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A Revisited Folding Reporter for Quantitative Assay of Protein Misfolding and Aggregation in Mammalian Cells

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Abstract

Protein misfolding and aggregation play important roles in many physiological processes. These include pathological protein aggregation in neurodegenerative diseases and biopharmaceutical protein aggregation during production in mammalian cells. In order to develop a simple non-invasive assay for protein misfolding and aggregation in mammalian cells, the folding reporter green fluorescent protein (GFP) system, originally developed for bacterial cells, was evaluated. As a folding reporter, GFP was fused to the C-terminus of a panel of human copper/zinc superoxide dismutase (SOD1) mutants with varying misfolding/aggregation propensities. Flow cytometric analysis of transfected HEK293T and NSC-34 cells revealed that the mean fluorescence intensities of the cells expressing GFP fusion of SOD1 variants exhibit an inverse correlation with the misfolding/aggregation propensities of the four SOD1 variants. Our results support the hypothesis that the extent of misfolding/aggregation of a target protein in mammalian cells can be quantitatively estimated by measuring the mean fluorescence intensity of the cells expressing GFP fusion. The assay method developed here will facilitate understanding of aggregation process of SOD1 variants and identifying aggregation inhibitors. The method also has great promise for misfolding/ aggregation study of other proteins in mammalian cells.

Keywords

green fluorescence protein; protein aggregation; protein misfolding; SOD1

1. Introduction

Protein misfolding and aggregation are often causative to the onset of numerous neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic lateral sclerosis (ALS) [1–7]. Therefore, modulating protein misfolding and aggregation are generally considered a promising strategy to treat neurodegenerative diseases [8–14]. Furthermore, intracellular aggregate formation and misfolding of biopharmaceuticals, such as α -galactosidase, α -glucosidase, antithrombin III, and angiopoietin-1 in mammalian cells are often obstacles in achieving high yield production of biopharmaceuticals [15–19]. Therefore, quantitative analysis of protein misfolding and aggregation in mammalian cells will facilitate enhanced understanding of the molecular mechanisms for various human diseases and improve recombinant protein quality produced in mammalian cells. In his pioneering work, Johnson et al. reported a novel split GFP complementation system to monitor aggregation of mutant tau proteins, which is

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associated with Alzheimer's disease, in mammalian cells [20]. The split GFP complementation is based on the assumption that a small GFP fragment fused to a target protein in aggregates has very limited accessibility to a large GFP fragment. Therefore, this method may not be effective when a target protein forms loosely packed aggregates enabling the small GFP fragment access to the large fragment. However, efficient complementation of the split GFP fragments requires a delicate control of relative (and absolute) expression levels of two split GFP fragments [20]. Therefore, the need remains to develop a simple but effective quantitative assay for protein misfolding and aggregation in mammalian cells. In order to address this need, intact GFP fusion to a target protein, originally developed to monitor protein aggregation/misfolding in bacterial cells, was reevaluated in mammalian cells.

For bacterial and yeast cells, this simple assay method for protein misfolding and aggregation has proven effective [21–25]. GFP folding for fluorescence is directly affected by folding of a target protein fused to the N-terminus of GFP. Any deviations from a correctly folded target protein structure such as misfolding and aggregation lead to a loss in the fluorescence of cells expressing the GFP fusion of the target protein in bacteria. With the aid of analytic tools that measure cellular fluorescence, including fluorescence microplate reader and flow cytometer, the extent of the deviation from correctly folded target protein structure can be quantitatively determined. However, GFP fusion for quantitative analysis of protein misfolding and aggregation has thus far been restricted to bacterial systems. Considering the general utility of GFP in both bacteria and mammalian cells as a reporter of cellular processes including protein expression, degradation, localization, and proteolysis [26–28], we hypothesize that GFP fusion to the C-terminus of a target protein is also effective in quantitatively monitoring protein misfolding and aggregation in mammalian cells.

