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Clearance and Phosphorylation of Alpha-Synuclein Are Inhibited in Methionine Sulfoxide Reductase A Null Yeast Cells

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Abstract

Aggregated α-synuclein and the point mutations Ala30Pro and Ala53Thr of α-synuclein are associated with Parkinson's disease. The physiological roles of α-synuclein and methionine oxidation of the α-synuclein protein structure and function are not fully understood. Methionine sulfoxide reductase A (MsrA) reduces methionine sulfoxide residues and functions as an antioxidant. To monitor the effect of methionine oxidation to α-synuclein on basic cellular processes, α-synucleins were expressed in *msrA* null mutant and wild-type yeast cells. Protein degradation was inhibited in the α-synuclein-expressing *msrA* null mutant cells compared to α-synuclein-expressing wild-type cells. Increased inhibition of degradation and elevated accumulations of fibrillated proteins were observed in SynA30P-expressing *msrA* null mutant cells. Additionally, methionine oxidation inhibited α-synuclein phosphorylation in yeast cells and in vitro by casein kinase 2. Thus, a compromised MsrA function combined with α-synuclein overexpression may promote processes leading to synucleinopathies.

Keywords

Oxidative stress; Posttranslation modification; Neurodegenerative diseases; Parkinson's disease; Antioxidants; Protein aggregation; Yeast; Synuclein

Introduction

One of the hallmarks of Parkinson's disease (PD) is the formation of Lewy bodies (Lucking and Brice 2000). These neuronal inclusion bodies are composed of aggregated proteins and consist mainly of α-synuclein fibrils (Baba et al. 1998). The α-synuclein protein is a presynaptic protein of unknown function and its involvement in the pathogenesis of PD is still not clear. However, the α-synuclein point mutations Ala30Pro and Ala53Thr are found

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in autosomal dominant forms of PD (Kruger et al. 1998; Polymeropoulos et al. 1997). Overexpression of α-synuclein is also associated with the risk of developing PD (Takeda et al. 1998), and α-synuclein locus triplication can cause PD (Singleton et al. 2003). When α-synuclein is abnormally expressed or modified, various studies indicate that it may cause alterations in mitochondrial and proteasomal function, protein aggregation, and accumulation of reactive oxygen species (ROS; Lindersson et al. 2004; Sawada et al. 2004; Snyder et al. 2005; Vekrellis et al. 2004).

Yeast cells have been used to study events related to α-synuclein biology and toxicity. This toxicity was shown to be mediated by increased cellular ROS and decreased proteasome function (Chen et al. 2004; Sharma et al. 2006; Willingham et al. 2003). The monomeric α-synuclein forms fibrils at a relatively slow rate. We hypothesize that modifications alter the physical properties and degradation rate of α-synuclein, overall promoting its oligomerization. This is consistent with the associated increase in oxidative stress among PD, aging, and other neurodegenerative diseases. Increased ROS and cellular α-synuclein levels promote its aggregation (Giasson et al. 2000; Hsu et al. 2000). The presence of ROS can cause posttranslational modifications, commonly to the sulfur-containing residues methionine (Met) and cysteine (Cys). The α-synuclein protein does not have Cys, but does contain four Met residues that can be readily oxidized to Met sulfoxide (MetO) in vitro. Under conditions of oxidative stress, MetO-α-synuclein can form fibrils when triggered by certain metals (e.g., Zn^{2+}, Al^{3+}, etc.) in vitro (Yamin et al. 2003). Furthermore, cellular conditions such as higher α-synuclein protein concentrations (Fink 2006) and a saline environment (Munishkina et al. 2004) could increase the rate of α-synuclein fibril formation. Posttranslational modifications can alter the solubility of α-synuclein, possibly decreasing the clearance of this short-lived protein. Cytoplasmic α-synuclein can be degraded by the ubiquitin–proteasome system, the autophagy system, and lysosomic pathway (Giorgi et al. 2006); however, degradation of α-synuclein is inhibited in PD. It is plausible that the latter inhibition decreases the clearance and promotes aggregation of α-synuclein. Modifications to α-synuclein may alter its solubility and interfere with its ability to be degraded, thereby resulting in a decreased clearance of α-synuclein.

The MetO modifications formed in the presence of ROS produce the enantiomers Met-*S*-O and Met-*R*-O. In proteins, MetO is reduced solely by Msr enzymes, which are MsrA (reduces Met-*S*-O) and MsrB (reduces Met-*R*-O; Moskovitz 2005; Oien and Moskovitz 2008). However, MsrA is thought to be the major Msr because it positively regulates MsrB expression (Moskovitz 2007; Moskovitz and Stadtman 2003). Lack of MsrA has been demonstrated in several organisms to cause a hypersensitivity to oxidative stress conditions (Oien et al. 2008; Pal et al. 2007) and premature death (Moskovitz et al. 2001; Oien and Moskovitz 2007). In contrast, overexpression of *msrA* may suppress dopaminergic cell death and protein aggregation (Liu et al. 2008). One hypothesis to explain the late onset of certain neurodegenerative diseases, such as PD, is that they occur in aging neurons when the capacity of the quality control system to cope with accumulating misfolded proteins is exceeded (Berke and Paulson 2003). This situation has been modeled in yeast by increasing α-synuclein expression (Outeiro and Lindquist 2003). Due to the lack of substantial information about MetO-α-synuclein in vivo, we further explored this by overexpressing α-synuclein and two mutants in *msrA* null (*msrA*-KO) yeast cells. The effects of the α-synucleins on protein fibril formation, degradation, and phosphorylation were monitored.

