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Familial amyotrophic lateral sclerosis-linked mutant SOD1 aberrantly interacts with tubulin

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ABSTRACT

Mutations in the Cu,Zn-superoxide dismutase (SOD1) gene cause 20–25% of familial amyotrophic lateral sclerosis (ALS). Mutant SOD1 causes motor neuron degeneration through toxic gain-of-function(s). However, the direct molecular targets of mutant SOD1, underlying its toxicity, are not fully understood. In this study, we found that α/β-tubulin is one of the major mutant SOD1-interacting proteins, but that wild-type SOD1 does not interact with it. The interaction between tubulin and mutant SOD1 was detected in the spinal cords of mutant G93A SOD1 transgenic mice before the onset of symptoms. Tubulin interacted with amino acid residues 1–23 and 116–153 of SOD1. Overexpression of mutant SOD1 resulted in the accumulation of tubulin in detergent-insoluble fractions. In a cell-free system, mutant SOD1 modulated tubulin polymerization, while wild-type SOD1 did not. Since tightly regulated microtubule dynamics is essential for neurons to remain viable, α/β-tubulin could be an important direct target of mutant SOD1.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by selective loss of motor neurons. Five to 10 percent of ALS cases are known to occur in familial forms. Dominant missense mutations in the gene that encodes the Cu/Zn-superoxide dismutase, SOD1, are associated with 20–25% of familial ALS cases (fALS). Mice overexpressing mutant SOD1 develop an ALS-like phenotype and have been used as a mouse model of ALS, whereas mice lacking SOD1 do not [1,2]. These studies indicate that mutant SOD1 causes motor neuron degeneration not by loss of function of SOD1, but by toxic gain-of-function(s) of mutant SOD1.

The toxic properties of mutant SOD1 may be exerted at least partly through the physical interactions of mutant SOD1 with other functional proteins. Recent studies suggest that the physical interactions of mutant SOD1 with other cellular proteins, such as Derlin-1, mediate the toxicity of mutant SOD1 [3]. However, the direct molecular targets of mutant SOD1, underlying its toxicity, are not fully understood. Therefore, elucidating novel mutant SOD1-interacting proteins is important for understanding the mechanisms that underlie the pathology of ALS, and for developing effective therapies for ALS.

In this study, we sought to identify novel mutant SOD1-interacting proteins using coimmunoprecipitation assays and mass spectrometry analyses. We found that α/β-tubulin is one of the major mutant SOD1-interacting proteins.

Materials and methods

Plasmid constructs and antibodies. These reagents are described in the Supplemental materials and methods.

Cell culture and transfection. HeLa, COS-7 and Neuro2a cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). Transient expression of each vector in cells was performed using Lipofectamine LTX (Invitrogen) or the FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Differentiation of Neuro2a cells was induced by adding 5 mM dibutyryl cAMP, 4 h after transfection, as described previously [4].

Preparation of recombinant proteins. Recombinant SOD1 proteins were prepared according to methods described previously [4]. Detailed procedures are described in the Supplemental materials and methods.

Immunoprecipitation. Immunoprecipitation was performed as previously described [5]. Detailed procedures are described in the Supplemental materials and methods.

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Mutant G93A SOD1 transgenic mice. Transgenic mice expressing G93A hSOD1 (G1L line of B6SJL-TgN(SOD1-G93A)1Gur^{dl}) were obtained from the Jackson Laboratory (Bar Harbor, ME), and backcrossed to C57BL/6J for more than 14 generations. These mice were propagated at the National Institute of Neuroscience, National Center of Neurology and Psychiatry. We observed that the G93A SOD1-transgenic mice we used in this study experienced disease onset around the age of 8 months (246.5 ± 5.8 days) and died around 9.5 months (288.5 ± 7.1. days, mean ± SEM, $n = 14$). For coimmunoprecipitation assays, brains and spinal cords from male mice at 65 days of age were homogenized in immunoprecipitation buffer. After the lysates were centrifuged at 20,000 *g* for 10 min at 4 °C, 1 ml of the supernatants (~1.5 mg/ml protein in immunoprecipitation buffer) were incubated with 15 μg of antibody for 15 h, and immunoprecipitation was performed using protein G Sepharose. All experiments using mice were approved by the institution's Animal Investigation Committee.