In order to validate the GFP fusion method's capacity to monitor protein misfolding/aggregation in mammalian cells, human copper/zinc superoxide dismutase (SOD1) mutants were chosen due to several features of SOD1. First, SOD1 mutants form intracellular aggregates in mammalian cells in a relatively short period time, usually within two to three days post-transfection [29]. Second, SOD1 mutants are known to form loosely packed aggregates inside mammalian cells [30] and so the split GFP complementation may not be effective in monitoring protein aggregation. Third, SOD1 mutants are associated with neurodegenerative disorder familial form of amyotrophic lateral sclerosis (fALS) [10, 31, 32]. Amyotrophic lateral sclerosis (ALS), often referred to as Lou Gehrig's disease, is a progressive neurodegenerative disorder that attacks and inevitably leads to the death of motor neurons in the brain, brain stem and spinal cord [31]. There are over one hundred natural SOD1 mutants with varying misfolding/aggregation propensities, though wild-type SOD1 forms a stable dimer [32–36]. It has been proposed that aggregation of the mutant SOD1 is linked to the gain of toxic function, eventually leading to the development of ALS. The most common mutation among mutant SOD1-associated fALS patients in the United States is alanine to valine at 4th residue (A4V). Researchers have biophysically characterized this mutation and have found that this mutation causes substantial destabilization at the dimer interface and the rapid development of aggregates in vitro and in vivo [33, 37, 38]. The progression of fALS has been linked to the level of aggregate formation in the spinal cord, implying SOD1 aggregation a potential drug target for preventing or curing ALS [39]. In order to screen molecules that inhibit mutant SOD1 aggregation, a simple, but effective way to monitor mutant SOD1 aggregation needs to be developed. Stevens et al. demonstrated that GFP tagging does not substantially alter properties of SOD1 variants inside cells using fluorescence microscopy [40]. Although this result supports the idea that GFP is safe to fuse to SOD1 variants, the fluorescence microscopy technique is not suitable to quantitatively monitor aggregation of mutant SOD1.

Therefore, we investigated whether flow cytometric analysis can be employed to quantitatively monitor aggregation of mutant SOD1s.

We chose wild-type SOD1 (SOD1^{WT}) and three aggregation-prone SOD1 mutants (SOD1^{A4V}, SOD1^{A4V/C57S}, and SOD1^{A4V/C111S}) as model proteins. As described earlier, SOD1^{A4V} is known to form aggregates in vitro and in vivo [33, 37, 38]. Among four cysteines (Cys6, Cys57, Cys111, and Cys146), two free cysteines Cys6 and Cys111 are known to form inter-molecular disulfide bond network with other SOD1 monomers containing free C6 or C111 during the aggregation process [29, 41–43]. Therefore, Cys to Ser mutation at residue 111 of SOD1 reduces SOD1 aggregation [29, 42]. SOD1^{A4V/C111S} mutant is more stable and soluble than SOD1^{A4V} mutant in cultured cells and in vitro [41, 43, 44], indicating that SOD1^{A4V/C111S} has a lower misfolding/aggregation propensity than SOD1^{A4V}. The other two cysteines, C57 and C147, are involved in an intra-subunit disulfide bond formation; an important post-translational modification of SOD1. It was reported that removing a Cys residue involved in the intra-subunit disulfide bond formation greatly destabilizes the protein and so enhances misfolding of the protein in cultured cells and in vitro [41, 44–46], indicating that the misfolding propensity of SOD1^{A4V/C57S} is higher than that of SOD1^{A4V}. In summary, for the four SOD1 mutants used in this study, the order of extent of deviations from correctly folded structure (misfolding/aggregation) is SOD1^{A4V/C57S} > SOD1^{A4V} > SOD1^{A4V/C111S} > and SOD1^{WT}.

Herein we investigate whether there is a direct correlation between misfolding/aggregation propensity of the four SOD1 variants and mean cellular fluorescence of transfected HEK293T cells expressing the corresponding SOD1 variant fused to GFP. Then, in order to generalize our hypothesis that GFP fusion is effective in quantitatively monitoring mammalian cell protein misfolding/aggregation, we also extend our studies to another cell line (neuroblastoma NSC-34 cells) and promoter (ubiquitin-C).

2. Materials and Methods

2.1 Materials

Anti-SOD1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- α tubulin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Anti-rabbit antibody was obtained from Invitrogen (Carlsbad, CA). HEK293T and NSC-34 cells were obtained from Invitrogen and CELLutions Biosystems (Burlington, Ontario, Canada), respectively. Restriction enzymes and DNA ligases were obtained from New England Biolabs (Ipswich, MA). All other chemicals were obtained from Sigma-Aldrich, unless otherwise noted.

2.2 Construction of Expression Vectors

The EGFP gene is located to 3' of either SOD1^{WT} or SOD1^{A4V} gene in pEGFP-N3-SOD1^{WT} or pEGFP-N3-SOD1^{A4V} plasmid, respectively [32]. In both plasmids, SOD1-EGFP fusion protein expression is under the control of Cytomegalovirus (CMV) constitutive promoter. Other detailed procedures to construct expression vectors are described in the Supplemental Information section.

