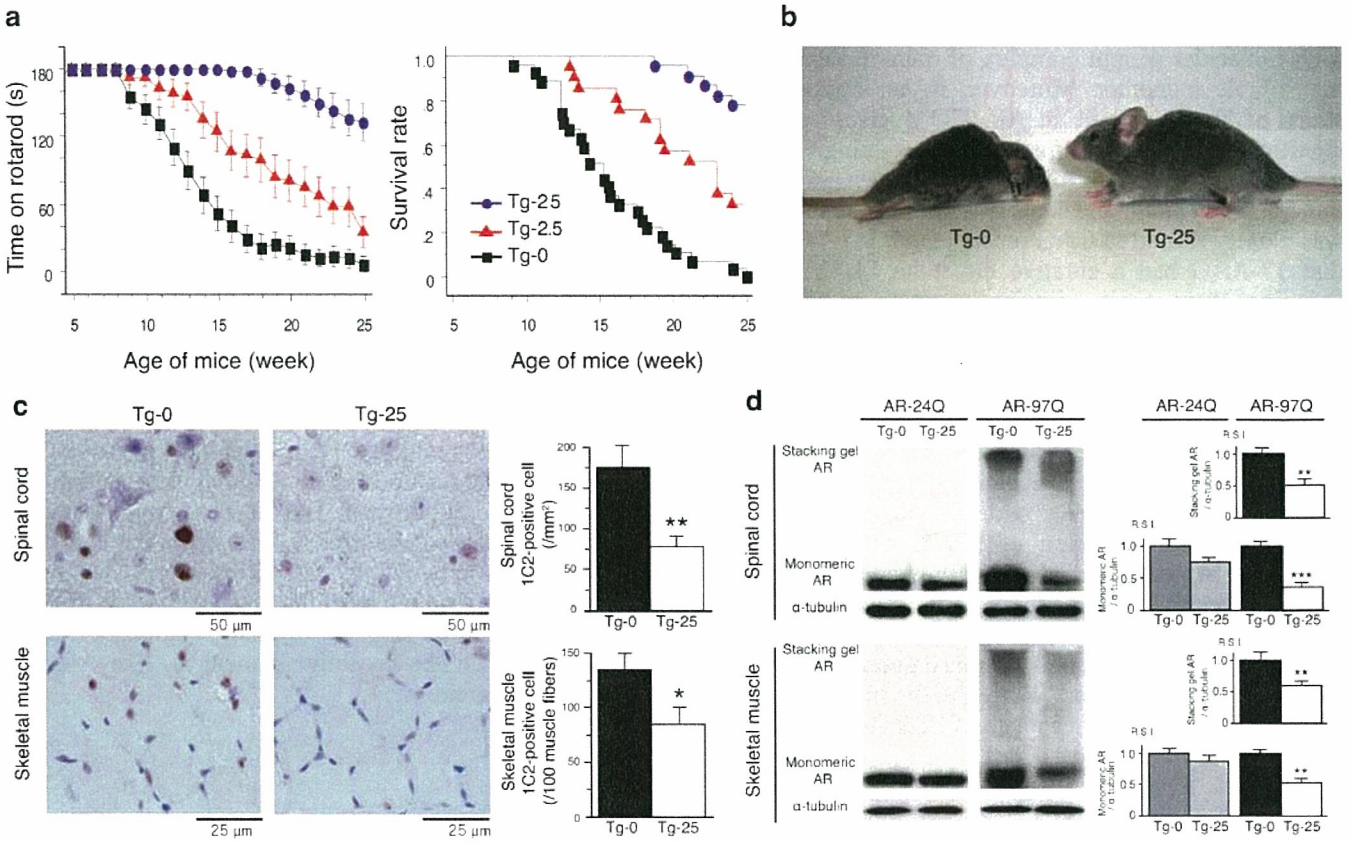

Fig. 3 **a–d** Effects of 17-AAG on transgenic SBMA mice. **a** Tg-0, Tg-2.5, and Tg-25 represent AR-97Q mice treated with vehicle alone and 2.5 and 25 mg/kg 17-AAG, respectively (each group, n=27). The Tg-25 remained longer on the Rotarod than the Tg-0 mice did. A Kaplan–Meier plot shows the prolonged survival of Tg-2.5 and Tg-25 mice compared with the Tg-0 mice, which were all dead by 25 weeks of age (P=0.004, P<0.001, respectively). 17-AAG was less effective at the dose of 2.5 than 25 mg/kg in all parameters tested. **b** Representative photographs of a 16-week-old Tg-0 mouse (*left*) reveal an obvious difference in size and illustrate muscular atrophy and kyphosis compared with an age-matched Tg-25 mouse (*right*). **c**. Immunohistochemical staining with 1C2 antibody showed marked differences in diffuse nuclear staining and nuclear inclusions between DMSO-treated mice (Tg-0) and 17-AAG-treated (Tg-25) mice in the spinal anterior horn and skeletal muscle, respectively. There was a significant reduction in 1C2-positive cell staining in the spinal cord (**P<0.01) and skeletal muscle (*P<0.05) in the Tg-25 compared with the Tg-0. Values are expressed as means±SE (n=6). **d** Western blot analysis of tissue from AR-24Q and AR-97Q mice probed with an AR-specific antibody. In both spinal cord and muscle of mice treated with 17-AAG, there was a significant decrease in the amount of complexed, mutant AR in the stacking gel and monomeric mutant AR in AR-97Q mice, but only slightly less monomeric wild-type AR in AR-24Q mice compared with that from their respective, untreated control mice. Results of a densitometric analysis demonstrated that the 17-AAG-induced reduction of monomeric mutant AR was significantly greater than that of the wild-type monomeric AR. 17-AAG resulted in a 64.4% decline in monomeric mutant AR in the spinal cord and a 45.0% decline in the skeletal muscle, whereas, there was only a 25.9% decline in the spinal cord and a 12.5% decline in the skeletal muscle of AR-24Q mice. These results show significant differences in the reduction rate between wild-type and mutant AR in both spinal cord and skeletal muscle. Values are expressed as means±SE (n=5). Statistical differences are indicated by *asterisks* (*P<0.05; **P<0.01; ***P<0.001)

mutant AR complex but also the monomeric mutant AR protein by preferential degradation of mutant AR.

17-AAG facilitates the degradation of monomeric mutant AR, reducing its aggregation; a therapeutic approach that directly targets the disease-causing protein

In both cultured cells and transgenic SBMA mice, we have demonstrated both the efficacy and safety of 17-AAG [15]. Among the other proposed therapeutic approaches we have previously examined [35, 37, 40, 41], the efficacy of 17-AAG most closely approximated the very successful hormonal therapy using the LH-RH analog, leuprolein [15]. But, unlike leuprorelin, the Hsp90 inhibitor 17-AAG holds enormous potential for application to a wide-range of neurodegenerative diseases in addition to SBMA as previously reported [52–54]. For development of Hsp90 inhibitor treatment in neurological disorders, we regard this general versatility as very important.

In neurodegenerative diseases, recent studies have shown that disease-causing proteins in the process of aggregating have more toxic consequences than they do in either the nascent state or when in NI [55]. NIs have been thought to be a beneficial coping response to toxic mutant protein [56]. We have accumulated several pieces of data demonstrating that 17-AAG is capable of reducing aggregated protein in animal models of SBMA [15]. In both Western blot and filter trap analyses in AR-97Q

models, 17-AAG significantly diminished the insoluble high molecular weight complex of mutant AR as well as the soluble monomer. Moreover, in an immunostaining study of nervous tissue in AR-97Q mice, 17-AAG also significantly reduced diffuse nuclear staining. In SBMA patients, the extent of diffuse nuclear accumulation of mutant AR in motor and sensory neurons of the spinal cord was closely related to CAG repeat length [6]. We consider that 17-AAG had a curative effect on SBMA mice by reducing these toxic proteins as well as the soluble monomeric form.

It is difficult to determine whether 17-AGG facilitates the degradation of, specifically, these toxic intermediate proteins, as 17-AAG has the potent ability to also degrade their precursors (i.e., the monomers). One possible mode of 17-AAG action is that it may inhibit the aggregation of mutant AR via Hsp70 and Hsp40 induction. The pharmacological induction of Hsp70 and Hsp40 using Hsp90 inhibitors has already been shown to inhibit polyQ-induced abnormal aggregation of the huntingtin protein [57]. However, as 17-AAG displayed only a limited ability to induce Hsp70 and Hsp40 in mouse tissue [15], we think that the large decrease in AR seen in the insoluble fraction in vivo, rather than being a result of HSP induction, may be due to 17-AAG's potent ability to degrade the soluble monomeric form of mutant protein, thereby preventing aggregation in the first place. There is no doubt that a reduction of the main culprit protein must have curative properties against various neurodegenerative diseases. In fact, one therapeutic approach that directly reduced abnormal protein using RNA interference has already proved beneficial in various mouse models of polyQ diseases and amyotrophic lateral sclerosis [58–60].

