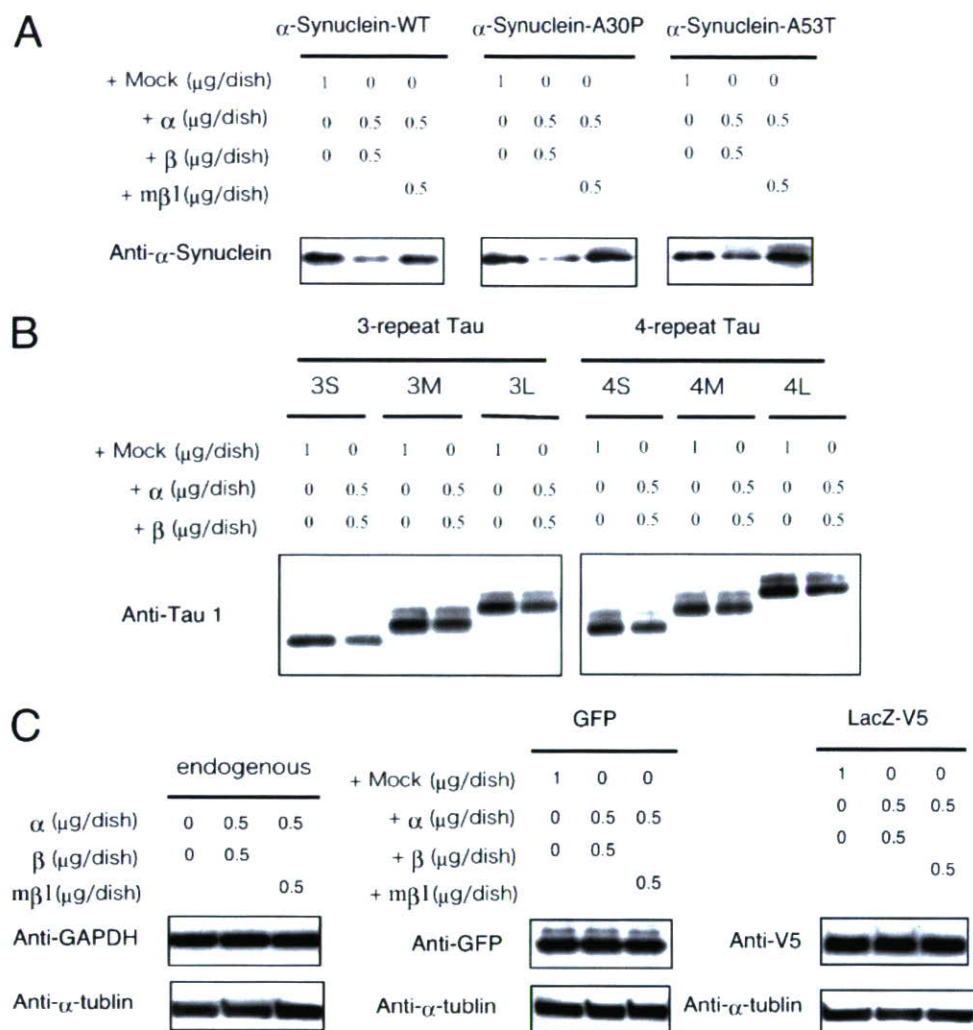
**FIGURE 6. *M. mazei* proteasome degrades mutant androgen receptor with expanded polyglutamine tract and reduces its cellular toxicity.** A, Neuro2a cells grown on 6-cm dishes and co-transfected with 1  $\mu\text{g}$  of AR containing either normal (24Q) or expanded (97Q) polyglutamine tract vectors and increasing doses of Mm proteasome subunits were analyzed. The levels of AR<sup>97Q</sup> proteins were reduced as Mm proteasome  $\alpha\beta$  increased. B, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of AR<sup>97Q</sup> proteins were decreased in the presence of Mm 20 S proteasome  $\alpha\beta$ . Transfected DNA dose/6-cm dish was as follows: AR<sup>97Q</sup> (1  $\mu\text{g}$ ),  $\alpha$ -subunit (0.5  $\mu\text{g}$ ),  $\beta$ -subunit (0.5  $\mu\text{g}$ ). C, the rescue effect of Mm proteasome  $\alpha\beta$  expression on cell viability in AR<sup>97Q</sup>-transfected HEK293 cells as shown in an MTS assay. The box plots show the median values (center line of box), the 25th (lower line of box), 75th (upper line of box), 10th (lower T bar), and 90th (upper T bar) percentiles in each group ( $n = 3 \times 6$  wells). The numbers indicate transfected DNA dose in a well of a 96-well plate ( $\alpha\beta$ , 0.1  $\mu\text{g}$ ;  $\alpha$ , 0.05  $\mu\text{g}$ ;  $\beta$ , 0.05  $\mu\text{g}$ ). The expression levels of AR,  $\alpha$ -subunit, and  $\beta$ -subunit at analyzed points are shown.

