ubiquitylated SOD1^{G85R} more effectively than did Dorfin or CHIP, while Dorfin-CHIP$^{D, E, F, J}$, and ^{K} did not (Fig. 4C). Thus, Dorfin-CHIP^{L} was the most potent candidate of the chimeric proteins.

Ubiquitylation of mutant SOD1 by Dorfin-CHIP^{L}

Dorfin specifically ubiquitylated mutant SOD1 proteins, but not SOD1^{WT} protein (Niwa et al., 2002; Ishigaki et al., 2004). Similarly, Dorfin-CHIP^{L} interacted with SOD1^{G93A}, SOD1^{G85R}, SOD1^{H46R}, and SOD1^{G37R}, but not SOD1^{WT}, in HEK293 cells. This was confirmed in N2a cells (Fig. 5A). In both HEK293 and N2a cells, Dorfin-CHIP^{L} also ubiquitylated mutant SOD1 proteins but not SOD1^{WT} (Fig. 5B).

Degradation of mutant SOD1 by Dorfin-CHIP chimeric proteins

To assess the degradation activity of Dorfin-CHIP^{L} against mutant SOD1s, we performed the pulse-chase analysis on N2a

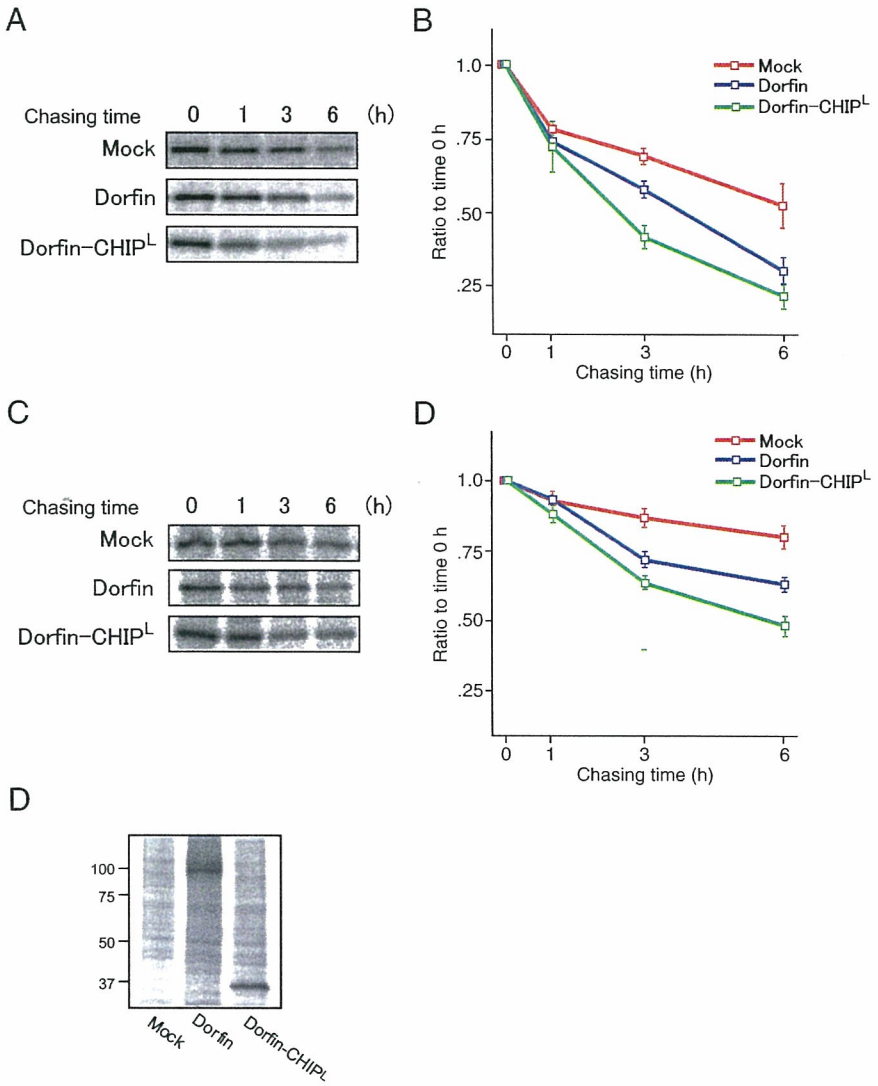


Fig. 6. Degradation of mutant SOD1 proteins with Dorfin-CHIP^{L}. (A) Pulse-chase analysis of SOD1^{G85R} with Dorfin-CHIP^{L}. N2a cells were coexpressed with SOD1^{G85R}-Myc and Mock, Dorfin, and Dorfin-CHIP^{L}. Pulse-chase experiments using [^{35}S]-Met/Cys were done. Immunoprecipitation using anti-Myc antibody and SOD-PAGE analysis revealed the degradation speed of SOD1^{G85R}-Myc. (B) Serial changes in the amount of SOD1^{G85R} coexpressed with Mock, Dorfin, or Dorfin-CHIP^{L}. Four independent experiments were performed and the amounts of SOD1^{G85R} were plotted. There were significant differences between Mock and Dorfin ($p<0.005$), Mock and Dorfin-CHIP^{L} ($p<0.005$), and Dorfin and Dorfin-CHIP^{L} ($p<0.05$) at 3 h, as well as between Mock and Dorfin ($p<0.05$), and Mock and Dorfin-CHIP^{L} ($p<0.05$) at 6 h after labeling. Values are the means±SE, $n=4$. Statistical analysis was done by one-way ANOVA. (C) Pulse-chase analysis of SOD1^{G93A} with Dorfin-CHIP^{L}. N2a cells were coexpressed with SOD1^{G93A}-Myc and Mock, Dorfin, and Dorfin-CHIP^{L} as in panel A. (D) Serial changes in the amount of SOD1^{G93A} coexpressed with Mock, Dorfin, or Dorfin-CHIP^{L}. Four independent experiments were performed and the amounts of SOD1^{G93A} were plotted. There were significant differences between Mock and Dorfin ($p<0.05$) and Mock and Dorfin-CHIP^{L} ($p<0.01$) at 3 h, as well as between Mock and Dorfin ($p<0.05$), Mock and Dorfin-CHIP^{L} ($p<0.01$), and Dorfin and Dorfin-CHIP^{L} ($p<0.05$) at 6 h after labeling. Values are the means±SE, $n=4$. Statistics were done by one-way ANOVA. (E) The equivalent protein expression levels of Dorfin and Dorfin-CHIP^{L}. Half of the volume of samples used in the pulse-chase analysis of panel C at 0 h was used for immunoprecipitation using anti-Flag M2 antibody. The following SOD-PAGE analysis revealed the amounts of Dorfin and Dorfin-CHIP^{L} in the experiment shown in panel C.

cells, using [^{35}S] labeled Met/Cys. The protein levels of SOD1^{G85R} and SOD1^{G93A} declined more rapidly with Dorfin coexpression. Dorfin-CHIP^{L} remarkably declined in both SOD1^{G85R} and SOD1^{G93A} (Figs. 6A, C). Dorfin and Dorfin-CHIP^{L} had similar expression levels at 0 h of this experiment (Fig. 6E). As compared to Mock, Dorfin showed significant declines of both SOD1^{G85R} at 3 h ($p<0.001$) and 6 h ($p<0.05$) after labeling, as shown in a previous study (Niwa et al., 2002). Dorfin-CHIP^{L} also significantly accelerated the decline of SOD1^{G85R} at 3 h ($p<0.001$) and 6 h ($p<0.05$) after labeling again as compared to Mock. At 3 h after labeling, a significant difference between Dorfin-CHIP^{L} and Dorfin was present with respect to SOD1^{G85R} degradation ($p<0.05$). As compared to Dorfin, Dorfin-CHIP^{L} also tended toward accelerated SOD1^{G85R} degradation at 6 h after labeling (Fig. 6B). Similarly, Dorfin showed significant declines of SOD1^{G93A} at 3 h ($p<0.05$) and 6 h ($p<0.05$) after labeling, and Dorfin-CHIP^{L} significantly accelerated the declines of SOD1^{G93A} at 3 h ($p<0.01$) and 6 h ($p<0.01$) after labeling as compared to Mock. A significant difference between Dorfin-CHIP^{L} and Dorfin was present at 6 h in SOD1^{G93A} degradation ($p<0.05$) (Fig. 6D).