Hsp70 is also known to accelerate proteasome-dependent degradation of polyQ abnormal protein [40, 61]. However, in our hands, mutant AR was markedly decreased after 17-AAG treatment even when Hsp70 and Hsp40 induction was completely blocked in the presence of a protein synthesis inhibitor [15], strongly suggesting that 17-AAG contributes to the preferential degradation of mutant AR mainly through Hsp90 chaperone complex formation and subsequent proteasome-dependent degradation rather than via Hsp70 and Hsp40 induction. Therefore, we think that, to reap the most therapeutic benefits, Hsp90 inhibitors should be applied against neurodegenerative diseases in which the causative protein is, like AR, an Hsp90 client protein.

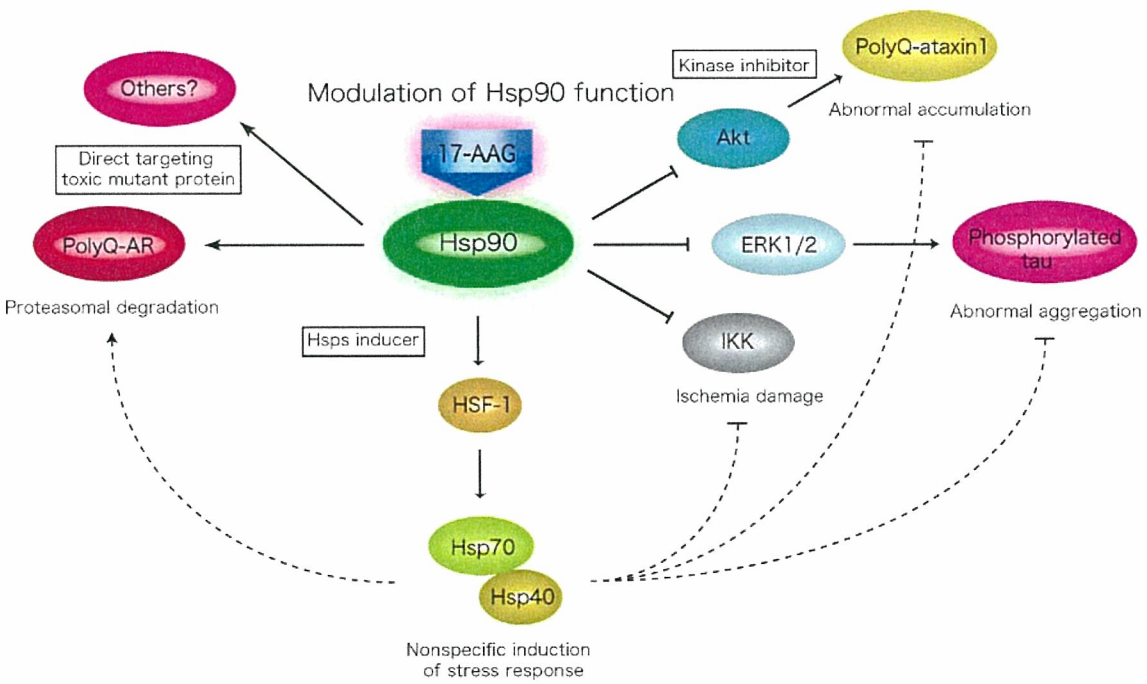

Fig. 4 Clinical application of Hsp90 inhibitors in neurological disorders. 17-AAG specifically binds to the Hsp90 ATP-binding site and disrupts its ATP-dependent function, resulting in inactivation and degradation of Hsp90 client proteins. Therapeutic approaches using Hsp90 inhibitors have now emerged that are unrelated to their role in inducing HSPs. In SBMA, where it may have its most effective potential, 17-AAG directly accelerates proteasomal degradation of the disease-causing protein, polyQ-expanded AR. However, it is still unclear whether other neurodegenerative disease-causing proteins are also Hsp90 client proteins. Many kinases involved in signal transduction do belong to the family of Hsp90 client proteins targeted by 17-AAG. ERK is associated with stabilizing phosphorylated tau. 17-AAG reduces the total amount of phosphorylated tau and its abnormal aggregation by inhibiting ERK activation. Following this same mechanism, 17-AAG might also reduce the abnormal accumulation of ataxin-1 by inhibiting Akt activation or alleviate cerebral infarction by inhibiting IKK activation, which is also an Hsp90 client protein, as well as by inducing HSPs. Hsp90 inhibitors are known to nonspecifically induce HSPs, although this effect was quite limited in our mouse model of SBMA. The induction of HSPs by Hsp90 inhibitors seems to play a supplementary role in neurodegenerative disorders

17-AAG functions with preserved UPS function

As we demonstrated in an in vitro study, 17-AAG-induced degradation requires a well-preserved proteasome function [15]. However, a question concerning the usefulness of this pharmacological approach to facilitate a self-clearing system has been raised [62]. It is generally accepted that the ubiquitin–proteasome system (UPS) is strongly involved in the pathology of polyQ diseases, as many components of the ubiquitin–proteasome and molecular chaperones are known to co-localize with polyQ-containing NIs [63, 64]. Previous reports of studies performed in cultured cell models suggested that an impairment of the UPS is probably a common pathology in polyQ diseases [65–67]. If this hypothesis were true, 17-AAG could not exert its pharmacological effect on polyQ diseases; its therapeutic effects are dependent on a preserved proteasome function [15, 47, 48, 62].

In this regard, recent studies using in vivo proteasome assays have raised serious questions concerning the impaired UPS hypothesis of polyQ diseases [68–70]. It has been reported that neuronal dysfunction developed without significant impairment of the UPS in a mouse model of SCA7 [69]. Consistent with this, it was also demonstrated that proteasome impairment did not contribute to pathogenesis in a mouse model of Huntington's disease (HD) [70]. Furthermore, in conditional mouse models of polyQ disease, genetic loss of the abnormal gene product led to a rapid clearance of pre-existing polyQ-mediated NIs and reversible improvement of the abnormal phenotypes [71, 72]. If the UPS were irreversibly damaged in polyQ diseases, then pre-existing NIs could not be diminished.

While it remains unclear what the difference is in proteasome function in in vitro and in vivo models, our research in a mouse model of SBMA indicates that impairment of the UPS is not a major etiology, at least in in vivo models of polyQ diseases. We therefore consider that treatment with 17-AAG, which takes advantage of a self-clearing system to target disease-causing proteins, is a reasonable therapeutic strategy against polyQ-related and other neurodegenerative diseases.

The expected beneficial effects of 17-AAG against other neurodegenerative diseases

Among neurodegenerative-disease-causing proteins, only AR in SBMA is established as an Hsp90 client protein at this time. It will be interesting to assess whether other neurodegenerative-disease-causing proteins also belong to the family of Hsp90 client proteins. Recent studies have already revealed that some Hsp90 client proteins exerted adverse influences on several neurological disorders [73–75], indicating that the clinical application of Hsp90 inhibitors could expand beyond the treatment of oncological diseases. With reference to previous reports, we now discuss the possibility that 17-AAG could be applicable to neurodegeneration other than SBMA (Fig. 4).

Using 17-AAG as an inducer of HSPs

Hsp90 inhibitors are known to possess the unique pharmacological effect of inducing a stress response and, in addition to their use as anti-cancer agents, have also been developed as pharmacological HSP inducers [52, 76]. This pharmacological effect has already been confirmed in human clinical trials [22]. Murakami et al. were the first to show that the Hsp90 inhibitor herbimycin had the ability to induce Hsp70 in various cultured cell models [77]. Thereafter, Hsp90 proved to be a repressor of heat transcription factor (HSF-1) [78]. Hsp90 inhibitors cause a disassociation of HSF-1 from the Hsp90 complex and a trimerization of HSF-1, thereby resulting in HSP activation. Based on the ability to induce HSPs, Hsp90 inhibitors have also been applied to non-oncological diseases [52].

A great number of reports revealed that forced overexpression of Hsp70 resulted in acquisition of tolerance against various types of stresses and protection against apoptosis in various disease models [79]. In a wide range of polyQ disease models, both genetic and pharmacological overexpression of HSPs has been shown to suppress aggregate formation and cellular toxicity [63, 80, 81]. There is no doubt that HSP induction is beneficial for various neurodegenerative diseases [54]. We have also demonstrated that both genetic and pharmacological overexpression of Hsp70 significantly ameliorated expression of the abnormal phenotype in a transgenic mouse model of SBMA [40, 82].

Taking advantage of HSP induction, many studies have already showed that Hsp90 inhibitors exerted potential neuroprotective effects in: a model of HD [57, 83, 84], tauopathies [28, 85–87], Parkinson's disease [88–90], stroke [91, 92], and autoimmune encephalomyelitis [93]. In addition, Hsp90 inhibitors themselves have been shown to have some neuroprotective effects against various stresses, such as drug-induced toxicity, oxidative stress, and oxygen-glucose deprivation [94–97]. As for polyQ diseases, Sittler et al. [57] first showed that GA significantly suppressed aggregation of mutant huntingtin in a cultured cell model of HD via induction of the Hsp70 and Hsp40 heat shock response. Thus, the enhancement of cellular defenses using Hsp90 inhibitors is a very reasonable clinical application for neurodegenerative diseases.