GFP-positive SOD1<sup>G93A</sup> aggregates are also anti-His positive, whereas the cells expressing wild-type SOD1-GFP are diffusely stained with anti-His antibody. There were no GFP-negative inclusion bodies stained with anti-His antibody, indicating that Mm proteasome co-localizes with the inclusion bodies consisting of mutant SOD1 in the vicinity of the nucleus. The percentages of aggregate-positive cells among the GFP-positive cells were determined in Fig. 5B. SOD1<sup>G93A</sup> aggregates were significantly reduced when co-expressed with Mm proteasome  $\alpha\beta$ .

***M. mazei* Proteasome Degrades Specifically Mutant Androgen Receptor with Expanded Polyglutamine Tract and Reduces Its Cellular Toxicity**—To demonstrate the ability of the Mm proteasome to degrade aggregation-prone proteins, we examined the AR with expanded polyglutamine tract (97-repeated glutamine; 97Q) protein, the causative protein of spinal and bulbar muscular atrophy. Similar to the results obtained with SOD1 proteins, Fig. 6A shows that in Neuro2a cells, the levels of mutant AR (97Q) were markedly reduced as the expression of



## Archaeal Proteasomes Degrade Aggregation-prone Proteins



**FIGURE 7. *M. mazei* proteasome degrades aggregation-prone but not non-aggregation-prone proteins.** Neuro2a cells grown on 6-cm dishes and co-transfected with Mm proteasome subunits vectors or mock and 1  $\mu$ g of  $\alpha$ -synuclein vectors (wild type, A30P, and A53T) (A), Tau vectors (six isoforms: three (3L, 3M, and 3S) or four (4L, 4M, and 4S) tubulin binding domains in the C-terminal portion and two (3L and 4L), one (3M and 4M), or no (3S and 4S) inserts of 29 amino acids each in the N-terminal portion) (B), or empty GFP vector or LacZ-V5 vector (C). A and B, the expression levels of all of  $\alpha$ -synuclein and tau proteins were reduced when co-transfected with the Mm proteasome  $\alpha\beta$ . C, the expression levels of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GFP, and LacZ-V5 proteins were not changed in the presence of the Mm proteasome  $\alpha\beta$ .

Mm proteasome  $\alpha\beta$  increased, but they were unaffected by the expression of the Mm proteasome  $\alpha\beta$ . On the other hand, wild-type AR (24-repeated glutamine; 24Q) levels were not affected by the expression of Mm proteasome  $\alpha\beta$ . Cycloheximide-chasing analysis demonstrated that the half-life of mutant AR (97Q) was reduced in the presence of the Mm proteasome but not in the presence of the mutant Mm proteasome (Fig. 6B). The viability of cells expressing mutant AR (97Q) was reduced compared with wild-type AR (24Q), and this reduction was attenuated by the co-transfection with Mm proteasome  $\alpha\beta$  (Fig. 6C). These results show that Mm proteasome  $\alpha\beta$  can accelerate the degradation of the aggregation-prone mutant AR with expanded polyglutamine tract and possibly protect the cells from its toxicities.

