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Cytotoxicity of α-synuclein amyloid fibrils generated with phage chaperonin OBP

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ABSTRACT

Chaperonins are known to be important players in the conversion of amyloidogenic proteins into amyloid precursors in a variety of neurodegenerative diseases. However, the mechanisms of their action is still poorly understood. In this work, we used a single-ring chaperonin of the bacteriophage OBP, which functions in an ATP-dependent manner but has a simpler structure than other chaperonins. The effect of the chaperonin OBP on the conversion of human α-synuclein mutant A53T into amyloid was studied and the cytotoxicity of the formed fibrils was investigated. The phage chaperonin OBP was expressed in HEK293T cells together with the human α-synuclein mutant A53T. Both proteins showed a diffuse distribution within the cell cytoplasm as determined by fluorescence microscopy using specific antibodies. Separate and co-expression of the two proteins did not result in the formation of distinguishable protein aggregates in the cells, nor did it have any effect on cell viability. However, the co-expression of chaperonin and α-synuclein did result in the appearance of some dimeric and oligomeric forms of α-synuclein in the insoluble fraction of the cell lysate. It can therefore be concluded that chaperonin OBP stimulates the amyloid transformation of α-synuclein A53T when both proteins are co-expressed in eukaryotic cells. A comparison of the cytotoxicity of mutant α-synuclein amyloid forms obtained *in vitro*, both during spontaneous fibrillation and with the participation of the chaperonin OBP, showed that the maximum effect on HEK293T and SH-SY5Y cells, resulting in the death of more than 50 % of the population, was exerted by α-synuclein fibrils formed under chaperonin action in the presence of ATP. In the context of recent data on the spread of amyloid α-synuclein from the gut to the brain, the role of phage chaperonins in the pathological aggregation of amyloidogenic proteins in the human body and the potential use of the OBP chaperonin in cellular models of synucleinopathies are discussed.

1. Introduction

A large number of chaperones are known to play an important role in the initiation and progression of neurodegenerative diseases [1–5]. In particular, chaperones have been shown to be associated with the development of Parkinson's disease (PD), which is characterised by the degeneration of dopaminergic neurons in substantia nigra pars compacta. In addition, the formation of extracellular Lewy bodies has been described in PD. These are known to be composed of large amounts of α-synuclein and several other proteins, including molecular chaperones. The effect of chaperones directly on α-synuclein aggregation and cytotoxicity has been shown. For example, Hsp70, clusterin, α_2-macroglobulin and αB-crystallin have been demonstrated to interact with α-synuclein, resulting in inhibition of its fibrillation [6–8]. A further six chaperones with highly divergent architectures have been shown to bind to α-synuclein via a canonical motif recognising α-synuclein N-terminus and Tyr39 site [9]. The question of whether chaperones prevent or induce pathological processes in synucleinopathies remains open. One of the main concepts is that α-synuclein–chaperon interaction could reduce cytotoxicity by inhibition of its fibrillation or by redirecting aggregation towards more easily degradable forms [7].

This concept makes chaperones an interesting object of research as potential therapeutic agents, and this is also true for non-eukaryotic chaperones. Thus, it has been shown that the apical domain of the bacterial chaperonin GroEL is able to inhibit the fibrillation of amyloidogenic proteins, including α-synuclein, *in vitro* [10]. Recently, the

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Pseudomonas fluorescens bacteriophage chaperonin OBP was found to prevent the fibrillation of the α-synuclein A53T mutant *in vitro* in the absence of ATP [11]. However, in the presence of ATP, the OBP chaperonin functions via the ATPase cycle and, in contrast, stimulates the fibrillation of α-synuclein monomers *in vitro*. Chaperonin OBP belongs to a group of GroEL-like chaperonins that function in an ATP-dependent manner but have a simpler structure [12]. A unique feature of phage chaperonins is that they do not require co-chaperonins for their function [13].

In this work, we investigated the effect of the single-ring phage chaperonin OBP on the fibrillation of the mutant form of α-synuclein A53T during their co-expression in eukaryotic HEK293T cells and evaluated the effect of such co-expression on cell viability. The effect of extracellular α-synuclein fibrils formed with the participation of the chaperonin OBP, with and without ATP, on the cytotoxicity of HEK293T and neuron-like SH-SY5Y cells was also tested.

2. Materials and Methods

2.1. Construction of plasmids for HEK293T and SH-SY5Y transfection

The gene 246 encoding the chaperonin OBP of bacteriophage OBP *P. fluorescence* was amplified by PCR from a plasmid previously constructed for expression of this gene in *E. coli* cells [14] as a DNA template. PCR was performed using Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) with the forward primer 5′-TTATGCTAGCACCATGTCTTCCATGTTAACTGA-3′ and the reverse primer 5′-CTGAGCAATAACTAGCAT-3’. The 246 amplicon was restricted with NheI and XhoI and cloned into pcDNA3.1 Hygro(+) vector (Invitrogen, USA) to obtain plasmid, called pcDNA-OBP.

The transfection vector for the expression of the human α-synuclein A53T mutant in eukaryotic cell lines was previously obtained [15]. Briefly, the plasmid was constructed by inserting the gene of human wild-type α-synuclein (NCBI Reference Sequence: NM_000345.3) into the pcDNA 3.1 Hygro (+) vector followed by site-directed mutagenesis using the Phusion site-directed mutagenesis kit (Thermo Scientific, USA) to obtain A53T mutation. The gene was inserted into the pcDNA 3.1 Hygro (+) polylinker region at the BamHI and NotI restriction sites with the addition of the Kozak consensus sequence in the 5’ region of the translation start site. The resulting vector, called pcDNA-syn_mut, carries the above gene under the control of the constitutive CMV promoter and the hygromycin resistance gene under the control of the constitutive SV40 promoter.