Attenuation of the toxicity of mutant SOD1 and decrease in the formation of visible aggregations of mutant SOD1 in cultured neuronal culture cells

The ability of Dorfin-CHIP chimeric proteins to attenuate mutant SOD1-related toxicity was analyzed by MTS assay using N2a cells. The expression of SOD1^{G85R}, as compared to that of SOD1^{WT}, decreased the viability of cells. Overexpression of Dorfin reversed the toxic effect of SOD1^{G85R}, whereas overexpression of CHIP did not. Dorfin-CHIP^{L} had a significantly greater rescue effect on SOD1^{G85R}-related cell toxicity than did Dorfin (Fig. 7A). We also measured the cell viability of N2a cells overexpressing Mock, Dorfin, and Dorfin-CHIP^{L} with various amounts of constructs, and found no difference in toxicity among them (Supplementary Fig. 2).

A structure that Johnston et al. (1998) called aggresome is formed when the capacity of a cell to degrade misfolded proteins is exceeded. The accumulation of mutant SOD1 induces visible macroaggregation, which is considered to be 'aggresome' in N2a cells. We examined the subcellular localizations of Dorfin, CHIP, and Dorfin-CHIP^{L} by immunostaining N2a cells expressing SOD1^{G85R}-GFP. Dorfin was localized in aggresomes with substrate proteins, as in our previous studies. Dorfin-CHIP^{L} was also seen in aggresomes, whereas the staining of CHIP was diffusely observed in the cytosol (Fig. 7B). We counted these visible aggregations with or without MG132 treatment. Dorfin decreased the number of aggregation-containing cells, as has been reported (Niwa et al., 2002), but Dorfin-CHIP^{L} did so more effectively. These effects were inhibited by the treatment of MG132 (Fig. 7C).

Discussion

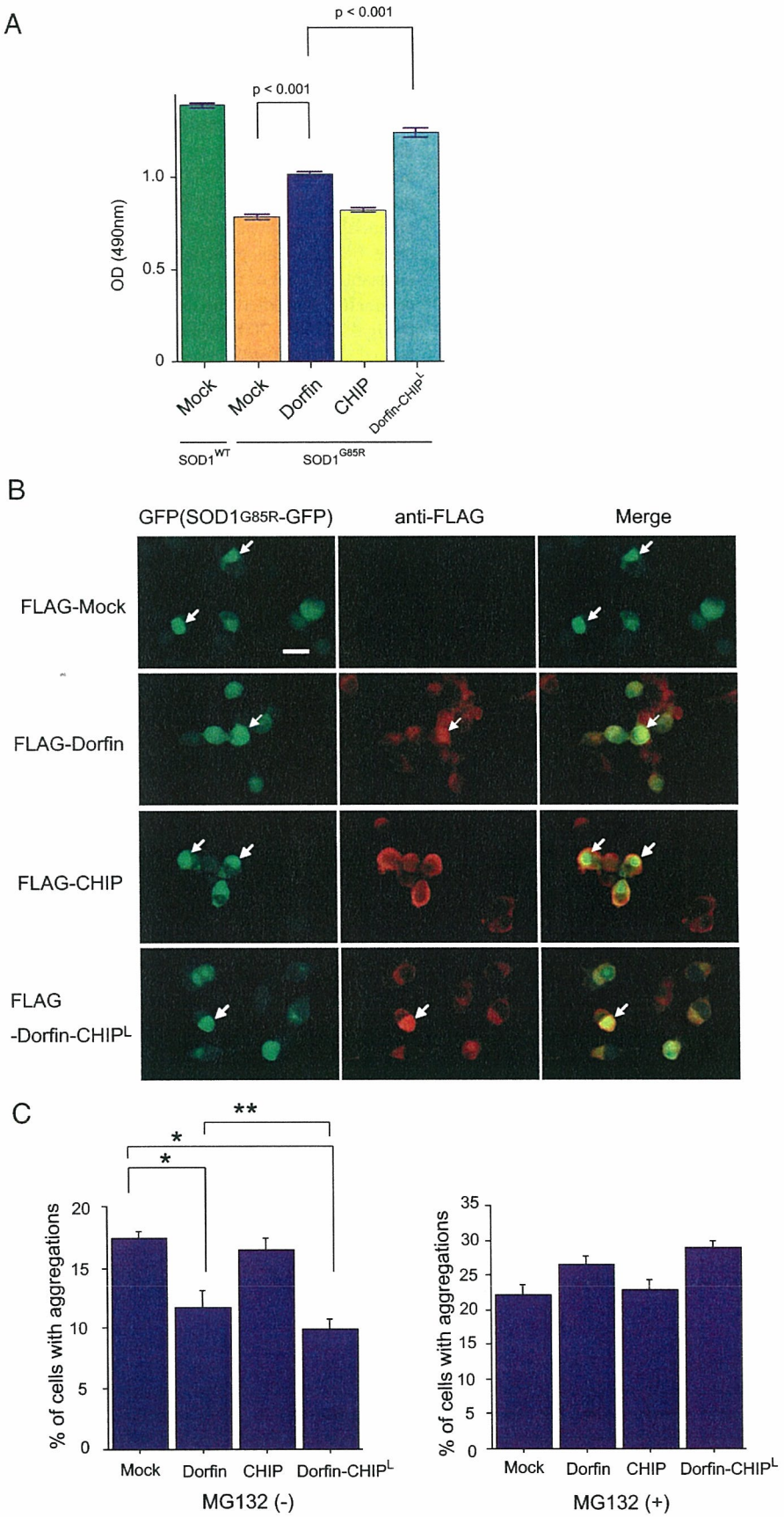
E3 proteins can specifically recognize and degrade accumulating aberrant proteins, which are deeply involved in the pathogenesis of neurodegenerative disorders, including ALS (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). For this reason, E3 proteins are candidate molecules for use in developing therapeutic technology for neurodegenerative diseases. Dorfin is the first E3 molecule that has been found specifically to ubiquitylate mutant SOD1 proteins as well as to attenuate mutant SOD-associated toxicity in cultured neuronal cells (Niwa et al., 2002).

