この差は $NaNO_2$ の N (m/z:14)がキヌレニンの 2 個のアミノ基の 1 個に 2H(m/z:2)を放出させて結合する変化に相当する。エールリッヒ試薬の反応結果とあわせるとキヌレニンのジアゾ化が誘導されたと考えられる。

7. BMDC によるキヌレニンのシステム L トランスポーターによる取込み

CpG で刺激した BMDC の培養上清のキヌレニン濃度をメタノールで除タンパクして測定した。CpG 刺激によってキヌレニン濃度は増加せず、培養系に加えたキヌレニン濃度は CpG 刺激をしなくても著しく低下した。CpG の取込みはシステム L のトランスポーターのインヒビターである BCH、トリプトファンのインヒビターの 1-methyltryptophan およびトリプトファンによって抑制された。これらの結果より、BMDC は芳香族アミノ酸を広範囲に取込むシステム L トランスポーターによりキヌレニンを取込むことが明らかとなった。

C. 考察

TCA による除タンパクは HPLC によるキヌレニン測定の前処理として広く行われている。本研究は NO が産生されるときは、TCA 処理によりキヌレニンとナイトライトが反応するためにキヌレニンの濃度測定において実際の値よりも低く評価する誤りが生じることを警告している。NO 産生は IDO を mRNA レベルとタンパクレベルで抑制することが報告されてきたが、IDO 活性の測定方法にも影響する。

キヌレニンの酸性化ナイトライトによる付加生成物がキヌレニンのジアゾ化によることは、次の結果から結論した。キヌレニンの付加生成物が芳香族のアミノ基と反応するエールリッヒ試薬で検出できなかったことより、このアミノ基にナイトライトが反応したと考えられる。マススペクトロメトリーによるキヌレニンの付加生成物は分子量がキヌレニンより 12 増加したが、これはナイトライトの N がキヌレニンの芳香族環のアミノ基の 2H と置換した変化に相当する。

CpG で誘導される BMDC の IDO は NO 産生によってタンパク発現ではなく活性が抑制されていた。BMDC は IDO タンパクを発現するが、キヌレニンを放出せず、NO を多く産生すること、また抗原提示能が高いことが $CD8^-$ DC と似ている。

BMDC の他の特徴は無刺激状態で細胞に加えられたキヌレニンを盛んに取込み、CpG 刺激でその取込みは促進されたことである。インヒビターを使用した実験から、BMDC はトリプトファンなどの芳香族アミノ酸のトンスポーターの L システムでキヌレニンを取込むことが明らかとなった。最近、免疫性の $CD8^-$ DC をキヌレニン存在下で IFN-γ で刺激すると、キヌレニンを取込んで IDO 非依存性に代謝産物のキノリン酸を産生して放出し、免疫抑制活性を示すようになることが報告された。我々は BMDC をキヌレニンと IFN-γ で刺激してもそのような免疫抑制活性を誘導することはできなかったが、細胞外のキヌレニンを取込むことは BMDC と $CD8^-$ DC の共通の性質である。IDO 活性が NO 産生によって抑制されている BMDC にとって、IDO 非依存性にキヌレニンを取込んでキヌレニン代謝経路を

働かせ、生存に必要な代謝産物の NAD 産生などに利用している可能性が考えられる。

E. 結論

BMDC が CpG 刺激で産生するナイトライトは酸性条件でキヌレニンをジアゾ化するのでメタノールで除タンパクしないと誤りが生じる。BMDC の IDO 活性は NO 産生によって抑制されており、外から加えたキヌレニンを細胞内に盛んに取込む。

F. 研究危険情報

なし。

G. 研究発表

（1） 論文発表

なし。

（2） 学会発表

1. Hara T., Hiramatsu R., Kawabe T., Nagase F. Acid-initiated reaction of kynurenine to nitrite produced by bone marrow-derived myeloid dendritic cells stimulated with CpG. 第 35 回日本免疫学会、2006 年 12 月（大阪）

2.Hara T., Hiramatsu R., Akimoto H., Takikawa O., Kawabe T., Nagase F. Trichloroacetic acid-triggered reaction of kynurenine to nitrite produced by bone marrow-derived myeloid dendritic cells stimulated with CpG. The 11th Meeting of International Study Group for Tryptophan Research p71, 2006 (Tokyo).

研究分担報告書

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研究要旨

様々な神経栄養因子受容体の下流で活性化されるセリン／スレオニンキナーゼ Akt（別名 PKB）が神経細胞の突起伸長および極性の決定に重要であることが最近報告されている。しかし、その分子機構については完全には明らかにされていない。われわれは、yeast two hybrid 法を用いて Akt の新規基質である Girdin (girders of actin filament の略)を発見し、Girdin が Akt の下流で細胞運動に重要な役割を果たしていることを明らかにした。機能解析により Girdin が新規アクチン結合蛋白であり、Akt によってリン酸化を受けるとアクチン線維の再構成を生じ、運動している細胞の先導端におけるラメリポディアの形成に関与していることを証明した。本年度はさらにラット褐色細胞腫由来の PC12D 細胞および海馬神経細胞を用いて Akt／Girdin のシグナル伝達経路が神経細胞の突起形成の制御に関与していることを示した。特に海馬神経細胞において Akt は極性決定に関わっており、Girdin はその機能の一端を担う可能性があると考えられた。

A.研究目的

神経栄養因子はそのレセプターを介して神経細胞の生存・分化さらに移動、極性決定に重要なシグナルを細胞内に伝達すると考えられている。最近、神経栄養因子受容体の下流で活性化されるセリン／スレオニンキナーゼ Akt が神経細胞の生存だけでなく、突起伸長および極性の決定に重要であることが報告された。当教室で同定された Akt の新規基質 Girdin はアクチン線維結合タンパク質であり、線維芽細胞において Akt によりリン酸化されることで細胞骨格の再構成や細胞運動に重要な機能を有する分子であることが明らかにされている。最近の研究によって Girdin の mRNA がマウス胎児の中枢神経系および後根神経節細胞に高発現していることが示された。本研究では、神経系の発生・分化における Akt および Girdin の機能について、マウス胚の固定組織標本と神経系培養細胞を用いて、形態学的、細胞生物学的手法により検討した。

A. 研究方法

(1) 胎生 13-17 日目のマウス胚の脳の固定組織切片を作成し、抗 Girdin 抗体を用いて免疫染色を行い、その発現様式を検討した。

(2) ラット褐色細胞腫由来の PC12D 細胞に Akt の恒常的活性型変異体（AktDA）あるいはベクター型 Girdin siRNA の導入し、形態変化を観察した。

