(13：00～15：15)

Ⅱ. 孤発性ALSをどう攻めるか

座長：中野　今治（自治医科大学神経内科）、郭　　伸（東京大学神経内科）

(1) 孤発性ALSの疾患範囲をどう捉えるか

プレゼンター　中野　今治（自治医科大学神経内科）

ディスカッサント　水澤　英洋（東京医科歯科大学神経内科）

(2) 孤発性ALSの病因 —glutamate受容体と神経細胞死—

プレゼンター　郭　　伸（東京大学神経内科）

ディスカッサント　高橋　良輔（京都大学神経内科）

(3) 遺伝子発現プロファイリング —孤発性ALSの分子標的治療に向けて—

プレゼンター　田中　章景（名古屋大学神経内科）

ディスカッサント　戸田　達史（大阪大学臨床遺伝学）

(15：20～16：20)

Ⅲ. 特別講演

座長：祖父江　元（名古屋大学神経内科）

『オートファジーと神経変性』

田中　啓二、小松　雅明（東京都臨床医学総合研究所）

厚生労働科学研究費補助金（難治性疾患克服研究事業）

筋萎縮性側索硬化症の画期的診断・治療法に関する研究班

（課題番号　H17 －難治－ 44）

平成 18 年度　班会議プログラム

日　時：平成 19 年 1 月 19 日(金)　10：00 ～ 16：05
場　所：全共連ビル　4 階　中会議室
東京都千代田区平河町 2-7-9
TEL 03-5215-9501

発表：口演時間 15 分（質疑・討論含む）

主任研究者　　祖父江　　元

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プ ロ グ ラ ム

10:00　主任研究者挨拶

10:05　厚生労働省疾病対策課御挨拶

10:10～10:55

セッションⅠ　【病態】　　座長：**谷口　直之**（大阪大学微生物病研究所）

1. ヒト Cu/Zn-SOD の Cys111 の酸化について

○藤原（ふじはら）　範子（のりこ）[1)]、松本　紋子[2)]、中堅三弥子[2)]、鈴木敬一郎[1)]、
谷口　直之[3)]

1）兵庫医科大学生化学
2）大阪大学生化学
3）大阪大学微生物病研究所疾患糖鎖学

2. MnSOD の運動ニューロン特異的ノックアウトマウス – 酸化的ストレスの運動神経変性への関与について

○三澤日出巳（みさわひでみ）[1),2)]、中田　和子[2)]、松浦　純子[2)]、森脇　康博[1)]、
川嶋紘一郎[1)]、清水　孝彦[3)]、白澤　卓二[3)]、高橋　良輔[4)]

1）共立薬科大学薬理学
2）都立神経科学総合研究所
3）東京都老人研老化ゲノムバイオマーカー研究チーム
4）京都大学神経内科

3. Co-chaperone Mrj を遺伝子導入した線虫の作製の試み

○小山（こやま）　信吾（しんご）[1)]、荒若　繁樹[1)]、加藤　丈夫[1)]、桑原　知樹[2)]、
岩坪　威[2)]

1）山形大学生命情報内科学
2）東京大学臨床薬学

10:55～11:40

セッションII 【病態・治療】　　座長：岡野　栄之（慶應義塾大学生理学）

4. 胚性幹細胞、神経幹細胞を用いた神経再生

○岡田（おかだ）　洋平（ようへい）[1),2)]、松本　有史[3),4)]、小川　大輔[1),5)]、
熊谷玄太郎[1),6),7)]、石井　聖二[1)]、中村　雅也[6)]、戸山　芳昭[6)]、
青木　正志[3)]、糸山　泰人[3)]、祖父江　元[2)]、岡野　栄之[1)]

1）慶應義塾大学生理学
2）名古屋大学神経内科
3）東北大学神経内科
4）国立病院機構宮城病院神経内科
5）香川大学脳神経内科
6）慶應義塾大学整形外科
7）弘前大学整形外科

5. EGF+FGF2 髄腔内投与による ALS モデルマウス脊髄での内在性未分化神経幹細胞増殖の試み

阿部　康二、○太田（おおた）　康之（やすゆき）、永井真貴子、村上　哲郎、
武久　　康、永田　哲也、奈良井　恒、倉田　智子、森本　展年、
宮崎　一徳、神谷　達司
岡山大学神経内科

6. ALS ラットモデル脊髄における再生阻害因子の解析

糸山　泰人[1)]、○割田（わりた）　仁（ひとし）[1)]、水野　秀紀[1)]
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1）東北大学神経内科学
2）慶應義塾大学生理学

11:40～13:00　　＊＊＊＊＊＊昼食・班員会議＊＊＊＊＊＊

13:00～13:30

特別講演　　座長：**祖父江　元**（名古屋大学神経内科）

【ALS における非自律性神経細胞死
－ ALS の発症と進行は運動ニューロンとグリアにより規定される】

山中　宏二（理化学研究所脳科学総合研究センター）

13:30～14:30

セッションⅢ　【病態・病態モデル】　　座長：**高橋　良輔**（京都大学　神経内科）

7.　オートファジーと封入体（異常蛋白質凝集体）形成機構

○田中（たなか）　啓二（けいじ）、小松　雅明
東京都臨床医学総合研究所

8.　ラット脊髄培養細胞を用いた運動ニューロン死の検討
－プロテアソーム障害と小胞体関連ストレス－

菊地　誠志[1)]、○辻（つじ）　幸子（さちこ）[2)]、田代　淳[2)]、新保　和賢[2)]、佐々木秀直[2)]
1）国立病院機構札幌南病院神経内科
2）北海道大学神経内科

9.　RNA 編集異常による孤発性 ALS モデルマウスの開発

郭　伸[1)]、○日出山拓人（ひでやまたくと）[1)]、西本　祥仁[1),2)]、伊藤　杏子[1)]、山下　雄也[1)]、辻　省次[1)]、高橋　良輔[3)]、三澤日出巳[4)]、鈴木　岳之[5)]
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2）慶應義塾大学神経内科
3）京都大学神経内科
4）共立薬科大学薬理学
5）共立薬科大学基礎生物学

10. 遺伝子発現プロファイリングに基づく孤発性 ALS モデルの作成

○田中（たなか） 章景（ふみあき）[1)]、黄　　哲[1)]、蔣　月梅[1)]、和座　雅浩[1)]、
曽根　　淳[1)]、熱田　直樹[1)]、飯島　正博[1)]、松尾　幸治[1)]、
足立　弘明[1)]、丹羽　淳一[1)]、山本　正彦[1),2)]、道勇　　学[1)]、
祖父江　元[1)]