NEDL1, a HECT type E3 ligase, has also been reported to be a mutant SOD1-specific E3 ligase and to interact with TRAPδ and dvl1 (Miyazaki et al., 2004). It has also been reported that ubiquitylation of mutant SOD1-associated complex was enhanced by CHIP and Hsp70 *in vivo* (Urushitani et al., 2004). CHIP ubiquitylated Hsp70-holding SOD1 complexes and degraded mutant SOD1, but did not directly interact with mutant SOD1 (Urushitani et al., 2004). Among these E3 molecules, Dorfin seems to be the most potentially beneficial E3 protein for use in ALS therapy since it is the only one that has been demonstrated to reverse mutant SOD1-associated toxicity (Niwa et al., 2002). Furthermore, Dorfin has been localized in various ubiquitin-positive inclusions such as Lewy bodies (LB) in PD, as well as LB-like inclusions in sporadic ALS and glial cell bodies in multiple-system atrophy. These findings indicate that Dorfin may be involved in the pathogenesis of a broad spectrum of neurodegenerative disorders other than familial ALS (Hishikawa et al., 2003; Ito et al., 2003; Ishigaki et al., 2004).

The half-life of Dorfin^{WT} is, however, less than 1 h (Fig. 1, Table 1). The amount of Dorfin is increased in the presence of MG132, a proteasome inhibitor, indicating that Dorfin is immediately degraded in the UPS. Since the nonfunctional RING mutant form of Dorfin, Dorfin$^{C132S/C135S}$, degraded more slowly than did Dorfin^{WT}, Dorfin seemed to be degraded by auto-ubiquitylation. The degradation of Dorfin$^{C132S/C135S}$ is also inhibited by MG132, suggesting that it is degraded by endogenous Dorfin or other E3s. This immediate degradation of Dorfin is a serious problem for its therapeutic application against neurodegenerative diseases.

Several reports have shown that engineered chimera E3s are able to degrade certain substrates with high efficiency. Protac, a chimeric protein-targeting molecule, was designed to target methionine aminopeptidase-2 to Skp1-Cullin-F box complex (SCF) ubiquitin ligase complex for ubiquitylation and degradation (Sakamoto et al.,

Fig. 7. Dorfin-CHIP chimeric proteins can attenuate toxicity induced by mutant SOD1 and decrease the formation of visible aggregation of mutant SOD1 in N2a cells. (A) N2a cells were grown in 96 collagen-coated wells (5000 cells per well) and transfected with 0.15 μg of SOD1^{WT} and 0.05 μg of Mock or 0.15 μg of SOD1^{G85R} and 0.05 μg of Mock, Dorfin, CHIP, or Dorfin-CHIP^{L}. After the medium was changed, MTS assays were done at 48 h of incubation. Viability was measured as the level of absorbance (490 nm). Values are the means±SE, $n=6$. Statistics were carried out by one-way ANOVA. There were significant differences between SOD1^{G85R}-expressing cells coexpressed with Mock and SOD1^{G85R}-expressing cells coexpressed with Dorfin ($p<0.001$), as well as between SOD1^{G85R}-expressing cells coexpressed with Dorfin and SOD1^{G85R}-expressing cells coexpressed with Dorfin-CHIP^{L} ($p<0.001$). (B) N2a cells were transiently expressed with SOD1^{G85R}-GFP and Mock, Dorfin, CHIP, or Dorfin-CHIP^{L}. Immunostaining with anti-FLAG antibody revealed that Dorfin, CHIP, and Dorfin-CHIP^{L} were localized with SOD1^{G85R}-GFP in macroaggresomes (arrows). Scale bar=20 μm (C) The visible macroaggregations in N2a cells expressing both SOD1^{G85R}-GFP and Mock, Dorfin, CHIP, or Dorfin-CHIP^{L} with or without MG132 treatment were counted and the ratio of cells with aggregations to those with GFP signals was calculated. Values are the means±SE, $n=4$. Statistics were done by one-way ANOVA. *$p<0.01$ denotes a significant difference between cells with Mock and Dorfin or Dorfin-CHIP^{L}. **$p<0.05$ denotes a significant difference between cells with Dorfin and Dorfin-CHIP^{L}.


A
p < 0.001
p < 0.001
OD (490nm)
1.0
0.5
0
Mock
Mock
Dorfin
CHIP
Dorfin-CHIP^L
SOD1^WT
SOD1^G85R
B
GFP(SOD1G85R-GFP)
anti-FLAG
Merge
FLAG-Mock
FLAG-Dorfin
FLAG-CHIP
FLAG
-Dorfin-CHIP^L
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*
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% of cells with aggregations
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% of cells with aggregations
35
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CHIP
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MG132 (+)

2001, 2003). Oyake et al. (2002) developed double RING ubiquitin ligases containing the RING finger domains of both BRCA and BARD1 linked to a substrate recognition site PCNA. Recently, Hatakeyama et al. developed a fusion protein composed of Max, which forms a heterodimer with c-Myc, and the U-box of CHIP. This fusion protein physically interacted with c-Myc and promoted the ubiquitylation of c-Myc. It also reduced the stability of c-Myc, resulting in the suppression of transcriptional activity dependent on c-Myc and the inhibition of tumorogenesis (Hatakeyama et al., 2005). This indicated that the U-box portion of CHIP is able to add an effective E3 function to a U-box-containing client protein.

We postulated that engineered forms of Dorfin could be stable and still function as specific E3s for mutant SOD1s. Dorfin has a RING/IBR domain in the N-terminal portion (amino acids 1-332), but has no obvious motif in the rest of the C-terminus (amino acids 333–838). In this study, we have demonstrated that the hydrophobic domain of Dorfin (amino acids 333–454) is both necessary and sufficient for substrate recruiting (Fig. 2B). In our engineered proteins, the RING/IBR motif of N-terminal Dorfin was replaced by the UPR domain of CHIP, which had strong E3 activity (Murata et al., 2001). Some of the engineered Dorfin-chimeric proteins, such as Dorfin-CHIP$^{D, G, J}$, and ^{L}, were degraded *in vivo* far more slowly than was wild-type Dorfin, indicating that they were capable of being stably presented *in vivo* (Fig. 3). However, Dorfin-CHIP^{G} failed to show strong ubiquitylation activity against SOD1^{G85R} in HEK293 cells. Since Dorfin-CHIP$^{D, J}$, and ^{L} were able to bind to SOD1^{G85R} more strongly than did Dorfin-CHIP^{G}, the binding activity was more important for the E3 activity than for the protein stability.

We next showed that although all of the Dorfin-CHIP chimeric proteins bound to mutant SOD1 *in vivo*, some of them, such as Dorfin-CHIP$^{B, C}$, and ^{I}, bound less than others (Fig. 4A). In HEK293 cells, Dorfin-CHIP$^{D, E, F, J, K}$, and ^{L} ubiquitylated SOD1^{G85R} more effectively than did Dorfin or CHIP; however, in N2a cells only Dorfin-CHIP^{L} had more effective E3 activity than did Dorfin or CHIP. This discrepancy may be due to differences between HEK 293 and N2a cells which could provide slight different environment for the E3 machinery. Therefore, Dorfin-CHIP^{L} was the most potent of the candidate chimeric proteins in degrading mutant SOD1 in the UPS in neuronal cells. We also showed that Dorfin-CHIP^{L} could specifically bind to and ubiquitylate mutant SOD1s but not SOD1^{WT} *in vivo*, as Dorfin had done (Niwa et al., 2002; Ishigaki et al., 2004) (Fig. 5). This observation confirmed that the hydrophobic domain of Dorfin (amino acids 333–454) is responsible for mutant SOD1 recruiting.