Immunoblotting analysis. SDS–PAGE was performed under reducing conditions. Immunoblotting was performed according to standard procedures as described previously [6]. The preparation of detergent (1% Triton X-100)-soluble and -insoluble fractions was performed as described previously [6].

Mass spectrometry analysis. Protein bands (55-kDa proteins in Fig. 1A) were sliced from gel and subjected to in-gel trypsin digestion, and liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed at Shimadzu (Kyoto, Japan) as a custom service.

Pull-down assay. A pull-down assay was performed as described previously [4]. Detailed procedures are described in the Supplemental materials and methods.

Tubulin polymerization assay. An in vitro tubulin polymerization assay was performed using a Tubulin Polymerization Assay kit, OD based, >99% pure tubulin (Cytoskeleton), according to the manufacturer's protocol. Recombinant SOD1 without a tag and tubulin were mixed to give a final concentration of ~0.04 mg/ml SOD1 and 3 mg/ml tubulin in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 5% glycerol), and subjected to a tubulin polymerization assay. As a control, vehicle was added instead of SOD1.

Statistical analysis. For comparison of two groups, the statistical difference was determined by the Student's *t*-test. For comparison

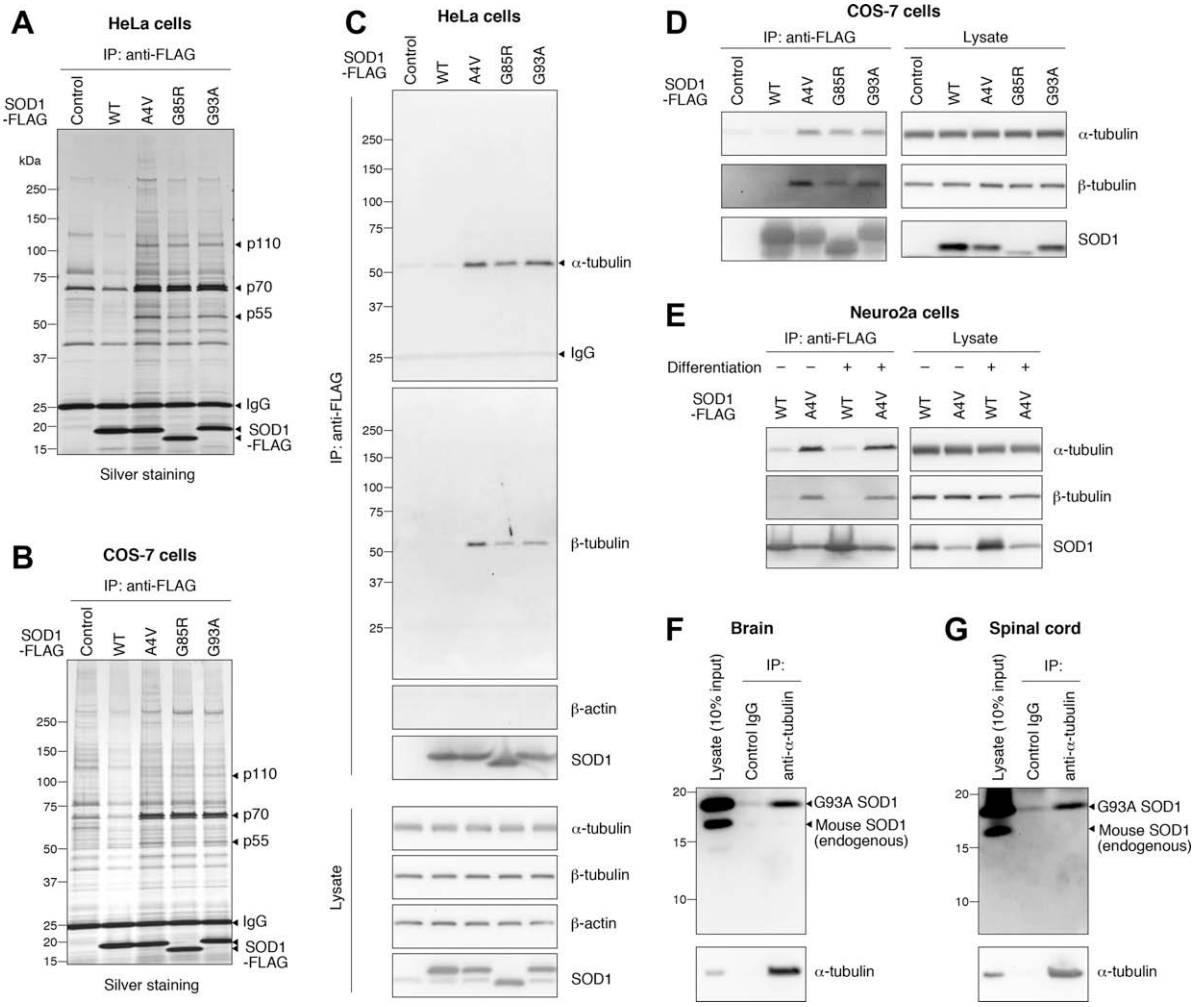

Fig. 1. Interaction between mutant SOD1 and tubulin. (A,B) Lysates of HeLa cells (A) or COS-7 cells (B) transfected with the indicated constructs (Control: empty vector) were immunoprecipitated with anti-FLAG antibody. The immunoprecipitants were analyzed by SDS–PAGE followed by silver staining. In the presence of FLAG-tagged mutant SOD1, mutant SOD1-interacting proteins were coimmunoprecipitated with mutant SOD1, while in the absence of mutant SOD1, proteins were nonspecifically precipitated with anti-FLAG beads. (C–E) HeLa (C), COS-7 (D) or Neuro2a (E) cells were transfected with the indicated constructs. Differentiation of Neuro2a cells was induced by adding 5 mM dibutyryl cAMP. Immunoprecipitation was performed using anti-FLAG antibody. The lysates and immunoprecipitants were analyzed by immunoblotting. (F,G) Lysates were prepared from the brains (F) and spinal cords (G) of mutant G93A transgenic mouse, and immunoprecipitated with anti-α-tubulin antibody or control IgG. The lysates and immunoprecipitants were analyzed by immunoblotting. Mutant SOD1 was clearly precipitated with α-tubulin. The coimmunoprecipitation of mutant SOD1 was also observed when immunoprecipitation was performed using anti-β-tubulin antibody (data not shown).