(3) 胎生 19 日目のラット胎児から海馬神経細胞を採取した。培養 1 日目にベクター型 siRNA を導入し、培養 5 日目（DIV5, Stage4）において Girdin のノックダウンが神経細胞分化に与える影響を観察した。

B. 研究結果

(1) 胎生 13-17 日目のマウス胚の脳の固定組織切片を作成し、抗 Girdin 抗体を用いて免疫染色を行ったところ、脳皮質等の神経線維に一致して Girdin およびアクチン線維の局在が観察された。Girdin が神経細胞で強く発現していることが明らかになった。

(2) ラット褐色細胞腫由来の PC12D 細胞は神経成長因子 (NGF) 刺激によって神経細胞へ分化し、突起を伸ばすことが知られている。PC12D 細胞に Akt の恒常的活性型変異体（AktDA）を強制発現したところ、NGF 刺激下で神経突起の数は減少しする一方、突起の長さが有意に延長する現象が観察された。よって、Akt は神経分化における神経突起の伸長および特異性を制御している可能性が示唆された。

さらに、PC12D 細胞に Girdin が発現していること、NGF 刺激下で Girdin が Akt 依存的にリン酸化されることをウエスタンブロットにより確認した。ベクター型 siRNA の導入により内因性の Girdin をノックダウンした細胞では、神経突起の数は変化しなかったが、伸長が有意に抑制された。AktDA の強制発現による上記の表現系は Girdin のノックダウンにより抑制される傾向が観察された。

(3) 胎生 19 日目のラット胎児から海馬神経細胞を採取し、培養 5 日目において Girdin の発現をウエスタンブロットおよび免疫染色にて確認した。培養 1 日目にベクター型 siRNA を導入し、培養 5 日目（DIV5, Stage4）において Girdin のノックダウンが神経細胞分化に与える影響を観察した結果、Girdin のノックダウンにより神経樹状突起の数が抑制され、tau-1 陽性の軸索の数も減少傾向がみられた。AktDA の発現により複数の軸索が形成されることが最近報告されているが、Girdin をノックダウンした細胞では AktDA による軸索の複数形成が阻害された。

C. 考察

本研究において、神経系の発生・分化においてAkt／Girdin のシグナル伝達経路が神経細胞の突起形成や極性決定の制御に関与していることが示唆された。しかしながら、このような生物学的現象には多くの分子が関与すことが知られており、Akt／Girdin シグナル伝達経路が他の機能分子とどのような相互作用を有するかを解析することが今後の課題である。

D. 結論

神経系の発生・分化において Akt／Girdin のシグナル伝達経路が神経細胞の突起形成の制御に関与していることが示唆された。特に海馬神経細胞において Akt は極性決定に関わっており、Girdin はその機能の一端を担う可能性があると考えられた。

E. 研究発表

1. Analyses of ultraviolet-induced focus formation of hREV1 protein.
Genes Cells 11: 193-205 (2006).

2. Takeda, K., Kawamoto, Y., Okuno, Y, Kato, M., Takahashi, M., Suzuki, H., Isobe, K. and Nakashima, I.
A PKC-mediated backup mechanism of the MXXCW motif-linked switch for initiating tyrosine kinase activities.
FEBS Lett. 580:839-843 (2006).

3. Asai, N., Jijiwa, M., Enomoto, A., Kawai, K., Maeda, K., Ichihara, M. and Takahashi M.
RET receptor signaling; Dysfunction in thyroid cancer and Hirschsprung's disease.
Pathol Int. 56: 164-172 (2006).

4. Kato, M., Takeda, K., Kawamoto, Y., Tsuzuki, T., Kato, Y., Ohno, T., Hossain, K., Iftakhar-E-Khuda, I., Ohgami, N., Isobe, K., Takahashi, M.and Nakashima, I.
Novelhairless RET-transgenic mouse line with melanocytic nevi and anagen hairfollicles.
J. Invest. Dermatol. 126: 2547-2550 (2006).

5. Uchida, M., Enomoto, A., Fukuda, T., Kurokawa, K., Maeda, K., Kodama, Y., Asai, N., Hasegawa, T., Shimono, Y., Jijiwa, M., Ichihara, M., Murakumo, Y. and Takahashi, M.
Dok-4 regulates GDNF-dependent neurite outgrowth through downstream activation of Rap1 and mitogen-activated protein kinase.
J. Cell Sci. 119: 3067-3077 (2006).

6. Asai, N., Fukuda, T., Wu, Z., Enomoto, A., Pachnis, V., Takahashi, M. and Costantini, F.
Targeted mutation of serine 697 in the Ret tyrosine kinase causes migration defect of enteric neural crest cells.
Development 133: 4507-4516 (2006).

7.. Murakumo, M., Jijiwa, M., Asai, N., Ichihara, M., and Takahashi, M.
RET and neuroendocrine tumors.
Pituitary 9: 179-192 (2006).

8. Enomoto, A., Jiang, P., and Takahashi, M.
Girdin, a novel actin-binding protein, and its

family of proteins posses versatile function in the Akt and Wnt signaling pathways.
Ann. NY Acad. Sci., 1086: 169-184 (2006).

9. Kato, M., Ohgami, N., Kawamoto, Y., Tsuzuki, T., Hossain, K., Yanagishita, T., Ohshima, Y., Tsuboi, H., Yamanoshita, O., Matsumoto, Y., Takahashi, M. and Nakashima, I.
Protective effect of hyperpigmented skin on UV-mediated cutaneous cancer development.
J. Invest. Dermatol. in press.

F. 学会発表

1. 内田真由美、榎本　篤、高橋雅英
神経分化におけるRETチロシンキナーゼとアダプタータンパク質Dok-4の機能解析
第95回日本病理学会学術総会　2006年5月
2. 浅井直也、高橋雅英
神経堤細胞の移動による腸管神経形成に作用するシグナルの解析
第95回日本病理学会学術総会　2006年5月
3. 石田麻紀、市原正智、村雲芳樹、高橋雅英
RET受容体型チロシンキナーゼのシグナル伝達系におけるSprouty蛋白質の機能解析
第65回日本癌学会学術総会　2006年9月
4. 石田麻紀、市原正智、村雲芳樹、高橋雅英
RET受容体型チロシンキナーゼのシグナル伝達系におけるSprouty蛋白質の機能解析
日本分子生物学会フォーラム 2006　2006年12月
5. 浅井直也、福田敏史、榎本　篤、高橋雅英
Retチロシンキナーゼの S697A 変異によって胎生期の腸管神経前駆細胞の移動が障害される
日本分子生物学会フォーラム 2006　2006年12月

III. 研究成果の刊行に関する一覧表

Ishigaki S, Niwa JI, Yamada SI, Takahashi M, Ito T, Sone J, Doyu M, Urano F, Sobue G.Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant sod1 proteins and reduce their cellular toxicity.
Neurobiol Dis. In press

Ito S, Kimura K, Haneda M, Ishida Y, Sawada M , Isobe KI. Induction of Matrix metalloproteinases (MMP3, MMP12 and MMP13) expression in the microglia by amyloid-b stimulation via the PI3K/Akt pathways Exp. Gerontology in press.

Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF, Kamalanathan S, Hattori Y, Ignarro LJ, Iguchi A. Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes.
Proc Natl Acad Sci U S A. 2006 Nov 7;103(45):17018-23.

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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

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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).

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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

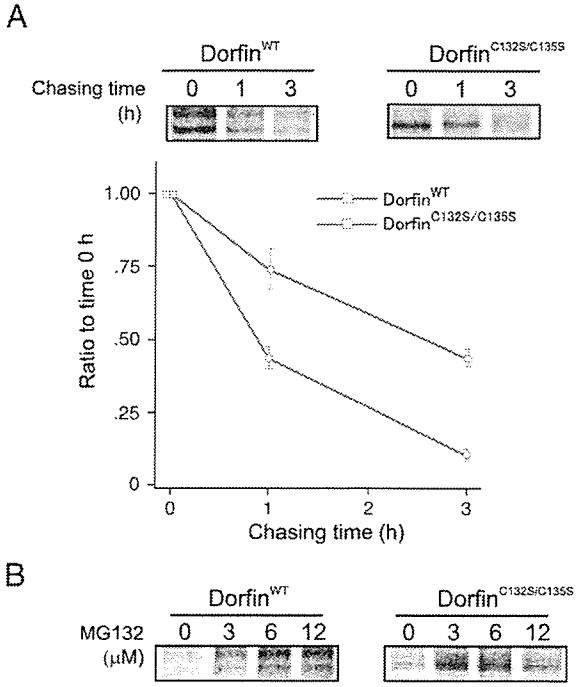


Fig. 1. Pulse-chase analysis of Dorfin^{WT} and Dorfin$^{C132S/C135S}$. (A) Dorfin^{WT} or Dorfin$^{C132S/C135S}$ was overexpressed in HEK293 cells. After overnight incubation, [^{35}S]-labeled Met/Cys pulse-chase analysis was performed. Cells were harvested and analyzed at 0, 1, or 3 h after labeling 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.

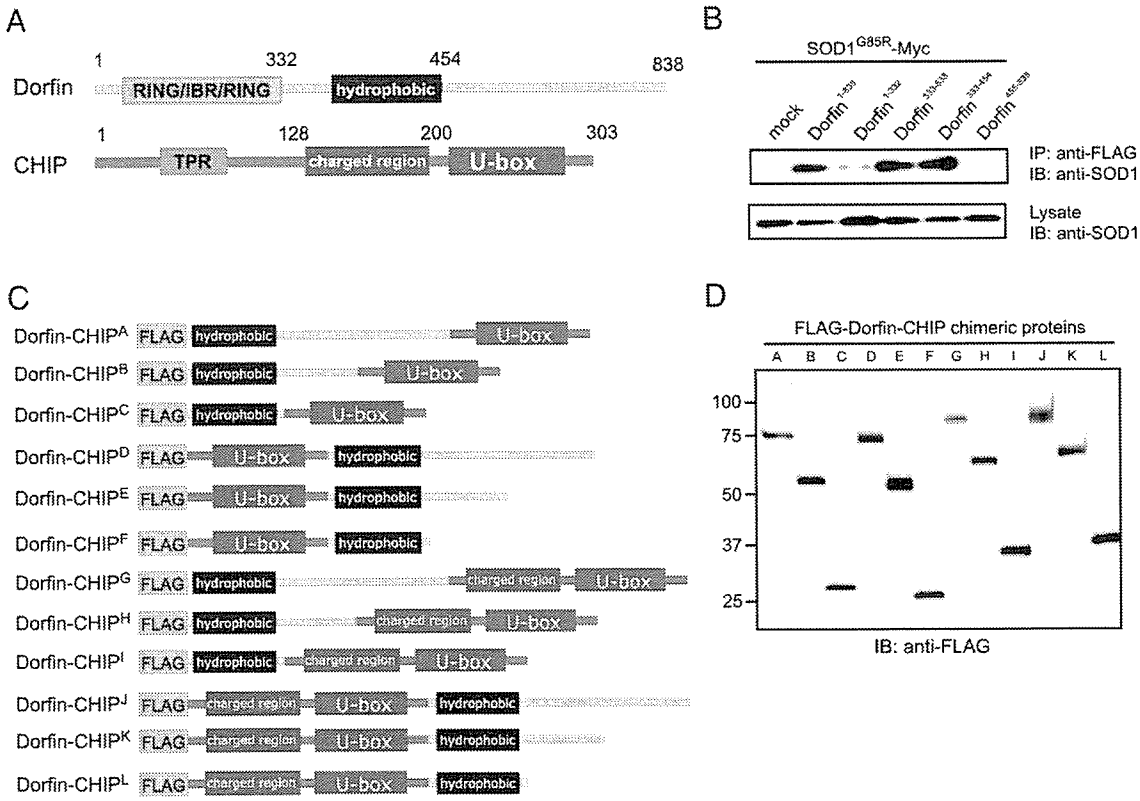


Fig. 2. Construction of Dorfin-CHIP chimeric proteins. (A) Dorfin has a RING/IBR domain in its N-terminus and a substrate-binding portion in the C-terminus. CHIP contains a TPR domain that binds to heat-shock proteins at the N-terminus; its C-terminal U-box domain has strong E3 ubiquitin ligase activity. (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.