2.2. Cell culture

The human cell lines SH-SY5Y and HEK293T were obtained from American Type Culture Collection (Manassas, USA). Cell lines were cultured in DMEM/F-12 medium (Paneco, Russia) supplemented with 10 % fetal bovine serum (HyClone, USA), L-glutamine (Paneco, Russia), 50 units/ml penicillin and 50 μg/ml streptomycin (Paneco, Russia). Cell cultures were grown in 5 % CO_2 humidified atmosphere at 37 °C and subcultured at 80–90 % confluence. Cells were transfected with the pcDNA-OBP and/or pcDNA-syn_mut plasmids using Lipofectamine 3000 (Invitrogen, USA) or TransIT-X2 (Mirus, USA) transfectants according to the manufacturer’s protocols.

2.3. Protein expression in HEK293T cells and immunoblotting

HEK293T cells were transfected either with a single plasmid or simultaneously with two plasmids (pcDNA-OBP and/or pcDNA-syn_mut) using TransIT-X2 (Mirus, USA) transfectant according to the manufacturer’s protocol. To confirm α-synuclein and chaperonin production after 2 days of incubation, transfected cells were lysed in PBS buffer (pH 7.4), 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, containing 1 % Triton X-100 and fractionated by centrifuging at 14000 g (Eppendorf, USA) for 10 min at 4 °C. The cell pellet was washed in cold PBS buffer and centrifuged under the same conditions as above. After removal of the supernatant, the cell pellet was resuspended in 200–300 μl of 1 × sample buffer and sonicated (Branson Digital Sonicator, USA). Aliquots of supernatant and pellet samples were analysed by SDS-PAGE on a 12 % or 15 % separating gel [16] followed by transfer to a PVDF membrane for 3 h at 150 mA. Additionally, the supernatant of pcDNA-OBP transfected cell lysates was analysed by native electrophoresis on a 5 % separating gel followed by transfer to a PVDF membrane. The membrane was blocked in a 3 % skim milk solution in PBS containing 0.05 % (v/v) Tween-20 (PBS-T). The membrane was stained with primary antibodies for 1 h at room temperature or overnight at 4 °C. Primary antibodies used were mouse monoclonal antibody against α-synuclein (1:1 000, clone LB509, Abcam, USA), mouse serum against recombinant OBP (1:10 000) or mouse monoclonal antibody against GAPDH (1:2 000, HyTest, Finland) for cellular GAPDH detection. Serum against OBP was prepared as previously described [17]. In all cases, 1 h incubation with the goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10 000, Jackson ImmunoResearch, USA) was used. Protein bands were visualised by Western blotting detection kit (Advansta WesternBrightECL, USA) using the ChemiDoc XRS+ (Bio-Rad, USA). As a control, the fractionation procedure with Triton X-100 containing buffer was performed on α-synuclein fibrils prepared as described in *2.6*.

2.4. Expression and purification of α-synuclein A53T

The recombinant human α-synuclein A53T mutant was obtained according to a previously published method [18]. Briefly, the protein was produced in *E. coli* BL21(DE3) cells and acid precipitation method was used to remove contaminating proteins. For this, the cell extract was adjusted to pH 2.8 by adding 9 % HCl and the denatured proteins were removed by centrifugation. The pH of the extract was returned to the range of neutral values (7.0–7.5) by adding 1 M potassium phosphate solution, pH 11. The recombinant protein was precipitated by addition of saturated ammonium sulfate to a final concentration of 40 % (w/v) followed by centrifugation. The protein pellet was dissolved in water, subjected to dialysis against water, lyophilised and stored at −20 °C. Prior to the experiment, the protein was dissolved in the required buffer and centrifuged at 15000 g for 10 min to remove aggregates. The protein concentration was determined spectrophotometrically at 280 nm using a specific absorption coefficient of $A^{0.1\ \%} = 0.412$.

2.5. Expression and purification of chaperonin OBP

The recombinant chaperonin OBP was produced in *E. coli* BL21(DE3) cells from the expression vector and purified as described previously [14]. Homogeneity of the purified recombinant protein was assessed by SDS-PAGE. The concentration of OBP was determined spectrophotometrically at 280 nm using a theoretical absorption coefficient of $A^{0.1\ \%} = 0.872$.

2.6. Fibrillation of α-synuclein A53T mutant in vitro

Fibrillation was carried out in a solution containing α-synuclein A53T mutant at a concentration of 50 μM. In the case of chaperonin-dependent aggregation, chaperonin was added to the solution at a molar ratio of 1:20 (chaperonin:α-synuclein) without and with 10 mM ATP. Samples were incubated at 37 °C with constant stirring at 600 rpm for 48 h in 50 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 0.02 % NaN_3. To detect the formation of amyloid structures during fibrillation, 5 μl-aliquots were taken from the reaction system at regular intervals and mixed with 95 μl of freshly prepared 40 μM Thioflavin T (ThT) solution in 50 mM Tris-HCl buffer (pH 7.5). After 15 min of incubation at 20 °C, ThT fluorescence in the wells of 96-well plate was determined (excitation at 435 nm, emission at 482 nm) using a

VICTOR X5 spectrofluorimeter (PerkinElmer, USA). Kinetics of fibrillation was analysed during incubation of samples in the presence of 0.5 mM ThT in a 96-well plate (Greiner, Kremsmuenster, Austria, Non-Binding, μClear®, black) with sealing film in 100 μl/well. The plate was incubated with orbital shaking at 37 °C and fluorescence was measured at 30-min intervals through the bottom of the plate using a CLARIOstar plate reader (BMG LABTECH GmbH, Germany). The lag phase time for each curve was calculated using the algorithm developed previously [19].

