Biochemical and Biophysical Research Communications 434 (2013) 521–526

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization and Hsp104-induced artificial clearance of familial ALS-related SOD1 aggregates

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ARTICLE INFO

Article history:
Received 18 March 2013
Available online 10 April 2013

Keywords:
Mutant SOD1
Amyotrophic lateral sclerosis
Hsp104
SOD1 aggregate
FLIP

ABSTRACT

Hsp104, a molecular chaperone protein, originates from *Saccharomyces cerevisiae* and shows potential for development as a therapeutic disaggregase for the treatment of neurodegenerative disorders. This study shows that aggregates of mutant superoxide dismutase 1 (SOD1), which cause amyotrophic lateral sclerosis (ALS), are disaggregated by Hsp104 in an ATP-dependent manner. Mutant SOD1 aggregates were first characterized using fluorescence loss in photobleaching experiments based on the reduced mobility of aggregated proteins. Hsp104 restored the mobility of mutant SOD1 proteins to a level comparable with that of the wild-type. However, ATPase-deficient Hsp104 mutants did not restore mobility, suggesting that, rather than preventing aggregation, Hsp104 disaggregates mutant SOD1 after it has aggregated. Despite the restored mobility, however, mutant SOD1 proteins existed as trimers or other higher-order structures, rather than as naturally occurring dimers. This study sheds further light on the mechanisms underlying the disaggregation of SOD1 mutant aggregates in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by weakness and degeneration of all muscles, apart from those controlling the eyes and bladder [1]. Loss of mobility stems from the death of motor neurons protruding from the anterior horn of the spine. The vast majority of cases are deemed idiopathic, while only 10% can be attributed to genetic causes [2]. Regardless of whether patients have known genetic risk factors, all types of ALS share certain common symptoms, raising questions about a possible common denominator between sporadic and familial ALS.

The primary genetic cause of ALS is a mutation in the gene encoding superoxide dismutase 1 (SOD1), also known as Cu/Zn superoxide dismutase [3]. The SOD1 protein converts oxygen radicals into hydrogen peroxide in a copper-mediated chemical reaction. Over 100 mutations have been reported to date, nearly all of which are point mutations, although other types, such as truncation mutations, have also been observed [4,5]. This large number of mutations, and the varied amino acid changes that result from them, cause ALS [6]. There are many hypotheses as to why mutations in SOD1 cause ALS: such hypotheses include oxidative stress, glutamate-related excitotoxicity, mitochondrial dysfunction, and axonal transport abnormalities [4,7]. The reasons are more complicated when one considers that molecular phenomena such as protein aggregation have multiple causes [8]. In addition, evidence suggests that protein aggregates are not necessarily pathogenic, which only complicates matters further [9]. The only thing that is certain is that ALS is not caused by a loss-of-function mutation in the SOD1 gene [10].

Budding yeast, *Saccharomyces cerevisiae*, contain an elaborate network of chaperones whose main function is protein quality control. Hsp104 rescues proteins that are denatured by environmental stress such as heat or chemical insult [11–13]. Yeast cells lacking Hsp104 survive normally until they encounter an adverse environment. Hsp104 stands apart from all other so-called disaggregates in that it functions cooperatively with human chaperones, such as Hsp90 [14], suggesting a possible role in human disease; however, neither Hsp104 nor any of its orthologs are found in *Homo sapiens*. On this basis, many research groups have studied Hsp104 with the aim of mitigating neurodegenerative diseases that accompany aggregation. Several reports regarding Huntington's, Parkinson's, and Alzheimer's diseases showed that Hsp104 dissolves protein aggregates and increases the survival of cells and/or model animals [15–17].

Abbreviations: ALS, amyotrophic lateral sclerosis; BiFC–FRET, Bimolecular fluorescence complementation–Förster resonance energy transfer; FLIP, fluorescence loss in photobleaching; ROI, region of interest; SOD1, superoxide dismutase 1.