of more than two groups, Dunnett's multiple comparison test was used.

Results

Mutant SOD1 interacts with α/β-tubulin

To understand the molecular mechanism underlying the gain-of-toxicity of mutant SOD1, we attempted to identify proteins that interact with mutant SOD1 using a coimmunoprecipitation assay. We observed that the SOD1 mutants specifically interacted with multiple proteins, although wild-type SOD1 did not (Fig. 1A). Among these interacting proteins, the band intensities of the 110-kDa, 70-kDa and 55-kDa proteins were considerably higher than those of other interacting proteins (Fig. 1A). The interactions of theses proteins with mutant SOD1 were also detected when we used COS-7 or Neuro2a cells (Fig. 1B and 3C).

The 110-kDa protein interacting with mutant SOD1 was previously reported to be heat-shock protein 105 [7]; mutant SOD1 was also previously reported to interact with the 70 kDa heat shock protein 70 and heat shock cognate protein 70 [8]. Using LC–MS/MS analysis, we confirmed that the 70-kDa band contained heat shock cognate protein 70 (data not shown). Using LC–MS/MS analysis, we found that the 55-kDa proteins interacting with mutant SOD1 in Fig. 1A are human α-tubulin and β-tubulin (Supplemental Fig. 1A and Supplemental files 1 and 2), indicating that mutant SOD1 physically interacts with endogenous α-tubulin and β-tubulin. The interactions of mutant SOD1 with α-tubulin and β-tubulin were also detected by immunoblotting (Fig. 1C). Consistent with the silver staining analysis (Fig. 1A), an interaction of wild-type SOD1 with tubulin was not detected (Fig. 1C). The interaction between mutant SOD1 and endogenous tubulin was also observed in COS-7 cells, and in both undifferentiated and differentiated Neuro2a cells (Fig. 1D and E). In contrast to tubulin, an interaction of β-actin with mutant SOD1 was not observed (Fig. 1C), indicating that the interaction between mutant SOD1 and tubulin is not a non-specific interaction of abundant proteins.