2.7. Immunocytochemistry

HEK293T cells were plated on 10-mm coverslips (Menzel-Glaser, Germany) in 24-well plates (Corning, USA) at $1.5*10^5$ cells per well. The next day cells were transfected with Lipofectamine 3000 (Invitrogen, USA) and stained 2 days after transfection. For this, the cells were washed in cold PBS, fixed with 3.7 % formaldehyde in PBS for 20 min, and permeabilised with 0.25 % Triton X-100 for 10 min. After blocking with 3 % normal goat serum (Thermo Fisher Scientific, USA) in PBS for 30 min, the cells were incubated with rabbit polyclonal antibodies against α-synuclein (1:500, Cell Signaling, USA) and/or with mouse serum against recombinant OBP (1:500) [17] for 1 h, washed with PBS, and incubated with Alexa Fluor 488-conjugated alpaca anti-rabbit IgG (1:500, Jackson ImmunoResearch, USA) and with Cy3-conjugated goat anti-mouse IgG (1:500; Jackson ImmunoResearch, USA) for 1 h. Cell nuclei were stained with DAPI (Sigma, USA) at 0.5 μg/ml for 10 min. The coverslips were mounted on glass slides with Mowiol (Sigma, USA) and analysed with an Axiovert 200 M fluorescent microscope (Carl Zeiss, Germany) equipped with a cooled ORCAII-ERG2 CCD camera (Hamamatsu Photonics, Japan).

2.8. Transmission electron microscopy (TEM)

The α-synuclein A53T samples after 27 h of fibrillation in the absence or presence of phage chaperonin OBP with 10 mM ATP were centrifuged at 3000 g for 3 min. The supernatants containing chaperonin were removed, and fibril pellets were resuspended in the same volume of 50 mM Tris-HCl buffer (pH 7.5). Samples were adsorbed onto a Formvar film attached to 200-mesh copper grids and contrasted with 1 % uranyl acetate aqueous solution. The specimens were observed in a JEOL JEM-1400 transmission electron microscope (JEOL, Akishima, Japan) at an accelerating voltage of 80 kV.

2.9. Evaluation of cell viability by the MTT-test

To study the viability of SH-SY5Y and HEK293T cells in the presence of extracellular α-synuclein A53T aggregates, $1.5*10^4$ cells per well were seeded onto 96-well cell culture treated plates (Biofil, China). After 24-h incubation the medium was replaced with tested samples of α-synuclein monomers/fibrils diluted with full DMEM/F12 complete medium to a final concentration of 0.8 μM. Cells exposed to full DMEM/F12 complete medium were also used as controls. After 24 h of incubation, cell viability was estimated by the reduction of the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, which was measured spectrophotometrically. The cell medium was replaced by DMEM/F12 with tetrazolium dye (to a final concentration of 0.375 mg/ml) and after 4 h of incubation formazan crystals were dissolved in 100 μl DMSO. After 15 min at 37 °C, the absorbance was registered at 570 nm using a VersaMax microplate reader (USA). A reference wavelength of 630 nm was used. Experimental procedures were performed in quadruplicate. Cell viability was determined by the ratio of the absorbance of the test samples to the absorbance of the control. To study the viability of SH-SY5Y and HEK293T cells after transfection with pcDNA-OBP and/or pcDNA-syn_mut vectors, $8*10^3$ cells per well were seeded onto 96-well cell culture treated plates (Biofil, China), transfected with Lipofectamine 3000 (Invitrogen, USA) 24 h post-seeding, and MTT-test was performed 72 h later. The results were presented as mean ± SD. One-way ANOVA with Bonferroni multiple comparison test was used to analyse the results. Statistical significance was set at adjusted p-value<0.05. GraphPad Prism software was used for statistical analysis.

3. Results

3.1. Co-expression of phage chaperonin OBP and α-synuclein A53T mutant in HEK293T cells

Phage chaperonins have previously been shown to stimulate the amyloid conversion of α-synuclein *in vitro*, but only in the presence of ATP [11]. In the absence of ATP, chaperonins prevented the formation of α-synuclein fibrils. To test the effect of phage chaperonins on the α-synuclein amyloid conversion under conditions more similar to those *in vivo*, investigated proteins were co-expressed in human embryonic kidney cell line HEK293T. The simplest of the ATP-dependent phage chaperonins, the single-ring chaperonin OBP, and the α-synuclein A53T mutant with an increased ability to form amyloid, were chosen for the experiments. The conversion of α-synuclein in eukaryotic cells was monitored by immunocytochemical analysis and Western blot analysis of Triton X-100 soluble and insoluble fractions of cell lysates.

First, to evaluate the experimental system, HEK293T cells were transiently transfected separately with a plasmid, containing the gene 246 encoding the chaperonin OBP or with a plasmid, containing the α-synuclein A53T mutant gene. Two days after transfection, the cells were lysed with PBS buffer, containing Triton X-100, and separated into pellet (insoluble) and supernatant (soluble) fractions by centrifugation. The fractions obtained were analysed by electrophoresis under denaturing conditions, followed by immunoblotting using specific antibodies (see Materials and Methods). As shown in Fig. 1A, transfection of cells with the pcDNA-OBP plasmid resulted in efficient production of the chaperonin OBP. This protein was detected in the supernatant fraction only. In addition, analysis of the same supernatant by native electrophoresis followed by immunoblotting showed that chaperonin OBP had the same mobility in the gel as the purified recombinant OBP produced by the bacterial cells (Fig. 1B). Since the latter was previously shown to be heptameric and functional [14], we assume that the chaperonin OBP produced in HEK293T cells is also heptameric and likely to have functional activity. Otherwise, native electrophoresis might show bands of different mobility, corresponding to different oligomeric or monomeric protein forms. Note that such bands are present in both samples, but as minor bands. When HEK293T cells were transfected with a plasmid containing the gene encoding the α-synuclein A53T mutant, the recombinant protein was efficiently expressed and was detected in the supernatant fraction only (Fig. 1C). Efficient separation of the Triton X-100 insoluble fraction was confirmed by the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a cytoplasmic marker, only in the supernatant (Fig. 1A–C).

