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

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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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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