To confirm the interaction of mutant SOD1 with tubulin *in vivo*, we performed coimmunoprecipitation assays using the spinal cords and brains of G93A SOD1 transgenic mice before the onset of the symptoms. The interaction of tubulin with G93A SOD1, but not with mouse wild-type SOD1, was clearly detected in the spinal cords and brains (Fig. 1F and G). Since the interaction between mutant SOD1 and tubulin was observed before the onset of symptoms, it is possible that this interaction partly plays a causative role in the pathogenesis of ALS.

To test whether mutant SOD1 directly interacts with tubulin, we purified recombinant mutant SOD1 proteins with a His tag (Supplemental Fig. 1B). Purified α/β-tubulin from bovine brain (Supplemental Fig. 1B) and His-tagged SOD1 proteins were mixed and pull-down assays were performed. Tubulin was precipitated with apo mutant SOD1 (Fig. 2A), indicating that mutant SOD1 can directly interact with tubulin. Tubulin was also precipitated with apo wild-type SOD1 (Supplemental Fig. 2).

The mutant SOD1-binding site within tubulin partly overlaps with the binding site for nocodazole

To determine the mutant SOD1-binding region within tubulin, we performed pull-down assays using the tubulin-binding agents paclitaxel, noscapine and nocodazole. We found that nocodazole inhibited the interaction between mutant SOD1 and tubulin, while paclitaxel and noscapine did not (Fig. 2A–C). The nocodazole binding site overlaps with that for colchicine [9]. Colchicine also inhibited the interaction between mutant SOD1 and tubulin (data not shown). The paclitaxel binding site within tubulin is distinct from those for noscapine and colchicine [10,11]. Thus the mutant SOD1-binding region of tubulin in part overlaps with the region(s) bound by nocodazole and colchicine, but does not overlap with the regions bound by paclitaxel and noscapine.

Amino acid residues 1–23 and 116–153 of SOD1 interact with tubulin

We next assessed whether tubulin interacts with the C-terminal deletion mutant T116X SOD1, which has been reported to cause ALS-like phenotype in mice [12]. Coimmunoprecipitation assays using FLAG-tagged T116X SOD1 (Fig. 2D) revealed that mutant T116X SOD1 interacts with tubulin (Fig. 2E), indicating that tubulin interacts with amino acid residues 1–115 of mutant SOD1. Silver staining analysis also showed that, similarly to A4V SOD1, mutant T116X SOD1 interacts with multiple proteins (Fig. 2F). This indicates that interacting with various proteins is a common abnormal property shared by ALS-associated mutant SOD1 proteins. We observed that tubulin also interacts with amino acid residues 116–153 of SOD1 (Fig. 2G and H). To determine the tubulin-binding region within residues 1–115 of SOD1, we further performed coimmunoprecipitation assays using deletion mutants of SOD1 (Fig. 2I). We found that amino acid residues 11–115 and 1–85 of SOD1 interact with tubulin, while amino acid residues 24–115 and 38–115 do not (Fig. 2J). Moreover, amino acid residues 1–10 and 1–23 of SOD1 both interacted with tubulin (Fig. 2K and L). These results suggest that tubulin interacts with amino acid residues 1–23 and 116–153 of mutant SOD1.

Cys-111 of mutant SOD1 affects the interaction with tubulin

SOD1 contains four cysteine residues, Cys-6, 57, 111 and 146. Recent studies have shown that, among these cysteine residues, Cys-111 in particular affects the aggregation and cytotoxicity of mutant SOD1 [13]. The substitution of Cys-111 to serine (C111S) significantly inhibits the aggregation or cytotoxicity of mutant SOD1 [13].

To examine the correlation between the cytotoxicity of mutant SOD1 and the interaction of mutant SOD1 with tubulin, we performed coimmunoprecipitation assays using mutant SOD1 containing serine substitutions at each cysteine residue. Interestingly, immunoblotting analyses showed that the C111S substitution in mutant SOD1 significantly decreased the interaction between tubulin and mutant SOD1, although C6S, C57S and C146S substitutions did not (Fig. 3A and B). Together with the report showing the role of Cys-111 in cytotoxicity and aggregation of mutant SOD1 [13], our results suggest that the cytotoxicity of mutant SOD1 is correlated with the degree of its interaction with tubulin. Silver staining analyses also showed that the C111S substitution in mutant SOD1 decreased the interaction between multiple proteins and mutant SOD1 (~50% decrease), while C6S, C57S and C146S substitutions did not (Fig. 3C).