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http://dx.doi.org/10.1016/j.bbrc.2013.03.107

Here, we investigated the effects of Hsp104 on aggregates of mutant superoxide dismutase 1, which cause amyotrophic lateral sclerosis (ALS). Our results may shed further light on the mechanisms underlying the disaggregation of SOD1 mutant aggregates in ALS.

2. Materials and methods

2.1. Cell culture and DNA transfection

Mouse neuroblastoma (N2a) cells (American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with 8% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). The polyethylenimine (PEI) method was used to transfect plasmids into cells as previously described [18]. The cells were incubated at 37 °C in a 5% CO_2 incubator.

2.2. Plasmid construction

SOD1 constructs were generated by sub-cloning from SOD1-FLAG plasmids [19] containing mutant A4V, G85R, G93A, or wild-type proteins. The SOD1 gene was flanked by *Kpn*I and *Eco*RI restriction sites, which yielded inserts that were subsequently cloned into the eGFP-N3 backbone (Clontech). A Site Directed Mutagenesis Kit (Stratagene) was used to generate Venus and Cerulean constructs containing the eGFP portion as previously described [20,21].

The cDNA fragments for Hsp104 were amplified by polymerase chain reaction (PCR) with pfu polymerase (Fermentas) using the first-strand yeast cDNAs as templates. The primers used for the fragments contained *Nhe*I (5′-cgcggagctagcatgaacgaccaaacgcaa-3′) and *Bam*HI (5′-ttattaggatccggttaatc taggtcatcatcaatttccatact-3′) restriction sites at the 5′ and 3′ ends, respectively. The amplified Hsp104 fragments were inserted into the pDsRed2-Mito plasmid (Clontech) after the mitochondrial marker gene was removed using the same set of restriction enzymes. Ax (ATP non-binding)- and TRP (ATP non-burning)-Hsp104 mutants were then generated by site-directed mutagenesis as previously described [22].

2.3. Visualization of aggregates using fluorescence loss in photobleaching (FLIP)

Laser confocal microscopy was employed to observe and measure protein mobility in conjunction with fluorescence loss in photobleaching (FLIP). About 5×10^4 cells were grown in 35 mm glass-bottomed dishes (SPL) for 14–16 h before transfection. The presence or absence of aggregates was recorded using a laser confocal microscope at 48 h post-transfection (Zeiss LSM 510 Meta, Carl Zeiss). To confirm that a group of GFP-tagged SOD1 proteins was aggregated and therefore immobile, a part of the fluorescence signal was directly targeted for constant bleaching by a 405 nm laser at 50–100% output for 10 iterations; the laser was focused on a specific region of interest (ROI). Any proteins suspected of forming aggregates were monitored using an argon 488 nm laser at a tube current of 5 A. All live imaging was performed at a speed of approximately 0.9 s per image. A persistent fluorescence signal outside the bleached ROI indicated the presence of aggregates, whereas an even fading of the green fluorescence signal throughout the cytoplasm suggested that they were absent.

2.4. Bimolecular fluorescence complementation–Förster resonance energy transfer (BiFC–FRET)

Approximately 5×10^4 cells were grown in 35 mm glass-bottomed confocal dishes. Forty-eight hours later they were cotransfected with three SOD1 bearing different tags: (a Cerulean tag, a Venus N-terminal tag or a Venus C-terminal tag) [23] and wild-type or mutant Hsp104. At 48 h post-transfection, the FRET between the Cerulean and Venus tags was measured to determine whether the Hsp104-processed mutant SOD1 still formed multimeric protein complexes. First, each fluorochrome was excited by its own excitation wavelength to confirm that the proteins were expressed and folded normally. Next, multimeric complex formation, which would suggest a propensity for aggregation, was explored by measuring the energy transfer.