In polyQ diseases, Bates and his colleagues [83] showed a progressive decrease in the expression of Hsp70 and Hsp40 in a mouse model of HD, which was also observed in our SBMA model [82]. The ability of Hsp90 inhibitors to significantly induce HSPs has been demonstrated only in cultured cell models and in the fly model, but not in mammals. Therefore, further investigation should be performed to address how much Hsp90 inhibitors can induce HSPs in mouse models of neurodegenerative disorders other than SBMA. Although it appears to be obvious that it would be advantageous for the treatment of neurodegeneration, in inducing HSPs by Hsp90 inhibitors, in view of our research finding in in vivo models, it would be unadvisable to rely only on the induction of nonspecific HSPs for human clinical trials.

Using 17-AAG as a kinase inhibitor in neurodegeneration

Phosphorylated tau is a representative disease-causing protein associated with tauopathies including fronto-temporal dementia, progressive supranuclear palsy, corticobasal degeneration, and multiple system atrophy. It is interesting to note that phosphorylated tau is a targeted protein of Hsp90 inhibitors [28, 85, 86]. Dou et al. recently showed that GA and 17-AAG indirectly blocked abnormal tau phosphorylation by inhibition of the Raf–MEK–extracellular signal-regulated kinase (ERK) pathway [98], of which upstream kinase Raf is also an Hsp90 client protein [10, 99]. ERK is known to mediate the activation and stabilization of phosphorylated tau [100, 101]. Along these same lines, LaFevre-Bernt and Ellerby [102] demonstrated that polyQ-expanded, mutant-AR-mediated neuronal cell death by ERK activation and that selective inhibition of the ERK pathway reduced polyQ-induced cell death. Based on this mechanism of inhibiting ERK activation, 17-AAG might also ameliorate abnormal phenotypic expression in the mouse model of SBMA. Furthermore, in other neurodegenerative disorders, evidence has accumulated suggesting that ERK activation is an important executor of neuronal damage [103–106]. Hsp90 inhibitors are well known to have the ability to inhibit various kinase activity [10]. This pharmacological effect of Hsp90 inhibitors, to reduce abnormal kinase activity, could be applied to neurodegenerative diseases as well as oncological diseases and could have far-reaching influence on the clinical application of Hsp90 inhibitors.

Zoghbi and colleagues demonstrated that Akt/PI3K was essential for stabilization and accumulation of mutant ataxin-1 in the polyQ-associated disease SCA1 [73, 107]. Akt is also an Hsp90 client protein, whose activity is significantly reduced by Hsp90 inhibition [108, 109]. Thus, reduction of Akt kinases activity by Hsp90 inhibition might be a therapeutic approach for SCA1. Although Akt/PI3K is believed to be a major pathway mediating survival signals in neuronal cells [110], their finding raises an issue about this hypothesis.

Hsp90 inhibitors have been found to have some neuroprotective effects such as on axonal regeneration in cultured cell models [94, 111]. Koprivica et al. demonstrated that epidermal growth factor receptor (EGFR) activation mediates inhibition of axon regeneration [74]. That EGFR is also an Hsp90 client protein [112, 113] might help explain how Hsp90 inhibition is related to axonal regeneration. In another example, GA markedly attenuates ischemic brain damage [91, 92] and IκB kinase (IKK), an Hsp90 client protein [114], plays an important role in ischemia-induced neuronal death [75, 115], suggesting that GA may ameliorate ischemia brain damage by reducing IKK activity as well as by inducing HSPs.

There is a caveat to this suggestion, however. If 17-AAG is to be applied clinically to treat neurodegenerative diseases with the expectation of reducing abnormal kinase activity, we should also keep in mind the possibility that 17-AAG might also inhibit some kinase activation that exerts cytoprotective effects against neuron degeneration. Taking HD as an example, the efficacy of GA has already been shown based on its ability to induce HSPs [57, 83]. However, a report recently released by Apostol et al. showed that ERK1/2 activation protects against mutant huntingtin-induced toxicity [116]. Furthermore, in a cultured cell model of HD carrying full-length huntingtin, various kinase activities were inhibited by mutant polyQ-expanded huntingtin, but not by normal huntingtin [117, 118]. If ERK activation plays a major role in protecting against HD phenotype expression, there is concern that 17-AAG might exert an adverse affect on HD by inhibiting ERK activation. Before applying 17-AAG to a neurological disorder, we should assess whether the kinase targeted by Hsp90 inhibitor is a true exacerbating factor for the pathology. While there may be some debate over whether 17-AAG should be used as a kinase inhibitor in neurodegeneration, if the application of 17-AAG is suitably performed, this agent would be expected to effectively inhibit abnormal kinase activity in several neurological disorders, possibly leading to cures for these diseases. Hence, this strategy is also of value to extend the versatility of Hsp90 inhibitors as therapeutic agents for neurological disorders.

Conclusion

When considering a role for molecular chaperones in neurological disorders, it should be said that Hsp70 and Hsp40 have received most of the attention, especially in neurodegenerative diseases, as these chaperones have the desirable ability to refold abnormal proteins or to carry them to degradation as a part of the system of protein quality control [54, 119]. Compared with this, Hsp90 is not known to directly fold non-native proteins but rather to bind to substrate proteins only at a late stage of folding [120]. Considering our research findings and those of the other above-mentioned reports, in addition to its role in malignancies, Hsp90 exerts an adverse influence on the nervous system in some situations. In this case, it is reasonable to consider modulating Hsp90 function appropriately.

The ability of 17-AAG to facilitate the degradation of abnormal toxic protein through the modulation of Hsp90 function would be directly applicable to SBMA and probably other neurodegenerative disorders as well. But we should keep in mind that 17-AAG is not a panacea for neurological disorders because it has only limited ability to induce Hsp70 and Hsp40 in vivo. 17-AAG is expected to exert the most effective therapeutic potential against diseases in which the main etiological factor is mediated by Hsp90 client proteins, like AR in SBMA. We believe that more research should be invested in examining the effects of Hsp90 inhibitors on neurodegeneration and that suitably modulating Hsp90 function has great potential to become a new molecular-targeted therapy against a wide range of neurodegenerative diseases.

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

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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 archaebacteria (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 α-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 α-type subunits, which are proteolytically inactive (3), form the outer rings, and the β-type subunits, which contain the active site (4), form the inner rings of the complex (5). The 20 S proteasome of archaebacteria (archaea) consists of only one type of each of the α- and β-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 α- and seven different β-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)[2] (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; α-synuclein, an accumulated protein in PD; and tau, an accumulated protein in AD.

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[2] 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.

