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Research article

Enzymatic *O*-GlcNAcylation of α-synuclein reduces aggregation and increases SDS-resistant soluble oligomers

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HIGHLIGHTS

- α-synuclein can be *O*-GlcNAcylated in bacteria co-expressed with sOGT;.
- Enzymatic *O*-GlcNAcylation blocks the aggregation of α-synuclein;.
- Enzymatic *O*-GlcNAcylation of α-synuclein increases the SDS-resistant soluble oligomers.

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ABSTRACT

Neurodegenerative diseases including dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and Parkinson's disease are associated with the aberrant aggregation of α-synuclein, which is influenced by several post-translational modifications (PTMs). *O*-GlcNAcylation is one PTM that has an important role in many fundamental processes. The *O*-GlcNAcylation of endogenous α-synuclein at residues 53, 64, 72 and 87 has been reported in an unbiased mass spectrum analysis. The consequences of *O*-GlcNAcylation at residues 72 or 87 have been studied by using a synthetic α-synuclein bearing *O*-GlcNAcylation at threonine residue 72 or serine 87, respectively. *O*-GlcNAcylation at Thr72 or Ser87 suppresses the aggregation of α-synuclein. However, the effect of enzymatic *O*-GlcNAcylation of α-synuclein at multiple residues is not clear. Here, we successfully generated *O*-GlcNAcylated α-synuclein by co-expressing a shorter form of OGT (sOGT) with α-synuclein. The *O*-GlcNAcylation inhibited α-synuclein aggregation and promoted the formation of soluble SDS-resistant and Thioflavine T negative oligomers. Our data warrant further studies on the role of *O*-GlcNAcylation in the progression/treatment of Parkinson's disease in animal models.

1. Introduction

Neurodegenerative diseases including dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and Parkinson's disease (PD) are associated with the aberrant aggregation of α-synuclein [1]. A common feature of synucleinopathy is the formation of α-synuclein-containing intracellular inclusions: Lewy bodies [2]. Therefore, factors that influence the aggregation of α-synuclein may be potentially therapeutic targets for these diseases.

α-Synuclein is subject to several post-translational modifications (PTMs), including ubiquitination [3], sumoylation [4], phosphorylation [5,6], nitration [7], and glycosylation [8]. Phosphorylation at Ser87, ubiquitination, or sumoylation inhibit α-synuclein aggregation [3,4,6]. In contrast, C-terminal truncations promote the aggregation of α-synuclein [9].

Abbreviations: AD, Alzheimer's disease; AFM, atomic force microscope; GlcNAc, *N*-acetylglucosamine; IPTG, isopropyl β-D-1-thiogalactopyranoside; OGT, *O*-GlcNAc transferase; PD, Parkinson's disease; PTM, post-translational modifications; sWGA, succinylated wheat germ agglutinin; sOGT, shorter form of OGT; ThT, thioflavine T; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetylglucosamine.

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O-GlcNAcylation is a dynamic biochemical process, in which N-acetylglucosamine (GlcNAc) from uridine 5′-diphospho-N-acetylglucosamine (UDP-GlcNAc) is transferred to the serine and threonine residues by *O*-GlcNAc transferase (OGT) and removed by *O*-GlcNAcase (OGA) [10]. *O*-GlcNAcylated proteins are widely distributed within cells, and involved in the regulation of transcription, translation, signal transduction, synaptic plasticity, the cell cycle and other biological functions [11]. There is a close relationship between *O*-GlcNAcylation and neurodegenerative diseases [12,13]. Tau is extensively *O*-GlcNAcylated, which inhibits its aggregation *in vitro* [14]. An OGA inhibitor increases the *O*-GlcNAcylation level in the tau transgenic mouse and reduces the number of neurofibrillary tangles [15].

In two unbiased mass spectrum studies, *O*-GlcNAcylation is identified at residues 53, 64, 72, and 87 of α-synuclein [16,17]. Pratt and colleagues performed a series of seminal studies to understand the functional consequences of *O*-GlcNAcylation on α-synuclein. First, they synthesize a peptide of α-synuclein comprsing residues 68–77, in which the Thr72 is *O*-GlcNAcylated. As compared with the unmodified peptide, the *O*-GlcNAcylated peptide inhibits full-length α-synuclein fibrillization [18]. Second, they synthesized full-length α-synuclein, with *O*-GlcNAcylation at Thr72. *O*-GlcNAcylation at Thr72 completely blocks the formation of both fiber and oligomer aggregates *in vitro* [19]. Recently, they synthesized full-length α-synuclein with *O*-GlcNAcylation at Ser87, which still aggregates but with slower kinetics than the unmodified protein [20].