3. Results and discussion

3.1. Mutant SOD1 but not wild-type SOD1 forms aggregates in N2a cells

Point mutations in SOD1 are known to cause ALS; we investigated SOD1 proteins harboring G93A, G85R, and A4V mutations in this study. N2a cells grown on confocal dishes were transfected with plasmids expressing wild-type or mutant SOD1 proteins bearing different fluorescent tags. After 48 h, the SOD1 proteins were observed under a laser confocal microscope. Constantly bleaching a given spot within the cytoplasm (no larger than 3 μm in diameter) with a 405 nm laser over ten iterations enabled us to monitor the mobility of the proteins.

Fig. 1A shows fluorescence intensity images of wild-type and G85R mutant SOD1 at 0, 72, and 108 s after bleaching. Green fluorescence generated by wild-type SOD1 was observed mainly in the cytoplasm at the start of bleaching and showed an even fading over time; however, the fluorescence generated by mutant SOD1 persisted.

We then recorded the change in fluorescence intensity over time (>5 min). A plane image was taken about every 0.9 s. Fig. 1B (left panel) shows that there was no significant difference in the pattern of fluorescence loss in the different ROIs, including the bleaching spot. The exception was the violet region. Loss of fluorescence in this area indicated that GFP-tagged wild-type SOD1 moved in and out of the bleaching spot in less than 0.9 s. The violet region was very pronounced in the nucleus (in which some (although not very high) fluorescence intensity was observed) and remained consistent after bleaching. Thus, it appears that SOD1 proteins were not easily able to move across the nuclear membrane.

A4V mutant SOD1 proteins formed single or multiple aggregates, which were condensed and highly fluorescent. The mutant aggregates in the red or green circle lost a certain amount of fluorescence intensity over time; however, this was much less than that observed for the wild-type (Fig. 1B, right panel). In addition, the fluorescence intensity within the blue circle (the spot directly targeted by the laser) was reduced upon bleaching and was not restored, indicating a lack of protein mobility. Taken together, these results strongly suggest that the SOD1 proteins are contained, or trapped, within one or more high-order structures from which they cannot break free. This state of reduced mobility is indicative of protein aggregation.

To determine the extent of protein aggregation in a given population of cells, we obtained low-magnification (20×) images (maximum 50 cells per image) and counted cells that contained or did not contain aggregates. The proportion of cells that contained aggregates varied from 20% (A4V mutant) to >40% (G93A mutant) (Fig. 1C). Taken together, these results demonstrate that the G93A, G85R, and A4V mutants, but not wild-type SOD1, formed aggregates within N2a cells. The results also show that wild-type SOD1 proteins are highly mobile within the cytoplasm and that