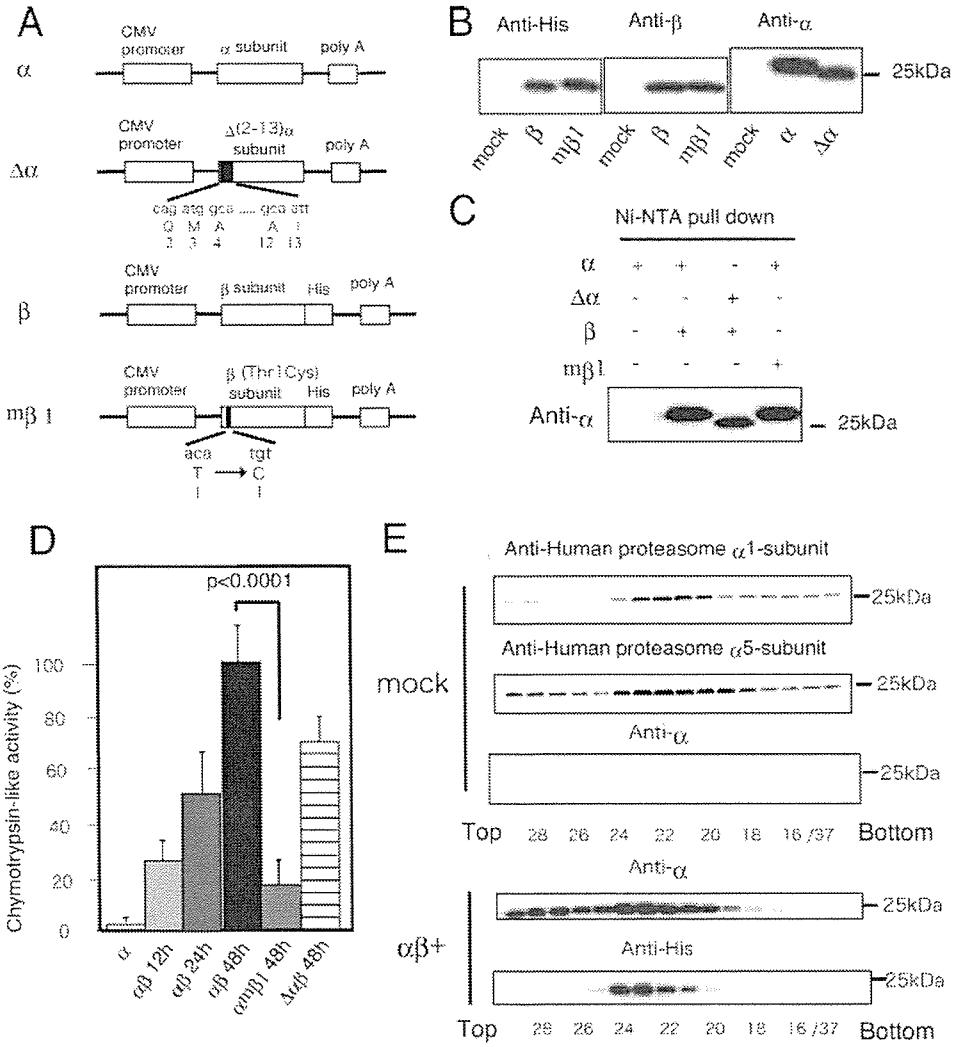

FIGURE 1. **Expression of *M. mazei* proteasome in mammalian cells.** *A*, schematic illustration of expression vectors used in this study. The deleted sequences of the Δα-subunit are depicted. The T1C β-subunit (mβ1) has three mutated base pairs (a to t, c to g, and a to t). *B*, Western blot analysis with anti-proteasome α-subunit, anti-proteasome β-subunit, and anti-His antibodies. *C*, Ni^{2+}-NTA pulldown assay. Pulled down proteins run on SDS-PAGE were probed with anti-proteasome α-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 α- and β-subunits fractionated into nearly the same fractions as did the human 20 S proteasome subunits α1 and α5, αβ− and αβ+, indicating that cells were transfected with mock and Mm proteasome αβ, respectively.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vectors: M. mazei Proteasome Subunits α, β, ΔN(2–13)α, and Mutant β (T1C)—The DNA fragment encoding the α-subunit protein (GenBank™ accession number 1480962) was amplified by PCR from the genomic DNA of *M. mazei* (ATCC) using the following primers: αF (5′-GCGGGTACCCCACCATGCAGATGGCACCACAGATG) and αR (5′-CGCCTCGAGTTATTCTTTGTTCTCATTTCCTTTGTG). The Δ(2–13) α-subunit (Δα) was amplified using the following primers: ΔαF (5′-GCGGGTACCCCACCATGACGGTTTTCAGCCCTGACGG) and αR. The amplified fragments were inserted into the KpnI and XhoI site of the pcDNA 3.1(+) vector (Invitrogen). The β-subunit (GenBank™ accession number 1479036) was amplified by PCR with the following primers: βF (5′-GCCTCTAGACCACCATGGATAATGACAAATATTTAAAG) and βR (5′-GCGACCGGTGTTTCCTAAAGCTCTTCTG) and inserted into the XbaI and AgeI site of the pcDNA3.1(+)/MycHis vector (Invitrogen) to fuse it to a His_6 tag. The mutated mβ1-subunit (T1C β-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-α-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-α-tubulin antibody (clone B-5-1-1; Sigma), anti-20 S proteasome β-subunit antibody (from *Methanosarcina thermophila*; Calbiochem), anti-20 S proteasome α-subunit antibody (from *M. thermophila*; Calbiochem), anti-AR antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-α-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 × *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 × *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-

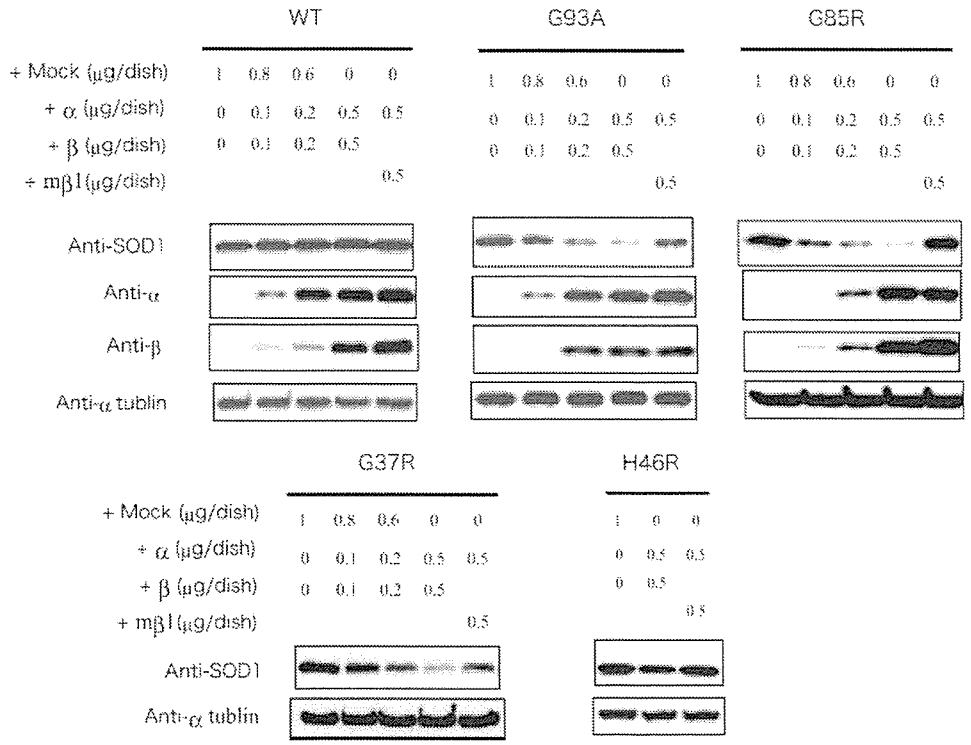

FIGURE 2. **Reduced expression levels of mutant SOD1 proteins in the presence of *M. mazei* proteasome.** Neuro2a cells grown on 6-cm dishes and co-transfected with 1 μg of SOD1-MycHis vector and increasing doses of Mm proteasome subunits were harvested and analyzed 48 h after transfection. The levels of mutant SOD1 proteins were gradually reduced as Mm proteasome αβ increased, whereas no changes in SOD1 proteins were seen with Mm proteasome αmβ1. *WT*, wild-type SOD1; *G93A*, $SOD1^{G93A}$; *G85R*, $SOD1^{G85R}$; *G37R*, $SOD1^{G37R}$; *H46R*, $SOD1^{H46R}$.

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^{2+}-NTA Pulldown—HEK 293 cells grown on 10-cm dishes, transfected with Mm proteasome α (as a control), αβ, Δαβ, and αmβ1, 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^{2+}-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 Mm proteasome α (as a control), αβ, Δαβ, and αmβ1. 12, 24, and 48 h after transfection, the cells were lysed and pulled down with Ni^{2+}-NTA. The chymotrypsin-like activity of 500 μl of the Ni^{2+}-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 Mm 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), Mm proteasome αmβ1 (0.3 μg each), or Mm 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-$SOD1^{G93A}$ with mock (0.6 μg) Mm proteasome αmβ1 (0.3 μg each) or Mm proteasome αβ (0.3 μg each). 24 h after transfection, cells were pulse-labeled with [^{35}S]Cys for 60 min and harvested at the indicated time points. After the immunoprecipitation by anti-FLAG antibody (M2; Sigma), the samples were subjected to SDS-PAGE, phosphor-imaged (Typhoon 9410; Genaral 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 Mm 20 S proteasome αβ, αmβ1, 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 Mm 20 S proteasome αβ, αmβ1, 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 Mm proteasome α-subunit (GenBank™ accession number 1480962) and β-subunit (GenBank™ accession number 1479036) from genomic DNA of Mm (Fig. 1*A*) and generated a mutant α-subunit lacking amino acids 2–13, Δ(2–13) α-subunit (Δα) (Fig. 1*A*). These amino acids (positions 2–13) nor-