***M. mazei* Proteasome Degrades Other Aggregation-prone Proteins but Not Non-aggregation-prone Proteins**—To determine whether the Mm proteasome degrades other aggregation-prone

proteins as well, we examined its effects on  $\alpha$ -synuclein (wild-type, A53T, and A30P) and six isoforms of wild-type tau protein in Neuro2a cells. The six tau isoforms contained either three (3L, 3M, and 3S) or four (4L, 4M, and 4S) microtubule binding domains in the C-terminal portion and two (3L, 4L), one (3M, 4M), or no (3S, 4S) inserts of 29 amino acids each in the N-terminal portion. Similar to the results obtained with the mutant SOD1 and AR with an expanded polyglutamine tract, the expression levels of all  $\alpha$ -synuclein and tau proteins were reduced in the presence of Mm proteasome  $\alpha\beta$  (Fig. 7, A and B). Although the degradations of wild-type SOD1 and AR proteins were not accelerated by Mm proteasome, the expression levels of  $\alpha$ -synuclein including wild-type and all of the six forms of wild-type tau were reduced.

We also examined whether Mm proteasomes degrade non-aggregation-prone proteins such as GFP or LacZ. Fig. 7C shows that the Mm proteasome does not affect the degradation of the exogenously expressed proteins, GFP and LacZ.

## DISCUSSION

In this study, we showed that the archaeal Mm proteasome  $\alpha$ - and  $\beta$ -subunits properly assembled to have proteolytic activity and accelerate the degradation of aggregation-prone, neurodegeneration-associated proteins in mammalian cells. Archaeal proteasomes contain 14 identical active sites that, although

originally classified as chymotrypsin-like, were later shown to cleave after acidic and basic residues (22), and they consist of only one type of each of the  $\alpha$ - and  $\beta$ -subunits (6). A comparison between archaeal and eukaryotic proteasomes *in vitro* showed that archaeal proteasomes are far more active in degrading poly(Q) peptides than are eukaryotic proteasomes (9). We utilized this potential power and manageability of archaeal proteasomes to degrade abnormal proteins that could not be effectively degraded by eukaryotic proteasomes. This is the first report showing that archaeal proteasomes can work to accelerate degradation of aggregation-prone proteins in mammalian cells.

Mm proteasomes promoted degradation of mutant SOD1, AR with an expanded polyglutamine tract, wild-type and mutant  $\alpha$ -synuclein, and six isoforms of wild-type tau. The first two proteins, mutant SOD1 and AR with an expanded polyglutamine tract, exhibit toxicity in cell culture models. Mice overexpressing these mutant proteins display abnormal aggrega-

## Archaeal Proteasomes Degrade Aggregation-prone Proteins

tions in their motor neurons and significant loss of motor functions, and they have been used as disease models (23, 24). Mm proteasomes accelerated the degradation of only the mutant forms of these two proteins and not that of the nonaggregating wild-type forms. Furthermore, chasing studies (Fig. 3, A and B) confirmed our belief that Mm proteasomes directly accelerate the degradation of mutant proteins.

However, both the wild-type and two mutants of  $\alpha$ -synuclein as well as six isoforms of wild-type tau were also degraded by Mm proteasomes (Fig. 7).  $\alpha$ -Synuclein and tau are pathogenically different proteins from SOD1 and AR, since they are known to accumulate as wild-type proteins in the affected lesions of PD and AD, respectively. Aggregation of the presynaptic protein,  $\alpha$ -synuclein, has been implicated in synucleinopathies, such as sporadic and familial PD, diffuse Lewy body disease, and multiple-system atrophy (25). In sporadic PD patients, wild-type  $\alpha$ -synuclein is accumulated, and increased expression of wild-type  $\alpha$ -synuclein is also observed (26). Proteasomal dysfunction has been thought to impair  $\alpha$ -synuclein degradation and thereby to facilitate its aggregation (27). Three- and four-repeat wild-type tau are among the proteins characteristically detected in neurofibrillary tangles formed by paired helical filaments in sporadic AD (28). Decreased proteasomal activity has been also reported in the AD brain (29).  $\alpha$ -Synuclein and tau are both relatively easily misfolded, which leads to the formation of aggregates, even in their wild-type forms (30, 31), thus possibly explaining why the Mm proteasomes degraded wild-type  $\alpha$ -synuclein and tau. Mm proteasomes might be able to recognize a wide range of aggregation-prone proteins, whereas they do not affect the degradation of exogenously expressed nonaggregating proteins, such as GFP and LacZ, or abundant endogenous proteins, such as  $\alpha$ -tubulin and glyceraldehyde-3-phosphate dehydrogenase (Fig. 7).