Data on the intracellular localisation of the investigated proteins after their expression in HEK293T cells were obtained by immunochemical staining with the specific antibodies (Fig. 2). When chaperonin OBP or a mutant form of α-synuclein A53T was expressed separately, an intense staining was observed with the corresponding antibodies. In both cases, the proteins showed a diffuse cytoplasmic distribution in the cells (Fig. 2a-d). It is noteworthy that no visible aggregates were observed during the production of the fibrillation-prone α-synuclein A53T mutant in this experimental system.

The effect of chaperonin OBP on the aggregation of mutant α-synuclein A53T in cells was then tested. When HEK293T cells were simultaneously transfected with both plasmids, efficient production of chaperonin OBP and the mutant form of α-synuclein A53T was observed. Fluorescence microscopy data on the intracellular localisation of chaperonin OBP and α-synuclein A53T during their co-production in HEK293T cells are shown in Fig. 2e–g. No significant changes in the

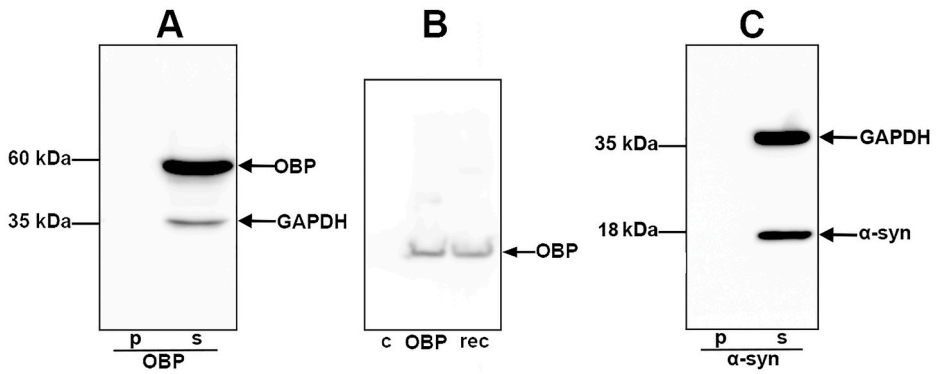

Fig. 1. Western blot analysis of HEK293T cell lysates after transfection with plasmids encoding chaperonin OBP (**A, B**) or α-synuclein A53T mutant (**C**). **A, C** - Triton X-100 pellet (p) and supernatant (s) fractions of cells 48 h post transfection with pcDNA-OBP (**A**) or pcDNA-syn_mut (**C**) plasmids after SDS-PAGE and immunoblotting with mouse serum against recombinant OBP (**A**) or mouse monoclonal antibodies against α-synuclein (**C**); **B** - Triton X-100 supernatant fractions of non-transfected HEK293T cells (c) and of HEK293T cells 48 h post-transfection with pcDNA-OBP (OBP) compared with purified recombinant chaperonin OBP produced in *E.coli* (rec). Native PAGE analysis of samples followed by immunoblotting with mouse serum against recombinant OBP is shown. The membranes were also stained with a primary antibody against GAPDH (**A, C**). Horseradish peroxidase-conjugated anti-mouse antibodies were used as secondary antibodies.

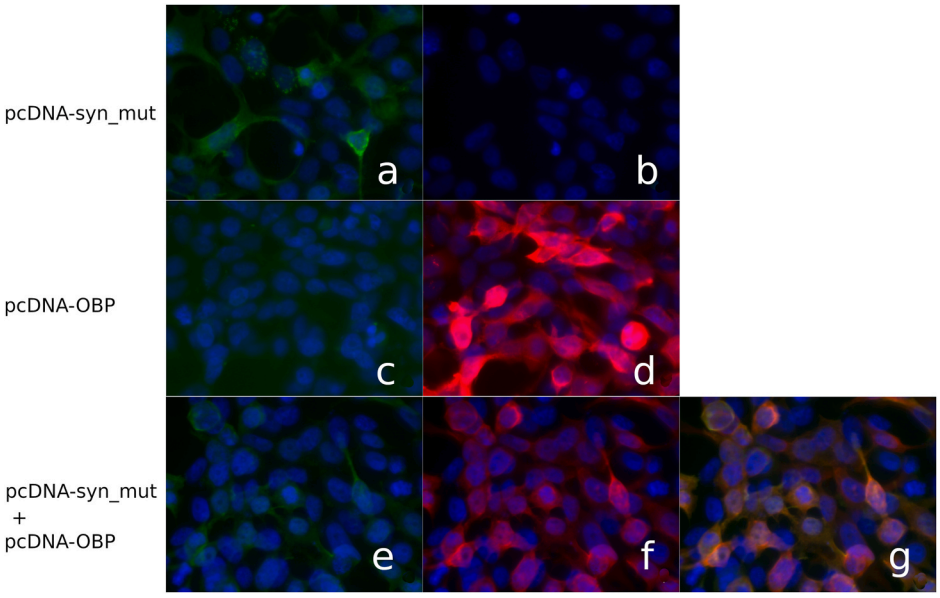

Fig. 2. Immunocytochemical analysis of HEK293T cells expressing α-synuclein A53T and/or the chaperonin OBP. **a, b** - Transient transfection of cells with plasmid containing the α-synuclein A53T mutant gene (pcDNA-syn_mut). Staining with antibodies against α-synuclein (*green* channel, **a**) and serum against recombinant OBP (*red* channel, **b**). **c, d** - Transient transfection of cells with plasmid containing the chaperonin OBP gene (pcDNA-OBP). Staining with antibodies against α-synuclein (*green* channel, **c**) and serum against recombinant OBP (*red* channel, **d**). **e, f, g** - Transient transfection of cells with a mixture of plasmids containing the chaperonin OBP gene and the α-synuclein A53T mutant gene (pcDNA-syn_mut + pcDNA-syn_mut). Staining with antibodies against α-synuclein (*green* channel, **e**) and serum against recombinant OBP (*red* channel, **f**). The merged image is also shown (*green* and *red* channel, **g**). The cell nuclei were stained with DAPI (*blue*, **a-g**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

localisation of either protein were observed during co-production compared to their separate production. They showed a diffuse cytoplasmic distribution, and no visible aggregates of α-synuclein were observed.