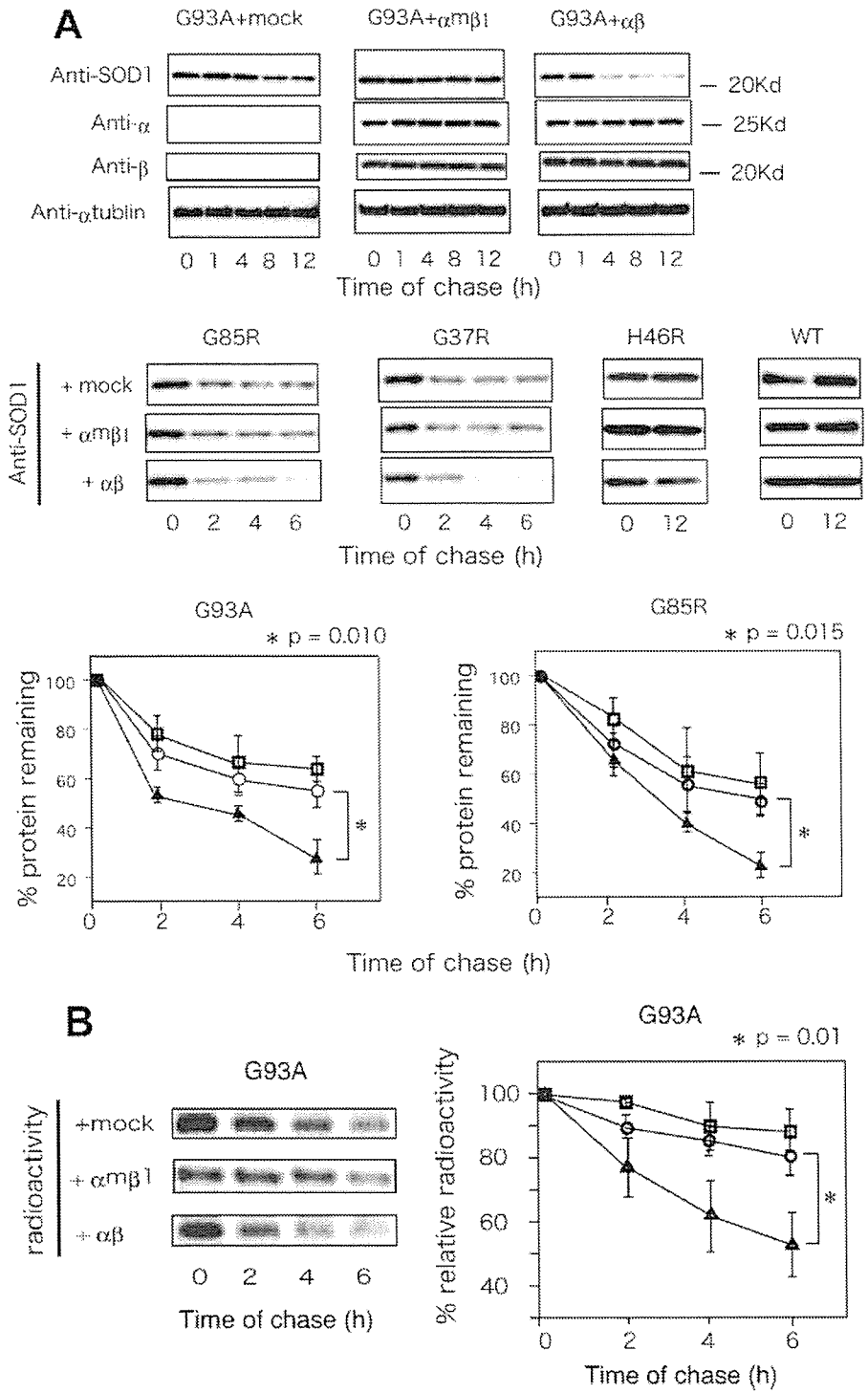

FIGURE 3. ***M. mazei* proteasome-accelerated degradation of mutant SOD1 proteins.** *A*, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of various mutant SOD1 proteins were reduced in the presence of Mm 20 S proteasome $\alpha\beta$. The *graphs* represent the percentage of degraded $SOD1^{G93A}$ and $SOD1^{G85R}$ proteins in three independent experiments. The *error bars* indicate S.D. *B*, pulse-chase analysis (see "Experimental Procedures") showing that the degradation of $SOD1^{G93A}$ 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 α-ring that regulates substrate entry into the 20 S proteasome (19). We also generated a mutant β-subunit with T1C ($m\beta 1$) (Fig. 1*A*). Thr-1 in the β-subunit of the archaeal proteasome is essential for proteolysis, and Thr-1 mutants lose their proteolytic activities (20). The 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, α, $\Delta\alpha$, β, or $m\beta 1$ were lysed, subjected to SDS-PAGE, and immunoblotted with anti-proteasome α-subunit, anti-proteasome β-subunit, and anti-His antibodies. Fig. 1*B* demonstrates that the α- and β-subunit antibodies detected the Mm proteasome α-subunit at 26 kDa, the $\Delta\alpha$-subunit around 25 kDa, and the β-subunit at 22 kDa, respectively, and faintly recognized endogenous human proteasome subunits. A Ni^{2+}-NTA pull-down assay showed that the Mm proteasome α- and $\Delta\alpha$-subunits co-sedimented with the Mm proteasome β- and $m\beta 1$-subunits but not with mock (Fig. 1*C*), 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. 1*D*). This protease activity was confirmed to become gradually higher after transfection (Fig. 1*D*).

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. 1*E*, data not shown for $\Delta\alpha\beta$ and $\alpha m\beta 1$). Moreover, of the anti-His-immunoblotted bands (Fig. 1*E*), 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-α staining as well suggests that about 80–90% of the β-subunit expression is incorporated into the Mm proteasome. These results suggested that the Mm proteasome α-, $\Delta\alpha$-, β-, and $m\beta 1$-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

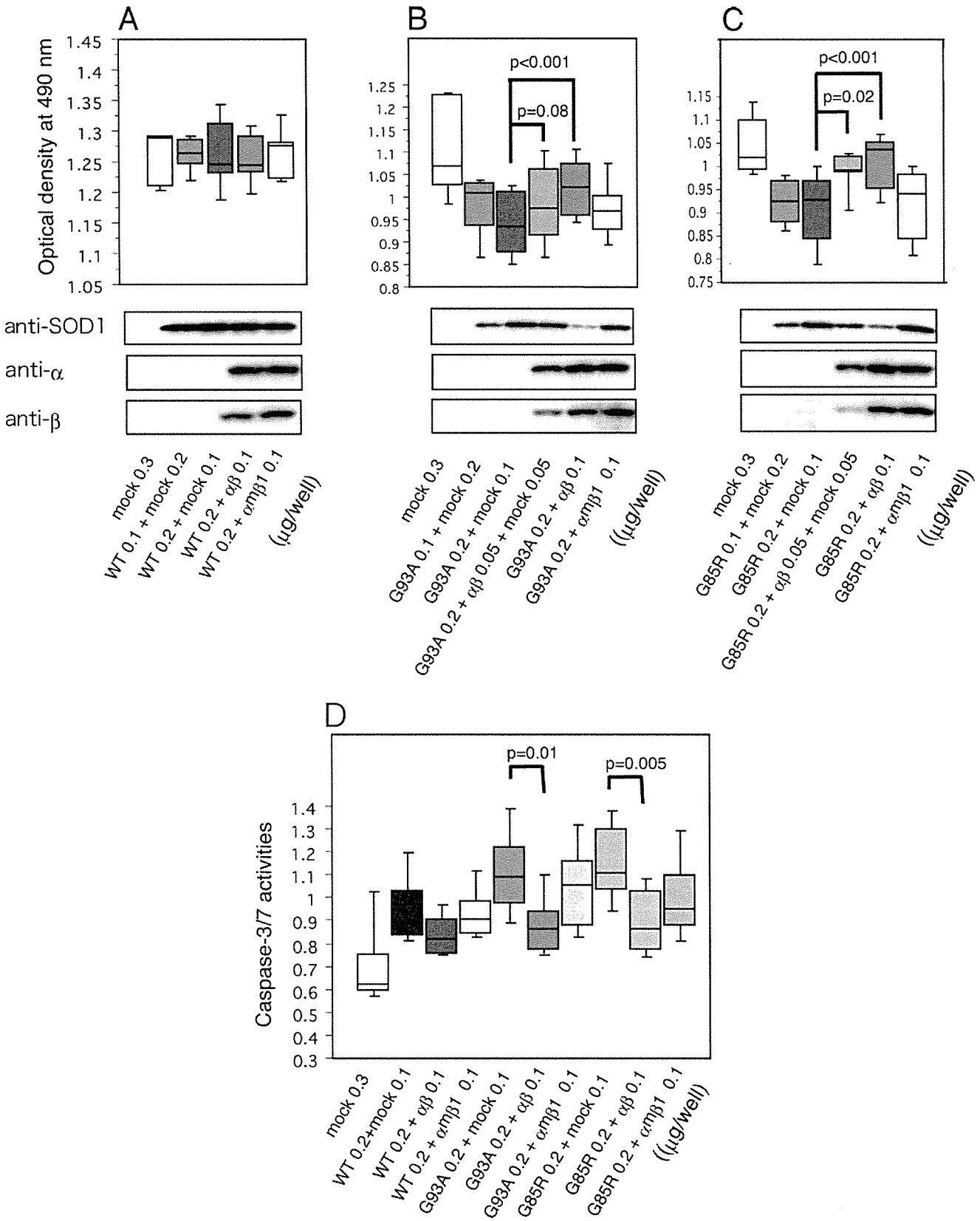

FIGURE 4. ***M. mazei* proteasome reduces the cellular toxicity of mutant SOD1.** The dose-dependent rescue effect of Mm proteasome $\alpha\beta$ expression on cell viability in $SOD1^{WT}$- (*A*), $SOD1^{G93A}$- (*B*), and $SOD1^{G85R}$-transfected HEK293 cells (*C*) as shown in MTS-based cell proliferation 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 μg; α, 0.05 μg; β, 0.05 μg). The expression levels of SOD1, α-subunit, and β-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 ± 0.2 (S.D.) ($n = 3 \times 4$ wells) (1 μM 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 ($SOD1^{G85R}$, $SOD1^{G37R}$, $SOD1^{G93A}$, and $SOD1^{H46R}$) expression. In cultured cells, mutant $SOD1^{G85R}$, $SOD1^{G37R}$, and $SOD1^{G93A}$ are more likely to form aggregates than is $SOD1^{H46R}$ (16), and cases of familial ALS expressing these mutant forms are also more severe than those expressing $SOD1^{H46R}$. 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 $SOD1^{H46R}$ was less affected by Mm proteasomal expression than other mutant SOD1 species may be associated with the lower toxicity of $SOD1^{H46R}$.

