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Antibodies against the C-terminus of α-synuclein modulate its fibrillation

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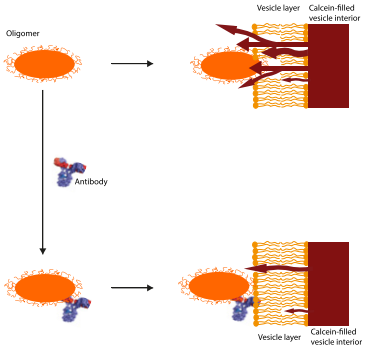
HIGHLIGHTS

- Antibodies targeting the C-terminus of alpha synuclein inhibit its fibrillation.
- Antibody-bound oligomers show reduced permeabilization of lipid membranes.
- Increased avidity for oligomers and fibrils is ascribed to multiple epitopes.

GRAPHICAL ABSTRACT



Binding of a bivalent antibody to an alpha-synuclein oligomer leads to a decrease in membrane permeabilization, as measured by a reduction in calcein release. The antibody is a space-filled cartoon model of an IgG1 molecule (PDB codes 1BJ1 and 1IGY) with the heavy chains colored purple and light chains colored red.

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ABSTRACT

The 140-residue natively disordered protein α-synuclein (aSN) is a central component in the development of a family of neurodegenerative diseases termed synucleinopathies. This is attributed to its ability to form cytotoxic aggregates such as oligomers and amyloid fibrils. Consequently there have been intense efforts to avoid aggregation or reroute the aggregation pathway using pharmaceutical agents such as small molecules, chaperones and antibodies. aSN's lack of persistent structure in the monomeric state, as well as the multitude of different oligomeric and even different fibrillar states, makes it difficult to raise antibodies that would be efficacious in neutralizing all conformations of aSN. However, the C-terminal 20–40 residues of aSN are a promising epitope for antibody development. It is primarily disordered in both monomeric and aggregated forms, and an anti-C-terminal antibody will therefore be able to bind all forms. Furthermore, it might not interfere with the folding of aSN into membranes, which could be important for its physiological role. Here we report a screen of a series of monoclonal antibodies, which all target the C-terminal of aSN. According to dot blot analyses, different antibodies

Abbreviations: Aβ, amyloid-β; aSN, α-synuclein; DOPG, dioleoyl-phosphatidylglycerol; EGCG, Epigallocatechin gallate; ELISA, enzyme-linked immunosorbent assay; LUV, large unilamellar vesicles; mAbs, monoclonal antibodies; NAC, non-Amyloid beta component; NC, negative control; PD, Parkinson's disease; RT, room temperature; ThT, Thioflavin T.

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bound different forms of aSN with different preferences and showed reduced binding to monomeric compared to aggregated (oligomeric and fibrillary) aSN. Consequently they have different effects on aSN's ability to fibrillate and permeabilize membranes. Generally, the antibodies with strongest binding to aggregated aSN in dot blot, also inhibited fibrillation and membrane permeabilization the most, and promoted formation of amorphous aggregates surrounded by small and thin fibers. This suggests that the development of antibodies that targets the C-terminus, exposed in the aggregated forms of aSN, may be beneficial for improved immunotherapy against PD.

1. Introduction

α-Synuclein (aSN) is an intrinsically disordered 140-residue presynaptic protein, which is abundant in Lewy Bodies in patients with Parkinson's disease (PD) [1,2]. The primary structure of aSN can be divided into three parts: the N-terminal region (residues 1–60), the non-Amyloid beta component or NAC region (61–95) and the acidic C-terminal tail (96–140) [3]. When aSN binds to negatively charged membranes, the N-terminal and a part of the NAC region, folds into an α-helical structure [4]. Residues 46–54 and 63–96 form the core of the fibrils, which have recently been shown to contain a Greek-key β-sheet topology [5], while the rest of the molecule is disordered. Under fibrillating conditions aSN appears to transiently exist in different oligomeric species that are both on- and off-pathway in fibril formation [6–8]. aSN fibrils have been a target for many years where especially small molecules such as flavonoids, *e.g.* Epigallocatechin gallate (EGCG), have been shown to inhibit the fibrillation of aSN and other intrinsically disordered proteins. There exists different ways to develop small-molecule inhibitors, including fragment-based combinatorial chemistry [9] and computational studies [10].

An alternative to small molecules is antibodies. Passive immunotherapy towards aSN was reviewed recently [11]. Anti-PD antibody therapy is challenged by the rather ill-defined nature of the pathogenic species. Both oligomers and fibrils of aSN have been suggested to be pathogenic [12], since both species have been shown to directly interact with cell membranes and to seed further aggregation of aSN in recipient cells. It is also possible that oligomerization, fibrillation and seeding could occur in the extracellular fluids, where aSN has been found [13–15]. Since aSN is not known to have any extracellular function, one therapeutic antibody strategy could be to identify an antibody that binds a motif accessible in monomers, oligomers and fibrils, *e.g.* the C-terminal tail [6]. Antibodies have previously been used to inhibit *in vitro* fibrillation of different aggregating proteins, disaggregate already formed aggregates *in vitro* and reduce cellular toxicity [16–19]. One such example is antibodies with conformational and linear epitopes in the N-terminal part of amyloid-β (Aβ), which inhibit *in vitro* Aβ1–42 fibril formation. Also an Aβ protofibril-selective antibody prevented amyloid formation in a mouse model for Alzheimer's disease [20]. It has been proposed that the mechanism behind antibody inhibition of fibril formation is simply binding to the monomers and thereby preventing the nucleation of the fibril reaction, stabilize and shield a specific type of aggregate or be able to disaggregate preformed oligomers or fibrils [21].