2.3 Transfection of HEK293T and NSC-34 cells

HEK293T cells were cultured on 6-well plates at 37° C in Dulbecco's modified Eagle's medium/High Glucose (DMEM/High Glucose, Thermo Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS), 100 μ g/mL of streptomycin sulfate and 100 units/ml of penicillin. NSC-34 cells were maintained in DMEM/12:1:1 modified containing 10% FBS. When the cells grew to 80–90% confluency, they were transfected

with appropriate plasmids via calcium phosphate precipitation [47, 48]. All samples were tested in triplicate, unless otherwise described.

2.4 Fluorescence Microscopy

At two days after transfection, the transfected HEK293T cells cultured on the 6-well plates were visualized by fluorescence microscopy using a VistaVision Inverted Fluorescence Microscope (VWR, Radnor, PA). Fluorescence microscopic images of the cells were taken with a DV-2C digital camera equipped on the microscope at the same magnification and camera settings. The fluorescence excitation wavelength is between 420 and 485 and the emission wavelength is 515 nm. The number of cells in the fluorescence microscopy images was manually counted. The percentage of transfected cells exhibiting SOD1-EGFP aggregates was determined by dividing the number of aggregates-containing cells by the number of total cells analyzed.

2.5 Flow Cytometric Analysis of Cellular Fluorescence

After incubating the desired amount of time, the fluorescence intensities of the HEK293T and NSC-34 cells expressing SOD1 variant-EGFP fusion protein were measured using the C6 flow cytometer (Accuri, Ann Arbor, MI). To prepare the cells for flow cytometric analysis, the HEK 293T cells and NSC-34 cells were trypsinized, washed twice with phosphate buffered saline buffer (PBS; pH 7.4) and diluted ten-fold in PBS to prevent cell aggregation. The excitation wavelength was 488 nm and the fluorescence emission was detected at 585 nm. Only GFP positive cells were used to calculate the mean cellular fluorescence. Transfection efficiency was determined by dividing the number of fluorescence positive cells by the number of total cells analyzed. The mean cellular fluorescence intensities were obtained by analyzing 20,000 to 50,000 cells. Each sample was prepared in triplicate and cellular fluorescence indicates mean cellular fluorescence, unless otherwise noted. Student's paired t-test was used for statistical analysis of fluorescence data. Gating strategy is described in Supplemental Information (Figure S1).

2.6 Dot-blotting

5×10^5 transfected HEK293T cells were trypsinized and centrifuged to obtain a cell pellet. The pellet was then washed with 1X PBS once and centrifuged to remove the buffer. The cell pellet was lysed in 100 μ L of lysis buffer containing 100mM NaH₂PO₄, 10mM Tris-Cl, 1x protease inhibitor cocktail (Sigma), and 8 M Urea at room temperature for 1 hour. The cell lysates were centrifuged at 14,000g for 30 minutes to remove the cell debris and the supernatant was collected. Protein concentration of the cell lysate was determined using BCA kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. Dot-blot assay of the cell lysate was performed as described earlier [13, 14] except antibodies used (anti- α tubulin and HRP-conjugated anti-GFP antibodies in this study). The blot images were captured using a BioSpectrum imaging system (UVP).

3. Results and Discussion

3.1 Fluorescence Microscopic Observation of Transfected HEK293T Cells Expressing SOD1 Variants

HEK293T cells were transfected with plasmids encoding each of the four SOD1-EGFP variants genes (SOD1^{WT}-EGFP, SOD1^{A4V/C111S}-EGFP, SOD1^{A4V}-EGFP, and SOD1^{A4V/C57S}-EGFP). At two days post-transfection, fluorescence images of the transfected cells were taken (Figure 1A). Transfected HEK293T cells expressing SOD1^{WT}-EGFP were brightest among the four different transfected cells. The order of brightness of the transfected cells expressing four SOD1 variants fused to EGFP is SOD1^{WT} > SOD1^{A4V/C111S} > SOD1^{A4V} > and SOD1^{A4V/C57S}, which is consistent with the inverse

order of misfolding/aggregation propensity of the four SOD1 variants in vitro and in cultured cells previously reported [41, 43, 44].