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 α- and β-subunits did not change (Fig. 3*A*). The degree of wild-type SOD1 degradation was not affected by the expression of Mm proteasome $\alpha\beta$. Pulse-chase experiments further confirmed that ^{35}S-labeled $SOD1^{G93A}$ degradation was significantly accelerated when co-expressed with Mm proteasome $\alpha\beta$ but not with Mm proteasome $\alpha m\beta 1$ or mock (Fig. 3*B*). These facts strongly suggest that the catalytic center in the Mm proteasome β-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, $SOD1^{G93A}$, and $SOD1^{G85R}$) 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. 4*A*). 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 activation of caspase-3/7, resulting in reductions of cellular toxicities of SOD1 proteins (Fig. 4*D*). These results show that Mm proteasome $\alpha\beta$ has a protective effect against the decrease in cellular viability evoked by mutant SOD1.

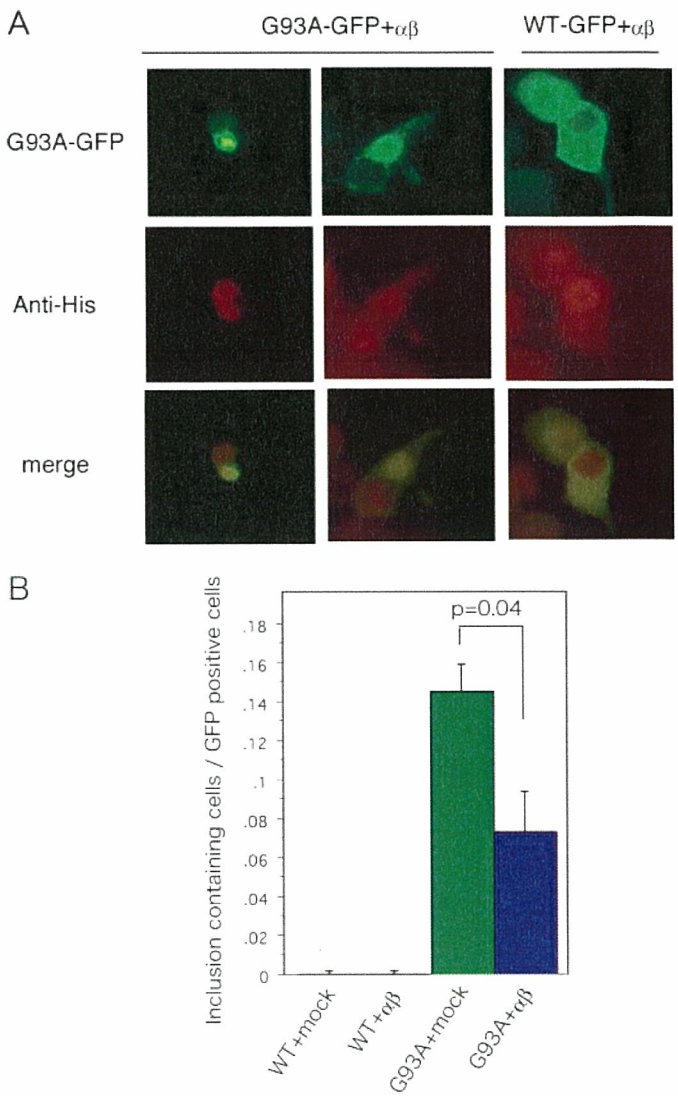

FIGURE 5. **Co-localization of mutant SOD1 and *M. mazei* proteasomes.** *A*, Neuro2a cells grown on glass coverslips were co-transfected with $SOD1^{WT}$-GFP or $SOD1^{G93A}$-GFP and Mm proteasome α- and His-tagged β-subunit. 48 h after transfection, cells were fixed, blocked, and incubated with anti-His antibody for 24 h. After washing, samples were incubated with Alexa-546-conjugated anti-mouse antibody. $SOD1^{G93A}$ and the Mm proteasome co-localized and formed aggregates together. *WT*, wild-type SOD1; *G93A*, $SOD1^{G93A}$. *B*, the percentages of aggregate-positive cells among the GFP-positive cells were determined. $SOD1^{G93A}$ 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.

M. mazei Proteasome Co-localizes with Aggregates Formed by Mutant SOD1—In the assembly process of the archaeal proteasome, α-subunit assembly is required for β-subunit incorporation into the proteasome (20), and since the anti-His-stained β-subunit is restricted largely to that incorporated into the Mm proteasome (Fig. 1*E*), 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. 5*A* shows that

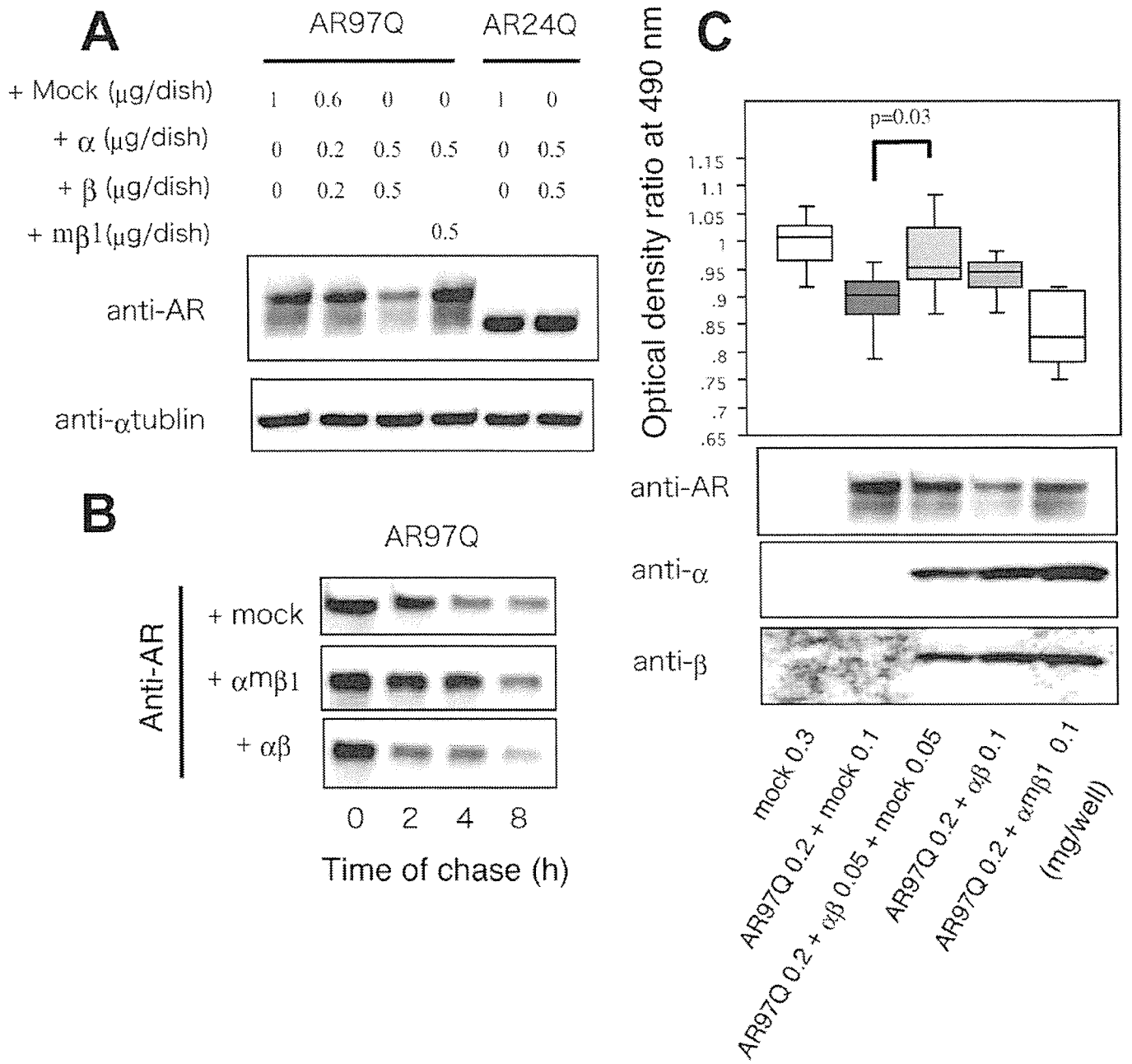

FIGURE 6. ***M. mazei* proteasome degrades mutant androgen receptor with expanded polyglutamine tract and reduces its cellular toxicity.** *A*, Neuro2a cells grown on 6-cm dishes and co-transfected with 1 μg of AR containing either normal (24Q) or expanded (97Q) polyglutamine tract vectors and increasing doses of Mm proteasome subunits were analyzed. The levels of AR^{97Q} proteins were reduced as Mm proteasome αβ increased. *B*, cycloheximide chase analysis (see "Experimental Procedures") showing that the half-lives of AR^{97Q} proteins were decreased in the presence of Mm 20 S proteasome αβ. Transfected DNA dose/6-cm dish was as follows: AR^{97Q} (1 μg), α-subunit (0.5 μg), β-subunit (0.5 μg). *C*, the rescue effect of Mm proteasome αβ expression on cell viability in AR^{97Q}-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 (αβ, 0.1 μg; α, 0.05 μg; β, 0.05 μg). The expression levels of AR, α-subunit, and β-subunit at analyzed points are shown.