Materials and Methods

Overexpressing Human α-Synuclein in Yeast

The human α-synuclein protein and its mutants were overexpressed in *msrA*-KO and WT *Saccharomyces cerevisiae* strains with the pYES6 expression vector used previously

(Moskovitz et al. 1997). This vector harbors the yeast GAL1 promoter for high-level inducible protein expression by galactose. It has a C-terminal peptide encoding a V5 epitope and a polyhistidine (6His) tag for detection and purification of the recombinant fusion α-synuclein. This vector contains the ampicillin and blasticidin resistance genes for selection of bacteria and yeast colonies harboring the plasmid, respectively. Three forms of the human α-synuclein cDNA (wild type (α-synuclein), Ala53Thr (SynA53T), and Ala30Pro (SynA30P)) were inserted into the vector keeping the sequence in frame with the 6His peptide. For that purpose, suitable 5′ forward and 3′ reverse complement primers (harboring *Hind* III and *Xba* I restriction sites, respectively) were used with each of the α-synuclein cDNAs (kindly provided by Dr. Mikiei Tanaka, NIH, NHLBI) in a routine polymerase chain reaction (PCR) procedure. The resulting PCR products were subcloned into the suitable complementary restriction sites on the pYES6 and transformed into bacterial cells (DH5α). Each plasmid was isolated from bacterial cells and transformed into WT and *msrA*-KO yeast. Vector-only transformed cells served as controls. Following growth selection on media containing blasticidin (Invivogen), all the transformed yeast strains (WT and *msrA*-KO containing either the vector only, vector containing α-synuclein, or vector containing either SynA53T or SynA30P) were grown in induction media containing 2% galactose and 1% raffinose for up to 24 h. At various time points, equal amounts of cells were taken from each culture (determined by optical density (OD_{600nm})) and extracted by glass beads and a bead-beater homogenizer apparatus (BioSpec Products, Bartlesville, OK, USA). The α-synuclein proteins were purified from the corresponding extracts by using of ProBond™ Nickel-Chelating Resin (Invitrogen). Equal volumes of purified α-synuclein proteins were subjected to sodium dodecyl sulfate (SDS)-gel electrophoresis and detected by Western blotting using anti-6His antibodies (Immunology Consultants Laboratories).

Total Protein Degradation and Synthesis in Yeast Expressing α-Synuclein

The procedure was performed as done previously (Chen et al. 2005) with modifications. Yeast cells (WT and *msrA*-KO) expressing the three α-synuclein variants (α-synuclein, SynA30P, SynA53T) were grown in yeast extract–peptone–dextrose (YPD; Fisher Scientific) medium to midlogarithmic phase while shaking at 30°C. The medium was removed by centrifugation and replaced with 2% galactose and 1% raffinose media lacking Met. Next, 50μCi [^{35}S]Met (Perkin-Elmer) was added and incubated for additional 30 min. The medium was removed and radioactive labeling was chased by media containing Met without radioactive isotopes. At different time intervals, equal aliquots of cells were removed from each culture and homogenized. The corresponding protein extracts were mixed with trichloroacetic acid (TCA) at a final concentration of 20% (*v/v*). After incubation at 4°C for 1 h, the samples were centrifuged, and the radioactivity (counts per minute) of the TCA-insoluble precipitates and soluble material was measured by liquid scintillation counting. For quantification of protein synthesis, the radioactivity (counts per minute) of the TCA-insoluble precipitate was measured by liquid scintillation counting after labeling with 50μCi [^{35}S]Met and immediate precipitation with TCA.

Phosphorylation of α-Synuclein in Yeast (Ex Vivo System)

The WT and *msrA*-KO yeast cells expressing α-synuclein, SynA30P, and SynA53T were grown in YPD medium to midlogarithmic phase shaking at 30°C. The medium was removed by centrifugation and substituted with induction media containing 2% galactose and 1% raffinose for 1 h. The cells were then incubated in media with only 10% of the nutrients for 1 h to reduce free phosphate. To each culture, 5μCi/mL [^{32}P]orthophosphoric acid (Perkin-Elmer) was added and incubated for 1 h. The cells were harvested and α-synuclein was isolated by resin affinity as described above with the addition of NaF and Na_2VO_4 as phosphatase inhibitors. The α-synuclein was eluted from the resin with 150 mM imidazole. Equal amounts of each eluted protein were subjected to an SDS-gel electrophoresis. At the

end of electrophoretic separation, the gel was stained with Gel-Code Blue (Thermo Scientific, Rockford, IL, USA). Bands corresponding to the molecular weight of the α-synucleins were excised, added to vials with liquid scintillation cocktail, and their counts per minute measurements were determined by liquid scintillation counting.

Phosphorylation of α-Synuclein In Vitro

Recombinant α-synuclein, SynA30P, and SynA53T (rPeptide) were incubated overnight in 200 mM H_2O_2 at 37°C in phosphate buffered saline buffer. The H_2O_2 was removed by adding catalase and incubating 30 min (oxidant removal verified by adding α-synuclein after oxidants were removed, this served as the nonoxidized α-synuclein control). To reduce MetO, recombinant yeast MsrA and PilB were produced as previously described (Moskovitz et al. 1997; Olry et al. 2002). These Msrs were added at a concentration of 0.3 mg/mL with 20 mM dithiothreitol for 1 h. Each α-synuclein was phosphorylated by adding 6 mCi [γ-^{32}P]-ATP (Perkin-Elmer), 20μM cold ATP, 10 mM $MgCl_2$, and 0.3 μg casein kinase 2 (Upstate) for 1 h. Reactions were stopped by adding an equal volume of protein gel sample buffer. Equal amounts of α-synuclein proteins were subjected to an SDS-gel electrophoresis. At the end of the electrophoretic separation, the gel was stained with Gel-Code Blue Stain reagent and exposed to X-ray film.