3.2 Flow Cytometric Analysis of Transfected HEK293T Cells Expressing SOD1 Variants

Fluorescence microscopic observation of cells expressing GFP fusion protein is a non-invasive method to qualitatively compare the fluorescence intensities of different cell samples but is not suitable for quantitative analysis. Therefore, we employed flow cytometric analysis of the transfected HEK293T cells to evaluate cellular fluorescence. At one and two days post-transfection of HEK293T cells with plasmids encoding four SOD1-EGFP fusion genes respectively, the mean fluorescence intensities of the four transfected cells were measured by flow cytometry (Figure 2A). The representative overlaid histograms of fluorescence of cells are shown in Figure S2 in Supplemental Information. In this article, the fluorescence intensities measured by flow cytometry indicate the mean cellular fluorescence intensities. As expected, the order of fluorescence intensity of the four transfected cells is $\text{SOD1}^{\text{WT}} > \text{SOD1}^{\text{A4V/C111S}} > \text{SOD1}^{\text{A4V}} > \text{SOD1}^{\text{A4V/C57S}}$, matching the inverse order of the misfolding/aggregation propensities of the four SOD1 variants. The cellular fluorescence intensity of $\text{SOD1}^{\text{A4V/C111S}}$ was significantly higher than that of SOD1^{A4V} ($P < 0.05$), whereas the cellular fluorescence intensity of $\text{SOD1}^{\text{A4V/C57S}}$ was significantly lower than that of SOD1^{A4V} ($P < 0.001$) (Figure 2A).

Although flow cytometric analysis of cellular fluorescence is a very convenient way to obtain information on cellular processes, caution should be taken to directly correlate the cellular fluorescence intensities to protein misfolding/aggregation. Other factors such as transfection efficiency and expression level of a target protein can also affect the fluorescence intensity of transfected cells. First, effects of transfection efficiencies on cellular fluorescence were investigated. The transfection efficiencies of the four SOD1 variants were determined by flow cytometry. The transfection efficiency was calculated by dividing the number of GFP positive cells by the number of total cells analyzed by flow cytometry. Although the transfection efficiencies vary from 42% to 55%, the differences are not large enough to explain the differences in cellular fluorescence of the four different transfected cells, supporting the idea that intensity is related to SOD1 properties. The transfection efficiencies of SOD1^{A4V} and $\text{SOD1}^{\text{A4V/C57S}}$ were 5 to 13% lower than those of SOD1^{WT} and $\text{SOD1}^{\text{A4V/C111S}}$, which is most likely due to the exclusion of some weak fluorescent cells in the samples. If the fluorescence intensity of the transfected cells are overlapped with that of untransfected cells (negative control), such weak fluorescent cells are not counted in determining the mean cellular fluorescence intensity after the background subtraction in flow cytometric analysis. According to the transfection efficiencies measured by flow cytometry (Figure 1), the maximum difference among the transfection efficiencies was 13%. However, this difference is too small to explain the more than 4-fold difference in the fluorescence intensities of cells expressing SOD1^{WT} and $\text{SOD1}^{\text{A4V/C57S}}$ (Figure 2A). Furthermore, the lower transfection efficiency of $\text{SOD1}^{\text{A4V/C57S}}$ cell sample is most likely made by the exclusion of weakly fluorescent cells from the transfection efficiency calculation due to the background subtraction in data analysis. If the weak fluorescent cells were included to correct the transfection efficiency, the cellular fluorescence intensities would decrease, making the difference in the cellular fluorescence intensities between SOD1^{WT} and $\text{SOD1}^{\text{A4V/C57S}}$ larger. Therefore, our results suggest that the low transfection efficiencies of SOD1^{A4V} and $\text{SOD1}^{\text{A4V/C57S}}$ are not the major cause of their low cellular fluorescence.

Second, we investigated whether the differences in the cellular fluorescence intensities resulted from differences in the expression levels of SOD1 variants; because the mean fluorescence intensities of cells can change according to SOD1-GFP fusion protein expression level. Therefore, expression levels of four different SOD1-EGFP fusion proteins

were compared using dot-blot assay using anti-SOD1 polyclonal antibody. Since HEK293T cells also express endogenous SOD1^{WT}, we first compared the endogenous SOD1^{WT} level in untransfected HEK293T cells and the total SOD1^{WT} level in transfected HEK293T cells over-expressing SOD1^{WT}-EGFP. As expected, anti-SOD1 immuno-reactivity of the untransfected cells was negligible compared to that of the transfected cells over-expressing SOD1^{WT}-EGFP (Figure S3 in Supplemental Information). Therefore, we ignored endogenous SOD1^{WT} expression level when over-expression levels of the four SOD1-EGFP fusion proteins are compared. Next, transfected cells expressing each of four SOD1-EGFP fusion proteins (SOD1^{WT}-EGFP, SOD1^{A4V/C111S}-EGFP, SOD1^{A4V}-EGFP, and SOD1^{A4V/C57S}-EGFP) were lysed and the total cell lysates were used for dot-blot assay. Anti- α tubulin antibody was used to ensure loading a comparable amount of the protein extracts to a nitrocellulose membrane (Figure 2B bottom). The immuno-reactivity of all four SOD1-EGFP fusion proteins was comparable (Figure 2A top), strongly indicating that the substantial differences in the cellular fluorescence do not result from differences in their expression levels.