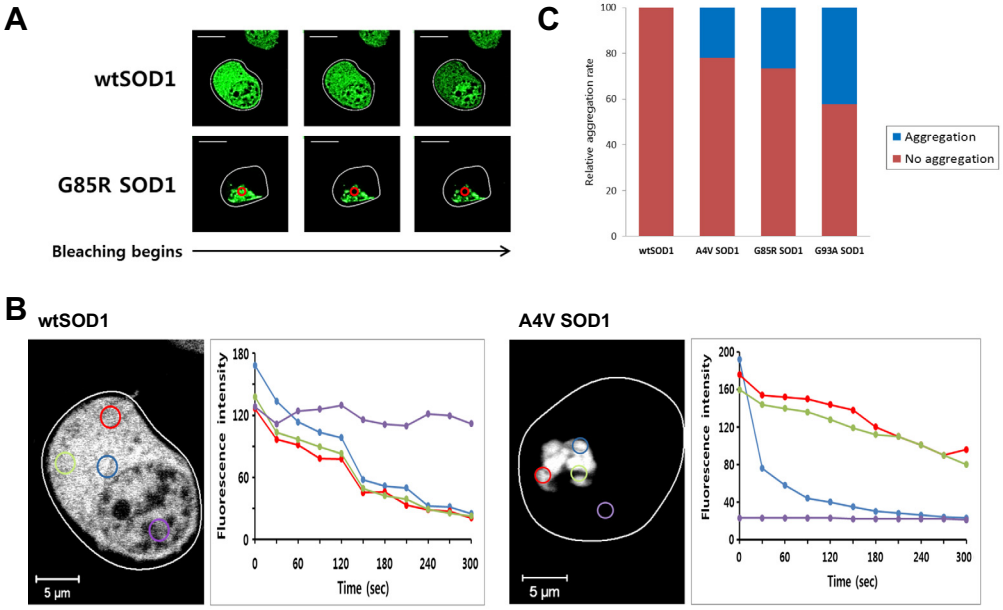

Fig. 1. Fluorescence loss in photobleaching (FLIP) of wild-type and mutant SOD1 fused to GFP. N2a cells were transfected with a mutant or wild-type SOD1–GFP fusion proteins and aggregation was observed by directly bleaching the green fluorescence signal with a 405 nm laser (100% output, 10 iterations) at 48 h post-transfection. (A) Each series of images shows the fluorescence intensity of wild-type or mutant (G85R) SOD1 at 0, 72, and 108 s after bleaching. (B) Fluorescence intensity of wild-type and mutant (A4V) SOD1 after consecutive bleaching periods. The blue circles indicate spots where bleaching was performed. Distant regions (yellow green, blue, or violet circles) were measured and plotted over time. All live imaging was taken every 0.9 s, but the fluorescence intensity was plotted every 30 s. A value of 20 (violet color) was considered the lowest value in the FLIP assays. (C) Percentage of cells containing aggregates. The presence or absence of aggregates was determined according to the persistence of the fluorescence signal outside the bleached ROI and the fading of the green fluorescence throughout the entire cytoplasm, respectively.

wild-type SOD1 proteins do not move, or very little, across the nuclear membrane.

3.2. Hsp104 dissolves SOD1 aggregates

The fungal chaperone, Hsp104, was cloned into a vector and cotransfected into N2a cells along with wild-type or mutant SOD1. SOD1 proteins were then observed under a laser confocal microscope after 48 h. When coexpressed with Hsp104, the G85R SOD1 mutant showed a pattern of fluorescence loss similar to that of the wild-type protein (Fig. 2A). A4V SOD1 was also cotransfected with Hsp104 (Fig. 2B) and a wild-type-like pattern of fluorescence loss was observed in all ROI regions (red, yellow, or green circles) distant from the bleached region (pink) after consecutive bleaching periods. These findings indicated that Hsp104 restored the mobility of the mutant SOD1 proteins.

Next, the number of cells that either contained or did not contain aggregates was counted. All mutant cells coexpressing Hsp104 (100%) showed restored mobility of mutant SOD1 proteins (Fig. 2C), indicating a lack of aggregate formation.

3.3. Hsp104 does not completely reverse the aggregation of mutant SOD1 proteins

A modified BiFC–FRET assay was designed to clarify the status of dissolved, formerly aggregated SOD1 proteins. The BiFC method was originally used to observe protein bimolecular complexes [23]. Addition of a FRET assay to the BiFC method enabled the detection of complexes containing more than three SOD1 proteins (Fig. 3A). Three SOD1 constructs (tagged with Cerulean, one tagged with the N-terminal fragment of Venus, and one tagged with the C-terminal fragment of Venus) were cotransfected into cells along with Hsp104 and the Cerulean-to-Venus FRET signal was visualized after 48 h. In this assay, no FRET signal is observed unless a stable trimer, or other higher-order complex, is present. In the previous experiments, no aggregates were observed in cells transfected with wild-type SOD1 plus Hsp104; likewise, no FRET signal was observed in the BiFC–FRET assay (wild-type SOD1 plus Hsp104; Fig. 3B, top panel). However, cells transfected with mutant SOD1 plus Hsp104 (which demonstrated a wild-type-like pattern of fluorescence loss after bleaching) showed a positive FRET signal (Fig. 3B, bottom panel). This suggests that mutant SOD1 exists as a trimer or other higher-order structure. Since SOD1 proteins naturally form dimers, the mutant SOD1 proteins in cells expressing Hsp104 may not be functional.