Protein Fibrillation in Yeast Strains Expressing α-Synuclein

In the presence of fibrils, Thioflavin T undergoes a “red shift” where it is excited at a wavelength of 442 nm and emits fluorescence at 482 nm. Yeast cells were grown in YPD and incubated in media with 2% galactose and 1% raffinose for 3 h. The cells were precipitated by centrifugation and resuspended in 1% Thioflavin T solution. Cells were washed in buffer and aliquots were diluted into a 96-well plate. Finally, Thioflavin T fluorescence was imaged and quantified using an IN Cell Analyzer 1000 (GE Healthcare) high content imaging system.

Statistics

Comparison and calculation of *P* values was performed using Student *t* test analysis. Values of $P<0.05$ were considered significant.

Results and Discussion

Overexpression of α-Synuclein in Yeast

To investigate the effects of MetO on α-synuclein and its point mutation variants (α-synuclein, SynA30P, SynA53T), they were separately overexpressed in WT or *msrA*-KO yeast cells. Cell aliquots were collected at various time points during the expression period of the α-synuclein proteins. Following conditional α-synuclein purification from the yeast cells, bands migrating at ~22 and ~16 kDa positions were detected by Western blot analysis (Fig. 1). The 22-kDa band corresponds to the full length of α-synuclein with the 6His tag moiety, which is known to cause slower migration in gel electrophoresis due to the added mass and positive charge. Accordingly, the 16-kDa and lower detected minor bands correspond to degradation products of the 22-kDa band. The three types of α-synuclein are degraded at an increased rate when expressed in the WT compared to the *msrA*-KO strain. The time-dependent degradation of the corresponding α-synucleins is trailing in the *msrA*-KO relative to WT strain without complete degradation of the 16-kDa band, which is completely degraded in WT strain (Fig. 1). Moreover, among all the types of α-synuclein examined, the SynA53T type seems to be the most resistant to degradation in the WT strain while the SynA30P appears to be the most resistant to degradation in the *msrA*-KO strain (Fig. 1). Interestingly, the degradation of the SynA30P into both the 16-kDA peptide and

lower mass peptides was inhibited more in the *msrA*-KO strain as shown by the relative lower amounts of the shorter peptides products with time (Fig. 1). The antibodies used in the Western blot recognize only α-synuclein protein and derived peptides that still contain the 6His tag, which provides recognition that is not based on α-synuclein conformation.

The expression patterns of α-synuclein suggests that the three types of α-synuclein are degraded faster in the WT compared to the *msrA*-KO strain (Fig. 1). Moreover, the SynA30P variant of α-synuclein was the most resistant to degradation in the *msrA*-KO strain (Fig. 1). This result is supported by previous studies showing that WT yeast cells overexpressing SynA30P are inhibited in their general protein degradation by the proteasome, compared to non-mutated α-synuclein (Chen et al. 2005). We suggest that the degradation of α-synuclein is inhibited more in *msrA*-KO cells compared to WT cells, which may be an enhanced inhibitory effect of the MetO on cellular degradation. Using matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) techniques, we have verified that SynA30P and SynA53T expressed in the *msrA*-KO cells had elevated levels of Met oxidation relative to same α-synucleins expressed in WT cells (data not shown).

Protein Degradation and Synthesis in Yeast Strains Overexpressing α-Synuclein

The accumulation of α-synuclein may be causing a general inhibition of degradation processes that is increased in the *msrA*-KO strain. Following pulse-chase labeling with [^{35}S] Met during the induction period of α-synuclein synthesis, all types of expressed α-synuclein lowered the percentage levels of acid-soluble labeled material, mostly in *msrA*-KO cells (Fig. 2a; labeled acid-soluble material, soluble degradation products; and labeled nonsoluble material, newly synthesized proteins; represent total labeled proteins). While SynA53T was the only type having a significant reduction effect on the WT total protein degradation rate, all types of α-synuclein reduced the total degradation rate in the *msrA*-KO strain compared to control (Fig. 2a).

It is noteworthy that the degradation rate over time was elevated in the *msrA*-KO versus WT yeast strain when sham vector was present. One possible explanation is that accumulation of MetO-containing proteins (the case in the *msrA*-KO strain) induces the action of the degradation machinery as an attempt to remove the faulty proteins (Stadtman and Berlett 1998). However, this response is inhibited by excess levels of specific α-synuclein types (Figs. 1 and 2a). The general inhibition of protein degradation was more pronounced in the *msrA*-KO cells expressing native and A30P α-synucleins (Fig. 2a). The overexpressed native α-synuclein effect on cellular degradation may be relevant to its accumulation that is thought to be a risk factor for PD, and specifically impaired α-synuclein degradation may be involved (Singleton et al. 2003).

The mechanism by which the nature of α-synuclein and MetO modifications to α-synucleins inhibits degradation is not known. The degradation pathways may be inhibited by the cellular localization of α-synuclein. In yeast, each α-synuclein mutant accumulated at the same level as non-mutated α-synuclein, but the cellular distributions differed profoundly. Similar to native α-synuclein, SynA53T is concentrated at the plasma membrane, while SynA30P dispersed throughout the cytoplasm (Brandis et al. 2006; Bussell and Eliezer 2003; Jo et al. 2002). Therefore, the cytoplasmic localization of SynA30P may cause the protein to be more prone to oxidation (compared to a membrane region) and accordingly less protected from the accumulation of MetO in the absence of cytoplasmic MsrA. This may also partly explain the special negative effect of MetO-α-synuclein accumulations on cytoplasmic degradation, as accumulations of oxidized proteins can inhibit protein degradation (Starke-Reed and Oliver 1989).

Inhibition of protein synthesis by native and Ala30Pro α-synuclein expressed in yeast cells was previously described (Chen et al. 2005). Therefore, we examined the contribution of *msrA* ablation to the α-synuclein inhibitory effect on protein synthesis. Expression of α-synuclein caused an overall inhibition of protein synthesis in both strains compared to control cells expressing the sham vector (Fig. 2b). The inhibition levels were similar in both yeast strains expressing either SynA30P or SynA53T. However, a combination of msrA ablation and native α-synuclein expression in *msrA*-KO cells caused a stronger inhibitory effect on protein synthesis compared to WT cells expressing native α-synuclein (Fig. 2b).