Third, besides transfection efficiency and expression level, there might be other factors that affect cellular fluorescence intensities. If there are other critical factors affecting the cellular fluorescence intensities, the results may not be reproducible. In order to confirm the reproducibility of the results, transfection and cellular fluorescence measurement were performed on two separate days with a month gap. Then, the mean fluorescence intensities of the transfected cells obtained from two independent experiments were compared (Figure 2C). For the four SOD1-EGFP variants (SOD1^{WT}-EGFP, SOD1^{A4V/C111S}-EGFP, SOD1^{A4V}-EGFP, and SOD1^{A4V/C57S}-EGFP), there is a linear correlation between the cellular fluorescence intensities of the cells transfected on two separate days ($R^2 = 0.99$), though there was a slight difference in the absolute fluorescence intensities (around 5%). Our results strongly indicate that the correlation between the cellular fluorescence intensities and the misfolding/aggregation propensities of the SOD1 variants is quite reproducible. However, since absolute fluorescence intensities of the transfected cells expressing EGFP fusion protein may vary each time, an accurate estimation of misfolding/aggregation status of a target protein requires a calibration using both highly stable and misfolding/aggregation-prone protein controls.

In order to compare the efficacy of EGFP fusion to that of split GFP complementation, split GFP complementation was performed on SOD1^{WT} and SOD1^{A4V} (experimental details are described in the Supplemental Information). The mean cellular fluorescence intensities of SOD1^{WT} and SOD1^{A4V} were not significantly different (Figure S4 in the supplemental Information), which is likely due to loosely packed structure of SOD1^{A4V} aggregates [30] allowing access of a small fragment of GFP fused to SOD1^{A4V} to a large GFP fragment. This finding suggests the idea that EGFP fusion is more effective in monitoring protein aggregation, which is loosely packed, than split GFP complementation.

3.3 Aggregate Formation of SOD1^{A4V} Variants in Transfected HEK293T Cells

Our results indicate that transfected cells expressing EGFP fusion of SOD1 variants exhibited significant disparity in the mean cellular fluorescence intensity; but the disparity was not caused by differences in the transfection efficiency or expression level of the SOD1 variants. Therefore, it is reasonable to assume that there are substantial differences in the structures of the SOD1 variants. We hypothesized that the disparity in fluorescence intensities of cells expressing the SOD1 variants result from differences in the extent of misfolding, aggregation, or both for the SOD1 variants. Transfected cells expressing four SOD1-EGFP variants were re-examined using fluorescence microscopy at a higher magnification (400X). Transfected cells expressing SOD1^{A4V} exhibited visible aggregates inside cells whereas cellular fluorescence of the transfected cells expressing SOD1^{WT} is

evenly distributed in the cytosol (Figure 1B), consistent with results previously reported [49]. Similar intracellular aggregates were found in both transfected cells expressing SOD1^{A4V/C111S} and SOD1^{A4V/C57S}, respectively (Figure S5 in the Supplemental Information). The percentage of cells containing intracellular aggregates was determined by examining the fluorescence microscopic images of the transfected cells. Over five hundred cells for each SOD1 variant were analyzed and the results were plotted (Figure 3). Around 12% of the transfected cells expressing SOD1^{A4V} exhibited intracellular aggregates, whereas none of the transfected cells expressing SOD1^{WT} showed any intracellular aggregates. However, approximately 5% of the cells expressing SOD1^{A4V/C111S} showed aggregates, consistent with the reduced aggregation of SOD1^{A4V/C111S} compared to SOD1^{A4V} in mammalian cells [41]. Therefore, the significant increase ($P < 0.05$) in the cellular fluorescence intensity of cells expressing SOD1^{A4V/C111S} over that of SOD1^{A4V} (Figure 2A) is attributed to the reduced aggregation of SOD1^{A4V/C111S} compared to SOD1^{A4V} (Figure 1B). In the case of SOD1^{A4V/C57S}, 10% of the transfected cells exhibited intracellular aggregates but the difference from SOD1^{A4V} is not statistically significant ($P > 0.05$). Therefore, the percentage of cells exhibiting SOD1-EGFP aggregates determined by fluorescence microscopy does not correlate well to the cellular fluorescence intensity. However, the enhanced misfolding propensity of SOD1^{A4V/C57S} over SOD1^{A4V} [41], which severely inhibits correct folding of GFP, is better attributed to a loss of fluorescence. In Figure 2A, the change in the cellular fluorescence caused by C57S mutation was greater than the change caused by C111S, which can be explained by the greater extent of structural perturbations made by aggregation and misfolding. Considering that the loss of an intra-subunit disulfide bond caused by C57S mutation generates very unstable monomeric SOD1s, structural perturbation of SOD1 caused by C57S mutation is likely greater than that of SOD1 caused by intermolecular disulfide network-mediated aggregation. In summary, mean cellular fluorescence intensity of cells expressing EGFP fusion of SOD1 variant is a good indicator of deviations (combined effects of misfolding and aggregation) from correctly folded structure. However, caution should be taken when correlating the cellular fluorescence intensity to only either aggregation or misfolding of SOD1 variants, because both aggregation and misfolding of SOD1 variant may not occur simultaneously.