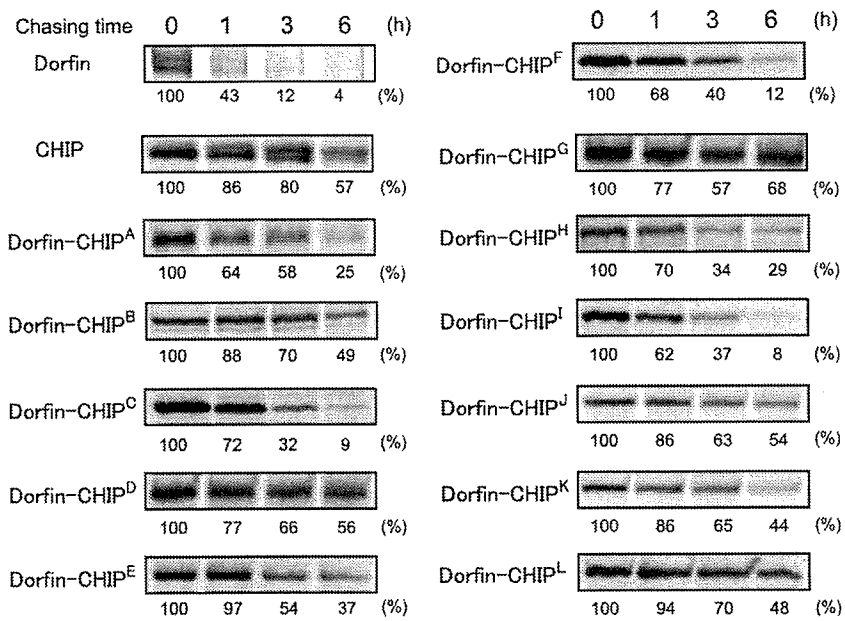


Fig. 3. The stability of Dorfin-CHIP chimeric proteins. Pulse-chase analysis using [^{35}S]-Met/Cys was performed. Dorfin, CHIP, and all the Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells and labeled with [^{35}S]-Met/Cys. Immunoprecipitation using anti-FLAG antibody and SOD-PAGE analysis 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*

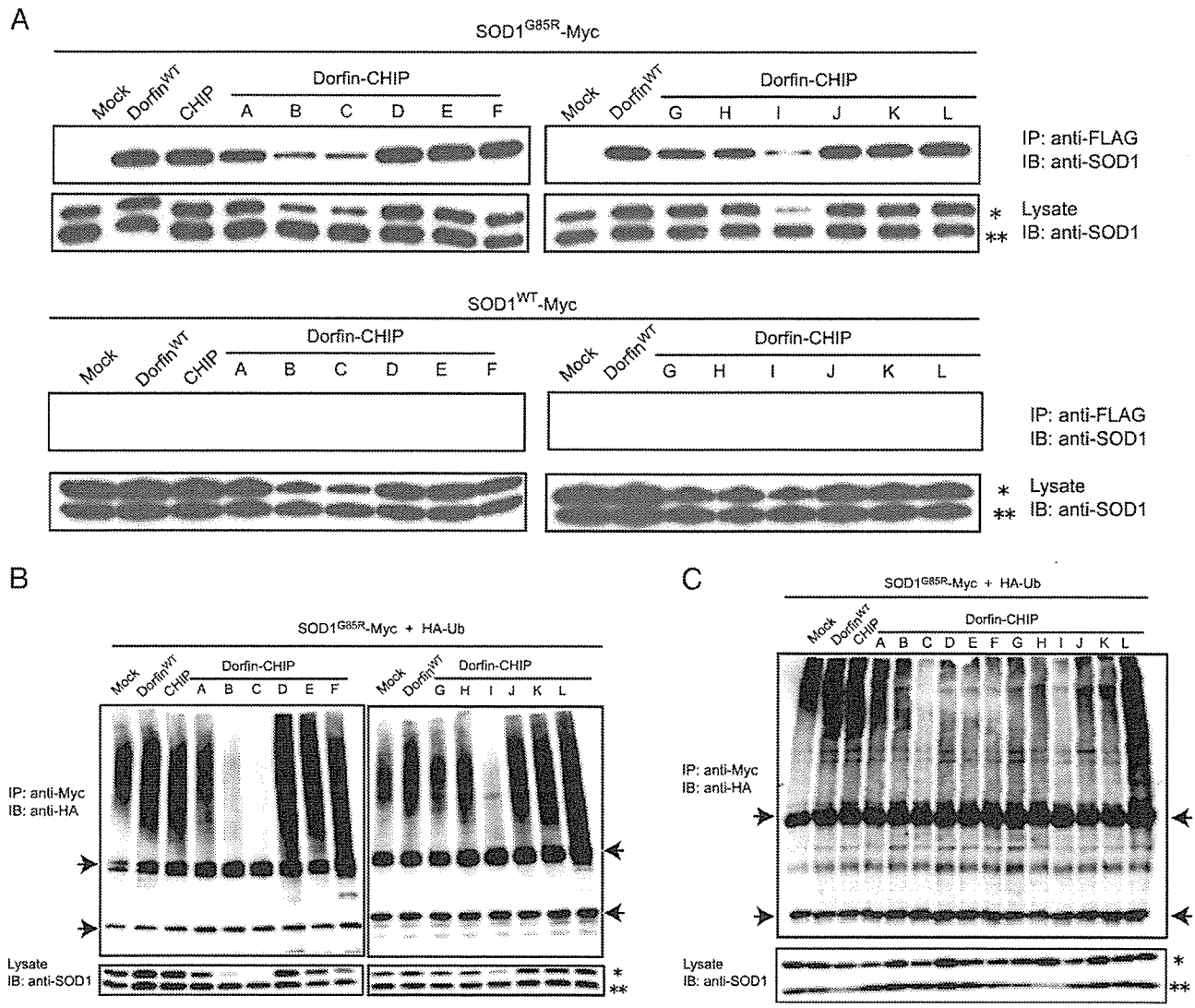


Fig. 4. The E3 activity of Dorfin-CHIP chimeric proteins on mutant SOD1 *in vivo*. (A) *In vivo* binding assay with both wild-type and mutant SOD1s. SOD1^{G85R}- or SOD1^{WT}-Myc and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoprecipitation was done using 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}