As discussed by other groups (Chen et al. 2005), the basis for decreased protein synthesis in cells expressing α-synuclein is unknown. They suggest that the impairments in this synthesis may either represent negative feedback (result of impaired protein degradation) or α-synuclein induction of endoplasmic reticulum stress impairing protein synthesis (Chen et al. 2005). It is possible that alterations to the structure and function of native α-synuclein expressed in *msrA*-KO cells are more effective in fostering inhibition of protein synthesis (Fig. 2b).

Accumulation of Fibrillated Proteins in Yeast Strains Overexpressing α-Synuclein

Fibrillation of protein, in general, and α-synuclein, in particular, is associated with posttranslational or genetic modifications to a protein. The observations above, suggesting an inhibition of degradation and accumulation of oxidized protein in cells expressing α-synuclein, led us to examine the contribution of these events to α-synuclein-mediated fibril formation. Yeast cells were stained with Thioflavin T, which is commonly used for detection of fibrillated proteins (Hawe et al. 2008). Cells were imaged by phase-contrast microscopy, and the staining of protein fibrils was detected with epifluorescence (Fig. 3a). In addition, quantification of stained cells per amount of cells present was determined (Fig. 3b). Expression of native α-synuclein in the WT strain had no effect on the percentile of cells exhibiting protein fibrillation compared to the sham vector. However, expression of SynA30P and, to a lower extent, SynA53T increased the percentile of cells containing protein fibrillation. Additionally, the *msrA*-KO strain shows an increased effect on protein fibrillation over the WT values when SynA30P is expressed and, to a lower extent, when native α-synuclein is expressed. In contrast, SynA53T expression had similar effect on the induction of protein fibrillation in both WT and *msrA*-KO strains. In general, it appears that expression of α-synuclein either with point mutations or in the absence of MsrA causes protein fibrillation.

Several posttranslational modifications can alter the solubility of α-synuclein. Oxidation will cause a Met residue to be more hydrophilic, but the overall MetO-protein may become more hydrophobic by exposing hidden hydrophobic residues (Chao et al. 1997). Degradation of α-synuclein is inhibited in PD, and thus, it is a plausible theory that this inhibition (also shown in Figs. 1 and 2) decreases the clearance of α-synuclein and promotes its aggregation, especially when the protein undergoes modifications that reduce its solubility. All types of α-synuclein caused enhanced cellular fibrillation in the absence of MsrA compared to the vector, while SynA30P had the most effect on forming fibrils in the *msrA*-KO strain (Fig. 3). This result would be the expected outcome of enhanced inhibited degradation of the α-synuclein A30P (Figs. 1 and 2). Since the SynA53T aggregation is less affected by the presence of MsrA (Fig. 3), it may reflect the enhanced aggregation found in PD patients carrying this mutation (Lee et al. 2002; Ostrerova-Golts et al. 2000). However, a combination of a compromised Msr system (from mutations or lowered expression with age) and overexpression of SynA30P could cause even greater levels of α-synuclein fibrils in PD patients.

Phosphorylation of α-Synuclein and Its Dependency on Methionine Sulfoxide Reductase

The functional role of phosphorylation to α-synuclein is not fully understood. Given our new data, it was intriguing to determine whether oxidation of Met could also affect the phosphorylation efficiency of α-synuclein. To follow the phosphorylation efficiency in both yeast strains expressing α-synuclein types, [^{32}P]ortho-phosphate was included in the expression induction media. Following the induction, the three types of α-synuclein protein were isolated from the corresponding yeast strains and phosphorylation was quantified by measuring the radiolabeling of the protein. All α-synuclein types isolated from *msrA*-KO cells showed significantly lower levels of phosphorylation relative to α-synuclein isolated from WT cells, especially in the native α-synuclein and SynA30P (Fig. 4a). The overall phosphorylation rate of SynA53T was significantly lower in both yeast strains, which may imply that this form of α-synuclein is more prone to oxidation even under normal function of the Msr system. Lower in vivo phosphorylation levels of α-synuclein in *msrA*-KO cells suggest that increased levels of MetO moiety in *msrA*-KO cells could partly inhibit the phosphorylation efficiency of α-synuclein. The two types of α-synuclein that are mostly affected by this phosphorylation decline in the *msrA*-KO strain are the native α-synuclein and SynA30P, similar to the effect of *msrA*-KO strain on protein fibrillation in the presence of these α-synuclein types (Fig. 3).

To further investigate the possibility that methionine oxidation of α-synuclein may contribute to the phosphorylation inhibition, we used recombinant α-synuclein proteins in reduced and oxidized form, [^{32}P]ATP, and casein kinase 2 (a major kinase for α-synuclein Ser^{129} phosphorylation; Ishii et al. 2007). To confirm that MetO formed in the oxidized α-synuclein is responsible for the observed effect on phosphorylation level, MsrA and PilB (naturally fused MsrA–MsrB protein from *Neisseria gonorrhoeae*) were added to the reaction mixture to reduce α-synuclein MetO residues. All samples were subjected to SDS-gel electrophoresis, gels were stained with GelCode Blue Stain (Fig. 4b), and phosphorylation was visualized by autoradiography. The oxidation of the three types of α-synuclein inhibited the phosphorylation by casein kinase 2 (Fig. 4c), while both MsrA and PilB were able to reverse this inhibition via MetO reduction. Both of the mutant α-synuclein proteins were more phosphorylated when reduced by the Msr enzymes (Fig. 4c). Thus, it is possible that conformational changes that may occur in these α-synuclein mutants may also facilitate MetO reduction by the Msr system due to better accessibility of the enzyme to MetO residues. It is important to note that casein kinase 2 phosphorylation activities on control samples were not altered when Msr enzymes were added. This excludes the possibility that the enhanced α-synuclein phosphorylation, shown by Msr enzymes, is due to their reducing activity on casein kinase 2. Also, using MALDI-MS, we have verified that the recombinant α-synucleins had elevated levels of oxidation relative to nonoxidized and Msr-reduced α-synucleins (data not shown).