The question raised here is what is the molecular mechanism of such selective, mutant species-dependant degradation. Archaeal 20 S proteasomes contain proteasome-activating nucleotidase, PAN, enabling substrates to enter the proteasomes easily and effectively (8). PAN has a chaperone-like activity to unfold aggregated proteins (32) and is thought to be an evolutionary precursor to the 19 S base in eukaryotic cells (8). Archaeal recognition tags (like ubiquitin tags in eukaryotic cells) have not been identified yet. However, archaeal 20 S proteasomes have been reported to rapidly degrade polyglutamine aggregates *in vitro*, without the help of PAN (9). Here we confirmed that this PAN-independent degradation by Mm 20 S proteasomes could occur in mammalian cells. Since the pore diameter of the closed gate in 20 S proteasomes is estimated to be much smaller than that of aggregated proteins (33), the question is, how do the unfolded substrate proteins enter the 20 S proteasomes? One hypothesis might be that the  $\alpha$ -ring in Mm proteasomes has chaperone-like activity to recognize and unfold the aggregation-prone proteins or misfolded proteins. The gated channel in the  $\alpha$ -ring of the archaeal 20 S proteasomes is thought to regulate substrate entry into the proteasomes and is assumed to be in either an open (34) or a closed state (2, 33) *in vitro*. In our experiments, the gate-free Mm 20 S proteasome  $\Delta\alpha\beta$  substantially reduced cell viability, but the Mm proteasome  $\alpha\beta$ , with the "gate," had little toxic effect on

the cells and, furthermore, accelerated the degradation of mutant proteins. This would be hard to explain if the gate is always in the closed state. There is a possibility that when Mm proteasomes gather, actively or passively, near aggregation-prone proteins, the  $\alpha$ -ring opens its gate and unfolds the aggregated proteins, enabling them to enter the proteasomes to be degraded.

Some kinds of molecular chaperones, such as Hsp90, -70, and -27, have been reported to assist in the selective degradation of mutant SOD1 and AR proteins in proteasome degradation pathways (35, 17). However, neither the protein levels of molecular chaperones (Hsp90, -70, -40, and -27) nor the ubiquitylation levels of mutant SOD1 and AR were changed in the presence of Mm proteasome  $\alpha\beta$  expression (data not shown), thus supporting the idea that endogenous ubiquitin-proteasome degradation pathways possibly did not play an important role in the accelerated degradation of mutant proteins. Further study is needed to elucidate the molecular mechanisms of selective recognition of misfolded aggregation-prone proteins by Mm proteasomes.

In this paper, we demonstrated that Mm proteasomes could effectively degrade neurodegenerative disease-related aggregation-prone proteins *in vivo*. Further studies are needed to determine whether archaeal proteasomes can be available to treat diseases in which toxic gain of proteins is causative.