Western blot analysis was also used to detect potential oligomers or aggregates of α-synuclein. Since aggregated α-synuclein is known to be less soluble in detergents, the presence of α-synuclein in the insoluble fraction after treatment of cells with Triton X-100 containing buffer would indicate its fibrillation [20]. Therefore, after co-expression of the two investigated proteins, the cells were lysed with a buffer containing Triton X-100, then centrifuged and the resulting fractions analysed by SDS-PAGE. Immunoblotting of the fractionated lysate samples with monoclonal antibodies against α-synuclein showed that after expression with or without the chaperonin OBP, most of the α-synuclein in the supernatant was present as a monomer. However, a minor band corresponding to the α-synuclein dimer was detected in the pellet, together with a smear that may represent its different oligomeric forms (Fig. 3A). For comparison, the same fractionation procedure, followed by immunoblotting, was performed on α-synuclein fibrils produced *in vitro* with the participation of the recombinant chaperonin OBP in the presence of ATP. In such experimental system, purified recombinant α-synuclein monomers with purified recombinant chaperonin OBP were incubated in test tube with shaking for 48 h at 37 °C (Materials and Methods, 2.6). It was found that in the Triton X-100-insoluble fraction of such fibrils, in addition to α-synuclein monomer and dimer bands, the bands of high molecular weight products were clearly detected (Fig. 3B).

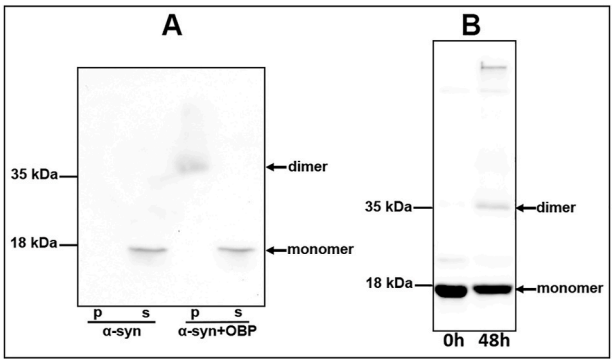

Fig. 3. Western blot analysis of fractionated HEK293T cell lysates after transfection with plasmid encoding α-synuclein A53T mutant (α-syn) and after co-transfection with plasmids encoding α-synuclein A53T mutant and chaperonin OBP (α-syn + OBP) (**A**). Western blot analysis of insoluble fractions of control fibrils produced *in vitro* with recombinant chaperonin OBP in the presence of ATP (**B**). Triton X-100 pellet (p) and supernatant (s) fractions of cells 48 h post- transfection after SDS-PAGE and immunoblotting with mouse monoclonal antibodies against α-synuclein (**A**). Horseradish peroxidase-conjugated anti-mouse antibodies were used as secondary antibodies. The same analysis of Triton X-100-insoluble fractions of control α-synuclein fibrils produced *in vitro* with recombinant OBP and ATP at the beginning (0 h) and at the end point (48 h) of fibrillation is shown (**B**).

3.2. Effect of chaperonin OBP on mutant α-synuclein A53T fibrillation in vitro

In addition, the effect of OBP on kinetics of α-synuclein amyloid transformation was studied *in vitro* on purified recombinant proteins. α-Synuclein amyloid transformation was monitored by an increase in fluorescence of thioflavin T after its interaction with β-sheet structures of fibrils formed. Fig. 4A shows the kinetics of fibrillation of α-synuclein monomers with the chaperonin OBP in the absence and presence of ATP compared to the spontaneous process. In the absence of chaperonin, α-synuclein monomers transform spontaneously into amyloid fibrils with a lag phase of 10.5 h (Fig. 4A, 'α-syn' curve). In the absence of ATP, the chaperonin OBP binds unstructured α-synuclein monomers, thereby preventing them from spontaneous fibrillation, as indicated by the low ThT fluorescence values (Fig. 4A, 'α-syn + OBP' curve). Since the concentration of chaperonin (2.5 μM) is lower than the concentration of α-synuclein (50 μM), it binds only a fraction of the monomers. The remaining unbound α-synuclein monomers spontaneously form fibrils, but their fibrillation occurs with a significantly increased lag phase, as evidenced by a slight increase in ThT fluorescence after 40 h of incubation (Fig. 4A, 'α-syn + OBP' curve).

In the presence of ATP, however, chaperonin OBP stimulates the fibrillation of α-synuclein monomers. The ATP-dependent action of chaperonin significantly shortens the lag phase to 3 h and leads to a dramatic increase in ThT fluorescence, which reaches maximum values after 30 h of incubation, indicating the amyloid transformation of α-synuclein (Fig. 4A, 'α-syn + OBP + ATP' curve). Note that ThT fluorescence is an order of magnitude higher when interacting with fibrils formed in the presence of chaperonin and ATP than with spontaneously formed fibrils.

According to the TEM data, the two types of α-synuclein fibrils, formed by chaperonin OBP participation in the presence of ATP (Fig. 4B) and by spontaneous fibrillation of α-synuclein monomers (Fig. 4C) appear to be morphologically similar. However, this does not exclude the possibility that they are structurally different. We therefore plan to investigate their structure at a higher resolution using cryo-electron microscopy in the future.