1）名古屋大学神経内科
2）愛知学院大学心身科学部健康科学科

休　憩（5 分）

14:35～15:20

セッションⅣ 【治療 1】　　**座長：野本　明男（東京大学微生物学）**

11. 2A プロテアーゼ遺伝子欠損ポリオウイルスの複製

○五十嵐博子（いがらしひろこ）、松田　法恵、吉野　泰子、大岡　静衣、
野本　明男
東京大学微生物学

12. ALS 遺伝子治療のための発現制御 AAV ベクターの開発

○村松（むらまつ） 慎一（しんいち）[1)]、新村　真則[2)]、三室　　淳[2)]、小澤　敬也[3)]、
坂田　洋一[2)]、中野　今治[1)]

1）自治医科大学神経内科
2）自治医科大学分子病態
3）自治医科大学遺伝子治療

13. ALS の shRNA を用いた遺伝子治療の問題点

水澤　英洋[1)]、○久保寺隆行（くぼでらたかゆき）[1)]、山田　宏美[1)]、村山　祥子[1)]、
大平　進嘉[1)]、海野　敏紀[1)]、笹栗　弘貴[1)]、横田　隆徳[1)]、
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1）東京医科歯科大学脳神経病態学
2）近畿大学先端技術研究所

15:20～16:05

セッションV 【治療2】 座長：**糸山 泰人**（東北大学神経内科）

14. 筋萎縮生側索硬化症における免疫補助分子 CD40 の解析

○中辻（なかつじ） 裕司（ゆうじ）[1)]、奥野 龍禎[2)]、佐古田三郎[1)]

1）大阪大学神経内科

2）大阪府立急性期・総合医療センター神経内科

15. ALS-Tg マウス肝臓・腎臓・心臓における一過性組織変性からの回復と c-Met/HGF 受容体のリン酸化調節：変性運動ニューロンに対する HGF 治療の戦略的拠点

○加藤（かとう） 信介（しんすけ）[1)]、加藤 雅子[2)]、船越 洋[3)]、角山 圭一[3)]、
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2）鳥取大学病院病理

3）大阪大学分子組織再生分野

4）東北大学神経内科

16. ALS 依存的な HGF 受容体（c-Met）の活性化制御機構の解析 ー ALS に対する安全な HGF 治療法の確立に向けてー

○角山（かどやま） 圭一（けいいち）、船越 洋、中村 敏一

大阪大学分子再生医学分野

VI. 研究者一覧

平成18年度厚生労働科学研究費補助金（難治性疾患克服研究事業）
「筋萎縮性側索硬化症の画期的診断・治療法に関する研究」班（課題番号　H17-難治・一般-044）
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Ⅶ. 研究成果の刊行物・別刷

厚生労働科学研究費補助金
難治性疾患克服研究事業

筋萎縮性側索硬化症の画期的診断・治療法に関する研究

平成18年度 総括研究報告書

（研究成果の刊行物・別刷）

主任研究者 祖父江 元
（名古屋大学大学院医学系研究科教授）

平成19（2007）年3月

2/2冊

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Neurobiology of Disease

www.elsevier.com/locate/ynbdi
Neurobiology of Disease 25 (2007) 331–341

Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant SOD1 proteins and reduce their cellular toxicity

Shinsuke Ishigaki,[a,b] Jun-ichi Niwa,[a] Shin-ichi Yamada,[a] Miho Takahashi,[a] Takashi Ito,[a] Jun Sone,[a] Manabu Doyu,[a] Fumihiko Urano,[b,c] and Gen Sobue[a,*]

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Received 19 May 2006; revised 8 September 2006; accepted 22 September 2006
Available online 6 December 2006

The ubiquitin–proteasome system (UPS) is involved in the pathogenetic mechanisms of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS). Dorfin is a ubiquitin ligase (E3) that degrades mutant SOD1 proteins, which are responsible for familial ALS. Although Dorfin has potential as an anti-ALS molecule, its life in cells is short. To improve its stability and enhance its E3 activity, we developed chimeric proteins containing the substrate-binding hydrophobic portion of Dorfin and the U-box domain of the carboxyl terminus of Hsc70-interacting protein (CHIP), which has strong E3 activity through the U-box domain. All the Dorfin-CHIP chimeric proteins were more stable in cells than was wild-type Dorfin (Dorfin^{WT}). One of the Dorfin-CHIP chimeric proteins, Dorfin-CHIP^{L}, ubiquitylated mutant SOD1 more effectively than did Dorfin^{WT} and CHIP *in vivo*, and degraded mutant SOD1 protein more rapidly than Dorfin^{WT} does. Furthermore, Dorfin-CHIP^{L} rescued neuronal cells from mutant SOD1-associated toxicity and reduced the aggresome formation induced by mutant SOD1 more effectively than did Dorfin^{WT}.

Keywords: Dorfin; ALS; SOD1; CHIP; Neurodegeneration; Ubiquitin–proteasome system

Amyotrophic lateral sclerosis (ALS), one of the most common neurodegenerative disorders, is characterized by selective motor neuron degeneration in the spinal cord, brainstem, and cortex. About 10% of ALS cases are familial; of these, 10%–20% are caused by Cu/Zn superoxide dismutase (SOD1) gene mutations (Rosen et al., 1993; Cudkowicz et al., 1997). However, the precise mechanism that causes motor neuron death in ALS is still unknown, although many have been proposed: oxidative toxicity, glutamate receptor abnormality, ubiquitin proteasome dysfunction, inflammatory and cytokine activation, neurotrophic factor deficiency, mitochondrial damage, cytoskeletal abnormalities, and activation of the apoptosis pathway (Julien, 2001; Rowland and Shneider, 2001).

Misfolded protein accumulation, one probable cause of neurodegenerative disorders, including ALS, can cause the deterioration of various cellular functions, leading to neuronal cell death (Julien, 2001; Ciechanover and Brundin, 2003). Recent findings indicate that the ubiquitin–proteasome system (UPS), a cellular function that recognizes and catalyzes misfolded or impaired cellular proteins (Jungmann et al., 1993; Lee et al., 1996; Bercovich et al., 1997), is involved in the pathogenesis of various neurodegenerative diseases, among them ALS, Parkinson's disease (PD), Alzheimer's disease, polyglutamine disease, and prion disease (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). The ubiquitin ligase (E3), a key molecule for the UPS, can specifically recognize misfolded substrates and convey them to proteasomal degradation (Scheffner et al., 1995; Glickman and Ciechanover, 2002; Tanaka et al., 2004).

Dorfin, an E3 protein, contains an in-between-ring-finger (RING-IBR) domain at its N-terminus. The C-terminus of Dorfin can recognize mutant SOD1 proteins, which cause familial ALS (Niwa et al., 2001; Ishigaki et al., 2002b; Niwa et al., 2002). In cultured cells, Dorfin colocalized with aggresomes and ubiquitin-positive inclusions, which are pathological hallmarks of neurodegenerative diseases (Hishikawa et al., 2003; Ito et al., 2003). Dorfin also interacted with VCP/p97 in ubiquitin-positive inclusions in

Abbreviations: ALS, amyotrophic lateral sclerosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, carboxyl terminus of Hsc70-interacting protein; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; FCS, fetal calf serum; IP, immunoprecipitation; LB, Lewy body; PD, Parkinson's disease; RING-IBR, in-between-ring-finger; SCF, Skp1-Cullin-F box complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD1, Cu/Zn super oxide dismutase; UPS, ubiquitin–proteasome system.