3.4 Comparison of the Cellular Fluorescence of Transfected NSC-34 and HEK293T Cells Expressing SOD1 Variants

In the transfected HEK293T cells expressing four SOD1-EGFP variants, there was good quantitative correlation between the cellular fluorescence intensity and the combined misfolding and aggregation propensities. In order to confirm that the correlation is valid in cells other than HEK293T cells, mouse neuroblastoma NSC-34 cells [50] were also examined at two days post-transfection using flow cytometric analysis similar to the transfected HEK293T cells. NSC-34 cell is a widely used cell line to study SOD1 expression in cultured cells [51–55]. Even in the transfected NSC-34 cells, the order of cellular fluorescence intensities matched well with the inverse order of misfolding/aggregation propensities of the SOD1 variants (Figure 4A). In order to determine whether there is a direct correlation between the mean fluorescence intensities of HEK293T and NSC-34 cells expressing the four SOD1-EGFP variants, the mean cellular fluorescence intensities of both cells were plotted (Figure 4B). The fluorescence ratios of SOD1 variant over SOD1^{WT} in NSC-34 cells were similar to those in HEK293T cells. Furthermore, a linear correlation between the fluorescence intensities of both transfected cells was observed ($R^2 = 0.98$), indicating that an inverse relationship between the cellular fluorescence intensity and the misfolding/aggregation propensity of SOD1 variants is valid in both cell lines. However, the fluorescence intensities of the transfected NSC-34 cells are about one half of that of the corresponding transfected HEK293T cells, which can be explained by weaker biosynthesis activities in slow growing NSC-34 cells. Therefore, in order to estimate extent of the

deviation of a target protein from its correctly folded structure in a specific cell line, cellular fluorescence intensities should be calibrated using appropriate positive and negative control samples.

3.5 Comparison of the CMV and Ubiquitin-C Promoters

Thus far SOD1-EGFP fusion proteins were expressed under control of CMV constitutive promoter. We chose CMV promoter because it is widely used to express recombinant proteins in mammalian cells due to its relatively strong promoter activity in diverse cell lines [56]. However, we can imagine situations where a different promoter should be used. Therefore, we examined whether the choice of promoter affects the correlation between the cellular fluorescence intensity and SOD1 variant misfolding/aggregation. In order to compare promoters, we chose Ubc promoter which is also widely used in mammalian cells [56]. SOD1^{WT}-EGFP and SOD1^{A4V}-EGFP genes were sub-cloned into pFUG-IP lentiviral vector plasmid [48] where a target gene is under the control of ubiquitin-C (Ubc) promoter and an internal ribosome entry site/puromycin resistance gene cassette is located at 3' of a target gene. HEK293T cells were transfected with the plasmids encoding either SOD1^{WT}-EGFP or SOD1^{A4V}-EGFP gene under the control of Ubc promoter. At two days post-transfection, fluorescence intensities of the transfected cells were determined by flow cytometry. The cellular fluorescence intensity of cells expressing SOD1^{A4V}-EGFP was about six-fold lower than that of cells expressing SOD1^{WT}-EGFP (Figure 5), indicating that Ubc promoter also allows distinction of cellular fluorescence intensities depending on their misfolding/aggregation propensity of SOD1 variants. The six-fold difference between the cellular fluorescence intensities of SOD1^{WT}-EGFP and SOD1^{A4V}-EGFP with Ubc promoter is greater than the two-fold difference with CMV promoter. Such a greater difference might be caused by the change of promoter from CMV to Ubc and/or the change of plasmid backbone from pEGFP-N3 to pFUG-IP. In order to investigate this, we replaced the Ubc promoter in the pFUG-IP plasmid backbone with CMV promoter. The cellular fluorescence intensity of cells transfected with the modified pFUG-IP plasmid carrying CMV-SOD1^{WT}-EGFP cassette was around four-fold greater than that of CMV-SOD1^{A4V}-EGFP cassette in the same plasmid backbone, which suggests that the pFUG-IP plasmid backbone plays an important role in increasing the difference between the cellular fluorescence intensities of SOD1^{WT}-EGFP and SOD1^{A4V}-EGFP. It is noteworthy that the pFUG-IP plasmid encodes a puromycin resistant gene as well as a target protein gene. Therefore, both puromycin resistant gene and SOD1-EGFP genes are over-expressed when pFUG-IP plasmid is used, whereas only SOD-EGFP is over-expressed when pEGFP-N3-SOD1 plasmid is used. Therefore, it is possible that chaperone proteins facilitating folding of unstable SOD1 are less available to SOD1 when pFUG-UP plasmid is used, because the chaperone proteins are also involved in the folding of co-expressed puromycin resistant protein. Furthermore, it was reported that several SOD1 mutants inhibit chaperone activities [57]. Therefore, we hypothesize that over-expression of another gene, as well as SOD1-EGFP, limits chaperone activity required for SOD1-EGFP folding. Such a shortage of chaperone activity is more detrimental to mutant SOD1 folding and leads to the greater reduction in the cellular fluorescence intensity. In order to investigate this hypothesis, we co-transfected HEK293T cells with both pEGFP-N3-SOD1^{A4V} and pAAV-Luc (a plasmid encoding luciferase gene under the control of CMV promoter) plasmids and also observed sixfold difference between the fluorescence intensities of SOD1^{WT}-EGFP and SOD1^{A4V}-EGFP with co-expression of luciferase protein (Figure S6 in the Supplemental Information). Therefore, when another protein is over-expressed, as well as the EGFP fusion of a target protein, caution should be taken to use the EGFP fusion to monitor aggregation/misfolding.