GFP-positive $SOD1^{G93A}$ 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. 5*B*. $SOD1^{G93A}$ aggregates were significantly reduced when co-expressed with Mm proteasome αβ.

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. 6*A* shows that in Neuro2a cells, the levels of mutant AR (97Q) were markedly reduced as the expression of

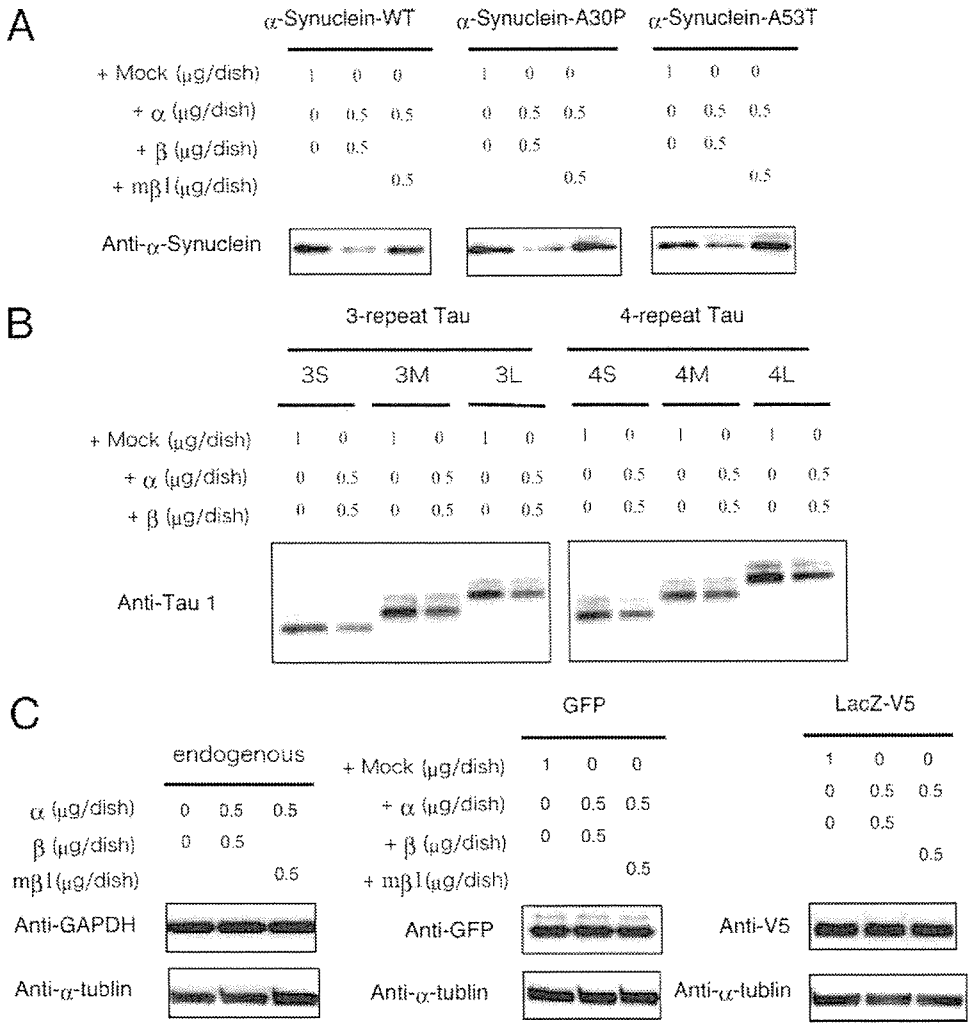

FIGURE 7. ***M. mazei* proteasome degrades aggregation-prone but not non-aggregation-prone proteins.** Neuro2a cells grown on 6-cm dishes and co-transfected with Mm proteasome subunits vectors or mock and 1 μg of α-synuclein vectors (wild type, A30P, and A53T) (*A*), Tau vectors (six isoforms: three (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 α-synuclein and tau proteins were reduced when co-transfected with the Mm proteasome αβ. *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 αβ.

Mm proteasome αβ increased, but they were unaffected by the expression of the Mm proteasome αmβ1. On the other hand, wild-type AR (24-repeated glutamine; 24Q) levels were not affected by the expression of Mm proteasome αβ. 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. 6*B*). 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 αβ (Fig. 6*C*). These results show that Mm proteasome αβ 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 α-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 α-synuclein and tau proteins were reduced in the presence of Mm proteasome αβ (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 α-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. 7*C* 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 α- and β-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 α- and β-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 α-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 over-expressing these mutant proteins display abnormal aggrega-

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 α-synuclein as well as six isoforms of wild-type tau were also degraded by Mm proteasomes (Fig. 7). α-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, α-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 α-synuclein is accumulated, and increased expression of wild-type α-synuclein is also observed (26). Proteasomal dysfunction has been thought to impair α-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). α-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 α-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 α-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 α-ring in Mm proteasomes has chaperone-like activity to recognize and unfold the aggregation-prone proteins or misfolded proteins. The gated channel in the α-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 α-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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14-3-3η is a novel regulator of parkin ubiquitin ligase

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Mutation of the *parkin* gene, which encodes an E3 ubiquitin-protein ligase, is the major cause of autosomal recessive juvenile parkinsonism (ARJP). Although various substrates for parkin have been identified, the mechanisms that regulate the ubiquitin ligase activity of parkin are poorly understood. Here we report that 14-3-3η, a chaperone-like protein present abundantly in neurons, could bind to parkin and negatively regulate its ubiquitin ligase activity. Furthermore, 14-3-3η could bind to the linker region of parkin but not parkin with ARJP-causing R42P, K161N, and T240R mutations. Intriguingly, α-synuclein (α-SN), another familial Parkinson's disease (PD) gene product, abrogated the 14-3-3η-induced suppression of parkin activity. α-SN could bind tightly to 14-3-3η and consequently sequester it from the parkin–14-3-3η complex. PD-causing A30P and A53T mutants of α-SN could not bind 14-3-3η, and failed to activate parkin. Our findings indicate that 14-3-3η is a regulator that functionally links parkin and α-SN. The α-SN-positive and 14-3-3η-negative control of parkin activity sheds new light on the pathophysiological roles of parkin.

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Introduction

In the last decade, people working in the field of Parkinson's disease (PD) witnessed a tremendous progress in uncovering the mechanisms of PD, and several familial PD genes were discovered in succession (Vila and Przedborski, 2004). Of these hereditary PD genes, *parkin* (*PARK2*), the causative gene of autosomal recessive juvenile parkinsonism (ARJP), is of a special interest because it encodes a ubiquitin ligase, a critical component of the pathway that covalently attaches ubiquitin to specific proteins with a polymerization step to form a degradation signal (Shimura *et al*, 2000). Indeed, parkin catalyzes the addition of ubiquitin to target proteins prior to their destruction via the proteasome, suggesting that the misregulation of proteasomal degradation of parkin substrate(s) is deleterious to dopaminergic neurons (Dawson and Dawson, 2003; Bossy-Wetzel *et al*, 2004; Kahle and Haass, 2004). Consequently, impaired protein clearance can induce dopaminergic cell death, supporting the concept that defects in the ubiquitin–proteasome system may underlie nigral degeneration in ARJP and perhaps sporadic forms of PD (McNaught and Olanow, 2003). On the other hand, it was recently reported that parkin also catalyzes the formation of the K63-linked polyubiquitylation chain, independent of proteasomal destruction, in which the K48-linked polyubiquitylation chain is necessary (Doss-Pepe *et al*, 2005; Lim *et al*, 2005). Thus, it is plausible that parkin shares two roles as an E3 ligase; that is, one linking to and the other independent of the proteasome.