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2006.09.017

ALS and PD (Ishigaki et al., 2004). Moreover, formation of this complex was found to be necessary for the E3 activity of Dorfin against mutant SOD1. These findings suggest that Dorfin is involved in the quality-control system for the abnormal proteins that accumulate in the affected neurons in neurodegenerative disorders.

Dorfin degrades mutant SOD1s and attenuates mutant SOD1-associated toxicity in cultured cells (Niwa et al., 2002). However, in Dorfin/mutant SOD1 double transgenic mice, we found only a modest beneficial effect on mutant SOD1-induced survival and motor dysfunction (unpublished data). These findings, combined with the short half-life of Dorfin protein, led us to hypothesize that the limiting effect of the Dorfin transgene may be a consequence of autodegradation of Dorfin, since Dorfin can execute autoubiquitilation *in vivo* (Niwa et al., 2001).

Carboxyl terminus of Hsc70-interacting protein (CHIP) is also an E3 protein; it has a TPR domain in the N terminus and a U-box domain in the C terminus. The U-box domain of CHIP is responsible for its strong E3 activity, whereas the TPR domain recruits heat shock proteins harboring misfolded client proteins such as cystic fibrosis transmembrane conductance regulator (CFTR), denatured luciferase, and tau (Meacham et al., 2001; Murata et al., 2001, 2003; Hatakeyama et al., 2004; Shimura et al., 2004).

To prolong the protein lifetime of Dorfin and thereby obtain more potent ubiquitylation and degradation activity against mutant SOD1s than is provided by Dorfin or CHIP alone, we generated chimeric proteins containing the substrate-binding domain of Dorfin and the UPR domain of CHIP substitute for RING/IBR of Dorfin. We developed 12 candidate constructs that encode Dorfin-CHIP chimeric proteins and analyzed them for their E3 activities and degradation abilities against mutant SOD1 protein in cultured cells.

Experimental procedures

Plasmids and antibodies

We designed constructs expressing Dorfin-CHIP chimeric protein. In these constructs, different-length fragments of the C-terminus portion of Dorfin, including the hydrophobic substrate-binding domain (amino acids 333–838, 333–700, and 333–454) and the C-terminus UPR domain of CHIP with amino acids 128–303 or without amino acids 201–303, a charged region was fused in various combinations as shown in Fig. 2C. Briefly, Dorfin-CHIP$^{A, B, C, G, H,}$ and ^{I} had the C-terminus portion of Dorfin in their N-terminus and the U-box of CHIP in their C-terminus; Dorfin-CHIP$^{D, E, F, J, K,}$ and ^{L} had the U-box of CHIP in their N-terminus and the C-terminus portion of Dorfin in their C-terminus.

We prepared a pCMV2/FLAG-Dorfin-CHIP chimeric vector (Dorfin-CHIP) by polymerase chain reaction (PCR) using the appropriate design of PCR primers with restriction sites (*Cla*I, *Kpn*I, and *XBa*I or *Eco*RI, *Cla*I, and *Kpn*I). The PCR products were digested and inserted into the *Cla*I–*Kpn*I site in pCMV2 vector (Sigma, St. Louis, MO). These vectors have been described previously: pFLAG-Dorfin^{WT} (Dorfin^{WT}), FLAG-Dorfin$^{C132S/C135S}$ (Dorfin$^{C132S/C135S}$), pFLAG-CHIP (CHIP), pFLAG-Mock (Mock), pcDNA3.1/Myc-SOD1^{WT} (SOD1^{WT}), pcDNA3.1/Myc-SOD1^{G93A} (SOD1^{G93A}), pcDNA3.1/Myc-SOD1^{G85R} (SOD1^{G85R}), pcDNA3.1/Myc-SOD1^{H46R} (SOD1^{H46R}), pcDNA3.1/Myc-SOD1^{G37R} (SOD1^{G37R}), pEGFP/SOD1^{WT} (SOD1^{WT}-GFP), and pEGFP/SOD1^{G85R} (SOD1^{G85R}-GFP) (Ishigaki et al., 2004). We used monoclonal anti-FLAG antibody (M2; Sigma), monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-HA antibody (12CA5; Roche, Basel, Switzerland), and polyclonal anti-SOD1 (SOD-100; Stressgen, San Diego, CA).

Cell culture and transfection

We grew HEK293 cells and neuro2a (N2a) cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin, and 50 μg/ml streptomycin. At subconfluence, we transfected these cells with the indicated plasmids, using Effectene reagent (Qiagen, Valencia, CA) for HEK293 cells and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for N2a cells. After overnight posttransfection, we treated the cells with 1 μM MG132 (Z-Leu-Leu-Leu-al; Sigma) for 16 h to inhibit cellular proteasome activity. We analyzed the cells 24–48 h after transfection. To differentiate N2a cells, cells were treated for 48 h with 15 μM of retinoic acid in 2% serum medium.

Immunological analysis

At 24–48 h after transfection, we lysed cells (4×10^5 in 6-cm dishes) with 500 μl of lysis buffer consisting of 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, and 1 mM ethylenediaminetetraacetic acid (EDTA), as well as a protease inhibitor cocktail (Complete Mini, Roche). The lysate was then centrifuged at $10{,}000 \times g$ for 10 min at 4°C to remove debris. We used a 10% volume of the supernatants as the lysate for SDS-PAGE. When immunoprecipitated, the supernatants were precleared with protein A/G agarose (Santa-Cruz). A specific antibody, either anti-FLAG (M2) or anti-Myc (9E10), was then added. We incubated the immune complexes, first at 4°C with rotation and with protein A/G agarose (Roche) for 3 h, after which they were collected by centrifugation and washed four times with the lysis buffer. For protein analysis, immune complexes were dissociated by heating in SDS-PAGE sample buffer and loaded onto SDS-PAGE. We separated the samples by SDS-PAGE (15% gel or 5%–20% gradient gel) and transferred them onto polyvinylidene difluoride membranes. We then immunoblotted samples with specific antibodies.

Immunohistochemistry

We fixed differentiated N2a cells grown in plastic dishes in 4% paraformaldehyde in PBS for 15 min. The cells were then blocked for 30 min with 5% (vol/vol) normal goat serum in PBS, incubated overnight at 4°C with anti-FLAG antibody (M2), washed with PBS, and incubated for 30 min with Alexa 496 nm anti-mouse antibodies (Molecular Probes, Eugene, OR). We mounted the cells on slides and obtained images using a fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP70; Olympus). Photographs were taken using DP Controller software (Olympus).