The fluorescence intensity of cells expressing SOD1^{WT}-EGFP under the control of Ubc promoter was about 5-fold lower than that of CMV promoter (Figure 5), likely due to

weaker activity of Ubc promoter than CMV promoter in HEK293T cells [56]. As expected, when the Ubc promoter in the pFUG-IP backbone was replaced with CMV promoter, the cellular fluorescence intensities of cells expressing both SOD1^{WT}-EGFP and SOD1^{A4V}-EGFP increased two fold (Figure 5). In summary, both CMV and Ubc promoters enable distinction of cellular fluorescence depending on misfolding/aggregation propensity of SOD1 variants.

4. Concluding remarks

Our investigation has conclusively established that EGFP fused to SOD1 variant C-terminus allows the extent of deviations to be determined from the correctly folded structure of SOD1 via cellular fluorescence measurement. The order of fluorescence intensities of transfected cells expressing four SOD1 variants correlates well to the inverse order of misfolding/aggregation propensities of the four SOD1 variants in both HEK293 and NSC-34 cells. Therefore, mean cellular fluorescence of cells expressing EGFP fusion of a target protein is a good indicator of misfolding/aggregation of the target protein. Flow cytometric monitoring of mutant SOD1 aggregation in cultured cells is a simple, but effective method that provides an array of structural information. This method could be used to screen for small molecule or protein-based drugs that inhibit mutant SOD1 aggregation. Screening of small molecule inhibitors in cultured cells is desirable to identify small molecule inhibitors of mutant SOD1 aggregation. If the small molecules identified were to inhibit mutant SOD1 aggregation, the fluorescence intensity of the cells will be near that of SOD1^{WT}. Combined with an automatic sampler in a 96-well plate format, large small molecule inhibitor libraries can be screened with moderate to high throughput. Protein-based therapeutics that inhibit mutant SOD1 aggregation can be also identified by screening protein libraries in a high-throughput manner using fluorescence activated cell sorter. With the rapid advancements in ALS research, this assay would serve as a powerful tool in understanding the pathological mechanism and developing treatments for fALS.

Generality of GFP tagging method will easily allow extension of this method to monitor other cytosolic proteins that readily form aggregates. Aggregation of recombinant protein expressed in mammalian cells can reduce recombinant protein production yield. Therefore, the folding reporter can be used to determine residues causing protein aggregation and to identify less aggregation-prone protein variants by screening protein libraries. Furthermore, the folding reporter can be used to develop a fusion tag/protein to enhance solubility of target proteins in mammalian cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALS	Amyotrophic lateral sclerosis
CMV	cytomegalovirus

DMEM	Dulbecco's modified Eagle's medium
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
GFP	green fluorescent protein
HEK	human embryonic kidney cell
SOD1	human copper/zinc superoxide dismutase
Ubc	ubiquitin-C

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Figure 1. Fluorescence microscopy images of the transfected HEK293T cells expressing SOD1 variants fused to EGFP
(A) Images of transfected HEK293T cells expressing EGFP fusion of SOD1^{WT}, SOD1^{A4VC111S}, SOD1^{A4V}, and SOD1^{A4V/C57S} taken at two days post-transfection. Transfection efficiency for each SOD1 variant was determined by calculating the percentage of GFP positive cells out of total cells analyzed using flow cytometry. Values represent means ± standard deviation (n = 3). Scale bars are 250 μm. (B) Magnified fluorescence microscopic images of the transfected HEK293T cells expressing EGFP fusion of SOD1^{WT} and SOD1^{A4V}. Arrows indicate the aggregates within the cell. Scale bars are 50 μm.