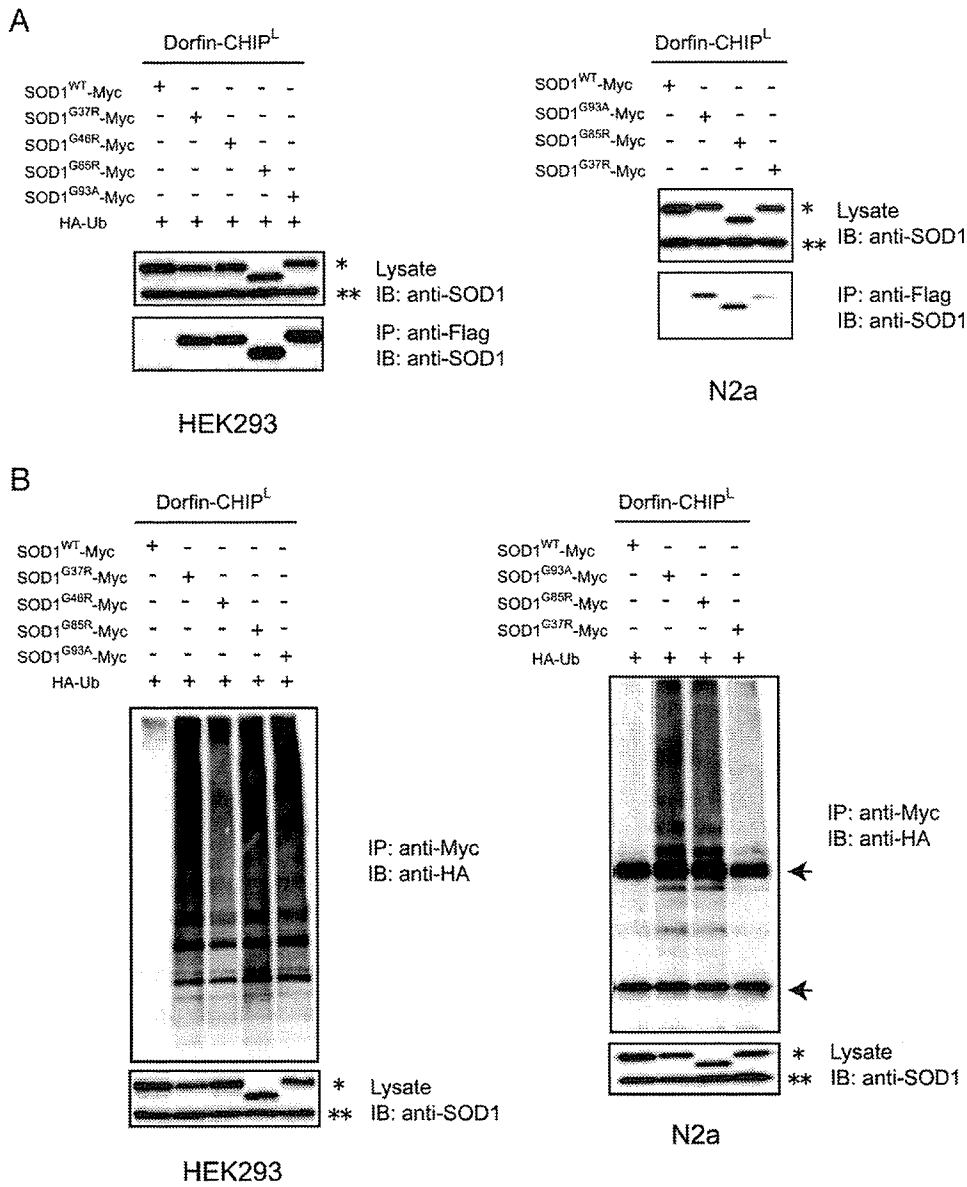


Fig. 5. Dorfin-CHIP^{L} specifically ubiquitylates mutant SOD1s *in vivo*. (A) *In vivo* binding assay with various mutant SOD1s. SOD1^{WT}-Myc, SOD1^{G93A}-Myc, SOD1^{G85R}-Myc, SOD1^{H46R}-Myc or SOD1^{G37R}-Myc, and FLAG-Dorfin-CHIP^{L} were coexpressed in HEK293 (left) and N2a cells (right). Immunoprecipitation 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

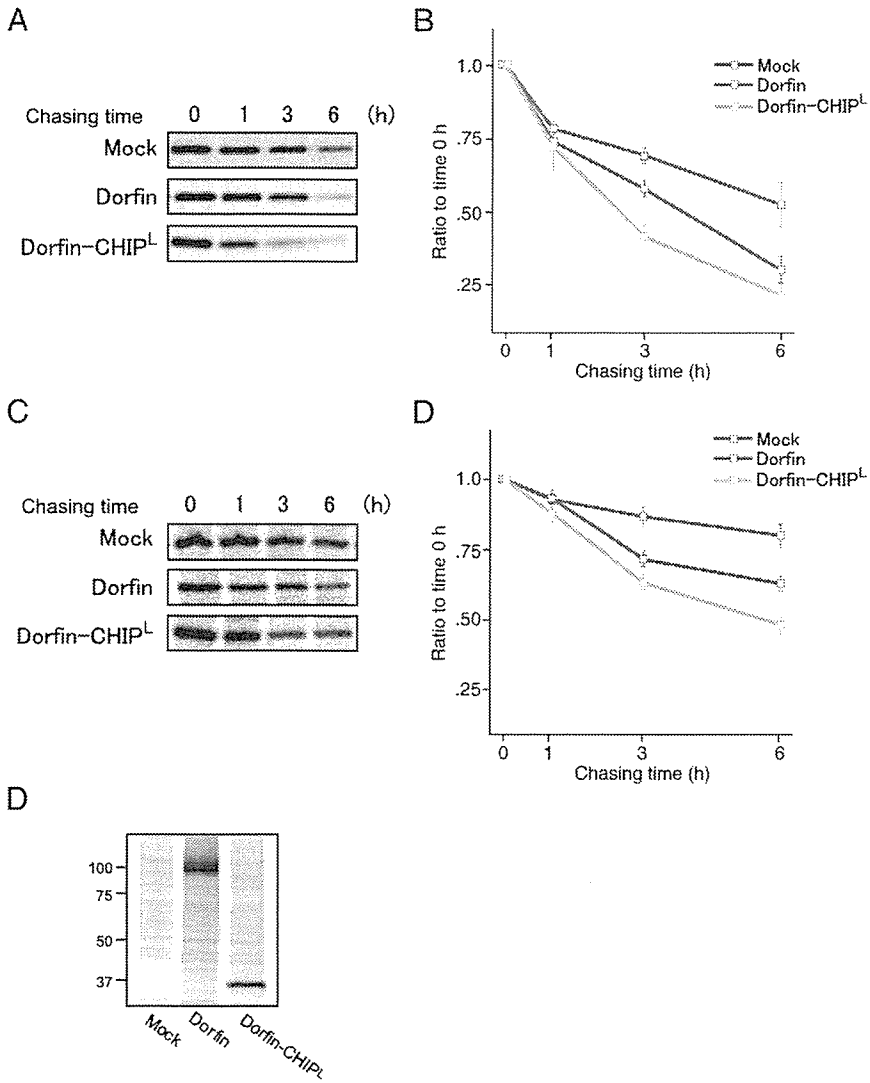


Fig. 6. Degradation of mutant SOD1 proteins with Dorfin-CHIP^{L}. (A) Pulse-chase analysis of SOD1^{G85R} with Dorfin-CHIP^{L}. N2a cells were coexpressed with SOD1^{G85R}-Myc and Mock, Dorfin, and Dorfin-CHIP^{L}. Pulse-chase experiments using [^{35}S]-Met/Cys were done. Immunoprecipitation using anti-Myc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