Analysis of protein stability

We assayed the stability of proteins by pulse-chase analysis using [^{35}S] followed by immunoprecipitation. Metabolic labeling was performed as described previously (Yoshida et al., 2003). Briefly, in the pulse-chase analysis of Dorfin proteins, HEK293 cells in 6-cm dishes were transiently transfected with 1 μg of

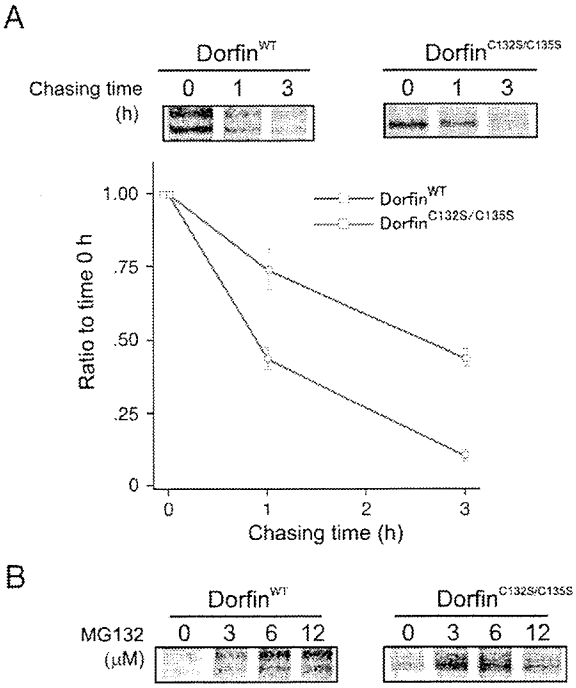


Fig. 1. Pulse-chase analysis of Dorfin^{WT} and Dorfin$^{C132S/C135S}$. (A) Dorfin^{WT} or Dorfin$^{C132S/C135S}$ was overexpressed in HEK293 cells. After overnight incubation, [^{35}S]-labeled Met/Cys pulse-chase analysis was performed. Cells were harvested and analyzed at 0, 1, or 3 h after labeling and immunoprecipitation by anti-FLAG antibody (upper panels). To determine serial changes in the amount of Dorfin^{WT} or Dorfin$^{C132S/C135S}$, four independent experiments were performed and the amounts of Dorfin^{WT} and Dorfin$^{C132S/C135S}$ were plotted. The differences between the amounts of Dorfin^{WT} and Dorfin$^{C132S/C135S}$ were significant at 1 h ($p<0.01$) and 3 h after labeling ($p<0.001$) (lower panels). Values are the means±SE, $n=4$. Statistics were done using an unpaired t-test. (B) Cells overexpressing Dorfin^{WT} or Dorfin$^{C132S/C135S}$ were treated with different concentrations of MG132 for 3 h after labeling.

FLAG-Dorfin^{WT} or FLAG-Dorfin$^{C132S/C135S}$. In pulse-chase experiments using SOD1^{G85R}, N2a cells in 6-cm dishes were transiently transfected with 1 μg of SOD1^{G85R}-Myc or SOD1^{G93A}-Myc and FLAG-Mock, FLAG-Dorfin, or FLAG-Dorfin-CHIP^{L}. FLAG-Mock was used as a negative control. After starving the cells for 60 min in methionine- and cysteine-free DMEM with 10% FCS, we labeled them for 60 min with 150 μCi/ml of Pro-Mix L-[^{35}S] *in vitro* cell-labeling mix (Amersham Biosciences). Cells were chased for different lengths of time at 37°C. In experiments with proteasomal inhibition, we added different amounts of MG132 in medium during the chase period. We performed immunoprecipitation using protein A/G agarose, mouse monoclonal anti-FLAG (M2), and anti-Myc (9E10). The intensity of the bands was quantified by ImageGauge software (Fuji Film, Tokyo, Japan).

MTS assay

We transfected N2a cells (5000 cells per well) in 96-well collagen-coated plates with 0.15 μg of SOD1^{G85R}-GFP and 0.05 μg of Dorfin, CHIP, Dorfin-CHIP^{L}, or pCMV2 vector (Mock) using Effecten reagent (Qiagen). Then we performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) assays using Cell Titer 96 (Promega) at 48 h after incubation. This procedure has previously been described (Ishigaki et al., 2002a).

Aggregation assay

We transfected N2a cells in 6-cm dishes with 1.0 μg of SOD1^{G85R}-GFP and 1.0 μg of FLAG-Mock, FLAG-Dorfin, FLAG-CHIP, or FLAG-Dorfin-CHIP^{L}. After overnight incubation, we changed the medium to 2% FCS containing medium with 15 μM retinoic acid (RA) for differentiation. In the MG132 (+) group, 1 μM of MG132 was added after 24 h of differentiation stimuli. After 48 h of differentiation stimuli, we examined the cells in their living condition by fluorescence microscopy. The transfection ratio was equivalent (75%) among all groups. Visually observable macro aggregation-harboring cells were counted as "aggregation positive" cells (Fig. 7C). All cells were counted in fields selected at random from the four different quadrants of the culture dish. Counting was done by an investigator who was blind to the experimental condition.

Results

Dorfin degradation by the UPS in vivo

We analyzed the degradation speed of FLAG-Dorfin by the pulse-chase method using [^{35}S] labeling, finding that more than half of wild-type Dorfin (Dorfin^{WT}) was degraded within 1 h (Fig. 1A). This degradation was dose-dependently inhibited by MG132, a proteasome inhibitor (Fig. 1B). On the other hand, the RING mutant form of Dorfin (Dorfin$^{C132S/C135S}$), which lacks E3 activity (Ishigaki et al., 2004), degraded significantly more slowly than did Dorfin^{WT} (Fig. 1A and Table 1). As shown in Fig. 1A, Dorfin^{WT} showed two bands, whereas Dorfin$^{C132S/C135S}$ had a single band. This was also seen in our previous study (Ishigaki et al., 2004) and may represent posttranslational modification.

Construction of Dorfin-CHIP chimeric proteins

It is known that the C-terminus portion of Dorfin can bind to substrates such as mutant SOD1 proteins or Synphilin-1 (Niwa et al., 2002; Ito et al., 2003). We attempted to identify the domain of Dorfin that interacts with substrates. Although there was no obvious known motif in the C-terminus of Dorfin (amino acids 333–838), its first quarter contained rich hydrophobic amino acids (amino acids 333–454) (Fig. 2A). Immunoprecipitation analysis revealed that the hydrophobic region of Dorfin (amino acids 333–454) was able to bind to SOD1^{G85R}, indicating that this hydrophobic region is responsible for recruiting mutant SOD1 in Dorfin protein (Fig. 2B).

To establish more effective and more stable E3 ubiquitin ligase molecules that can recognize and degrade mutant SOD1s, we

Table 1
Serial changes in the amounts of Dorfin^{WT}, Dorfin$^{C132S/C135S}$, and Dorfin-CHIP^{L}

	0 h (%)	1 h (%)	3 h (%)
Dorfin^{WT}	100	43.7±7.0	10.3±4.4
Dorfin$^{C132S/C135S}$	100	73.9±13.8	43.7±1.9
Dorfin-CHIP^{L}	100	89.0±5.7	47.5±5.3

Values are the mean and SD of four independent experiments.