B

Figure 2. Comparison of the cellular fluorescence intensities and expression levels of EGFP fusion of SOD1 variants

(A) Time course of fluorescence intensities of the transfected HEK293T cells expressing EGFP fusion of SOD1 variants. Fluorescence intensities of untransfected cells (day 0) and transfected cells expressing EGFP fusion of four SOD1 variants (SOD1^{WT}, SOD1^{A4V/C111S}, SOD1^{A4V}, and SOD1^{A4V/C57S}) at day 1 and 2 were determined by flow cytometry. Values and error bars represent mean cellular fluorescence and standard deviations, respectively (n = 3). Two-sided Student's t-tests were applied to the data (*: P < 0.05; **: P < 0.001). (B) Dot-blot images of the transfected cell lysates of EGFP fusion of SOD1^{WT} (WT), SOD1^{A4V/C111S} (A4V/C111S), SOD1^{A4V} (A4V), and SOD1^{A4V/C57S} (A4V/C57S) using anti-SOD1 polyclonal antibody. (α-tubulin; loading control) (C) Comparison of the mean cellular fluorescence of HEK293T cells expressing SOD1 variants that were transfected at two separate days. At two days post-transfection, cellular fluorescence of transfected HEK293T cells expressing EGFP fusion of SOD1 variants (SOD1^{WT}, SOD1^{A4V/C111S}, SOD1^{A4V}, and SOD1^{A4V/C57S}) was determined by flow cytometry. The data were fitted to a straight line (R² = 0.99). (a.u. = arbitrary unit) Numerical values of the mean cellular fluorescence intensities are shown in Table S1 in Supplemental Information.

Figure 3. The percentage of transfected HEK293T cells exhibiting SOD1 aggregates

The number of cells exhibiting intracellular SOD1 aggregates was determined by analyzing fluorescence microscopy images of the transfected cells expressing EGFP fusion of four SOD1 variants (SOD1^{WT}, SOD1^{A4V/C111S}, SOD1^{A4V}, and SOD1^{A4V/C57S}) at day two post-transfection. Values and error bars represent mean and standard deviations, respectively (n = 3). Two-sided Student's t-tests were applied to the data (*: P < 0.05). Numerical values of the mean cellular fluorescence intensities are shown in Table S2 in Supplemental Information.

A

B

Figure 4. Comparison of the orders of fluorescence intensities of transfected HEK293T and NSC-34 cells expressing EGFP fusion of SOD1 variants

(A) Fluorescence intensities of the transfected NSC-34 cells expressing EGFP fusion of SOD1 variants (SOD1^{WT}, SOD1^{A4V/C111S}, SOD1^{A4V}, and SOD1^{A4V/C57S}) at two days post-transfection were determined by flow cytometry. Values and error bars represent mean cellular fluorescence and standard deviations, respectively (n = 3). (a.u. = arbitrary unit) Two-sided Student's t-tests were applied to the data (*: P < 0.05; **: P < 0.01). Numerical values of the mean cellular fluorescence intensities are shown in Table S1 in Supplemental Information. (B) Comparison of the cellular fluorescence of the transfected HEK293T at day two and NSC-34 cells at day three post-transfection. The cellular fluorescence of transfected HEK293T and NSC-34 cells expressing EGFP fusion of SOD1 variants (SOD1^{WT}, SOD1^{A4V/C111S}, SOD1^{A4V}, and SOD1^{A4V/C57S}) was determined by flow cytometry. The data were fitted to a straight line (R² = 0.98). FL(SOD1^X)/FL(SOD1^{WT}) indicates the ratio of fluorescence intensity of cells expressing a SOD1 variant (SOD1^X) over the fluorescence intensity of cells expressing SOD1^{WT}.

Figure 5. Comparison of CMV and Ubc promoter activities for the fluorescence intensities of transfected HEK293T cells expressing EGFP fusion of SOD1 variants

Cellular fluorescence of HEK293T cells expressing EGFP fusion of SOD1^{WT} and SOD1^{A4V} under the control of either CMV or Ubc promoter was determined by flow cytometry. Values and error bars represent mean cellular fluorescence and standard deviations, respectively (n = 3). (a.u. = arbitrary unit) Numerical values of the mean cellular fluorescence intensities are shown in Table S1 in Supplemental Information.