However, α-synuclein is *O*-GlcNAcylated at multiple residues *in vivo* [16,17], and modification at different residues may have different effects. For instance, Ser129 phosphorylation promotes, whereas Ser87 phosphorylation inhibits α-synuclein aggregation [6,21]. Pratt and colleagues tried to obtain enzymatically *O*-GlcNAcylated α-synuclein by co-expressing OGT and α-synuclein in bacterial, however, no *O*-GlcNAcylation was identified in their assay. To study the functional consequences of enzymatic *O*-GlcNAcylation of α-synuclein, we co-expressed sOGT and α-synuclein in bacterial and demonstrated *O*-GlcNAcylation of α-synuclein by a more sensitive Click-iT assay. Our study indicated that the enzymatically *O*-GlcNAylated α-synuclein blocked the aggregation and increased production of SDS-resistant oligomers.

2. Materials and methods

2.1. Plasmids

DNA encoding human α-synuclein was introduced into pET25b (EMD Millipore, USA) using the Nde I and Hind III restriction sites, resulting in pET25b-syn. The pET24b vector containing sOGT (pET24b-sOGT) was a generous gift from Dr. Walker at Harvard Medical School [22].

2.2. Expression and purification of α-synuclein

The pET25b-syn plasmid (Fig. 1A) was transformed into *E. coli* BL21 (DE3), and ampicillin-resistant colonies were selected. To generate the *O*-GlcNAcylated α-synuclein, we co-transformed pET25b-syn and pET24b-sOGT into BL21 (DE3), and colonies resistant to both kanamycin and ampicillin were used for the following experiments.

Freshly transformed BL21 (DE3) colonies were inoculated into 1L of LB medium. Expression was induced by treatment with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C for 18 h. Cells were harvested and re-suspended in 10 ml of 30 mM Tris-HCl, 2 mM EDTA, 40% sucrose, pH7.2. After incubation for 10 min at room temperature, the suspension was centrifuged at 10,000*g* for 10 min at 25 °C. The pellet was then re-suspended in 11.25 ml of cold H_2O. The suspension was incubated on ice for 3 min after adding 4.7 ml of 2 M $MgCl_2$. Then the cold suspension was centrifuged at 20,000*g* for 15 min at 4 °C. Streptomycin (final concentration 10 mg/ml) was added to the supernatant and the mixture centrifuged at 20,000*g* for 15 min at 4 °C. Tris-HCl (final concentration 1 mM, pH8.0) and β-mercaptoethanol (final concentration 10 mM) were added to the supernatant. After boiling at 100 °C for 10 min, the supernatant was centrifuged at 20,000*g* for 10 min at 4 °C to remove the pellet. Then two sequential ammonium sulfate precipitations (30% and 50% saturation) were applied to further remove other proteins. α-Synuclein was precipitated at 50% saturation. The pellet was re-suspended in 10 mM Tris-HCl and loaded onto a DEAE sepharose (GE healthcare, USA) column. The column was eluted in 0–1 M NaCl, and α-synuclein was eluted in 300 mM NaCl.

2.3. Succinylated wheat germ agglutinin (sWGA) enrichment of O-GlcNAcylated proteins

The purified α-synuclein (50 μg) was incubated with 30 μl of sWGA beads in sWGA lysis buffer (10 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1% NP-40, 1 mM PMSF) overnight at 4 °C. The beads were collected by centrifugation. After washing 10 times with PBS, the beads were boiled for 10 min in 2 × SDS loading buffer, and subjected to immunoblotting with an α-synuclein antibody (Cat#2642S, Cell Signaling Technology, USA).

2.4. Detection of O-GlcNAcylation with Click-iT assay

The Click-iT *O*-GlcNAc Enzymatic Labeling System and Click-iT Protein Analysis Detection Kits (Invitrogen, USA) were used to detect *O*-GlcNAcylation according to the manufacture's instructions. The labeled proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were electro-blotted onto polyvinylidene fluoride (PVDF) membranes, which were blocked by 5% BSA. HRP-labeled streptavidin (Cell Signaling Technology, USA) was used to detect biotin generated by the Click-iT assay in *O*-GlcNAcylated proteins.