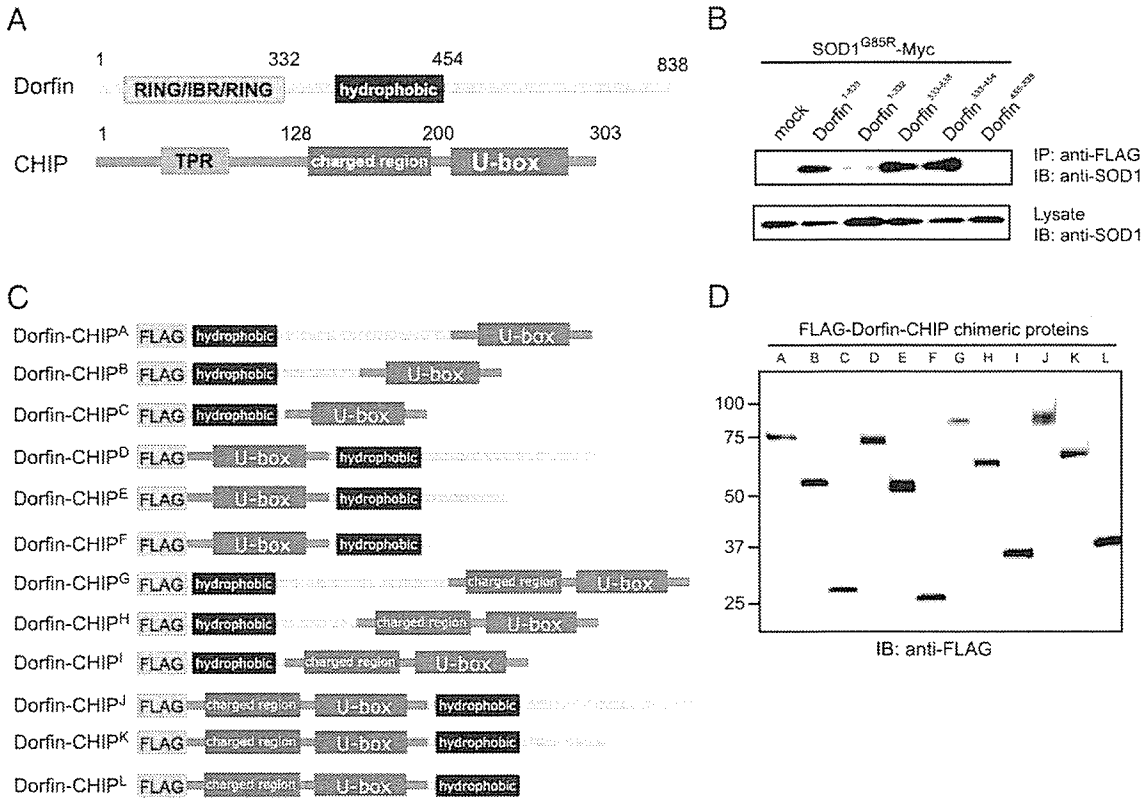


Fig. 2. Construction of Dorfin-CHIP chimeric proteins. (A) Dorfin has a RING/IBR domain in its N-terminus and a substrate-binding portion in the C-terminus. CHIP contains a TPR domain that binds to heat-shock proteins at the N-terminus; its C-terminal U-box domain has strong E3 ubiquitin ligase activity. (B) SOD1^{G85R}-Myc and FLAG-Dorfin derivatives were overexpressed in HEK 293 cells. Cell lysates were immunoprecipitated with anti-myc antibody. Immunoblotting showed that FLAG-Dorfin derivatives containing Dorfin$^{333-454}$ bound to SOD1^{G85R}-Myc, indicating that the hydrophobic region of Dorfin (Dorfin$^{333-454}$) is essential for interaction with mutant SOD1 *in vivo*. (C) Scheme of engineered Dorfin-CHIP chimeric proteins. Three different lengths of C-terminal Dorfin containing the hydrophobic region of Dorfin (Dorfin$^{333-454}$) and the U-box domain of CHIP with or without the charged region were fused. (D) Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells. Harvested cells were lysed and analyzed by immunoblotting using anti-FLAG antibody.

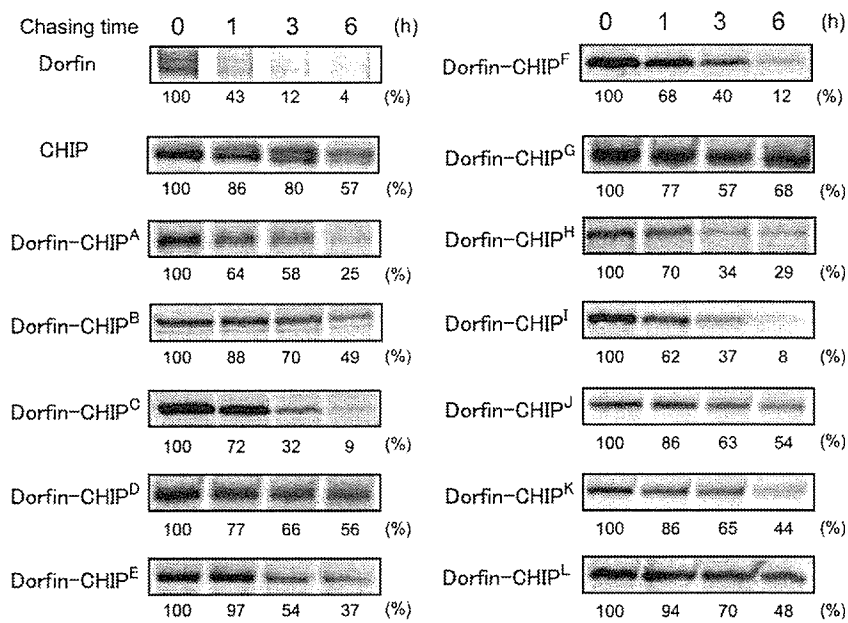


Fig. 3. The stability of Dorfin-CHIP chimeric proteins. Pulse-chase analysis using [^{35}S]-Met/Cys was performed. Dorfin, CHIP, and all the Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells and labeled with [^{35}S]-Met/Cys. Immunoprecipitation using anti-FLAG antibody and SOD-PAGE analysis revealed the degradation speed of FLAG-Dorfin-CHIP chimeric proteins. The amount of each Dorfin-CHIP chimeric protein was measured by quantifying the band using ImageGauge software.

designed Dorfin-CHIP chimeric proteins containing both the hydrophobic substrate-binding domain of Dorfin and the U-box domain of CHIP, which has strong E3 activity (Fig. 2C). We verified that all of the 12 candidate chimeric proteins were expressed in HEK293 cells (Fig. 2D).