Overexpression of mutant SOD1 causes accumulation of tubulin in insoluble fractions

Given that mutant SOD1 physically interacts with tubulin, and that mutant SOD1 forms insoluble aggregates when overexpressed in cells, we hypothesized that mutant SOD1 causes aggregation of tubulin. To examine the effects of mutant SOD1 on the aggregation of tubulin, we prepared 1% Triton X-100-soluble and insoluble fractions from cells overexpressing wild-type or mutant SOD1, and the amount of tubulin in each fraction was analyzed. An increased level of SOD1 was detected in the insoluble fraction of cells overexpressing mutant SOD1 compared with that of cells

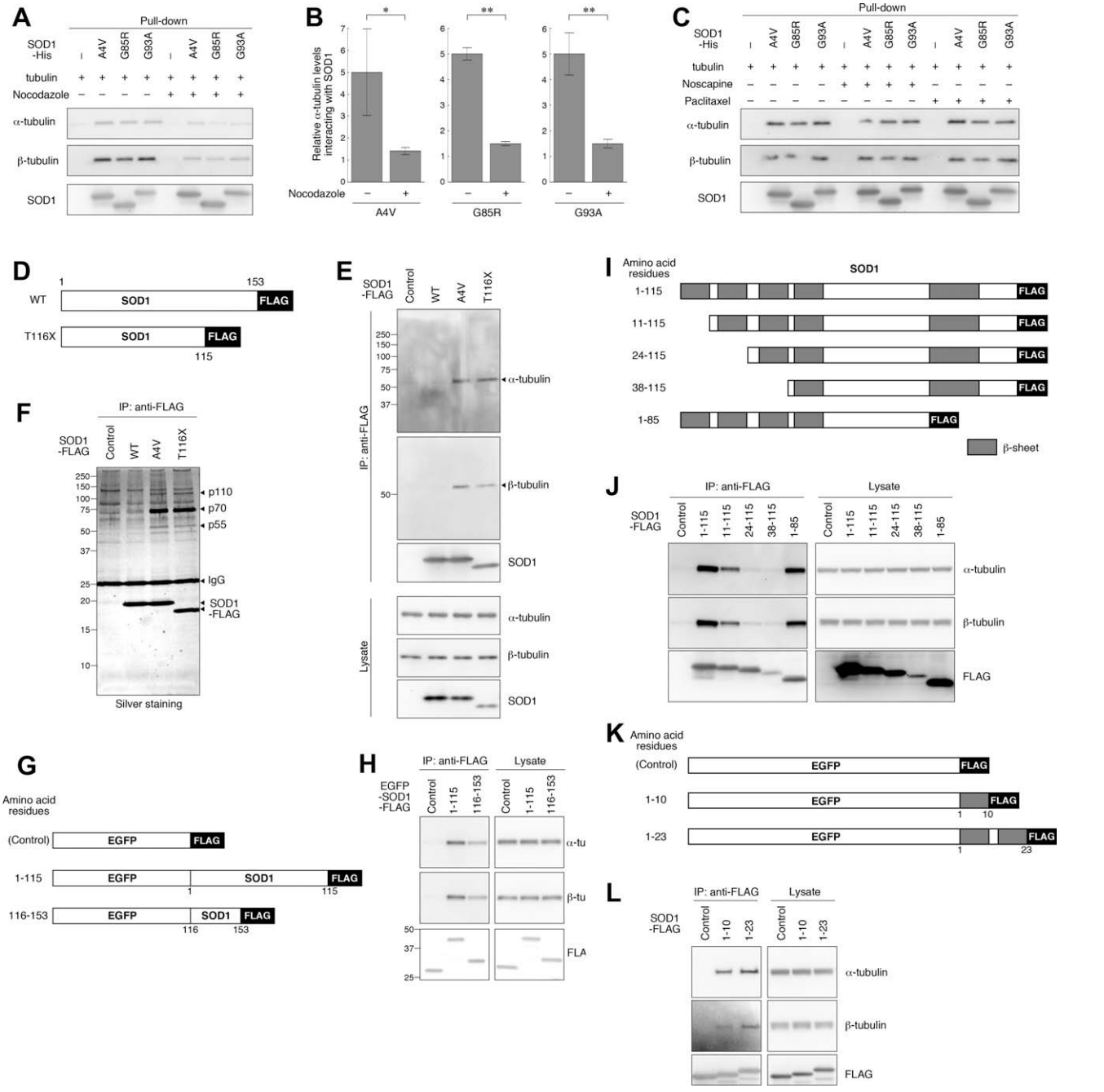

Fig. 2. Binding site within tubulin and mutant SOD1. (A–C) Forty micromolars of nocodazole (A), paclitaxel (C), noscapine (C) or vehicle was mixed with the indicated purified proteins and pull-down assays were performed. Precipitants were analyzed by immunoblotting. The relative levels of α-tubulin interacting with mutant SOD1 were quantified by densitometry. Mean values are shown with SD ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$ (B). (D, G, I, and K) Schematic representation of deletion mutants of SOD1. (E, F, H, J, and L) Lysates of COS-7 cells transfected with the indicated constructs were immunoprecipitated with an anti-FLAG antibody and analyzed by immunoblotting (E, H, J, and L) or silver staining (F).

overexpressing wild-type SOD1 (Fig. 3D) We found that overexpression of mutant SOD1 resulted in increased levels of α- and β-tubulin in the insoluble fraction, but had no effect on the protein levels in the soluble fraction (Fig. 3D and E). By contrast, overexpression of wild-type SOD1 did not alter the levels of α- and β-tubulin in either fraction (Fig. 3D and E). In both fractions, the level of β-actin, which does not interact with mutant SOD1 (Fig. 1C), was not affected by the overexpression of wild-type or mutant SOD1 (Fig. 3D). These results indicate that the accumulation of tubulin in insoluble fractions is mediated by the interaction between mutant SOD1 and tubulin. Since the insoluble aggregates associated with neurodegenerative diseases exhibit protease resistance [14], it is possible that aggregated tubulin exhibits resistance to constitutive degradation, resulting in the accumulation of insoluble tubulin in cells overexpressing mutant SOD1.

Mutant SOD1 directly modulates tubulin polymerization

To elucidate the direct effect of mutant SOD1 on microtubule dynamics, we used a cell-free tubulin polymerization assay. We confirmed that paclitaxel, a microtubule-stabilizing agent, promotes tubulin polymerization, and that nocodazole, a microtu-