2.5. In vitro aggregation

The purified α-synuclein was filtered through a 0.22 mm filter unit to remove any aggregation seeds. The assembly reaction was initiated by incubating α-synuclein at 1 mg/ml in PBS solution at 37 °C with constant shaking (900 rpm, Thermo Scientific, Fluoroskan Ascent). In order to monitor fibrillization, thioflavine T (ThT, final concentration 10 μM) was added. Fluorescence was recorded (with excitation at 440 nm and emission at 485 nm) every 10 minutues over a 9 day period. After 9 days of incubation, we used an atomic force microscope (AFM) to monitor the formation of amyloid fibrils. The reaction was centrifuged at 20,000*g* for 30 min, and the supernatant was analyzed by Western blot with an antibody against α-synuclein.

3. Results and discussion

3.1. Expression and purification of α-synuclein

To obtain large amounts of α-synuclein protein, we cloned an α-synuclein-coding gene into the bacterial expression plasmid pET25b (Fig. 1A); the expression of α-synuclein protein was induced by IPTG. As shown in Fig. 1B, a protein of ~15 kDa was significantly induced by IPTG (Lane 2). The α-synuclein was purified by boiling at 100 °C for 10 min, followed by two sequential ammonium sulfate precipitations and anion-exchange chromatography.

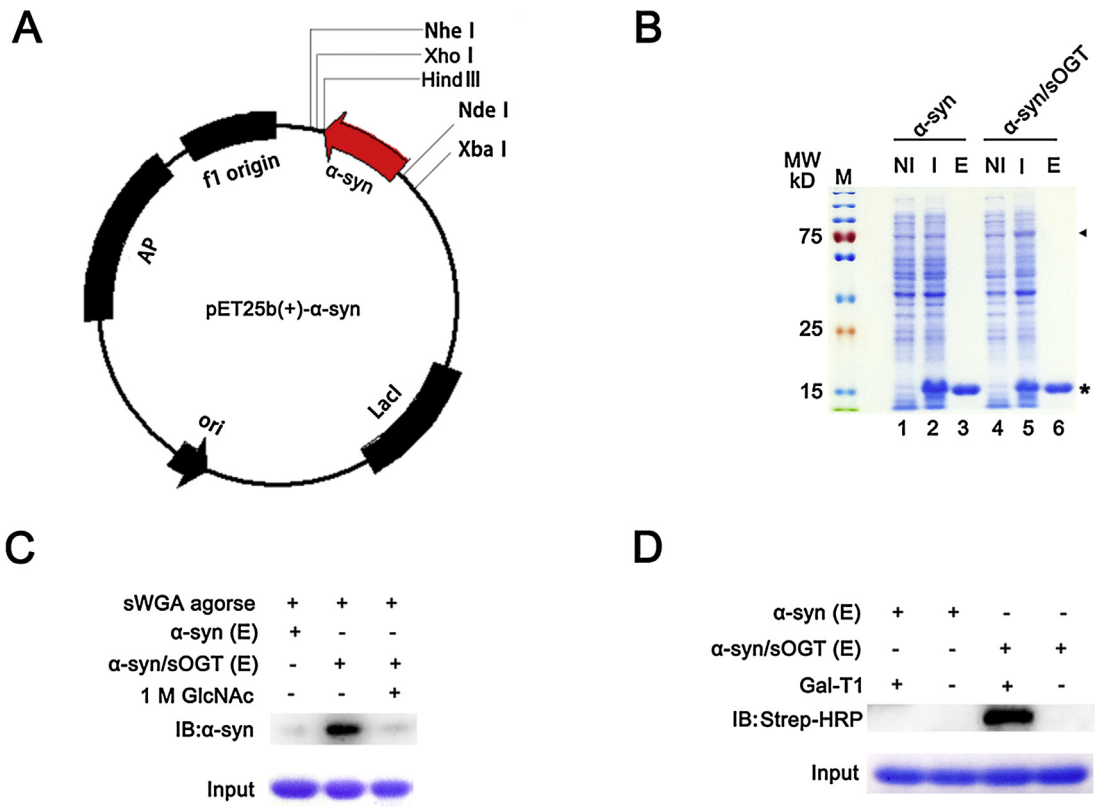

Fig. 1. Co-expression with sOGT led to *O*-GlcNAcylation of α-synuclein. (A) A diagram of the pET25b-α-synuclein plasmid. DNA encoding human α-synuclein was introduced into pET25b using the Nde I and HindIII restriction sites. (B) Expression and purification of α-synuclein. α-Synuclein expressed alone (Lanes 1–3) or together with sOGT (Lanes 4–6) were visualized by Coomassie blue staining. NI: proteins from untreated bacteria; I: proteins induced in bacteria treated with IPTG; E: proteins eluted from DEAE-sepharose. Triangles denote sOGT; asterisks,α-synuclein. (C) Purified α-synuclein was subjected to precipitation with sWGA. Samples were analyzed by Western blotting with an antibody against α-synuclein. Coomassie blue stained gels are presented to show even loading. (D) *O*-GlcNAcylation detected by the Click-iT chemoenzymatic method. Purified α-synuclein was labeled by Click-iT and analyzed by Western blot using HRP-labeled streptavidin.

As a result, ~10 mg of α-synuclein with 90–95% purity (as estimated by Coomassie blue staining) was obtained from 1 l of culture.

To generate *O*-GlcNAcylated α-synuclein, we co-expressed sOGT with α-synuclein in bacteria. IPTG induced the expression of both α-synuclein (~15 kDa) and sOGT (~75 kDa) (Fig. 1B, Lane 4). After applying the same purification procedure as above, we obtained ~10 mg α-synuclein with 90–95% purity from 1 l of culture.