The role of phosphorylation of α-synuclein (especially at Ser^{129}) is controversial. The phosphorylation α-synuclein at Ser^{129} by casein kinase 2 forms fibrils faster than unmodified α-synuclein in vitro (Fujiwara et al. 2002) and increases the formation of cytoplasmic inclusion bodies in cell culture models of synucleinopathies (Smith et al. 2005). However, in vivo studies showed no obvious correlation between phosphorylated Ser^{129} and of α-synuclein fibril formation (Chen and Feany 2005). In contrast, another study demonstrated that phosphorylation at Ser^{129} inhibits α-synuclein fibril formation in vitro (Paleologou et al. 2008). With regard to the vicinity of Met^{127} to Ser^{129}, we have examined the effect of MetO modifications on the rate of the in vivo phosphorylation of α-synuclein and casein kinase 2-mediated phosphorylation of α-synuclein. The observed decrease in α-synuclein phosphorylation due to MetO following MsrA ablation in cells (Fig. 4a) may be relevant to processes that regulate α-synuclein phosphorylation. As the provided yeast system is only a model to investigate the role of MsrA in maintaining α-synuclein function,

further investigations in a parallel mammalian system will greatly contribute to clarify the physiological role of MsrA on α-synuclein phosphorylation.

A functional Msr system and Met oxidation may have a role in regulating α-synuclein and the phosphorylation of other proteins. Recent studies suggested that MetO formation on inhibitory factor of nuclear factor-kappaB (IkBα) prevents its subsequent degradation. Similar to α-synuclein, the IkBα Met^{45} residue is close to the phosphorylation sites Ser^{32} and Ser^{36}. The MetO formation, particularly within helices, can cause major conformational rearrangements (Bigelow and Squier 2005), and the oxidation of Met^{45} may cause a structural disruption that makes the protein a less effective kinase target (Kanayama et al. 2002; Midwinter et al. 2006; Mohri et al. 2002). In another recent study, MsrA was shown to suppress dopaminergic protein aggregation induced by mutant α-synuclein via MetO repair. The MsrA was able to reduce several oxidized Met residues in recombinant α-synuclein (Liu et al. 2008). Overall, it is suggested that oxidation of Met in α-synuclein inhibits phosphorylation, which may be involved in processes relevant to its degradation or aggregation depending on cellular environment.

In summary, both the nature of α-synuclein and presence of MsrA determined the overall protein and α-synuclein degradation in yeast. Furthermore, Met oxidation of α-synuclein inhibits α-synuclein phosphorylation. This occurs parallel to increasing protein fibrillation, especially when SynA30P is expressed in *msrA*-KO cells. To establish the relationships between α-synuclein overexpression and MsrA function in mammals, similar studies will be conducted in transgenic α-synuclein mice on an $MsrA^{-/-}$ background.

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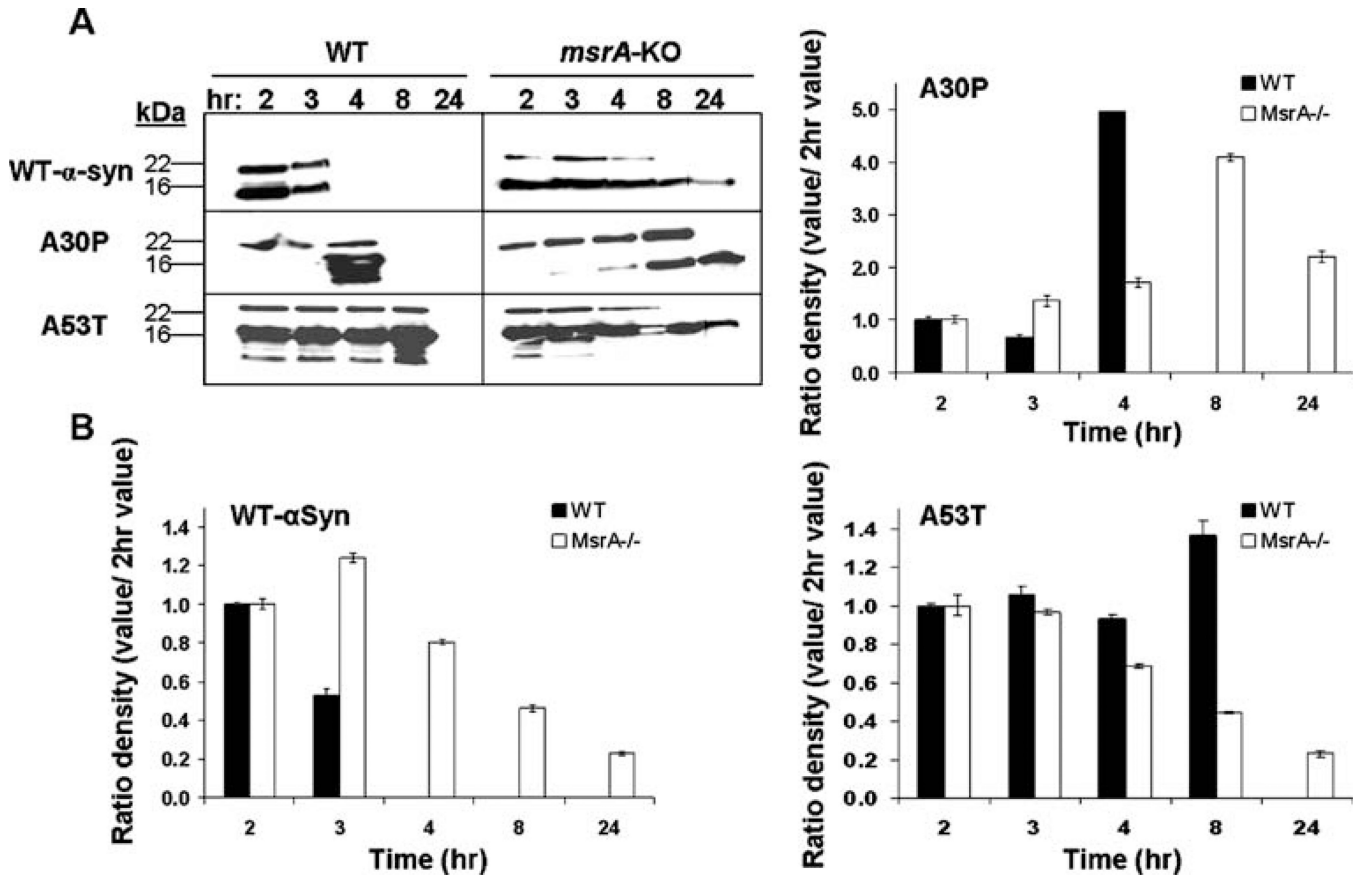