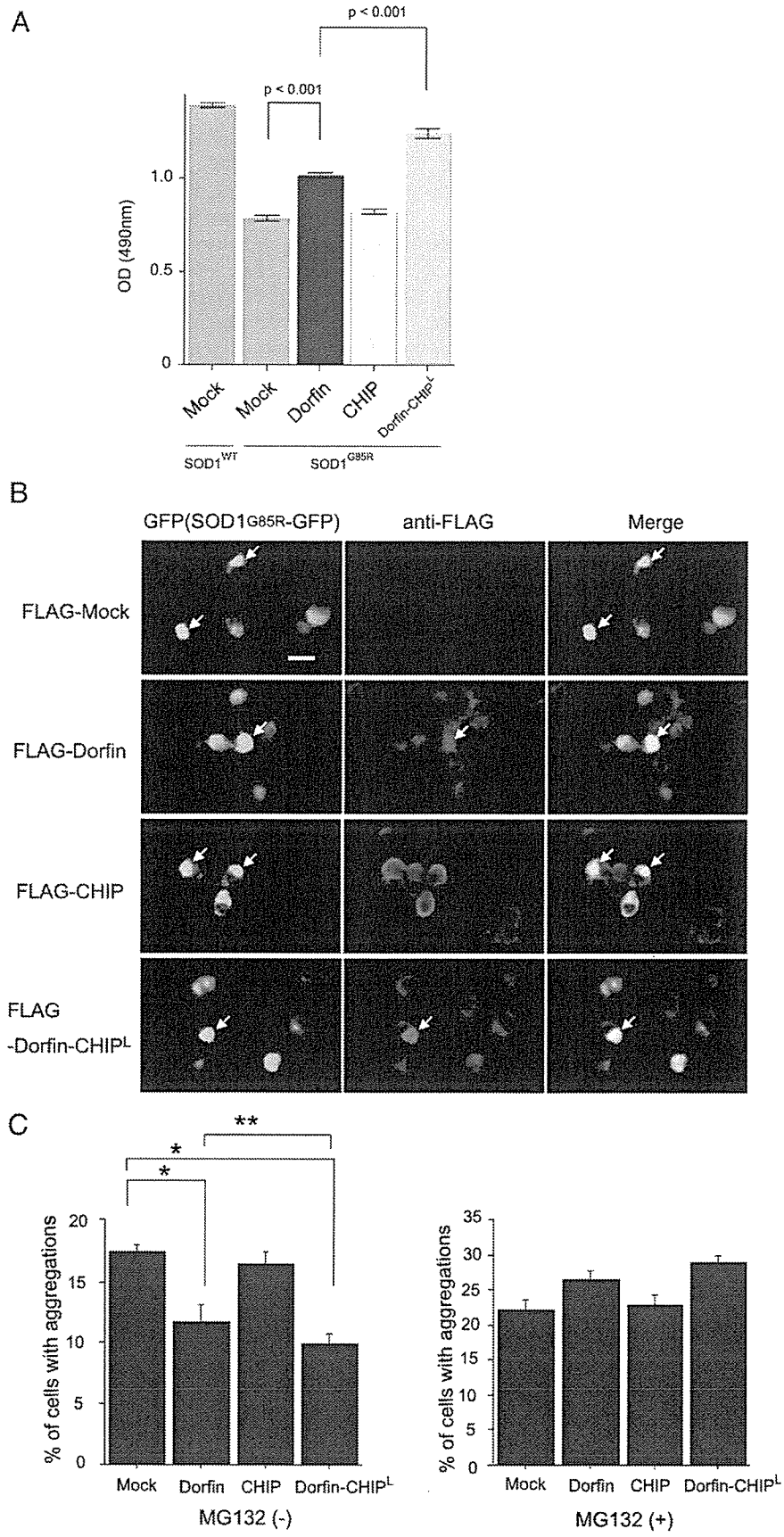
E3 proteins can specifically recognize and degrade accumulating aberrant proteins, which are deeply involved in the pathogenesis of neurodegenerative disorders, including ALS (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). For this reason, E3 proteins are candidate 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(SOD1^{G85R}-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 (+)

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

We gratefully thank Dr. Shigetsugu Hatakeyama at Hokkaido University for his advice about the construction of Dorfin-CHIP chimeric proteins. This work was supported by the Nakabayashi Trust for ALS Research; a grant for Center of Excellence (COE) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and grants from the Ministry of Health, Welfare and Labor of Japan.

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.

References

Alves-Rodrigues, A., Gregori, L., Figueiredo-Pereira, M.E., 1998. Ubiquitin, cellular inclusions and their role in neurodegeneration. Trends Neurosci. 21, 516–520.

Bercovich, B., Stancovski, I., Mayer, A., Blumenfeld, N., Laszlo, A., Schwartz, A.L., Ciechanover, A., 1997. Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70. J. Biol. Chem. 272, 9002–9010.

Ciechanover, A., Brundin, P., 2003. The ubiquitin proteasome system in

neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron 40, 427–446.

Cudkowicz, M.E., McKenna-Yasek, D., Sapp, P.E., Chin, W., Geller, B., Hayden, D.L., Schoenfeld, D.A., Hosler, B.A., Horvitz, H.R., Brown, R.H., 1997. Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. Ann. Neurol. 41, 210–221.

Glickman, M.H., Ciechanover, A., 2002. The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.

Hatakeyama, S., Matsumoto, M., Kamura, T., Murayama, M., Chui, D.H., Planel, E., Takahashi, R., Nakayama, K.I., Takashima, A., 2004. U-box protein carboxyl terminus of Hsc70-interacting protein (CHIP) mediates poly-ubiquitylation preferentially on four-repeat Tau and is involved in neurodegeneration of tauopathy. J. Neurochem. 91, 299–307.

Hatakeyama, S., Watanabe, M., Fujii, Y., Nakayama, K.I., 2005. Targeted destruction of c-Myc by an engineered ubiquitin ligase suppresses cell transformation and tumor formation. Cancer Res. 65, 7874–7879.

Hishikawa, N., Niwa, J., Doyu, M., Ito, T., Ishigaki, S., Hashizume, Y., Sobue, G., 2003. Dorfin localizes to the ubiquitylated inclusions in Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, and amyotrophic lateral sclerosis. Am. J. Pathol. 163, 609–619.