3.3. Cytotoxicity of α-synuclein A53T co-expressed with chaperonin OBP in eukaryotic cells and cytotoxicity of α-synuclein A53T amyloid forms produced in vitro with chaperonin OBP and ATP

Within the cell, α-synuclein can exist in several forms, including monomers, oligomers and fibrils. Oligomers are thought to be the most cytotoxic species [21]. Since the co-expression of α-synuclein with the chaperonin OBP leads to the production of oligomeric forms of α-synuclein, the next step in our work was to test the viability of transfected cells. As cellular α-synuclein aggregation pathways are of interest for the modelling of pathological processes in synucleinopathies, in addition to cytotoxicity in the HEK29T cell line, the effect of co-expression of α-synuclein with the chaperonin OBP on the survival of the SH-SY5Y cell line was evaluated as an accepted neuron-like model [22]. According to the results of the MTT test 3 days after transfection, there was no difference in the cell viability of the two cell lines when α-synuclein and the chaperonin OBP were expressed separately or co-expressed (Fig. 5). The data obtained suggest that the amount of oligomeric forms accumulated in the cell during co-expression of α-synuclein A53T with OBP is insufficient for a significant cytotoxic effect, or that such forms have different properties from those of α-synuclein oligomers formed in the absence of chaperonin.

Next the cytotoxicity of different amyloid forms of α-synuclein A53T produced *in vitro* on the HEK293T and SH-SY5Y neuroblastoma cell lines was evaluated. For this, samples of α-synuclein A53T obtained after 24 h incubation with or without chaperonin OBP and control α-synuclein A53T sample without incubation were tested (Fig. 6A and B). Comparable results were obtained for both cell lines: α-synuclein monomers, as well as chaperonin OBP alone, had no significant effect on cell viability, whereas the fibrils obtained with chaperonin in the presence of ATP showed the highest toxicity. The higher toxicity of fibrillar forms of α-synuclein compared to its monomers is in accordance with data from other experimental systems [23]. It was found that α-synuclein co-incubated with the chaperonin OBP in the presence of ATP was more toxic than spontaneously fibrillating α-synuclein. In the absence of ATP, the toxicity of the co-incubated sample is statistically indistinguishable from that of spontaneously fibrillating α-synuclein. The fibrillation kinetics data, based on ThT fluorescence, suggest that α-synuclein conversion is more effective under ATP-dependent chaperonin action, resulting in a greater amount of amyloid fibrils that provide high sample toxicity (Fig. 4A).

4. Discussion

A large body of data suggests that chaperonins may play an important role in the neurodegeneration process. For example, there is an evidence that the mitochondrial chaperonin Hsp60 may be involved in the development of Parkinson's disease (PD) [24]. In a mouse model of 6-OHDA-induced disease, the expression of *Hspd1*, which encodes Hsp60, was changed. In addition, the chaperonin was released extracellularly and promoted microglial activation [25,26]. It has also been shown that PD-related cellular stress is associated with the induction and secretion of Hsp60 in SH-SY5Y cells [27]. Hsp60 knockdown significantly reduces paraquat-induced microglial inflammation, which induces α-synuclein aggregation in mouse models [28]. In cellular models of PD based on SH-SY5Y cells, the expression of the chaperonin-containing TCP-1 subunit 2 (CCT2 or CCTβ) is induced in addition to the mitochondrial chaperonin [29].

It is also well known that a variety of chaperones, including chaperonins, have different effects on the fibrillation of amyloidogenic proteins. The structural features of chaperones and their affinity for substrate proteins are important determinants. Members of the Hsp60 family, the eukaryotic TRiC and the bacterial GroEL, have been shown to inhibit fibrillation of amyloidogenic proteins both *in vivo* and *in vitro* [30,31]. The effect is more pronounced in GroEL with the Gly192Trp mutation, which causes a rotation of the apical domains relative to the