Expression of Dorfin-CHIP chimeric proteins in cells

The half lives of all the Dorfin-CHIP chimeric proteins were more than 1 h. In some of these proteins, such as Dorfin-CHIP$^{D, G, J}$, and ^{L}, moderate amounts of protein still remained at 6 h after labeling, indicating that they were degraded much more slowly than was Dorfin^{WT} (Fig. 3). Repetitive experiments using Dorfin-CHIP^{L} yielded a significant difference between the amount of Dorfin^{WT} and Dorfin-CHIP^{L} at 1 h and 3 h (Table 1).

E3 activity of Dorfin-CHIP chimeric proteins against mutant SOD1

Immunoprecipitation analysis demonstrated that Dorfin and CHIP bound to mutant SOD1^{G85R} in equivalent amounts and that all of the Dorfin-CHIP chimeric proteins interacted with mutant SOD1^{G85R} *in vivo*. Dorfin-CHIP$^{A, D, E, F, J, K}$, and ^{L} bound to the same or greater amounts of SOD1^{G85R} than did Dorfin, whereas Dorfin-CHIP$^{B, C, G, H}$, and ^{I} did not (Fig. 4A, upper panel). None of the Dorfin-CHIP chimeric proteins bound to SOD1^{WT} *in vivo*

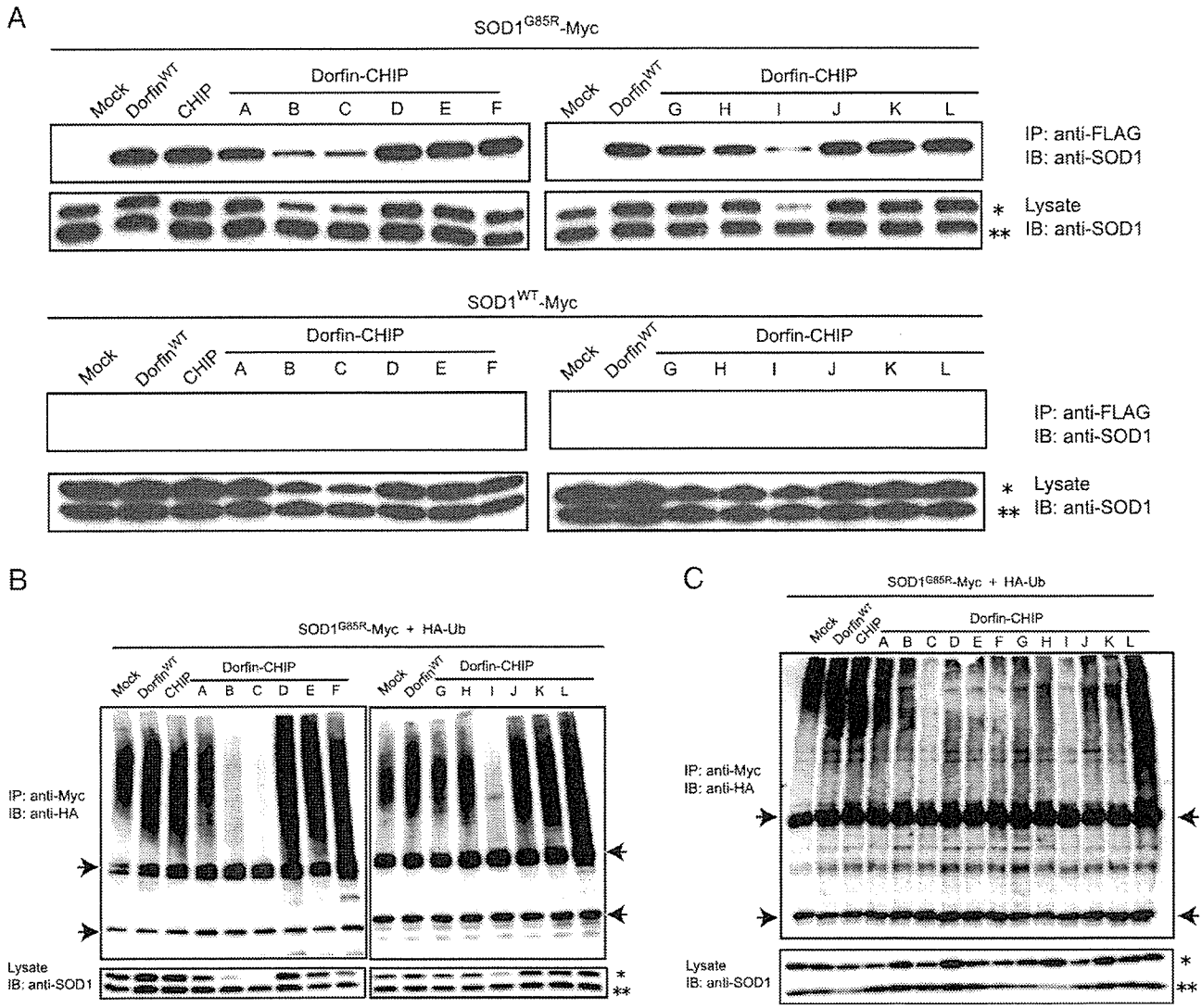


Fig. 4. The E3 activity of Dorfin-CHIP chimeric proteins on mutant SOD1 *in vivo*. (A) *In vivo* binding assay with both wild-type and mutant SOD1s. SOD1^{G85R}- or SOD1^{WT}-Myc and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoprecipitation was done using anti-Myc antibody. Immunoblotting with anti-FLAG antibody revealed that all the Dorfin-CHIP chimeric proteins bound *in vivo* to SOD1^{G85R}-Myc but not to SOD1^{WT}-Myc. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively. (B) *In vivo* ubiquitylation assay in HEK293 cells. SOD1^{G85R}-Myc, HA-Ub, and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoblotting with anti-HA antibody demonstrated the ubiquitylation level of SOD1^{G85R}-Myc by FLAG derivatives of Dorfin-CHIP chimeric proteins *in vivo*. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed SOD1 and mouse endogenous SOD1, respectively. (C) *In vivo* ubiquitylation assay in N2a cells. SOD1^{G85R}-Myc, HA-Ub, and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in N2a cells. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively.

(Fig. 4A, lower panel). Some Dorfin-CHIP chimeric proteins, such as Dorfin-CHIP$^{B, C}$, and ^{I}, had lower amounts of both SOD1^{WT} and SOD1^{G85R} in the lysates. We performed quantitative RT-PCR using specific primers for SOD1-Myc, finding that coexpression of Dorfin-CHIP$^{B, C,}$ or ^{I} suppressed the mRNA expression of overexpressed SOD1 gene (Supplementary Fig. 1). Considering the possibility that these Dorfin-CHIP chimeric proteins might have unpredicted toxicity for cells by affecting gene transcription via unknown mechanisms, we excluded them from further analysis. Other Dorfin-CHIP proteins did not affect SOD1-Myc gene expression, which validated the comparison among IPs and ubiquitylated mutant SOD1 in Figs. 4A–C.