Figure 1.

Presence of α-synuclein over time isolated from *msrA*-KO and WT yeast cells. Soluble α-synucleins (WT-α-syn, A30P, A53T) were isolated from cells at specified time points during α-synuclein protein induction. Equal amounts of cells were taken from each culture and α-synuclein proteins were isolated by resin from the corresponding extracts. **a** The α-synuclein proteins were detected by Western blotting using antibodies specific to the 6His tag. *Left side*: α-synuclein from WT yeast cells. *Right side*: α-synuclein from *msrA*-KO yeast cells. *Horizontal axis*: time from start of α-synuclein induction period (2, 3, 4, 8, and 24 h). **b** Densitometry analysis performed on the protein bands shown in **a**. To compare between the types of synuclein expressed and cells, the pattern of resistance to degradation over time was demonstrated as a percent ratio of the protein densitometry for each time point relative to the densitometry value of the 2-h point level (represented 100% expression level)

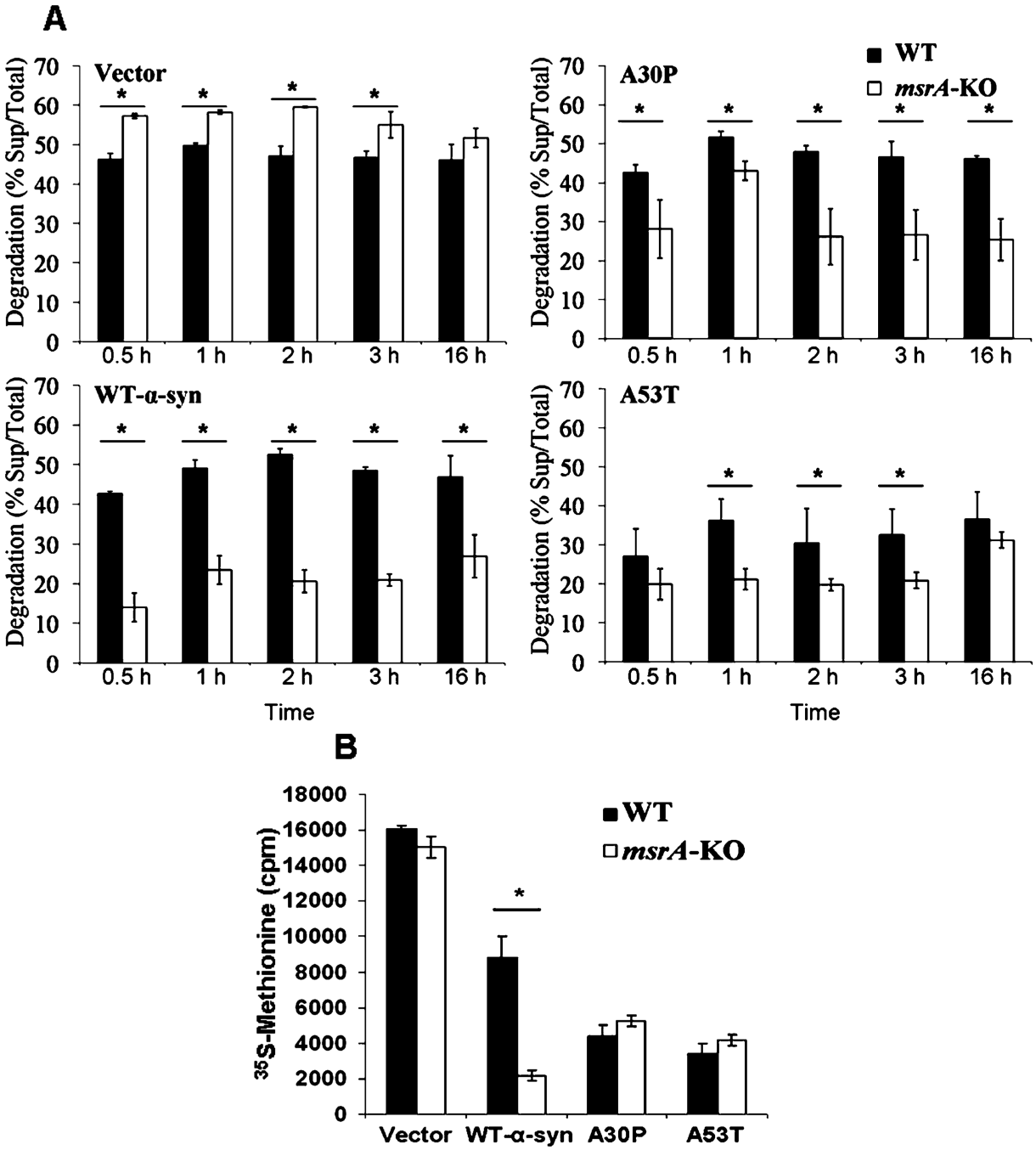

Figure 2.