3.4. Hsp104 requires ATP hydrolysis to disassociate SOD1 aggregates

Of the two most studied Hsp104 mutants, one cripples ATP binding and the other allows ATP binding while blocking its hydrolysis. The former, called ‘ATP non-binding’ (K218T/K620T) and the latter, called ‘ATP non-burning’ (E285Q/E687Q or trap mutant), were used in studies of alpha synuclein and amyloid beta, respectively [16,24]. Both of these mutants were cotransfected into cells along with wild-type SOD1 or the A4V SOD1 mutant. Neither of these Hsp104 mutants was able to disaggregate SOD1 complexes as well as wild-type Hsp104 (Fig. 4A), indicating that the ATPase-deficient Hsp104 mutants would not restore mobility. In other words, Hsp104 must complete the reaction cycle to dissociate mutant SOD1 aggregates. This strongly suggests that Hsp104 disaggregates mutant SOD1 complexes after the latter have formed, rather than by preventing their formation in the first place (Fig. 4B).

The term “aggregate” can either indicate a state of total disarray, as observed for incorrectly folded/aggregated proteins found in heat-shocked yeast cells (which Hsp104 evolved to deal with) or it can also indicate a high-order structure composed of denatured proteins, such as a prion fibril. In either case, a common finding is that the mobility of the predominant protein within the

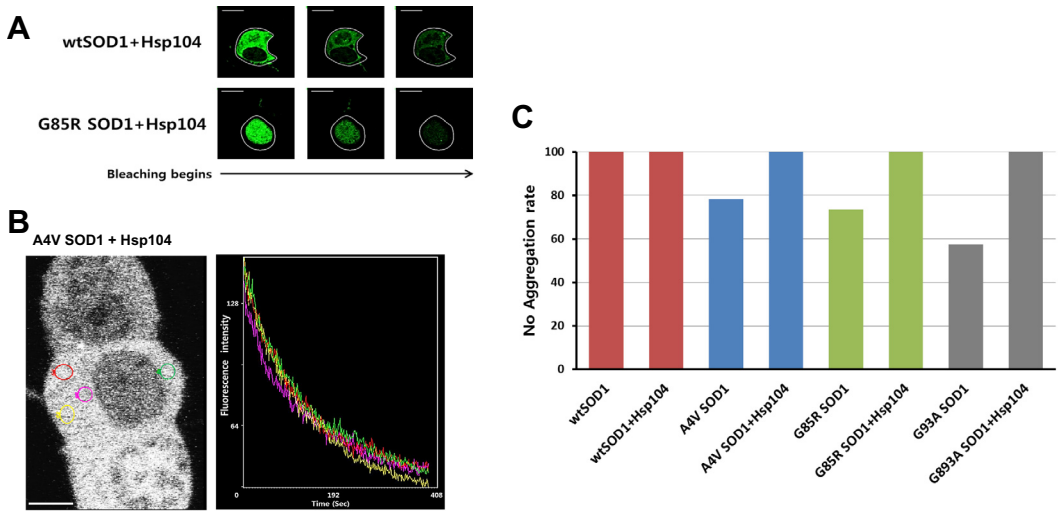

Fig. 2. Hsp104 disassociates mutant SOD1 aggregates. Fluorescence loss in photobleaching in cells cotransfected with a GFP-fused mutant (A4V) SOD1 and Hsp104-DsRed. N2a cells were transfected with SOD1–GFP gene fusion plasmids, either with or without Hsp104 and aggregation was observed by directly bleaching the green fluorescence signal with a 405 nm laser (100% output, 10 iterations) at 48 h after transfection. (A) Each image series shows the fluorescence intensity of wild-type and mutant (G85R) SOD1 in the presence of Hsp104 at 0, 100, and 200 s after bleaching. (B) Fluorescence intensity of wild-type and mutant (A4V) SOD1 in the presence of Hsp104 after consecutive bleaching periods. Distant regions (red, yellow, or green circles) were measured and plotted every 0.9 s. The scale bar represents 5 μm. The pink circle indicates the bleached area. (C) Percentage of cells containing aggregates in the presence of Hsp104. The presence or absence of aggregates was determined according to the persistence of a fluorescence signal outside the bleached spot and by the fading of the green fluorescence throughout the entire cytoplasm, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