Pulse-chase analysis using N2a cells showed that Dorfin-CHIP^{L} degraded SOD1^{G85R} and SOD1^{G93A} more effectively than did Dorfin (Fig. 6). This is compatible with the finding that Dorfin-CHIP^{L} had a greater effect than Dorfin did on the ubiquitylation against mutant SOD1. The cycloheximide assay verified that the degradation ability of Dorfin-CHIP^{L} against SOD1^{G85R} was stronger than that of Dorfin or CHIP in HEK293 cells (data not shown).

Dorfin-CHIP^{L} also reversed SOD1^{G85R}-associated toxicity in N2a cells more effectively than did Dorfin (Fig. 7). This therapeutic effect of Dorfin-CHIP^{L} was expected from its strong E3 activity and degradation ability against SOD1^{G85R}. Visible protein aggregations have been considered to be hallmarks of neurodegeneration. Increased understanding of the pathway involved in protein aggregation may demonstrate that visible macroaggregates represent the end-stage of a molecular cascade of steps rather than a direct toxic insult (Ross and Poirier, 2004). Two facts that Dorfin-CHIP^{L} decreased aggregation formation of SOD1^{G85R} and that this effect was inhibited by a proteasome inhibitor should reflect the ability of Dorfin-CHIP^{L} to degrade mutant SOD1 in the UPS of cells.

Based on our present observations, Dorfin-CHIP^{L}, an engineered chimeric molecule with the hydrophobic substrate-binding domain of Dorfin and the U-box domain of CHIP, had stronger E3 activity against mutant SOD1 than did Dorfin or CHIP. Indeed, it not only degraded mutant SOD1 more effectively than did Dorfin or CHIP but, as compared to Dorfin, produced marked attenuation of mutant SOD1-associated toxicity in N2a cells. This protective effect of Dorfin-CHIP^{L} against mutant SOD1 has potential applications to gene therapy for mutant SOD1 transgenic mice because this protein has a long enough life to allow the constant removal of mutant SOD1 from neurons. Since Dorfin was originally identified as a sporadic ALS-associated molecule (Ishigaki et al., 2002b) and is located in the ubiquitin-positive inclusions of various neurodegenerative diseases (Hishikawa et al., 2003), this molecule is an appropriate candidate for future use in gene therapy not only for familial ALS, but also for sporadic ALS and other neurodegenerative disorders.

So far, most reports on engineered chimera E3s have targeted cancer-promoting proteins. Dorfin-CHIP chimeric proteins are the first chimera E3s to be intended for the treatment of neurodegenerative diseases. Since the accumulation of ubiquitylated proteins in neurons is a pathological hallmark of various neurodegenerative diseases, development of chimera E3s like Dorfin-CHIP^{L}, which can remove unnecessary proteins, is a new therapeutic concept. Further analysis, including transgenic overexpression and vector delivery of Dorfin-CHIP chimeric proteins using ALS animal models will increase our understanding of the potential utility of Dorfin-CHIP chimeric proteins as therapeutic tools.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2006.09.017.

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Gene Expression Profiling toward Understanding of ALS Pathogenesis

FUMIAKI TANAKA, JUN-ICHI NIWA, SHINSUKE ISHIGAKI, MASAHISA KATSUNO, MASAHIRO WAZA, MASAHIKO YAMAMOTO, MANABU DOYU, AND GEN SOBUE

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

ABSTRACT: **Although more than 130 years have gone by since the first description in 1869 by Jean-Martin Charcot, the mechanism underlying the characteristic selective motor neuron degeneration in amyotrophic lateral sclerosis (ALS) has remained elusive. Modest advances in this research field have been achieved by the identification of copper/zinc superoxide dismutase 1 (SOD1) as one of the causative genes for rare familial ALS (FALS) and by the development and analysis of mutant SOD1 transgenic mouse models. However, in sporadic ALS (SALS) with many more patients, causative or critical genes situated upstream of the disease pathway have not yet been elucidated and no available disease models have been established. To approach genes causative or critical for ALS, gene expression profiling in tissues primarily affected by the disease has represented an attractive research strategy. We have been working on screening these genes employing and combining several new technologies such as cDNA microarray, molecular indexing, and laser capture microdissection. Many of the resultant genes are of intense interest and may provide a powerful tool for determining the molecular mechanisms of ALS. However, we have barely arrived at the starting point and are confronting an enormous number of genes whose roles remain undetermined. Challenging tasks lie ahead of us such as identifying which genes are really causative for ALS and developing a disease model of SALS with due consideration for the expression changes in those genes.**

KEYWORDS: **ALS; SOD1; gene expression analysis; cDNA microarray; molecular indexing; laser capture microdissection**

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative and fatal human disorder characterized by loss of motor neurons in the spinal cord, brain stem,

Address for correspondence: Dr. Gen Sobue, Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Voice: +81-52-744-2385; fax: +81-52-744-2384.
e-mail: sobueg@med.nagoya-u.ac.jp

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and motor cortex, presenting as weakness of the limbs, speech abnormalities, and difficulties in swallowing.[1] The terminal phases of the disease involve respiratory insufficiency and half of the patients die within 3 years after the onset of symptoms. ALS can be inherited as an autosomal dominant trait in a subset of individuals who make up 5% to 10% of the total population of those affected. In addition, 20% to 30% of familial ALS (FALS) cases are associated with a mutation in the copper/zinc superoxide dismutase 1 gene (SOD1).[2] However, more than 90% of ALS patients are sporadic, not showing any familial trait. Since there have been no available disease models for sporadic ALS (SALS) as of now, transgenic mouse models or cell culture models[3] of ALS associated with SOD1 mutations have proven very useful in studying the initial mechanisms underlying this neurodegenerative disease of unknown etiology. The use of an animal model makes it possible and easy to investigate the different stages of disease progression including the early preclinical phase.

One of the experimental approaches toward a more comprehensive understanding of the molecular changes occurring in ALS is gene expression study[4] employing array-based methods or a differential display and its related techniques. Using transgenic mouse models expressing the SOD1 gene with a G93A mutation, we performed cDNA microarray analysis[5] to reveal the transcriptional profiles of affected tissues, namely, spinal anterior horn tissues. This analysis revealed an upregulation of genes related to an inflammatory process together with a change in apoptosis-related gene expression at the presymptomatic stage prior to motor neuron death.

Next, we extended our gene expression study from mouse to human postmortem spinal anterior horn tissues obtained from SALS patients. In this analysis, we employed a molecular indexing technique, a modified version of the differential display developed by Kato in 1995.[6] These PCR-based screening procedures have the advantage of being able to cover an unrestricted range of expressed genes including even hitherto unknown ones. As a result, we have successfully cloned a novel gene designated "dorfin,"[7] the expression of which was upregulated in SALS spinal cords.