In the current study, we have raised a number of different monoclonal antibodies (mAbs) against aggregated aSN, in order to ascertain whether we can modulate the aggregation process of aSN. It turns out that all these antibodies target the C-terminal region of aSN. Interestingly, despite having common epitopes, the antibodies have different effects on aSN's aggregation, indicating that different modes of binding, or subtle differences in the epitope, can have substantial effects. Several of the antibodies inhibited fibrillation *in vitro*, and the inhibition appeared to depend on the apparent binding affinity evaluated by dot blot analysis. The antibodies, which showed the highest degree of binding to monomer, oligomers and fibrils in dot blots, were also the best to reduce (albeit incompletely) membrane permeabilization by oligomers. This suggests that a strong binding affinity might be important for the development of mAbs for improved immunotherapy against PD.

2. Materials and methods

All experiments with aSN were carried out in Phosphate Buffered Saline (PBS) (20 mM sodium phosphate, 137 mM NaCl, pH 7.4). Wild-type (wt) α-synuclein (aSN) was purified as described [22]. Lyophilized aSN was dissolved in PBS and filtered through a 0.22 μm filter (Q-Max® RR Syringe Filters, Frisenette ApS, Denmark) and the aSN concentration was measured on NanoDrop1000, Thermo Scientific, USA, at the absorbance wavelength 280 nm using a theoretical extinction coefficient of 0.412 $(mg/mL)^{-1}\ cm^{-1}$ to determine the concentration. Room temperature (RT) is defined as 21–23 °C.

Antibody purification: Monoclonal antibodies against aSN were generated by immunizing mice with different aSN monomer-aggregate mixtures, including aggregates cross-linked with reactive aldehydes as described [23]. The antigens were made from recombinant lyophilized aSN from Rpeptide (4241 Mars Hill Road, Bogart, GA 30622, USA). To make the aggregated antigen, aSN was dissolved in PBS to a concentration of 70 μM aSN (1 mg/mL), and incubated for 18 h at 37 °C. Three female mice (4–7 weeks old) were each immunized with aSN mixtures and boosted up to three times. Tail-bleeds were taken and screened for anti-aSN antibodies by enzyme-linked immunosorbent assay (ELISA) against monomeric aSN. Mice showing a titer greater than 1:50,000 over control were selected for fusion. Harvested splenocytes were fused to SP2/0 mouse myeloma cells, diluted and plated from single cell fusions. Antibody-positive fusions were analyzed for binding to aSN by ELISA. Cultures of hybridoma clones were expanded and mouse monoclonal antibodies were purified from the cultured supernatants using protein G chromatography. Six anti-aSN antibodies (A1–A6) were included in the study. A control antibody not binding to aSN was included as negative control (NC). The antibody 4B12 (alpha Synuclein Antibody MA1-90346, Thermo Fisher Scientific, Waltham, MA, USA) is a commercial antibody to alpha-synuclein and was included as positive control in the Western blots. The antibody concentration was measured by Bradford dye-binding method (BCA kit, Bio Rad Laboratories, Hercules, CA, USA) using human IgG as standard.

Oligomer purification: aSN oligomers were prepared as described [6, 24] with the modification that we used 5 h of incubation instead of 6 h and used a PBS buffer at pH 7.4. To remove insoluble material, the sample was then centrifuged for 10 min at RT and 16,060 x g. The supernatant was loaded on a Superose 6 Prep Grade column, GE healthcare Life Sciences, Sweden, in PBS at 2.5 mL/min. Oligomer fractions eluted at ~250 mL were collected and pooled, and concentrated with 15 mL Amicon® Ultra Centrifugal Filters with 30 kDa cut-off (Merck Millipore, Billerica, MA, USA) to the desired concentration.

Dot-blot assay: aSN sample (2 μL) was deposited onto a nitrocellulose membrane (Optitran BA-S 85 Reinforced NC, 0.45 μm, GE Healthcare and Life Sciences), after which the membrane was blocked in 5% skimmed milk solution (FLUKA analytical, Sigma) in PBS-T buffer (PBS pH 7.5, 0.1% w/v Tween-20) for 30 min at room temperature. The membrane was incubated with 100 ng/mL anti-aSN antibody in PBS-T buffer overnight at 4 °C, washed three times with PBS-T buffer, incubated with secondary antibody (HRP-tagged Rabbit Anti-Mouse IgG, DAKO (P0260), 1:5000 dilution to 0.26 μg/mL) for 1 h at RT, washed three times with PBS-T buffer. Signal was developed by chemiluminescence (Super signal West Dura substrate (#34076), 5 min incubation) according to the manufacturers' instructions and visualized on a Fuji Film LAS-