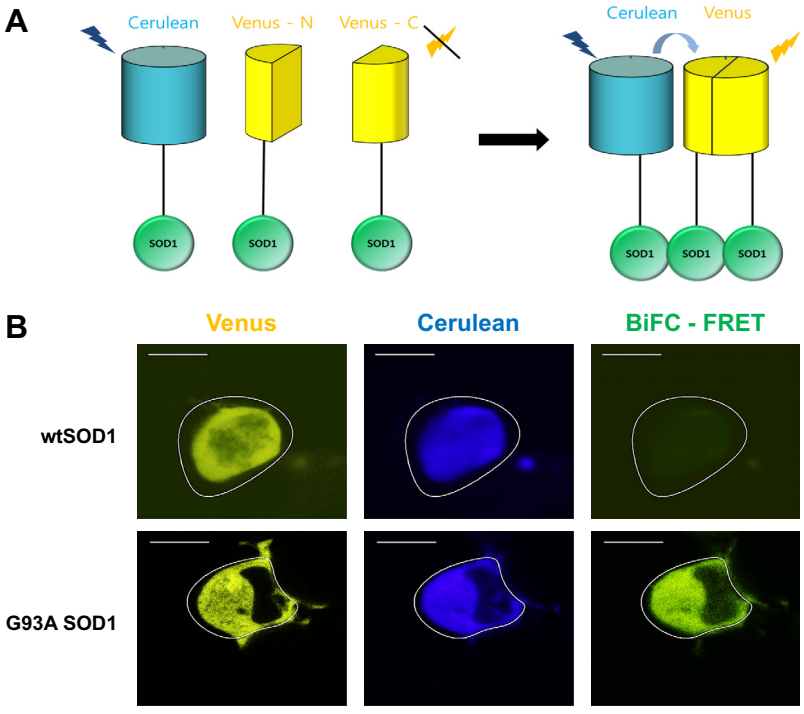

Fig. 3. BiFC–FRET in cells cotransfected with mutant SOD1 and Hsp104. N2a cells were cotransfected with mutant SOD1 (fused to the N- or C-terminal fragments of Venus (bimolecular fluorescence complementation) or to Cerulean) and Hsp104. The presence of higher-order structures (other than dimers) was observed by Förster resonance energy transfer. Cells were maintained, transfected, and observed with laser confocal microscopy as described in "Section 2". (A) Schematic diagram of Venus-based BiFC. (B) Fluorescence intensity of wild-type and mutant (A4V) SOD1 in the presence of Hsp104.

complex is markedly decreased. The bleaching method used in the present study relies on mobility differences between species, which is particularly useful because the mobility of wild-type SOD1 is quite high due to its small size. Fluorescence recovery after photobleaching (FRAP) is often used to study protein mobility. Its validity depends partly on whether the image obtained immediately after bleaching shows a blackened spot, which results from a rapid and sudden loss of fluorescence. However, the high mobility of wild-type SOD1 makes FRAP difficult, because SOD1 moves too quickly to obtain reliable and accurate data. Therefore, we chose a method that involves constant bleaching, even though it necessitates the ceaseless bombardment of cells with a high-power laser beam. This practical consideration was not an issue for mutant SOD1s, as they move much more slowly. The loss of fluores-