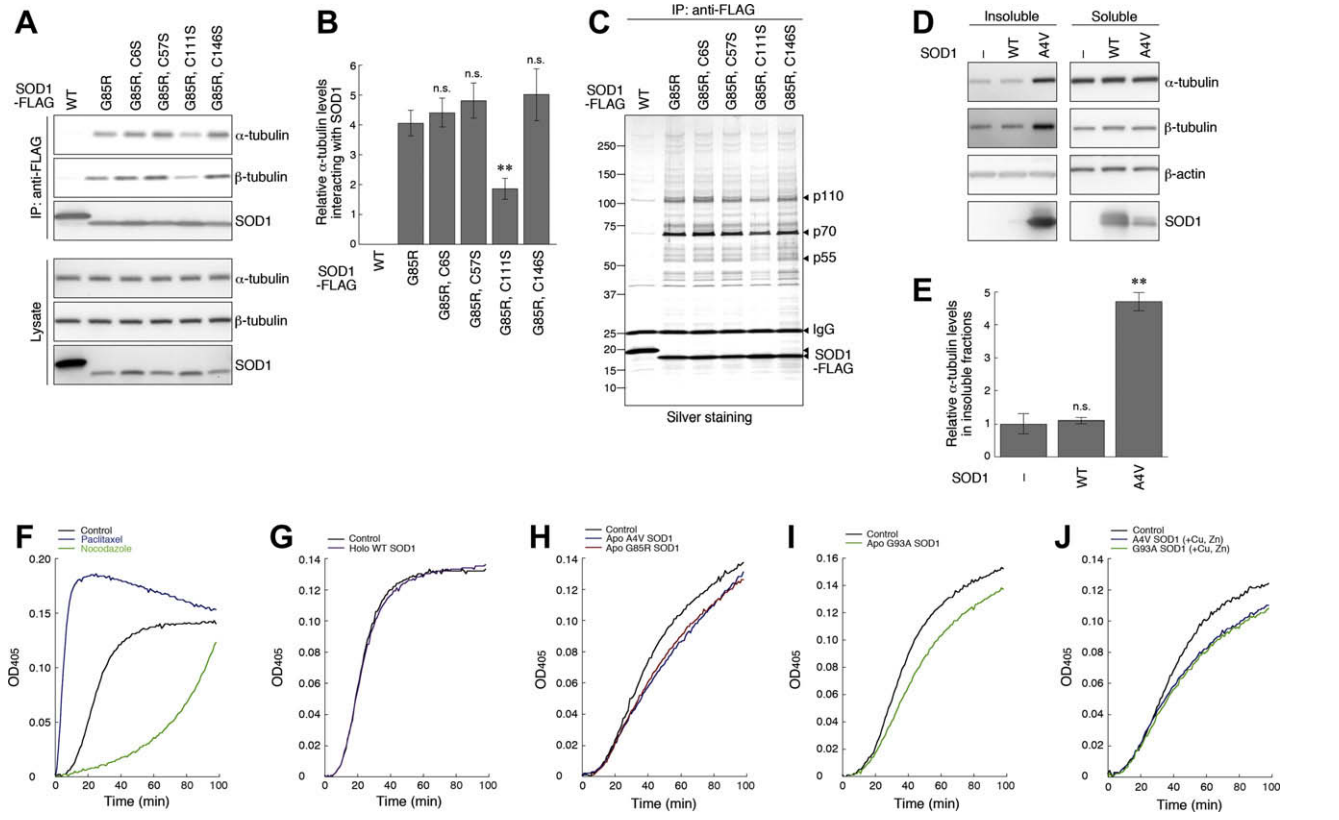

Fig. 3. Effects of mutant SOD1 on tubulin. (A–C) Lysates of Neuro2a cells transfected with the indicated constructs were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting (A) or silver staining (C). The relative levels of α-tubulin interacting with mutant SOD1 were quantified by densitometry (B). Mean values are shown with SD ($n = 3$). n.s., not significant; $^{**}p < 0.01$ (B, Dunnett's multiple comparison test). (D,E) pEF-hSOD1 (WT or A4V) or empty vector was transfected into COS-7 cells. Three days after transfection, soluble and insoluble fractions were prepared and analyzed by immunoblotting (D). The relative levels of α-tubulin in the insoluble fractions were quantified by densitometry. Mean values are shown with SD ($n = 3$). n.s., not significant; $^{**}p < 0.01$ (E, Dunnett's multiple comparison test). (F) A tubulin polymerization assay was performed in the presence of 10 μM paclitaxel, 10 μM nocodazole or vehicle (control, DMSO). Light scattering was used to measure the amount of polymerized microtubules. (G–J) Four microliters of 1 μg/μl holo wild-type SOD1 or vehicle (200 μM $CuSO_4$, 200 μM $ZnCl_2$ in 20 mM sodium phosphate buffer, pH 8.0) was mixed with 100 μl of 3 mg/ml tubulin (G). Twenty microliters of 0.2 μg/μl apo mutant SOD1 or vehicle (20 mM sodium phosphate buffer, pH 8.0) was mixed with 80 μl of 3.75 mg/ml tubulin (H,I). Twenty microliters of 0.2 μg/μl holo mutant SOD1 or vehicle (200 μM $CuSO_4$, 200 μM $ZnCl_2$ in 20 mM sodium phosphate buffer, pH 8.0) was mixed with 80 μl of 3.75 mg/ml tubulin (J). Then tubulin polymerization assays were performed. The assays were performed at least three times; representative results are shown. Wild-type SOD1 did not inhibit tubulin polymerization when we used 20 μl of 0.2 μg/μl holo wild-type SOD1 (data not shown).