3.2. *Co-expression with sOGT led to* O-*GlcNAcylation of α-synuclein*

To see if co-expression of sOGT leads to *O*-GlcNAcylation of α-synuclein, the purified α-synuclein was precipitated with agarose conjugated with succinylated wheat germ agglutinin (sWGA), a lectin that specifically recognizes *O*-GlcNAc. The precipitated protein was then detected with an antibody against α-synuclein. As shown in Fig. 1C, sWGA was able to pulldown α-synuclein only when it was co-expressed with sOGT. Importantly, no α-synuclein was pulled down in the presence of 1 M GlcNAc. We then performed a Western blot on the purified α-synuclein with an *O*-GlcNAcylation antibody, CTD110.6. Similar to the reports by Marotta et al. [18], no specific band was detected (data not shown).

Therefore, we used a Click-iT chemoenzymatic approach to detect *O*-GlcNAcylation. Using this assay, *O*-GlcNAcylated proteins can be enzymatically labeled by the mutant galactosyltransferase GalT1 Y289L, which transfers azido-modified galactose (GalNAz) from UDP-GalNAz to *O*-GlcNAc residues on the target proteins. The azide-labeled proteins can then be chemically tagged with biotin alkyne; the biotin adduct thus formed is specifically detected by HRP-labeled streptavidin [23]. As shown in Fig. 1D, *O*-GlcNAcylation was detected in α-synuclein co-expressed with sOGT, but not in α-synuclein expressed alone. Our data demonstrate that α-synuclein can be *O*-GlcNAcylated in bacterial in the presence of sOGT.

3.3. O-*GlcNAcylation inhibits α-synuclein aggregation* in vitro

Recombinant monomeric α-synuclein can form amyloid fibrils *in vitro*, which are similar to the α-synuclein aggregates isolated from patients with synucleinopathies [24]. We used an *in vitro* assay to compare the aggregation propensity of α-synuclein modified or not by *O*-GlcNAcylation. Thioflavin T (ThT) fluorescence was used to monitor the *in vitro* fibrillation process over a period of 9 days. As shown in Fig. 2A, un-modified α-synuclein started to aggregate from the second day, and reached a plateau after 6-7 days. In contrast, fibril formation of *O*-GlcNAcylated α-synuclein was completely abolished.

After 9 days of incubation, we used an atomic force microscope (AFM) to monitor the fibrils and identified amyloid fibrils in un-*O*-GlcNAcylated α-synuclein, but not in the *O*-GlcNAcylated α-synuclein samples (Fig. 2B). We also centrifuged these samples and performed Western blots on the supernatants with an antibody against α-synuclein. Interestingly, SDS-resistant oligomers were identified in the *O*-GlcNAcylated samples, but not in the un-*O*-GlcNAcylated ones (Fig. 2C).