Total protein degradation and synthesis in *msrA*-KO and WT yeast cells. **a** Total degradation of soluble cytosolic proteins. Soluble protein fraction was precipitated by TCA following pulse-chase labeling with [^{35}S]Met during α-synuclein protein induction. Values represent the percent of counts per minute of the TCA-soluble material divided by the total counts per minute values (TCA-soluble material plus precipitated TCA pellet at specified time points). *Black* bars represent WT yeast cells; *white bars* represent *msrA*-KO yeast cells. *Top left*: cells expressing sham vector. *Bottom Left*: cells expressing α-synuclein (WT-α-syn). *Top right*: cells expressing A30P α-synuclein. *Bottom right*: cells expressing A53T α-synuclein. **b** For quantification of protein synthesis, the radioactivity (counts per minute) in

the TCA-insoluble material was measured following labeling with [^{35}S]Met. *Black bars* represent WT; *white bars* represent *msrA*-KO yeast cells. The annotation for the type of expression (vector or α-synuclein) is the same as in **a** *Error bars* represent standard error of three experiments. *$P<0.05$

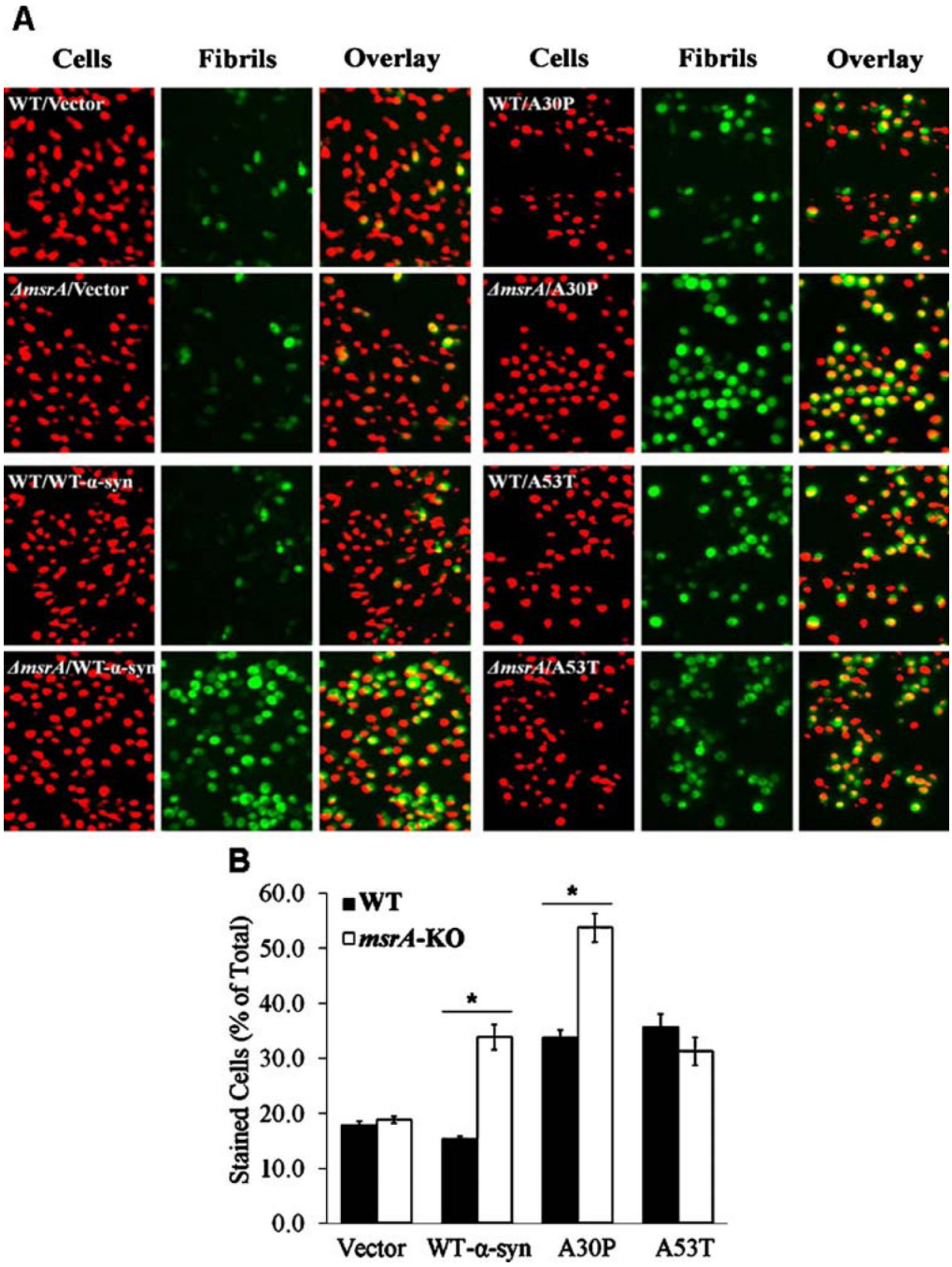

Figure 3.