Using spinal anterior horn tissues of SOD1 mutant mice or SALS patients as starting materials, these gene expression studies[5,7] have shed considerable light on the pathogenesis of FALS and SALS. However, in the spinal anterior horn tissues of ALS spinal cords, there are reduced numbers of motor neurons with glial cell proliferation. The alteration of the gene expression in the spinal anterior horn tissues could reflect the number of motor neurons and glial cells during disease progression. Such a disadvantage in using anterior horn tissues as starting materials prompted us to try to extract a pure motor neuron-specific gene expression profile. To this end, we employed the technology of laser capture microdissection[8] combined with T7-based RNA amplification and cDNA microarrays, which culminated in the successful detection of a total of 196 genes considered important for the SALS molecular mechanism.[9]

GENE EXPRESSION ANALYSIS FOR MUTANT SOD1 MOUSE MODEL OF ALS

We analyzed both temporal and differential gene expressions in the lumbar spinal anterior horn tissues of the transgenic mouse models expressing the SOD1 gene with a G93A mutation and the controls.[5] In this analysis, we detected a significant upregulation of 30 specific transcripts and downregulation of 7 transcripts in the spinal cords of mutant SOD1 mice[5] (TABLE 1). Before 11 weeks of age, mutant SOD1 mice are free of a disease phenotype, but they begin to decline rapidly in motor function after 14 weeks. The employment of mice for gene expression analysis provides a great advantage in obtaining data in the preclinical stage.

Interestingly, we found an upregulation of genes related to an inflammatory process together with a change in apoptosis-related gene expression at 11 weeks of age in the preclinical stage prior to motor neuron death.[5] The representative inflammatory-related genes elevated in their expression at this stage were the tumor necrosis factor (TNF)-α gene, which is a proinflammatory cytokine, and the Janus tyrosine-protein kinase 3 (JAK3), a necessary component of cytokine receptor signaling (TABLE 1). At a subsequent disease stage of 14 or 17 weeks of age, many more genes associated with an inflammatory process such as cathepsin D, serine protease inhibitor (SPI) 2–4, and cystatin C precursor, CD68, CD147, and clusterin increased their expression (TABLE 1). A histopathological evaluation showed glial cell activation and proliferation as early as 11 weeks of age and continuing to advance until 17 weeks.[10] A temporal increase in the expression level observed in these genes might reflect an inflammatory response with activated microglia and reactive astrocytes.

On the other hand, caspase-1, an initiator of the neuronal apoptotic cascade, was also upregulated at a presymptomatic 11 weeks of age (TABLE 1). An interrelationship between the inflammatory reaction and apoptotic pathway has been demonstrated. In addition to its role as an initiator of neuronal apoptotis, extracellular caspase-1 converts interleukin-1β (IL-1β) into a mature form. Thus, caspase-1 activation in motor neurons contributes to an inflammatory pathway with early astrocytosis and microglial activation in mutant SOD1 mice. In contrast, there is strong evidence for an inflammatory response involving microglial activation that leads to neuronal apoptosis.[11] Activated microglia express neurotoxic cytokines and substances such as TNF-α, proteases, oxyradicals, and small reactive molecules.[12] A nearly simultaneous upregulation of genes related to an inflammatory process and apoptotic initiation at the preclinical stage might contribute to the relentless neurodegenerative process making for a detrimental cycle. At 14 weeks of age, an early phase of the symptomatic stage, a key executioner of apoptosis, caspase-3, resulting from caspase-1 activation, began to be upregulated.[13] This finding agrees with

TABLE 1. Differentially expressed predominant genes detected in spinal anterior horn or spinal motor neurons from SOD1 mutant mice and sporadic ALS patients

Analysis object	SOD1 G93A mutant mice[5,13] spinal anterior horn	SALS patients[7,18] spinal anterior horn	SALS patients[9] spinal anterior horn	SALS patients[9] spinal motor neuron
Analysis method	cDNA microarray	Molecular indexing	cDNA microarray	cDNA microarray
	30/1176	46/entire mRNA	37/4845	52/4845
Upregulated genes in ALS	TNF-α*†	dorfin*	KIAA0231	death receptor 5 (DR5)*
	JAK3†	TAFII30*	fibrinogen A a polypeptide	cyclin A1, cyclin C*, ephrin A1*
	cathepsin D	neugrin*	presenilin 1	caspase 1, caspase 3, caspase 9
	serine protease inhibitor (SPI) 2–4		ephrin A1	acetyl-coenzyme A transporter*, NF-κB
	cystatin C precursor		transcription factor NF-Atc	ciliary neurotrophic factor (CNTF)
	CD68*, CD147		SH3-binding protein 2	hepatocyte growth factor (HGF)
	clusterin		integrin alpha E precursor (ITGAE)	glial cell line-derived neurotrophic factor (GDNF)
	caspase-1*†, caspase-3		cysteine dioxygenase, type 1	KIAA0231*
	GFAP*, vimentin			glutamate receptor subunit 2 (GLUR-2)
	Bcl-xL			interleukin-1 receptor antagonist
	c-fos, junD			TNF receptor-associated factor 6 (TRAF6)
	7/1176	38/entire mRNA	8/4845	144/4845
	XIAP	metallothionein-3*	glutamate receptor, metabotropic 6	dynactin 1 (p150)*, TRK-C*, midkine, musashi 1
Downregulated genes in ALS	$GABA_A$-receptor-α1	MRP8*	cholecystokinin A receptor	microtubule-associated protein 1A, 4
		ubiquitin-like protein 5*	signal recognition particle 14kD	microtubule-associated protein tau
			syntaxin 1B	early growth response 3 (EGR3)*
			sex-determining region Y (SRY)-box 11	BCL2-antagonist/killer 1 (Bak)*
				cellular retinoic acid-binding protein 1 (CRABP1)*
				retinoic acid receptor-α

Principal genes showing expression changes of 3.0-fold increase and 0.3-fold decrease are listed. Fold-change is calculated by dividing the fluorescence signals of each ALS sample by those of control samples.*Gene expression changes were confirmed by other methods such as reverse transcription-polymerase chain reaction (RT-PCR) or *in situ* hybridization.†Genes upregulated in 11-week-old mice.

the result that at 14 weeks of age XIAP mRNA downregulation occurred in the spinal cords of mutant SOD1 mice (TABLE 1) since XIAP is a direct inhibitor of caspase-3, -7, and -9.[14]

DISCOVERY OF NOVEL GENES ASSOCIATED WITH ALS PATHOGENESIS

To identify genes differentially expressed in the anterior horn tissues of the human SALS spinal cord, we adopted molecular indexing, a modified version of the differential display.[6] The entire mRNA population is identified and displayed by 3′ end cDNA fragments generated by class IIS restriction enzyme digestion and PCR.[6] Accordingly, molecular indexing provides a significant advantage in expression analysis for unknown genes. Among 84 fragments differentially expressed in SALS cloned in the first screening procedure, we noticed a fragment with an unknown sequence overexpressed in SALS spinal cords. We cloned it using RACE methods and named it dorfin (double ring-finger protein)[7] (TABLE 1).

Dorfin contains a RING-IBR (in between ring finger) domain at its N terminus and mediated ubiquitin ligase (E3) activity.[7] Interestingly, dorfin is predominantly localized and overexpressed in the ubiquitinated neuronal hyaline inclusion bodies found in the motor neurons of SALS patients as well as FALS patients with a SOD1 mutation and of mutant SOD1-transgenic mice.[15,16] An *in vitro* assay revealed that dorfin physically bound and ubiquitylated various SOD1 mutants and enhanced their degradation, and that its overexpression protected neural cells against the toxic effects of mutant SOD1 and reduced SOD1 inclusions.[15,17] These findings suggest that dorfin, an E3 ligase, may play some protective role in the pathogenesis of FALS and SALS via the ubiquitylation and degradation of its substrates, mutant SOD1, and others yet to be identified.

Besides dorfin, we have detected 30-kDa TATA binding protein-associated factor (TAFII30) and neugrin as upregulated genes in the SALS spinal cord[18] (TABLE 1). On the other hand, metallothionein-3, macrophage-inhibiting factor-related protein-8 (MRP-8) and ubiquitin-like protein 5 were downregulated in their expression[18] (TABLE 1).