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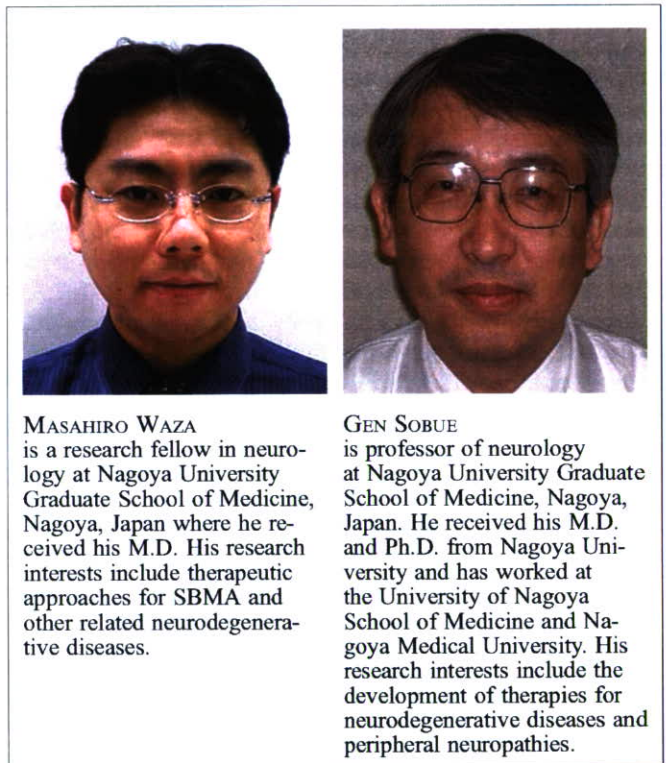
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Masahiro Waza · Hiroaki Adachi · Masahisa Katsuno ·  
Makoto Minamiyama · Fumiaki Tanaka ·  
Manabu Doyu · Gen Sobue

## Modulation of Hsp90 function in neurodegenerative disorders: a molecular-targeted therapy against disease-causing protein

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**Abstract** Abnormal accumulation of disease-causing protein is a commonly observed characteristic in chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and polyglutamine (polyQ) diseases. A therapeutic approach that could selectively eliminate would be a promising remedy for neurodegenerative disorders. Spinal and bulbar muscular atrophy (SBMA), one of the polyQ diseases, is a late-onset motor neuron disease characterized by proximal muscle atrophy, weakness, contraction fasciculations, and bulbar involvement. The pathogenic gene product is polyQ-expanded androgen receptor (AR), which belongs to the heat shock protein (Hsp) 90 client protein family. 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a novel Hsp90 inhibitor, is a new derivative of geldanamycin that shares its important biological activities but shows less toxicity. 17-AAG is now in phase II clinical trials as a potential anticancer agent because of its ability to selectively degrade several oncoproteins. We have recently demonstrated the efficacy and safety of 17-AAG in a mouse model of SBMA. The administration of 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. 17-AAG accomplished the preferential reduction of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation. 17-AAG induced Hsp70 and Hsp40 *in vivo* as previously reported; however, its ability to induce HSPs was limited, suggesting that the HSP induction might support the degradation of mutant protein. The ability of 17-AAG to preferentially



**MASAHIRO WAZA** is a research fellow in neurology at Nagoya University Graduate School of Medicine, Nagoya, Japan where he received his M.D. His research interests include therapeutic approaches for SBMA and other related neurodegenerative diseases.

**GEN SOBUE** is professor of neurology at Nagoya University Graduate School of Medicine, Nagoya, Japan. He received his M.D. and Ph.D. from Nagoya University and has worked at the University of Nagoya School of Medicine and Nagoya Medical University. His research interests include the development of therapies for neurodegenerative diseases and peripheral neuropathies.

degrade mutant protein would be directly applicable to SBMA and other neurodegenerative diseases in which the disease-causing proteins also belong to the Hsp90 client protein family. Our proposed therapeutic approach, modulation of Hsp90 function by 17-AAG treatment, has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases. This review will consider our research findings and discuss the possibility of a clinical application of 17-AAG to SBMA and other neurodegenerative diseases.

**Keywords** Hsp90 inhibitor · Hsp90–client protein complex · Proteasomal degradation · Polyglutamine · Neurodegenerative diseases

M. Waza · H. Adachi · M. Katsuno · M. Minamiyama ·  
F. Tanaka · M. Doyu · G. Sobue (✉)  
Department of Neurology,  
Nagoya University Graduate School of Medicine,  
65 Tsurumai-cho, Showa-ku,  
466-8550 Nagoya, Japan  
e-mail: sobueg@med.nagoya-u.ac.jp  
Tel.: +81-52-7442385  
Fax: +81-52-7442384