Detection of fibrils present in *msrA*-KO and WT yeast cells by Thioflavin T staining. Yeast cells were stained with Thioflavin T after α-synuclein (WT-α-syn, A30P, A53T) induction of expression and transferring equal aliquots of cells into a 96-well plate. Cells were detected by phase-contrast microscopy and staining was detected with epifluorescence, both using a Nikon 40X objective of 1.3 N. A. and a standard DAPI/FitC/ TxRed B filter set and dichroic (Brightline filters, Semrock). **a** Representative images of cells collected by IN Cell Analyzer (GE Healthcare). For visualization, Image J software was used to make cells appear as *red*, fibrils appear as *green*, and to make an overlay image. **b** Quantification of

stained cells per number of cells present determined by IN Cell Analyzer provided software. Automated image segmentation was achieved independently on each image channel using the Otsu "global" method of thresholding. *Black bars* represent WT yeast cells; *white bars* represent *msrA*-KO yeast cells. *Error bars* represent standard deviation of six experiments. **P*<0.0001

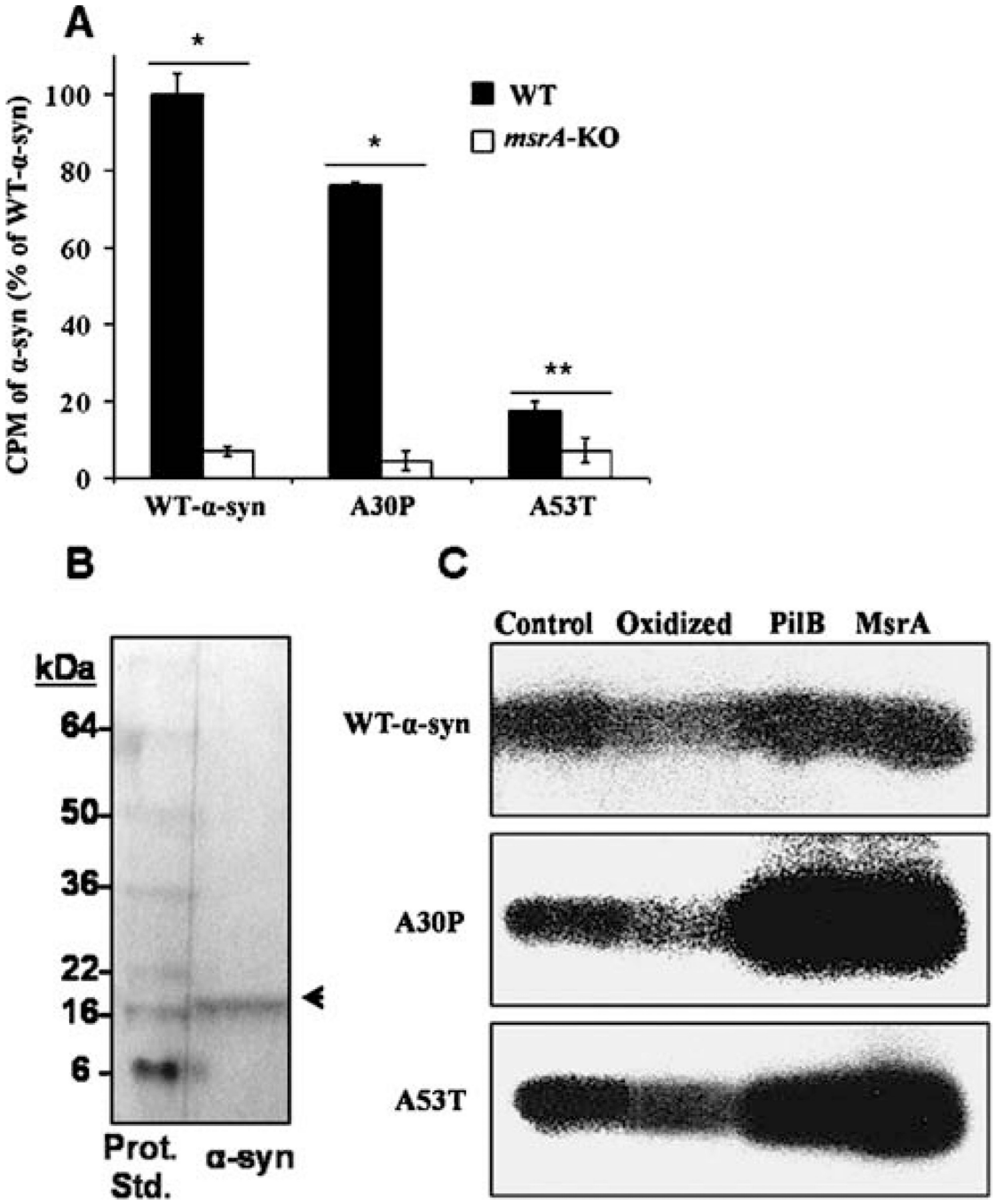

Figure 4.

Phosphorylation of α-synuclein isolated from *msrA*-KO and WT yeast cells. **a** Each yeast strain expressing each α-synuclein type was induced and then incubated in medium containing ortho-[^{32}P] phosphoric acid. Expressed α-synuclein proteins (WT-α-syn, A30P, A53T) were isolated by resin binding and phosphorylation was detected by measuring counts per minute. Values represent percentage of counts per minute value relative to WT-α-syn counts per minute value in WT yeast cells. *Black bars* represent WT yeast cells; *white bars* represent *msrA*-KO yeast cells. *Error bars* represent standard error of four experiments. *P<0.001; **P<0.05. **b** A representative gel stained with GelCode Blue Stain reagent. *Left*

lane: SeeBlue Plus2 protein standard (Invitrogen). *Right lane*: typical separation of pure commercial recombinant human α-synuclein proteins (WT-α-syn shown as a representative α-synuclein; *arrow* indicates the band corresponding to α-synuclein). c Pure commercial recombinant human α-synuclein proteins (WT-α-syn, A30P, A53T) were exposed to H_2O_2 overnight (*Oxidized*). Oxidant was removed by catalase and Msr enzymes were added where indicated (PilB, MsrA). The removal of oxidants was verified by adding α-synuclein after oxidants were removed and served as the nonoxidized form of α-synuclein (*Control*). All samples were phosphorylated with [γ-^{32}P]-ATP and casein kinase 2. Equal amounts of α-synuclein proteins were subjected to an SDS-gel electrophoresis and the gel was exposed to X-ray film, followed by developing for visualization