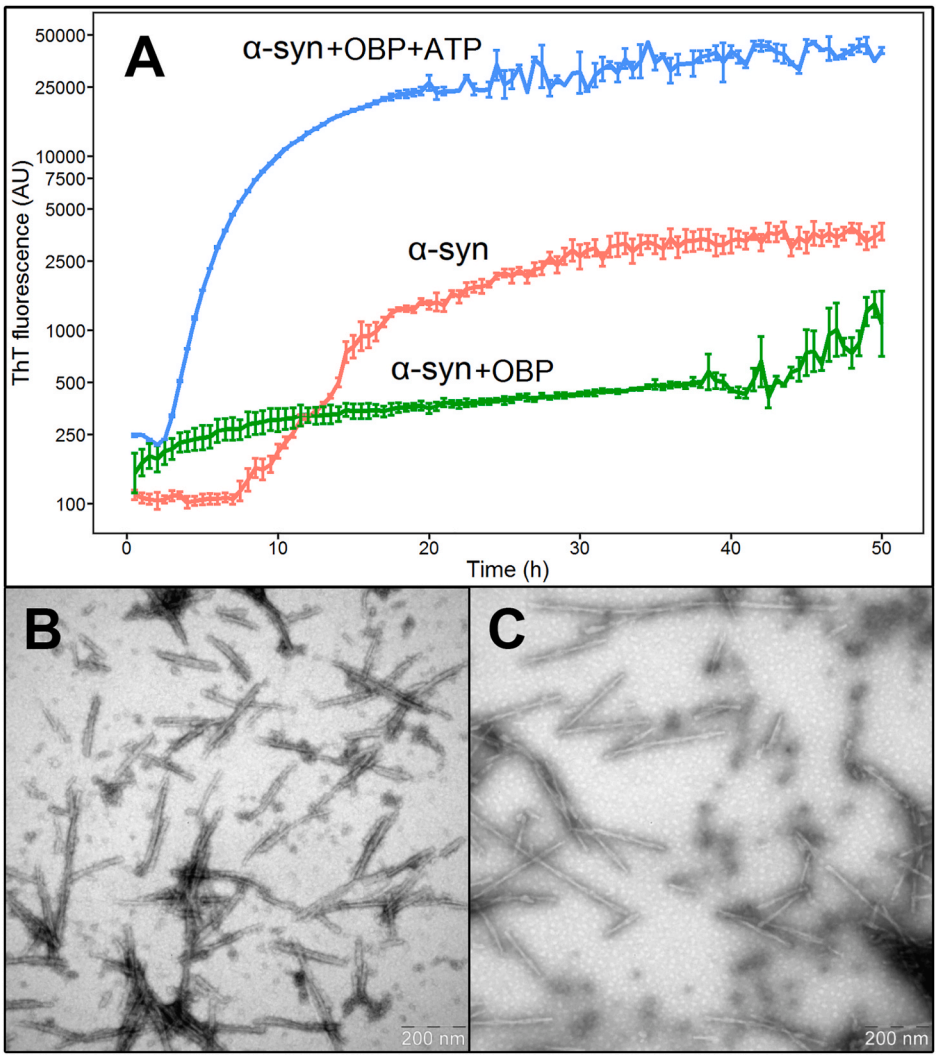

Fig. 4. Effect of chaperonin OBP on the fibrillation of α-synuclein monomers in the presence and absence of ATP compared to spontaneous fibrillation *in vitro*. **A** - Changes in ThT fluorescence during incubation of α-synuclein monomers without chaperonin (α-syn) and with chaperonin in the absence (α-syn + OBP) and in the presence of ATP (α-syn + OBP + ATP). **B** -TEM image of α-synuclein fibrils formed by the chaperonin OBP in the presence of ATP after 24 h incubation. **C** - TEM image of α-synuclein fibrils formed by spontaneous transformation of its monomers after 24 h incubation.

intermediate domains, opening the internal chaperonin cavity. As a result, its hydrophobic interface becomes more accessible for binding of unfolded polypeptide chains, thereby increasing the affinity of the chaperonin for the protein substrates [32]. A similar effect was found for the GlyTrp mutation in mitochondrial Hsp60 [33]. The apical domain of GroEL itself, the mini-chaperone, was shown to inhibit the fibrillation of some amyloidogenic proteins including α-synuclein *in vitro* [10]. These observations suggest the possibility of using chaperonins to prevent and treat amyloid neurodegenerative diseases. However, it will be necessary to demonstrate a reduction in the cytotoxicity of the amyloidogenic protein forms produced by chaperonin action.

A convenient model for studying the effect of chaperonins on amyloidogenic proteins is the bacteriophage-encoded GroEL-like chaperonins, which act in an ATP-dependent manner but do not require co-chaperonins to function [12,13]. We previously found that phage chaperonins, the single-ring OBP and the double-ring EL, inhibited fibrillation of the α-synuclein A53T mutant *in vitro* in the absence of ATP [11]. Unlike bacterial GroEL and mitochondrial Hsp60 chaperonins, which only have this property as open-mimic mutants, the phage chaperonin OBP itself has an open conformation. This is because the OBP sequence contains two large polar residues, Glu-191 and Asn-376, at the positions of two conserved Gly residues that form the hinge between the apical and intermediate domains. These substitutions prevent the rotation of the apical domains during chaperonin function. The inner cavity of the OBP chaperonin is therefore open at different stages of its ATPase cycle, which simplifies its mechanism of action in comparison with that of the Hsp60 family of chaperonins [34]. Perhaps because of this unique feature, the internal cavity of the phage chaperonin OBP is available for the binding/release of unstructured proteins such as α-synuclein. The results obtained *in vitro* suggest that, in the absence of ATP, the chaperonin OBP binds unstructured α-synuclein monomer and forms a stable complex with it, thereby preventing its involvement in spontaneous fibrillation. In the presence of ATP, chaperonin exhibits chaperone activity and stimulates the conversion of α-synuclein monomers into amyloid. In this case, chaperonin appears to help the unfolded α-synuclein polypeptide chain to undergo a conformational change and transform into a stable aggregate with β-structures, the so-called nucleation center, which initiates further fibril growth. In the case of α-synuclein fibrillation in the absence of chaperonin, the formation of the β-structures proceeds spontaneously, but at a much slower rate, as evidenced by a rather prolonged lag phase. The ATP-dependent action of chaperonin significantly reduces the time required for the transformation of unfolded monomers into nucleation centers, resulting in a shorter initial lag phase compared to the

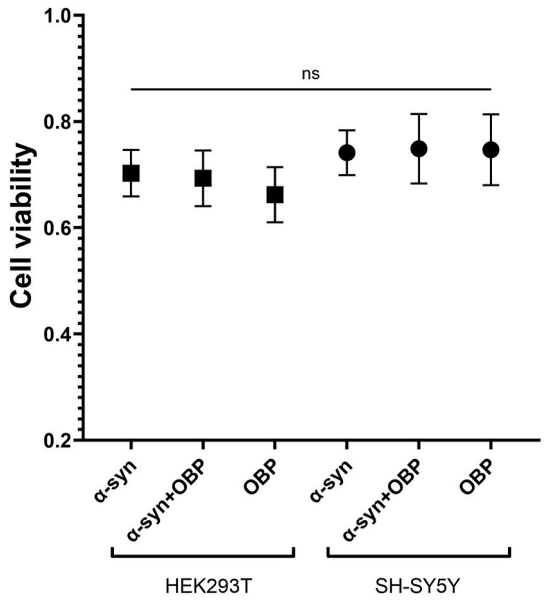

Fig. 5. Effect of transfection of HEK293T and SH-SY5Y cell lines with plasmid encoding α-synuclein A53T, with plasmid encoding chaperonin OBP or with a mixture of these plasmids on cell viability using the MTT assay. The proportions of functional cells 3 days post-transfection are shown in comparison with non-transfected cells. Differences within each cell line are not statistically significant ('ns').

spontaneous process.

The results obtained upon co-expression of genes encoding the α-synuclein A53T mutant and the chaperonin OBP in eukaryotic cells show that chaperonins do not prevent the pathological transformation of the amyloidogenic protein, but rather stimulate it. In the future, we will compare the efficiency of α-synuclein fibrillation under chaperonin action in cells with different ATP levels. We also plan to co-express α-synuclein with a mutant OBP lacking ATPase activity. Such experiments will allow us to test the involvement of ATP-dependent chaperonin in amyloid protein conversion.