Among the products of major familial PD genes (Vila and Przedborski, 2004), α-synuclein (α-SN) is a product of familial PD gene (*PARK1*) identified as a presynaptic protein of unknown function. α-SN is considered in the molecular mechanisms of PD mainly because it is one of the major components of the cytoplasmic Lewy body (LB) inclusion present in the remaining nigral dopaminergic neurons of PD patients, which is the pathological hallmark of sporadic and some familial PDs (Forno, 1996). Although various studies have been conducted on α-SN (Dawson and Dawson, 2003; Bossy-Wetzel *et al*, 2004; Kahle and Haass, 2004), its pathophysiological role(s) and the interplay between α-SN and parkin are largely unknown.

To date, little is known about the role of parkin as a ubiquitin E3 ligase with respect to the underlying molecular mechanism(s) of ARJP or PD. Here we report for the first time that 14-3-3η, a member of the 14-3-3 family (β/α, γ, ε, η, ζ/δ, σ, and τ/θ) (Berg *et al*, 2003; Bridges and Moorhead, 2004; Mackintosh, 2004) identified in LB (Kawamoto *et al*, 2002; Ubl *et al*, 2002), binds primarily to the linker region of parkin and functions as a novel negative regulator of parkin. We also show that α-SN relieves parkin activity suppressed by 14-3-3η, indicating that 14-3-3η is a novel molecule handling both parkin and α-SN, and that functionally links the two familial PD gene products.

Results

Parkin specifically interacts with 14-3-3η but not with other 14-3-3 isoforms

We first examined the physical association of parkin with 14-3-3 isoforms, which are abundantly expressed in the brain (Martin *et al*, 1994; Baxter *et al*, 2002). Parkin was immunoprecipitated from mouse brain extracts, and the presence of 14-3-3 was analyzed by Western blotting (Figure 1A). 14-3-3 was clearly detected in the parkin immunoprecipitant, but not in those of control IgG or parkin antibody preabsorbed

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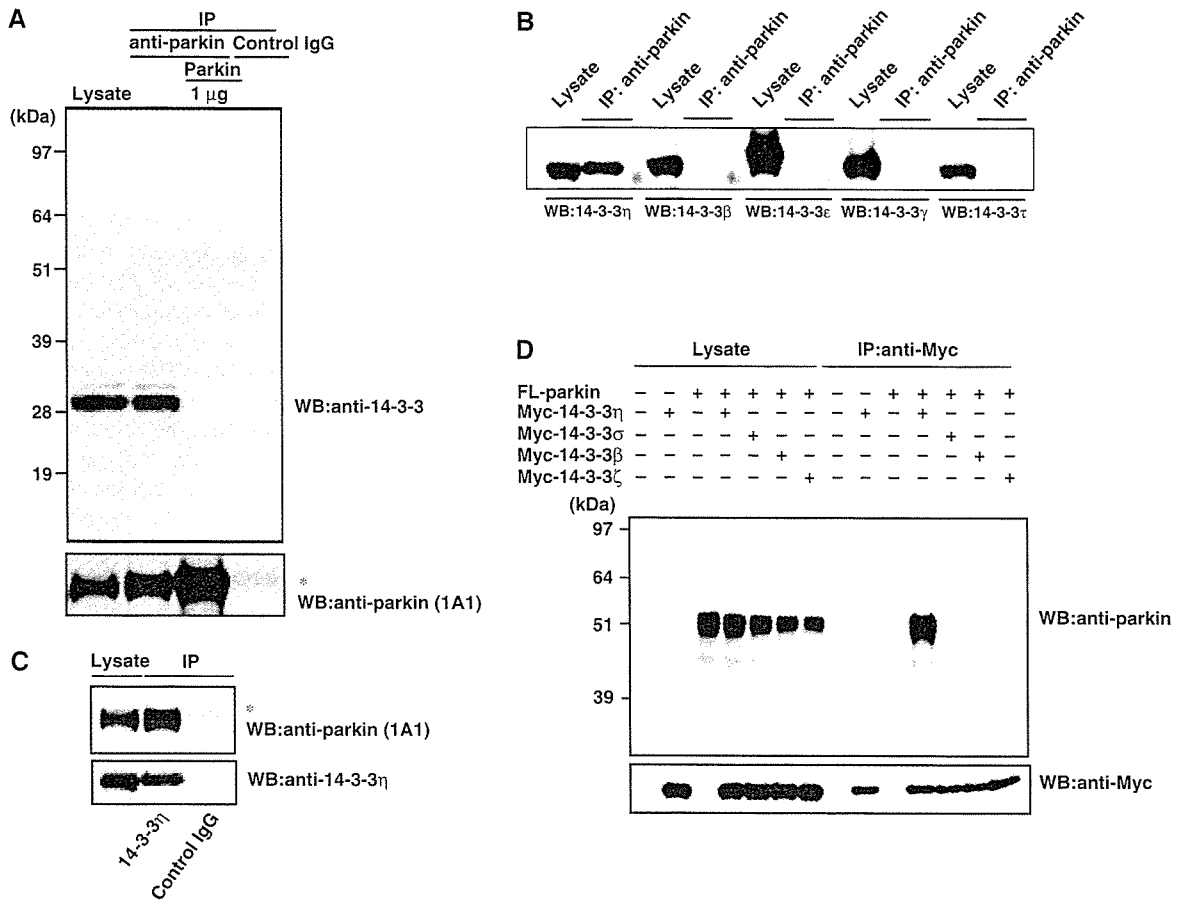

Figure 1 Physical interaction between parkin and 14-3-3η. (**A**) Immunoprecipitation by anti-parkin antibody in the mouse brain. Mouse brain lysates were prepared and treated with anti-parkin or control IgG as described in Materials and methods. The resulting immunoprecipitates were subjected to SDS–PAGE, followed by Western blotting with anti-14-3-3 and parkin (1A1) antibodies. In all, 1 μg of recombinant parkin was pretreated with anti-parkin prior to immunoprecipitation. Left lane: the brain lysate (1.5% input). Asterisk denotes an IgG heavy chain. (**B**) Specificity analysis of 14-3-3 species. The immunoprecipitation with anti-parkin and subsequent SDS–PAGE were carried out as in (A). Western blotting was conducted with antibodies against various 14-3-3 isoforms as indicated for lysates and anti-parkin immunoprecipitates. (**C**) Immunoprecipitation by anti-14-3-3η antibody. After immunoprecipitation with anti-14-3-3η or control IgG of the brain lysate, the immunoprecipitates were analyzed by Western blotting with anti-parkin (1A1) and 14-3-3η antibodies, similar to (A). Left lane: the brain lysate (1.5% input). Asterisk denotes an IgG heavy chain. (**D**) Interaction between parkin and 14-3-3η in HEK293 cells. FL-parkin (5 μg), Myc-14-3-3η, σ, β, or ζ (2 μg) plasmids were transfected as indicated into HEK293 cells. After 48 h, the cell lysate was prepared and used for immunoprecipitation with anti-Myc antibody. The immunoprecipitates and the lysate (7.5% input) were analyzed by Western blotting with anti-parkin and Myc antibodies, as in (A).

with recombinant parkin protein (1 μg). Intriguingly, two 14-3-3 signals were evident: a faint band and a strongly stained band, indicating that the 14-3-3 may form homo- and/or hetero-dimers. Subsequently, we determined the type(s) of 14-3-3 species that interacts with parkin in the mouse brain in more detail. In the parkin immunoprecipitant, 14-3-3η, but not other 14-3-3 isoforms examined, that is, β, γ, ε, and τ, was detected (Figure 1B). In the next step, we examined whether parkin is coimmunoprecipitated with anti-14-3-3η antibody and found parkin in the 14-3-3η immunoprecipitant (Figure 1C). These reciprocal immunoprecipitation experiments revealed that parkin is associated with 14-3-3η in the mouse brain.

To confirm the specific interaction of parkin with 14-3-3η, Myc-tagged 14-3-3η, σ, β, or ζ was cotransfected with FLAG (FL)-parkin into HEK293 cells, and their interactions were tested. FL-parkin was detected in the immunoprecipitant of Myc-14-3-3η, but not those of Myc-14-3-3σ, β, and ζ (Figure 1D). Taken together with the results of Figure 1B, our data indicate that parkin mainly interacts with 14-3-3η.

Parkin domain interacts with 14-3-3η

We next investigated the region of parkin necessary for interaction with 14-3-3η. Structurally, parkin is characterized by the presence of the N-terminal ubiquitin-like domain (UBL) (which is highly homologous to ubiquitin), the C-terminal RING box, consisting of two RING finger motifs, RING1 and RING2, flanked by one IBR (in between RING finger) motif, and a linker region, which connects these N- and C-terminal regions (Shimura *et al*, 2000). In these experiments, various deletion mutants of FL-tagged parkin were expressed in HEK293 cells and immunoprecipitated by FL-antibody beads (Figure 2A). FL-parkin or its derivatives on the beads were further incubated with cell lysates that expressed Myc-14-3-3η, and then the amounts of Myc-14-3-3η bound to the beads were determined (Figure 2B).