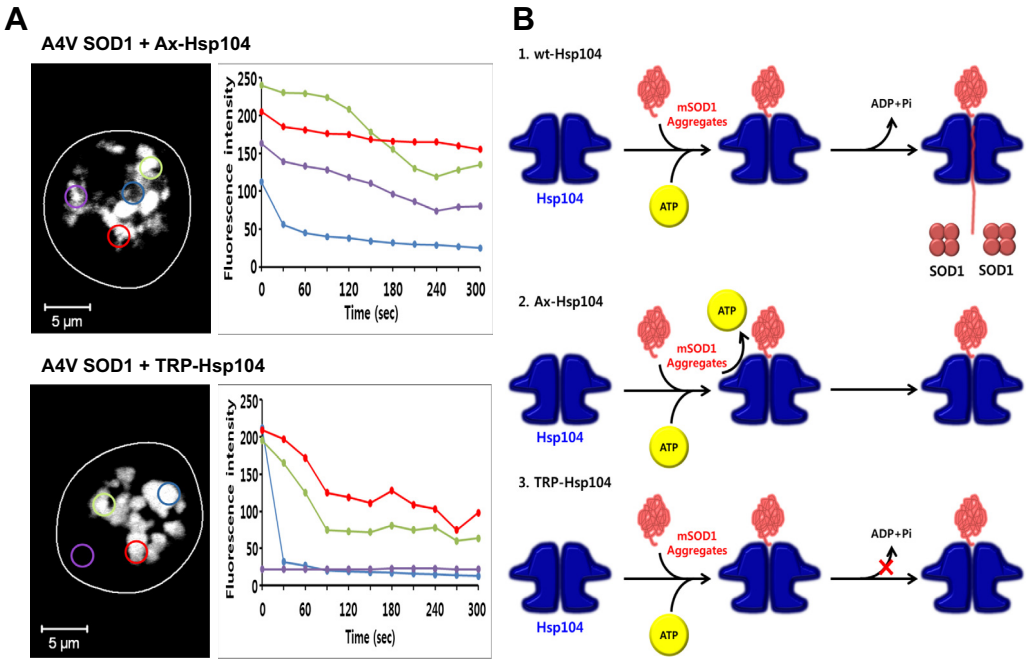

Fig. 4. Hsp104 requires ATP hydrolysis. (A) FLIP of cells cotransfected with a GFP-fused mutant (A4V) SOD1 plus a Hsp104 mutant that cannot bind to ATP or a Hsp104 mutant that cannot hydrolyze ATP. N2a cells were maintained in DMEM supplemented with 8% fetal bovine serum and transfected with a SOD1–GFP gene fusion plasmids either with or without Hsp104. Aggregation was observed by directly bleaching the fluorescence signal with a 405 nm laser (100% output, 10 iterations) at 48 h post-transfection. The blue circles indicate the bleached areas. The fluorescence in distant regions (circles) were measured and plotted over time. All live imaging was taken every 0.9 s, but the fluorescence intensity was plotted every 30 s. The scale bar represents 10 μm. (B) Schematic diagram showing the mechanism underlying Hsp104-mediated disaggregation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cence intensity observed in the mutant SOD1 aggregates indicates that some SOD1 molecules within the aggregates can escape, albeit slowly, which is consistent with the findings of a previous report [25].

Hsp104 leads to a non-localized pattern of fluorescence loss from mutant SOD1 proteins, similar to that shown by wild-type SOD1. Once decreased, the mobility of SOD1 can be restored by Hsp104, although a fully functional ATP hydrolysis cycle is required. If any of the steps involved in ATP hydrolysis are blocked, either by binding [26] or by hydrolysis [27], the effect of Hsp104 on SOD1 aggregates is nullified. Taken together, these findings suggest that Hsp104 acts by "burning" ATP to disaggregate SOD1 protein complexes rather than by preventing the aggregation of SOD1 monomers or dimers. In the latter scenario, ATP non-burning Hsp104 should have an effect comparable with that of wild-type Hsp104. The observation that binding alone is insufficient indicates that prevention is less likely than reversal. Liberated SOD1 is, however, not comparable with wild-type SOD1. SOD1 normally exists as a dimer. Higher-order states are observed only in ALS patients. Hsp104-treated SOD1 remains in a trimeric or higher state. Thus, the present study suggests that disaggregating SOD1 proteins will have only a limited effect in preventing cell death.

Acknowledgments

This work was supported by the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, the Republic of Korea (A101254), and by NRF grant funded by the Korea government (MEST) (No. R1203241).

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