Ishigaki, S., Liang, Y., Yamamoto, M., Niwa, J., Ando, Y., Yoshihara, T., Takeuchi, H., Doyu, M., Sobue, G., 2002a. X-Linked inhibitor of apoptosis protein is involved in mutant SOD1-mediated neuronal degeneration. J. Neurochem. 82, 576–584.

Ishigaki, S., Niwa, J., Ando, Y., Yoshihara, T., Sawada, K., Doyu, M., Yamamoto, M., Kato, K., Yotsumoto, Y., Sobue, G., 2002b. Differentially expressed genes in sporadic amyotrophic lateral sclerosis spinal cords—Screening by molecular indexing and subsequent cDNA microarray analysis. FEBS Lett. 531, 354–358.

Ishigaki, S., Hishikawa, N., Niwa, J., Iemura, S., Natsume, T., Hori, S., Kakizuka, A., Tanaka, K., Sobue, G., 2004. Physical and functional interaction between Dorfin and Valosin-containing protein that are colocalized in ubiquitylated inclusions in neurodegenerative disorders. J. Biol. Chem. 279, 51376–51385.

Ito, T., Niwa, J., Hishikawa, N., Ishigaki, S., Doyu, M., Sobue, G., 2003. Dorfin localizes to Lewy bodies and ubiquitylates synphilin-1. J. Biol. Chem. 278, 29106–29114.

Johnston, J.A., Ward, C.L., Kopito, R.R., 1998. Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. 143, 1883–1898.

Julien, J.P., 2001. Amyotrophic lateral sclerosis. unfolding the toxicity of the misfolded. Cell 104, 581–591.

Jungmann, J., Reins, H.A., Schobert, C., Jentsch, S., 1993. Resistance to cadmium mediated by ubiquitin-dependent proteolysis. Nature 361, 369–371.

Lee, D.H., Sherman, M.Y., Goldberg, A.L., 1996. Involvement of the molecular chaperone Ydj1 in the ubiquitin-dependent degradation of short-lived and abnormal proteins in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 16, 4773–4781.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., Cyr, D.M., 2001. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat. Cell Biol. 3, 100–105.

Miyazaki, K., Fujita, T., Ozaki, T., Kato, C., Kurose, Y., Sakamoto, M., Kato, S., Goto, T., Itoyama, Y., Aoki, M., Nakagawara, A., 2004. NEDL1, a novel ubiquitin–protein isopeptide ligase for dishevelled-1, targets mutant superoxide dismutase-1. J. Biol. Chem. 279, 11327–11335.

Murata, S., Minami, Y., Minami, M., Chiba, T., Tanaka, K., 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. 2, 1133–1138.

Murata, S., Chiba, T., Tanaka, K., 2003. CHIP: a quality-control E3 ligase collaborating with molecular chaperones. Int. J. Biochem. Cell Biol. 35, 572–578.

Niwa, J., Ishigaki, S., Doyu, M., Suzuki, T., Tanaka, K., Sobue, G., 2001. A novel centrosomal ring-finger protein, dorfin, mediates ubiquitin ligase activity. Biochem. Biophys. Res. Commun. 281, 706–713.

Niwa, J., Ishigaki, S., Hishikawa, N., Yamamoto, M., Doyu, M., Murata, S., Tanaka, K., Taniguchi, N., Sobue, G., 2002. Dorfin ubiquitylates mutant SOD1 and prevents mutant SOD1-mediated neurotoxicity. J. Biol. Chem. 277, 36793–36798.

Oyake, D., Nishikawa, H., Koizuka, I., Fukuda, M., Ohta, T., 2002. Targeted substrate degradation by an engineered double RING ubiquitin ligase. Biochem. Biophys. Res. Commun. 295, 370–375.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.

Ross, C.A., Poirier, M.A., 2004. Protein aggregation and neurodegenerative disease. Nat. Med. 10, S10–S17 (Suppl.).

Rowland, L.P., Shneider, N.A., 2001. Amyotrophic lateral sclerosis. N. Engl. J. Med. 344, 1688–1700.

Sakamoto, K.M., Kim, K.B., Kumagai, A., Mercurio, F., Crews, C.M., Deshaies, R.J., 2001. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. Proc. Natl. Acad. Sci. U. S. A. 98, 8554–8559.

Sakamoto, K.M., Kim, K.B., Verma, R., Ransick, A., Stein, B., Crews, C.M., Deshaies, R.J., 2003. Development of Protacs to target cancer-promoting proteins for ubiquitination and degradation. Mol. Cell Proteomics 2, 1350–1358.

Scheffner, M., Nuber, U., Huibregtse, J.M., 1995. Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. Nature 373, 81–83.

Sherman, M.Y., Goldberg, A.L., 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29, 15–32.

Shimura, H., Schwartz, D., Gygi, S.P., Kosik, K.S., 2004. CHIP–Hsc70 complex ubiquitinates phosphorylated tau and enhances cell survival. J. Biol. Chem. 279, 4869–4876.

Tanaka, K., Suzuki, T., Hattori, N., Mizuno, Y., 2004. Ubiquitin, proteasome and parkin. Biochim. Biophys. Acta 1695, 235–247.

Urushitani, M., Kurisu, J., Tateno, M., Hatakeyama, S., Nakayama, K., Kato, S., Takahashi, R., 2004. CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70. J. Neurochem. 90, 231–244.

Yoshida, Y., Tokunaga, F., Chiba, T., Iwai, K., Tanaka, K., Tai, T., 2003. Fbs2 is a new member of the E3 ubiquitin ligase family that recognizes sugar chains. J. Biol. Chem. 278, 43877–43884.

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Induction of matrix metalloproteinases (MMP3, MMP12 and MMP13) expression in the microglia by amyloid-β stimulation via the PI3K/Akt pathway

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Received 21 June 2006; received in revised form 2 November 2006; accepted 21 November 2006

Abstract

Alzheimer's disease is characterized by the presence of senile plaques in the brain composed primarily of amyloid-β peptide. Microglia have been reported to surround these Aβ plaques, which have opposite roles, provoking a microglia-mediated inflammatory response that contributes to neuronal cell loss or the removal of Aβ and damaged neurons. We herein analyzed the process of expression of Matrix metalloproteinases induced by Aβ stimulation. We found that Aβ1-42 induces a high level of MMP3, MMP12 and MMP13 in the microglia. The signal transduction pathway for the expression of these MMPs mRNA induced by Aβ1-42 depends on PI3K/Akt.