MOTOR NEURON-SPECIFIC GENE EXPRESSION PROFILE IN SALS

As noted above, even using spinal anterior horn tissues consisting of heterogeneous cell types including motor neurons and glial cells as starting materials, gene expression studies have successfully shed light on the genes related to the pathogenesis of FALS and SALS.[5,7,18] However, the constitution

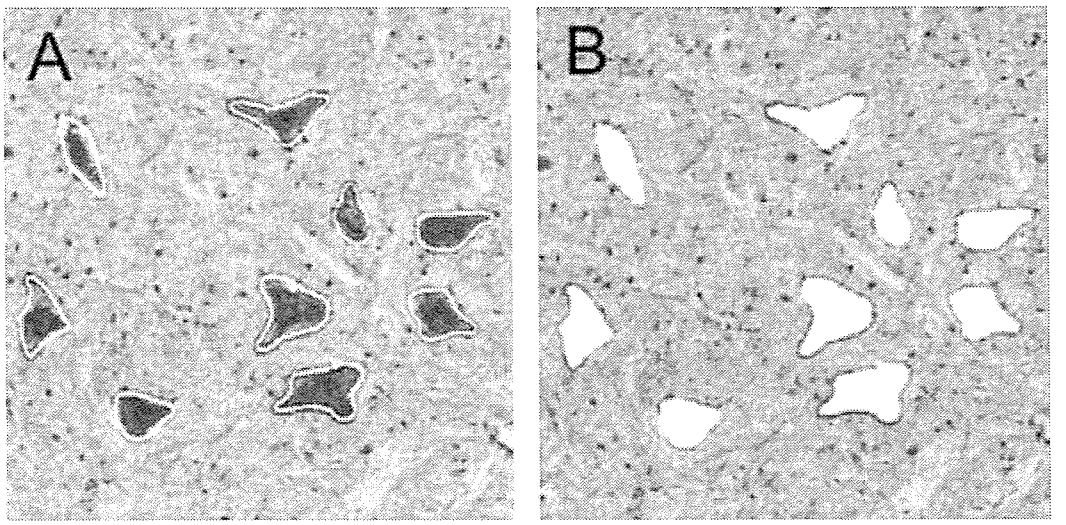

FIGURE 1. Laser microdissection of motor neurons in spinal anterior horn. sections were stained with hematoxylin and margins of motor neurons were dissected by the laser beam (**A**); motor neurons were isolated from slides by laser pressure catapulting (**B**).

of spinal anterior horn tissues is overwhelmingly dominated by glial cells in comparison with motor neurons. Furthermore, in the lesions of ALS spinal cords, there are reduced numbers of motor neurons with glial cell proliferation. When the genes display a dramatic change of expression in ALS motor neurons, they can be detected (TABLE 1) even by using spinal anterior horn tissues. In fact, we have successfully cloned dorfin overexpressed in SALS motor neurons[7] as described above. However, a small change of gene expression in motor neurons might be masked by a large quantity of glial cells and such genes might be those we are seeking as the essential ones for ALS pathomechanisms. The technologies of laser capture microdissection have been developed to provide a reliable method of procuring pure populations of cells from specific microscopic regions of tissue sections under direct visualization.[8,19] The pulsed laser microbeam cut precisely around the targeted motor neurons in the spinal anterior horn (FIG. 1). Each laser-isolated specimen was subsequently transferred to the cap of a PCR tube that was activated by laser pulses.

Using this technology combined with T7 RNA polymerase-based RNA amplification[20] and cDNA microarrays, we have obtained motor neuron-specific gene expression profiles of SALS patients[9] (TABLE 1). Simultaneously, we also conducted conventional gene expression analysis using spinal anterior horn tissues and validated the differential characteristics[9] (TABLE 1). As a result, spinal motor neurons showed a gene expression profile distinct from that of spinal anterior horn tissues (TABLE 1). Of the genes examined 3% (144/4845) were downregulated and 1% (52/4845) were upregulated in motor neurons. Downregulated genes included those associated with cytoskeleton/axonal

transport, transcription, and cell surface antigens/receptors such as dynactin 1, microtubule-associated proteins, and early growth response 3 (EGR3). In contrast, cell death-associated genes were mostly upregulated. Promoters for a cell death pathway, death receptor 5 (DR5), cyclins A1 and C, and caspases-1, -3, and -9, were upregulated as were cell death inhibitors, acetyl-CoA transporter, and NF-κB (TABLE 1). Moreover, neuroprotective neurotrophic factors such as ciliary neurotrophic factor (CNTF), hepatocyte growth factor (HGF), and glial cell line-derived neurotrophic factor (GDNF) were upregulated. However, inflammation-related genes such as those belonging to the cytokine family were not significantly upregulated in SALS motor neurons.

One of the interesting genes downregulated in motor neurons was dynactin 1, recently identified as a causative gene for human motor neuron disease.[21–23] Other motor proteins including the kinesin family responsible for antegrade axonal transport and dyneins for retrograde axonal transport were not changed significantly, but the expression levels of microtubule-associated proteins (MAPs) 1A, 4, and tau were reduced (TABLE 1). The impairment of axonal transport is thought to be an early event in motor neuron degeneration, and the protein levels of MAPs 1A and tau have especially been reported to decrease well before the onset of symptoms in mutant SOD1 transgenic mice also.[24]

As shown in the examples of MAPs 1A and tau, gene expression profiles of SALS patients may share some features with those of SOD1 mutant mice. However, taking into account our overall differential gene expression profiles between mice and humans drawn from spinal anterior horn tissues (TABLE 1), the disease in transgenic mouse may mimic but not be identical to the pathophysiology in human SALS. Consequently, we should be cautious about applying the research results of the pathophysiological process or therapeutic strategy obtained from SOD1 mutant mice to human SALS patients.

Seen in this light, the gene expression data of SALS motor neurons obtained by our analysis are of particular value and contribute a starting point for clarifying the pathomechanisms of a great many more SALS than FALS. At present, it is not easy to determine the genes of primary pathological significance from a total of 144 downregulated and 52 upregulated genes in SALS motor neurons. The primary molecular events should occur in the preclinical phase of the disease. Unlike the case of mice, it is impossible to obtain human spinal cord specimens at a preclinical stage. However, even in postmortem tissue, some motor neurons remain intact and have not yet started to degenerate. From this standpoint, a detailed investigation of the gene expression level, particularly in motor neurons, verified to be intact by reliable neurodegenerative markers would lead to the successful detection of genes related to primary molecular events. Detecting such genes would provide a first step toward a new molecular targeted therapy for SALS by developing animal or cell models mimicking those upstream and primary molecular events determined in human SALS patients.

INTEGRATED RESEARCH FOR NEURODEGENERATION AND TUMORIGENESIS

Among the genes in which we have detected an alteration in their expression in SOD1 mutant mice or SALS patients, a number of them are well known to be related to tumorigenesis rather than neurodegeneration (TABLE 1). Evidence has been steadily accumulating for the existence of many common molecular pathways between neurodegeneration and tumorigenesis.[25] Based on the concept of "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" proposed by The 21st Century Center of Excellence (COE) Program at Nagoya University,[26] the contribution of these tumor-related genes to the molecular mechanism of ALS should be clarified to advance our understanding of this devastating disease.