To assess the effectiveness of the E3 activity of Dorfin-CHIP chimeric proteins, we did an *in-vivo* ubiquitylation analysis by coexpression of SOD1^{G85R}-Myc, HA-Ub, and Dorfin-CHIP chimeric proteins in HEK293 cells. We found that Dorfin and CHIP enhanced the ubiquitylation of SOD1^{G85R} protein and that the ubiquitylation levels of these two E3 ligases were almost equivalent. Moreover, Dorfin-CHIP$^{D, E, F, J, K}$, and ^{L} ubiquitylated SOD1^{G85R} more effectively than did Dorfin or CHIP (Fig. 4B).

Performing the same *in-vivo* ubiquitylation assay using N2a cells, we observed that the levels of ubiquitylation of SOD1^{G85R} by Dorfin and CHIP were equivalent, as they were in HEK293 cells. Among Dorfin-CHIP chimeric proteins, only Dorfin-CHIP^{L}

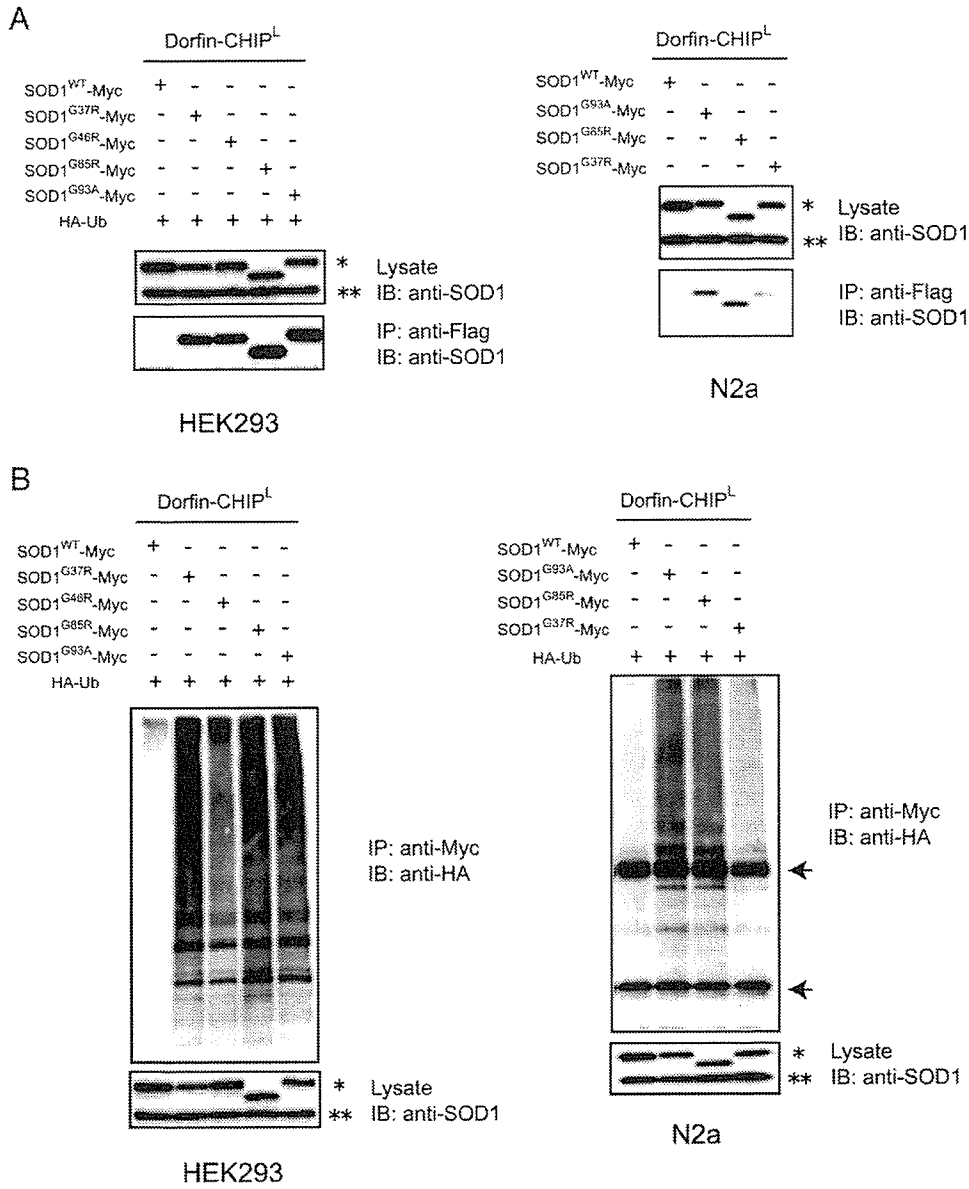


Fig. 5. Dorfin-CHIP^{L} specifically ubiquitylates mutant SOD1s *in vivo*. (A) *In vivo* binding assay with various mutant SOD1s. SOD1^{WT}-Myc, SOD1^{G93A}-Myc, SOD1^{G85R}-Myc, SOD1^{H46R}-Myc or SOD1^{G37R}-Myc, and FLAG-Dorfin-CHIP^{L} were coexpressed in HEK293 (left) and N2a cells (right). Immunoprecipitation was done using anti-Myc antibody. Immunoblotting with anti-FLAG antibody showed that both chimeric proteins specifically bound to mutant SOD1s *in vivo*. Single and double asterisks indicate overexpressed SOD1 and mouse endogenous SOD1, respectively. (B) *In vivo* ubiquitylation assay. SOD1^{WT}-Myc, SOD1^{G93A}-Myc, SOD1^{G85R}-Myc, SOD1^{H46R}-Myc or SOD1^{G37R}-Myc, as well as FLAG-Dorfin-CHIP^{L} and HA-Ub, was coexpressed in HEK293 (left) and N2a cells (right). Immunoblotting with anti-HA antibody showed the specific ubiquitylation of mutant SOD1-Myc by FLAG-Dorfin-CHIP^{L} *in vivo*. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively.