Keywords: Microglia; Alzheimer's disease; Amyloid β; MMP; Akt

1. Introduction

Alzheimer's disease (AD) is characterized by the presence of senile plaques in the brain composed primarily of amyloid-β peptide (Aβ). Microglia have been reported to surround such Aβ plaques (Haga et al., 1989; Itagaki et al., 1989). Microglia stimulated with Aβ may promote the death of neurons by producing free radicals or cytokines (Meda et al., 1995; Ishii et al., 2000; McDonald et al., 1997). On the contrary, microglia may clear Aβ through phagocytosis (Frautschy et al., 1992; Wyss-Coray et al., 2001; Rogers et al., 2002). In previous work we have shown that Aβ induces proliferation of microglia and produces M-CSF (Ito et al., 2005). These results suggest that innate immune responses may work as pathogenesis of AD.

Microglia belongs to the family of tissue macrophages. Monocytes/macrophages are prominent cells at sites of chronic inflammation and have been shown to produce Matrix metalloproteinases (MMPs), when activated by agents such as LPS, Con A (Wahl and Lampel, 1987; Lu and Wahl, 2005). MMPs have been implicated as being of pathological significance in the extracellular matrix degradation seen in rheumatoid arthritis, osteoarthritis, atherosclerosis, asthma and inflammatory bowel disease (Mahmoodi et al., 2005; Gueders et al., 2005; Naito and Yoshikawa, 2005; Maier et al., 2004). The relationship between MMPs and AD has been suggested. MMPs may prevent disease progression by degradation of Aβ. On the other hand MMPs may engage the disease progression by degrading brain matrix. Here, we investigate the several kinds of MMPs, which are induced by microglia. Further we examined the signaling pathways, which induce the expression of MMPs by Aβ.

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doi:10.1016/j.exger.2006.11.012

Please cite this article in press as: Ito, S. et al., Induction of matrix metalloproteinases (MMP3, MMP12 ..., Exp. Gerontol. (2007), doi:10.1016/j.exger.2006.11.012

2. Materials and methods

2.1. Materials

Synthetic human Aβ1-42 and Aβ25-35 were obtained from Peptide Institute Inc. Aβ25-35 was dissolved in H_2O and Aβ1-42 was dissolved in 0.1% NH_3 according to the manufacturer's instructions. Anti-MMP3 monoclonal antibody was from R&D Systems, Inc. and anti-MMP12 polyclonal antibody was from Santa Cruz Biotechnology, Inc. Anti-phospho-Akt (Serine 473), anti-Akt, antibodies were from Cell Signaling. Wortmannin and PD98059 were from Calbiochem. SB203580 was from Promega. All inhibitors were resolved in DMSO. Mouse recombinant granulocyte-macrophage colony-stimulating factor (mrGM-CSF) was from BD Bioscience Pharmingen.

2.2. Cell culture

The microglial cell line Ra2 was cultured in MGI medium [Eagle's MEM supplemented with 0.2% glucose, 5 μg/ml bovine Insulin (Sigma–Aldrich), 10% fetal bovine serum (FBS, Invitrogen)] and 0.8 ng/ml mrGM-CSF (Ito et al., 2005). Before Aβ-treatment, the Ra2 cells were cultured in an MGI medium without mrGM-CSF for 16 h. Primary microglia and primary astrocytes were prepared using newborn C57BL/6 mice and cultured as described previously (Ito et al., 2005), and cultured in MGI medium containing 0.8 ng/ml mrGM-CSF. Primary neurons were obtained from the cortex of 14-day-old C57BL/6 mouse embryos as described previously (Ito et al., 2005).

2.3. RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Conventional RT-PCR was performed at the condition of 30 cycles for MMPs or 23 cycles for β-actin at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Quantitative real-time PCR was performed on Mx3000P (Promega) using the following program: 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 20 s at 60 °C, and a dissociation reaction. The reactions were carried out using 0.5 μl cDNA with SYBR Premix EX Taq (Takara). Specificity of the PCR product was confirmed by examination of dissociation reaction plots. The values were expressed as the relative expression was normalized to β-actin mRNA. For RT-PCR and real-time quantitative PCR, the primers for mouse MMPs and β-actin genes were listed in Table 1.

Table 1
RT-PCR primers

Target (product size)		Sequence (5′-3′)
MMP2 (203 bp)	Sense	CACACCAGGTGAAGGATGTG
	Antisense	AGGGCTGCATTGCAAATATC
MMP3 (173 bp)	Sense	CAGACTTGTCCCGTTTCCAT
	Antisense	GGTGCTGACTGCATCAAAGA
MMP8 (250 bp)	Sense	CCTATTTCTCGTGGCTGCTC
	Antisense	CCCACGGAGTGTGGTAGTAG
MMP9 (228 bp)	Sense	GAAGGCAAACCCTGTGTGTT
	Antisense	AGAGTACTGCTTGCCCAGGA
MMP12 (184 bp)	Sense	CCAAGCATCCCATCTGCTAT
	Antisense	GGTCAAAGACAGCTGCATCA
MMP13 (286 bp)	Sense	TGATGAAACCTGGACAAGCA
	Antisense	TCCTCGGAGACTGGTAATGG
MMP20 (270 bp)	Sense	CTCGTCCTTTGATGCAGTGA
	Antisense	CTTGGGAACCCGAAGTCATA

2.4. Immunoblotting

The cells were lysed in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 5% bromo phenol blue). Next, 50 μg of total protein were resolved by SDS–PAGE and then were transferred to PVDF membranes (Millipore). Cell-conditioned medium was centrifuged at 2000 rpm to remove dead cells and debris, and concentrated 5 times using Biomax-10 (Millipore). Immunoblotting was performed with the appropriate antibody using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia).

2.5. Statistical analysis

The results are expressed as the means ± SD. A statistical analysis was done using the two-tailed student's *t*-test. A *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Induction of MMPs by Aβ in microglia

To identify MMPs induced by Aβ stimulation in microglia, we examined mRNA expression of microglial cell line Ra2 at 16 h after 10 μM Aβ1-42 treatment by Microarray analysis (data not shown). Microarray analysis revealed several MMPs mRNA species responsive to Aβ1-42 over this time frame (Table 2). These MMPs have been shown to be secreted by inflammatory macrophages. To further analyze MMPs induced by Aβ, Ra2 was treated with 10 μM Aβ1-42 for 16 h, and the expression of MMPs was examined by quantitative real-time PCR. Among several MMPs examined, we found the expression of MMP3 (Stromelysin 1), MMP12 (Macrophage elastase) and MMP13 (Collagenase 3) mRNA to be highly increased by Aβ1-42 stimulation (Fig. 1). We examined the time course of the induction of MMPs. As shown in Fig. 2A, induction of MMP3 or MMP12 by Aβ1-42 stimulation was appeared at 6 h and lasted for 24 h. Aβ25–35 was also

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