bule-destabilizing agent inhibits tubulin polymerization in a cell-free system (Fig. 3F). We prepared wild-type and mutant SOD1 proteins without a tag (Supplemental Fig. 1C and Supplemental Table 1), and performed tubulin polymerization assays. Because apo wild-type SOD1 interacts with tubulin, we used holo wild-type SOD1 as a control native SOD1. Wild-type SOD1 had almost no effect on tubulin polymerization (Fig. 3G). Interestingly, mutant SOD1 inhibited tubulin polymerization in tubulin polymerization assays (Fig. 3H–J). These results are consistent with the results showing that tubulin interacts with mutant SOD1, but not with wild-type SOD1, and indicate that mutant SOD1 directly affects the stability of microtubules.

Discussion

The microtubule-destabilizing agent colchicine has been reported to induce neuronal cell death in mice [15]. Microtubule-stabilizing agents such as paclitaxel are effective chemotherapeutic agents for the treatment of many cancers. However, neuropathy is a major adverse effect of microtubule-stabilizing agent-based chemotherapy [16]. Paclitaxel is also reported to induce cell death in cortical neurons [17]. Thus tightly regulated microtubule dynamics, namely controlled tubulin polymerization/depolymerization, are required for neurons to remain viable, and our results may indicate that the physical interaction of mutant SOD1 with α/β-tubulin at least partly underlies the neurodegeneration mediated by mutant SOD1.

A recent report has shown that microtubule dynamics are increased in the motor neurons of mutant G93A SOD1 transgenic mice, and that this increase in microtubule dynamics in motor neurons is associated with altered axonal transport and motor neuron degeneration [18]. We have shown that mutant SOD1 directly interacts with tubulin (Fig. 2A), and that mutant SOD1 has a similar effect on microtubules to a microtubule-destabilizing agent, but to a lesser extent (Fig. 3F–J). Since microtubules in neurons are relatively stable due to their interactions with microtubule-associated proteins, destabilization of microtubules may cause increased microtubule dynamics in neurons. Taken together, our results suggest that mutant SOD1 can directly increase microtubule dynamics through its destabilizing effect on microtubules in neurons.

Using tubulin-binding agents, we have shown that the mutant SOD1-binding site within tubulin in part overlaps with the sites bound by colchicine and nocodazole (Fig. 2A–C). Considering that colchicine destabilizes microtubules through the interaction with tubulin at microtubule ends [19], mutant SOD1 may also modulate microtubule dynamics through the interaction with tubulin at microtubule ends. Structural analysis of the interaction of SOD1 with tubulin, such as X-ray crystallography, is required for further understanding of the mechanism that underlies the modulation of microtubule dynamics by mutant SOD1. Native SOD1 forms a sta-

ble homodimer, while mutant SOD1 partly exists as a monomer [20]. We showed that amino acid residues 1–23 and 116–153 in SOD1 interact with tubulin (Fig. 2D–L). These amino acid residues contain residues 4–8 and 143–151, which are sequestered in the SOD1 dimer interface and are inaccessible in native SOD1 [20]. We also showed that tubulin interacts with apo wild-type SOD1 (Supplemental Fig. 2). Apo SOD1 has been reported to exist substantially as a monomer [8]. Together, our results suggest that tubulin interacts with monomeric SOD1. The inhibitory effects of holo mutant SOD1 on tubulin polymerization (Fig. 3J) may be due to the misfolded or remaining apo SOD1 in the recombinant SOD1 sample, considering that the enzymatic activities of holo mutant SOD1 were reduced compared with that of wild-type SOD1 (Supplemental Table 1).

In addition to tubulin, we observed that SOD1 mutants specifically interact with multiple proteins (Fig. 1A and B and 3C). The C111S substitution in mutant SOD1 decreases the degree of interaction between mutant SOD1 and various proteins (Fig. 3C), suggesting that most mutant SOD1-interacting proteins bind to SOD1 in a similar way. These other interactors may also be involved in the mechanism underlying SOD1-mediated neurodegeneration. We have identified some of these interactors, and these proteins are currently under investigation.

Our present study demonstrates that mutant SOD1 can directly modulates tubulin polymerization by physically interacting with tubulin. The findings in the present study provide novel insights into the mechanisms underlying the pathology of ALS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.138.

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