Leong et al. reported that under physiological conditions, α-synuclein associates into small oligomers and proto-fibrils, which further elongate into mature amyloid fibrils. However, dopamine induces α-synuclein to form soluble, SDS-resistant and ThT nega-

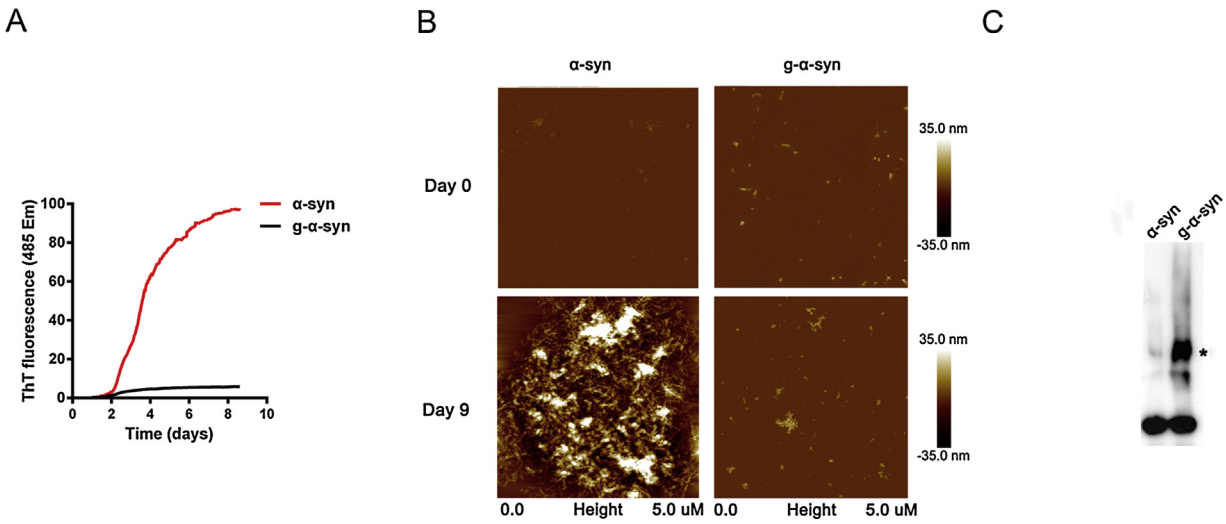

Fig. 2. *O*-GlcNAcylation inhibits α-synuclein aggregation *in vitro*. (A) ThT fluorescence was monitored over a 9 day period in α-synuclein samples (1 mg/ml) incubated at 37 °C with constant shaking (900 rpm). (B) An AFM was used to monitor fibril formation and identify amyloid fibrils before (day 0) and after 9 days of incubation (day 9). (C) After 9 days of incubation, the samples were centrifuged and the supernatant was analyzed by Western blot with an α-synuclein antibody. An asterisk denotes SDS-resistant oligomers. g-α-syn: *O*-GlcNAcylated α-synuclein.

tive oligomers, species which do not participate in amyloid fibril formation [25]. In this study, we found that *O*-GlcNAcylation of α-synuclein also promoted the formation of SDS-resistant oligomers, which may underlie the reduced aggregation observed.

Several lines of evidence indicate that α-synuclein oligomers are pathological elements. First, PD-related α-synuclein variants are prone to form oligomers, instead of fibers [26,27]. Second, experiments employing well-defined recombinant α-synuclein species demonstrate that pre-fibrillar assemblies can cause a wide range of damaging effects [28,29]. Third, oligomers also have been found in patients affected by synucleinopathies [30]. Therefore, *O*-GlcNAcylation may increase the toxicity of α-synuclein by promoting oligomer formation.

In summary, we successfully generated *O*-GlcNAcylated α-synuclein by co-expressing sOGT with α-synuclein. The *O*-GlcNAcylation inhibited α-synuclein aggregation and promoted the formation of soluble, SDS-resistant and ThT negative oligomers. Our data warrant further studies on the role of *O*-GlcNAcylation in the progression/treatment of Parkinson's disease in animal models.

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