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

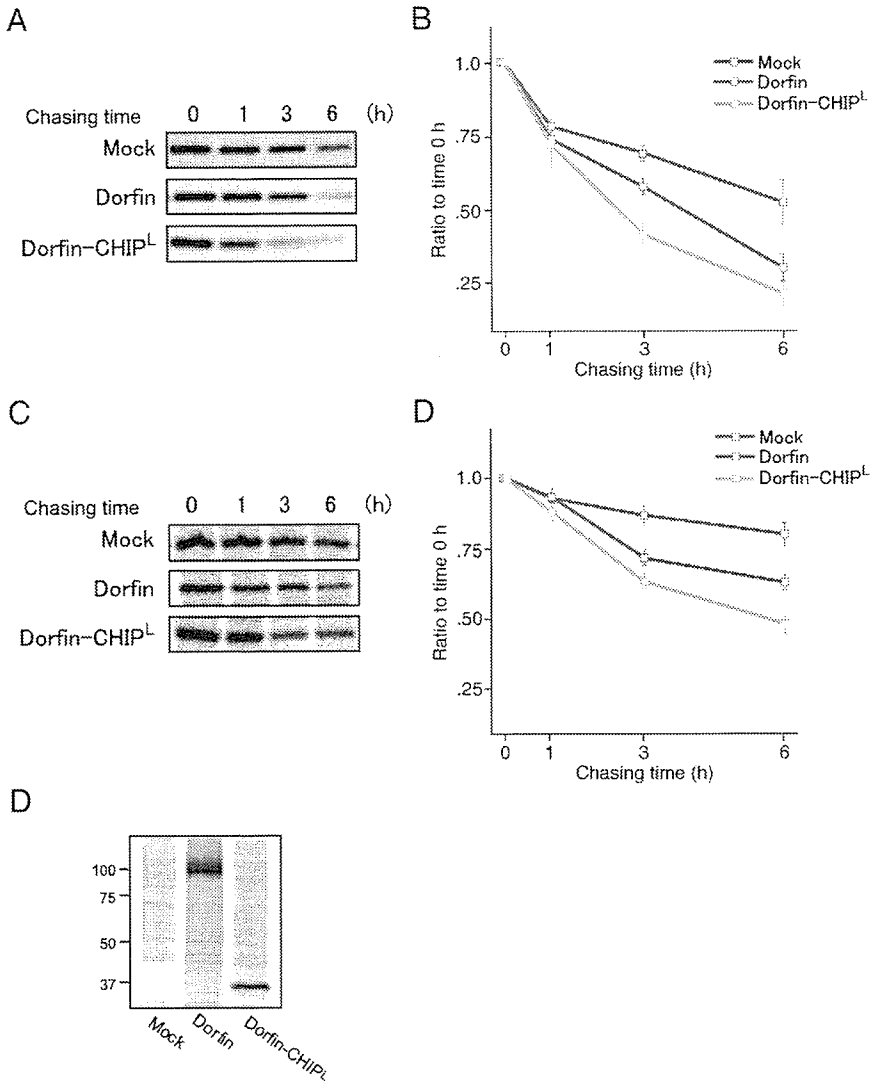


Fig. 6. Degradation of mutant SOD1 proteins with Dorfin-CHIP^{L}. (A) Pulse-chase analysis of SOD1^{G85R} with Dorfin-CHIP^{L}. N2a cells were coexpressed with SOD1^{G85R}-Myc and Mock, Dorfin, and Dorfin-CHIP^{L}. Pulse-chase experiments using [^{35}S]-Met/Cys were done. Immunoprecipitation using anti-Myc 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

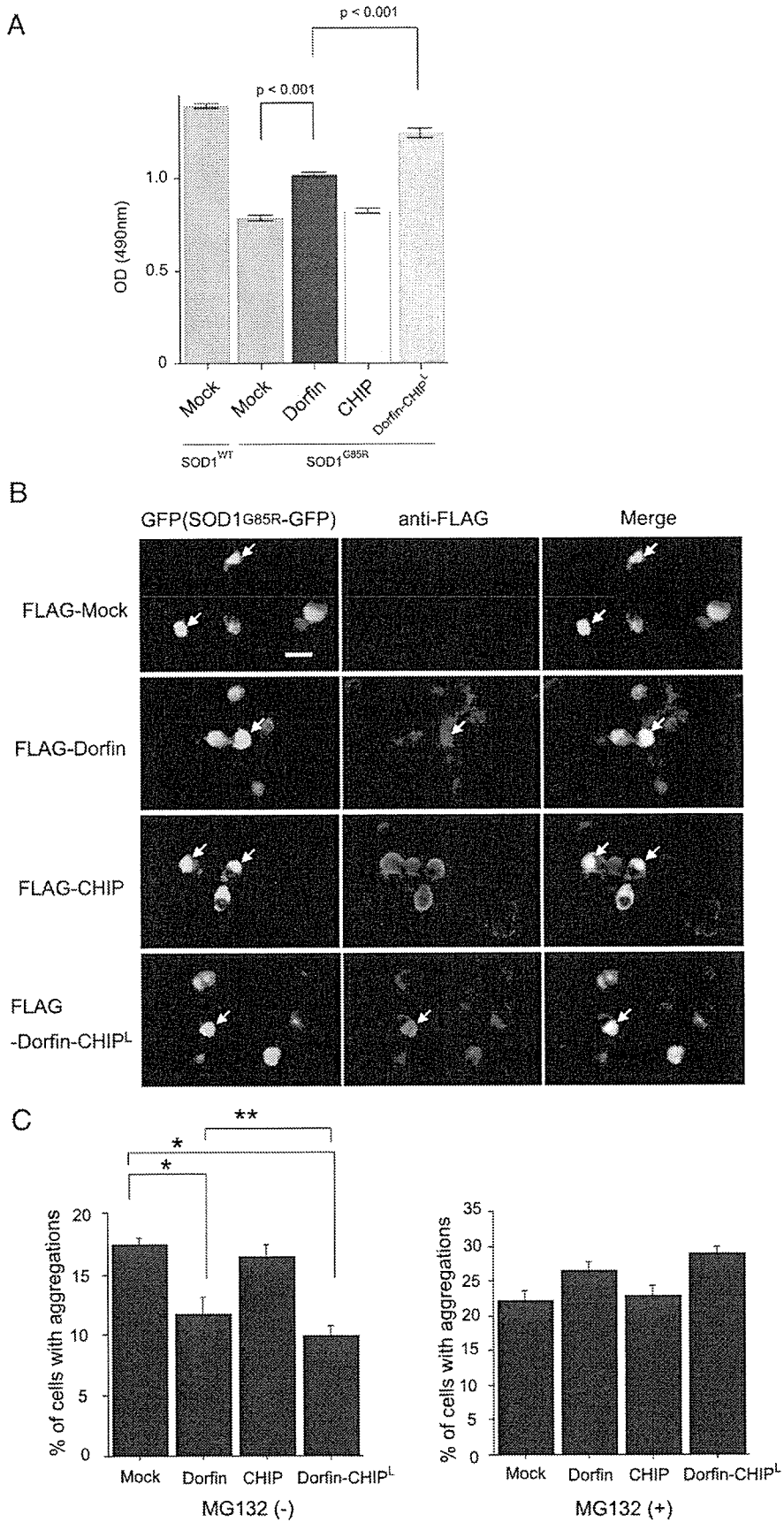
E3 proteins can specifically recognize and degrade accumulating aberrant proteins, which are deeply involved in the pathogenesis of neurodegenerative disorders, including ALS (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). For this reason, E3 proteins are candidate 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
C
**
*
*
% of cells with aggregations
20
15
10
5
0
Mock
Dorfin
CHIP
Dorfin-CHIP^L
MG132 (-)
35
30
25
20
15
10
5
0
Mock
Dorfin
CHIP
Dorfin-CHIP^L
MG132 (+)