In the present work, we have used the single-ring ATP-dependent chaperonin OBP as a model to study the role of chaperonins in α-synuclein transformation. However, it cannot be excluded that the chaperonin OBP may also stimulate the amyloidisation of α-synuclein *in vivo*. In recent years, more and more information has become available on the presence of α-synuclein in various biological fluids, both in a soluble form and incapsulated in the exovesicles [35,36]. It is thought that α-synuclein enters the nervous system via the gastrointestinal tract, as it occurs in prion diseases [37–39]. It is possible that phage chaperonins, especially in severe bacterial infections, encounter α-synuclein and stimulate its pathological transformation.

The observation that chaperonin significantly accelerates the conversion of α-synuclein into amyloid may have practical implications. The study of the anti-amyloid effect of various compounds is complicated by the very slow rate of spontaneous fibrillation of the wild-type α-synuclein. The process can be accelerated by using the A53T mutant form of α-synuclein, which has structural features that result in a much shorter lag phase during spontaneous fibrillation. The addition of the readily available chaperonin OBP can shorten the lag phase of fibrillation from 10.5 h to 3 h and significantly increase the sensitivity of the method.

Investigation of phage chaperonins has shown that, depending on their functional state, chaperonins can either inhibit or stimulate the pathological conversion of α-synuclein. Based on these results, it is possible to specifically generate mutant recombinant phage chaperonins with anti-amyloid activity and to predict the effect of other chaperonins on pathological processes.

5. Conclusions

The phage chaperonin OBP stimulates the amyloid conversion of the

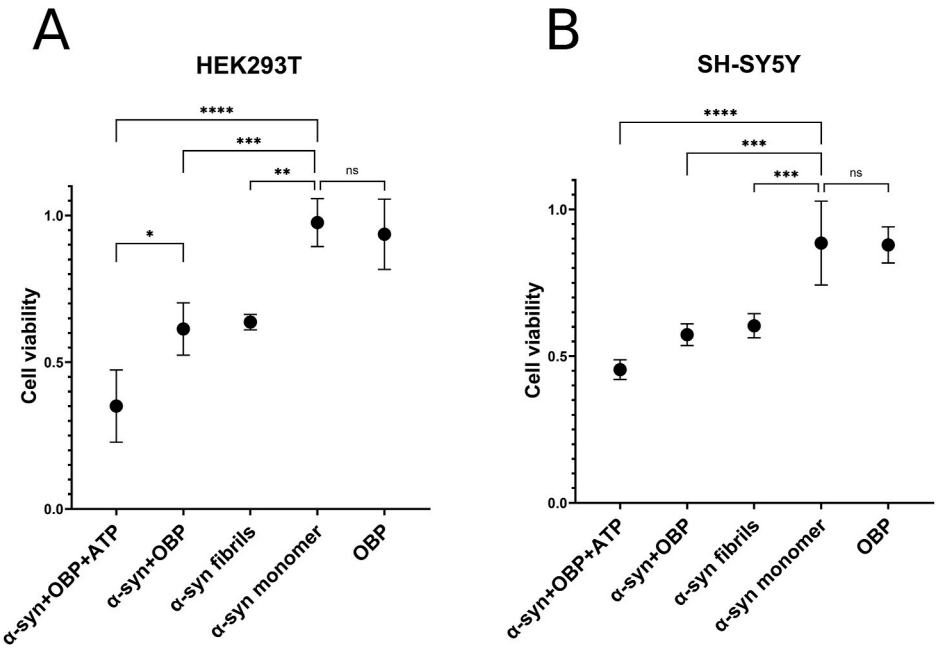

Fig. 6. Effect of monomers and amyloid forms of α-synuclein A53T on the viability of HEK293T (**A**) and SH-SY5Y (**B**) cell lines using the MTT assay. Cells were exposed to DMEM/F12 complete media containing the following α-synuclein samples at a concentration of 0.8 μM: control sample of α-synuclein before *in vitro* fibrillation (α-syn monomer), sample of α-synuclein after 24 h incubation with the chaperonin OBP and ATP (α-syn + OBP + ATP), sample of α-synuclein after 24 h incubation with chaperonin OBP without ATP (α-syn + OBP), sample of α-synuclein after 24 h spontaneous fibrillation (α-syn fibrils) and control sample of chaperonin OBP (OBP). Statistical significance: * adjusted p-value<0.05; ** adjusted p-value<0.01; *** adjusted p-value<0.001; **** adjusted p-value<0.0001, ns – non-significant.

A53T mutant form of α-synuclein in an ATP-dependent manner. The formation of oligomeric forms of α-synuclein under the action of chaperonin OBP occurs during the co-expression of chaperonin and α-synuclein in eukaryotic cells. The formation of oligomeric forms of α-synuclein co-expressed with chaperonin OBP occurs in cells even under conditions in which separately expressed α-synuclein does not undergo transformation. The α-synuclein fibrils that are formed *in vitro* under the action of the chaperonin OBP are significantly more cytotoxic than the spontaneously formed fibrils. The marked acceleration of α-synuclein fibrillation under the action of phage chaperonin allows the creation of more efficient systems for testing anti-amyloid compounds, reducing experimental time by an order of magnitude.

CRediT authorship contribution statement

Denis V. Pozdyshev: Writing – review & editing, Investigation, Conceptualization. **Evgeniia V. Leisi:** Writing – review & editing, Visualization, Investigation. **Vladimir I. Muronetz:** Writing – original draft, Project administration, Conceptualization. **Sergei A. Golyshev:** Writing – review & editing, Visualization, Investigation, Funding acquisition. **Lidia P. Kurochkina:** Writing – review & editing, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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