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Alleviating Neurodegeneration by an Anticancer Agent

An Hsp90 Inhibitor (17-AAG)

MASAHIRO WAZA, HIROAKI ADACHI, MASAHISA KATSUNO, MAKOTO MINAMIYAMA, FUMIAKI TANAKA, AND GEN SOBUE

Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya 466–8550, Japan

ABSTRACT: **Heat shock proteins (HSPs) that function mainly as molecular chaperones play an important role in the folding and quality control of proteins. Compared with these chaperones, Hsp90 is unique in that it binds to substrate proteins, called Hsp90 client proteins. Hsp90 is involved in the folding, activation, and assembly of its client proteins in association with its co-chaperones. Because numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded by Hsp90 inhibitors, 17-allylamino-17-demethoxygeldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now under clinical trials as a novel molecular-targeted agent for a wide range of malignancies. In spinal and bulbar muscular atrophy (SBMA), the pathogenic gene product is polyglutamine (polyQ)-expanded androgen receptor (AR), which belongs to the Hsp90 client protein family and is known to be degraded by 17-AAG. We have recently demonstrated that administration of an anticancer agent 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant AR. The ability of 17-AAG to 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 using a novel anticancer agent 17-AAG has emerged as a candidate for molecular-targeted therapies for neurodegenerative diseases.**

KEYWORDS: **neurodegeneration; polyglutamine; anticancer agent; Hsp90 inhibitor**

INTRODUCTION

In chronic neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and polyglutamine (polyQ) diseases, abnormal

Address for correspondence: Gen Sobue, M.D., Ph.D., Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan. Voice: +81-52-744-2385; fax: +81-52-744-2384.
e-mail: sobueg@med.nagoya-u.ac.jp

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accumulation of disease-causing protein is a commonly observed characteristic.[1] A selective elimination of disease-causing protein would be a promising remedy for neurodegenerative disorders.

Among these neurodegenerative disorders, it is well confirmed that polyQ diseases are caused by the expansion of a trinucleotide CAG repeat encoding glutamine in the causative genes, and to date, nine disorders have been identified as polyQ diseases.[2] In polyQ diseases, spinal and bulbar muscular atrophy (SBMA) was first identified among these diseases[3] and is characterized by premature muscular exhaustion, slow progressive muscular weakness, atrophy, and fasciculation in bulbar and limb muscles.[4] In SBMA, the pathogenic gene product is the androgen receptor (AR), which contains an abnormally expanded polyQ. The number of polymorphic CAG repeats in the *AR* gene is normally 14–32, but ranges from 40 to 62 CAGs in SBMA patients.[5] A correlation exists between the number of CAG repeats and disease severity.[6,7] The pathologic features of SBMA are motor neuron loss in the spinal cord and brain stem,[4] and diffuse nuclear accumulations and nuclear inclusions (NIs) containing the mutant AR in the residual motor neurons and certain visceral organs.[8]

Heat shock protein (Hsp)90 is essential for function and stability of the AR, the C-terminus of which has a high affinity for Hsp90, inducing the conformational change required for its nuclear translocation after ligand activation.[9–11] Hsp90 functions in a multicomponent complex of chaperone proteins including Hsp70, Hop (Hsp70 and Hsp90 organizing protein), cdc37, and p23. In addition, Hsp90 is involved in the folding, activation, and assembly of several proteins known as Hsp90 client proteins.[12] Numerous oncoproteins belonging to the Hsp90 client protein family are selectively degraded in the ubiquitin–proteasome system (UPS) by Hsp90 inhibitors. 17-allylamino-17-demethoxygeldanamycin (17-AAG), a first-in-class Hsp90 inhibitor, is now in clinical trials as a novel molecular-targeted agent for a wide range of malignancies.[13] AR also belongs to the Hsp90 client protein family and is degraded in the presence of Hsp90 inhibitors.[14–16]

We have recently demonstrated that 17-AAG markedly ameliorated polyQ-mediated motor neuron degeneration through degradation of mutant AR.[17] We consider that the ability to facilitate degradation of disease-causing protein would be of value when applied to SBMA and other related neurodegenerative diseases. This review discusses our research findings and other studies, and the clinical application of Hsp90 inhibitors to neurodegenerative diseases beyond neoplastic ones.

GENERATION OF LESS TOXIC HSP90 INHIBITOR, 17-AAG

The most classical Hsp90 inhibitor is geldanamycin (GA), a natural product that was developed as an antifungal agent.[18] Later, GA was also found to have

a potent and selective antitumor effect against a wide range of malignancies.[19] Although GA showed potential as a novel anticancer agent,[20] it was also found to have intolerable liver toxicity.[21] To overcome this GA-induced liver toxicity, scientists at the U.S. National Cancer Institute (NCI) succeeded in developing a new derivative of GA, 17-AAG, that shares its important biological activities[22] but shows less toxicity.[23] Owing to this promising derivative, Hsp90 inhibitors have taken a major developmental leap in their clinical applications, and 17-AAG is now in Phase II clinical trials with encouraging results as an anticancer agent.[24–28]

The antitumor effect of Hsp90 inhibitors is due to their ability to specifically bind to the Hsp90 adenosine 5'-triphosphate (ATP)-binding site, thereby modulating Hsp90 function and proteasomal degradation of Hsp90 client proteins.[29–31] Because numerous oncoproteins were shown to belong to the family of Hsp90 client proteins,[12] Hsp90 inhibitors are expected to become part of a new strategy in antitumor therapy.[20] Hsp90 inhibitors including GA and 17-AAG have been shown to have a higher selectivity for tumor cells compared with general antitumor agents.[32,33] This selectivity is due to the high affinity of 17-AAG for the Hsp90 client oncoproteins when they are incorporated in the Hsp90-dependent multichaperone complex, thereby increasing their binding affinity to 17-AAG more than 100-fold.[34] This high selectivity of 17-AAG to the incorporated Hsp90 client protein eventually minimizes its toxic side effects.[31] We think that this selectivity of Hsp90 inhibitors would also be advantageous for the treatment of neurodegenerative diseases.

Hsp90 complexes are thought to exist in two main forms; one complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with cdc37 and p23 (FIG. 1A).[16,35–37] As for AR, Hsp90 is essential to maintain its high ligand-binding affinity and its stabilization.[9,14] In practice, Hsp90 inhibitors reduce androgen ligand-binding affinity and induce the degradation of AR.[14,15] We also confirmed that 17-AAG resulted in the shifting of the AR-Hsp90 chaperone complex from a mature stabilizing form with p23 to a proteasome-targeting form with Hop.[17]

17-AAG'S DEGRADATION OF MUTANT AR DEPENDS ON PROTEASOME

To determine whether or not 17-AAG promotes the degradation of the polyQ-expanded mutant AR, we treated SH-SY5Y cells highly expressing the wild-type (AR-24Q) or mutant (AR-97Q) AR for 6 h with 36 μM 17-AAG or with dimethylsulfoxide (DMSO) as control, in the absence or presence of the proteasome inhibitor, MG132. Immunoblot analysis demonstrated that the monomeric mutant AR decreased significantly more than did the wild type, suggesting that the mutant AR is more sensitive to 17-AAG than is the wild type. The degradation of wild-type and mutant AR by 17-AAG was completely

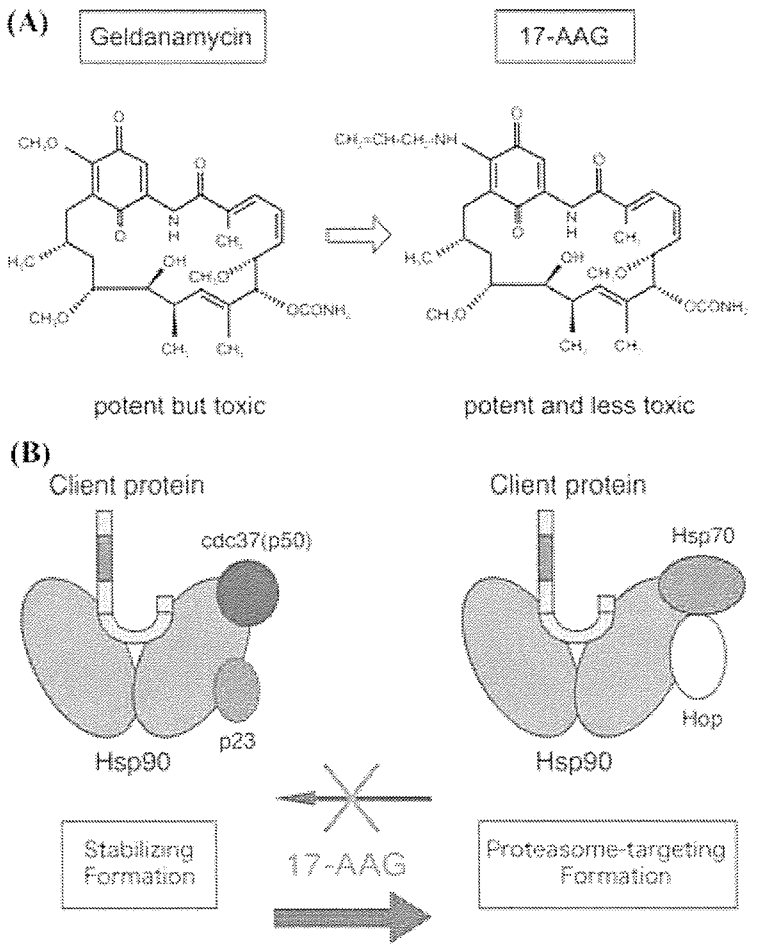

FIGURE 1. Hsp90 inhibitors and the pharmacological effect. (**A**) Geldanamycin (GA) is the most classical Hsp90 inhibitor. Although GA has a potent and selective antitumor effect against a wide range of malignancies, this agent has intolerable liver toxicity. The 17-AAG, a new derivative of GA, shares its important biological activities but shows less toxicity. (**B**) The 17-AAG specifically binds ATP-binding site of Hsp90, resulting in a shift of the Hsp90 complex. Two main forms of this complex exist. One complex is a proteasome-targeting form associated with Hsp70 and Hop, and the other is a stabilizing form with cdc37 and p23. Hsp90 inhibitors block the progression of the Hsp90 complex toward the stabilizing form and shift it to the proteasomal-targeting form. This figure is modified from a model proposed by Neckers.[16]

blocked by MG132 (FIG. 2A), suggesting that 17-AAG-faciiltated degradation was dependent on the proteasome system as previously reported.[38,39]

To determine whether the decrease in AR was due to protein degradation or to changes in RNA expression, the turnover of wild-type and mutant AR was then assessed with a pulse-chase labeling assay. In the presence of 17-AAG, the mutant AR and the wild-type AR had half-lives of 2 h and 3.5 h, respectively (FIG. 2B), while mRNA levels for both the wild-type and mutant AR were quite similar.[17] These data indicate that 17-AAG preferentially degrades the mutant AR protein without altering mRNA levels.

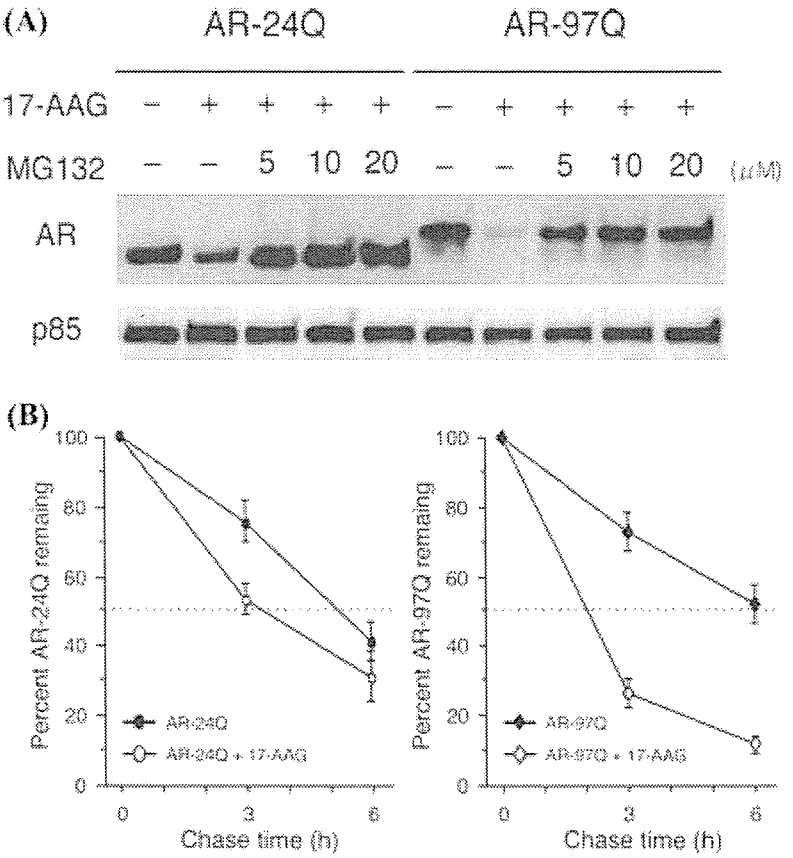

FIGURE 2. The 17-AAG's degradation of mutant AR depends on proteasome function. (**A**) 17-AAG treatment (36 μM, 6 h) of transfected SH-SY5Y cells reduced the levels of mutant AR (AR-97Q) significantly more than wild-type AR (AR-24Q), however, both decreases were completely blocked by the proteasomal inhibitor, MG132. (**B**) Effects of 17-AAG on the half-life of wild-type and mutant AR assessed from pulse-chase experiments. Amounts of AR-24Q remaining in the absence and presence of 17-AAG are indicated by closed circles (•) and open circles (○), respectively. Amounts of AR-97Q remaining in the absence and presence of 17-AAG are indicated by closed (♦) and open (◇) diamonds, respectively. Mutant AR was degraded more rapidly than the wild-type AR in the presence of 17-AAG. Values are expressed as means ± SE ($n = 4$).

Thus, these *in vitro* studies indicated that the mutant AR was a good target protein of 17-AAG. To determine whether 17-AAG has the ability to degrade the toxic disease-causing protein *in vivo*, we next examined the effects of 17-AAG in SBMA transgenic mice.

17-AAG AMELIORATES THE PHENOTYPE IN A MOUSE MODEL OF SBMA WITHOUT DETECTABLE TOXICITY

Referring to previous reports,[15,36,40] we administered 17-AAG thrice a week on alternate days at doses of 2.5 or 25 mg/kg to males of the transgenic mouse model carrying full-length human AR with either 24Q or 97Q. The disease