Ubiquitination of α-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function

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Running title: Ubiquitination of modified α-synuclein in Lewy bodies

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Abbreviations: LB, Lewy body; PD, Parkinson’s disease; DLB, Dementia with Lewy bodies; AD, Alzheimer’s disease; SN, substantia nigra; 2-DE, two dimensional gel electrophoresis
Ubiquitination of modified α-synuclein in Lewy bodies

Abstract:
Lewy bodies are intracellular fibrillar inclusions comprised of α-synuclein. They constitute the pathological hallmark of Parkinson’s disease, Dementia with Lewy bodies and other neurodegenerative diseases. Although the majority of Lewy bodies are stained for ubiquitin by immunohistochemistry, the substrate for this modification is poorly understood. Insoluble, urea-soluble α-synuclein was separated from soluble fractions and subjected to 2-dimensional gel electrophoresis, to further characterize pathogenic α-synuclein species from disease brains. Using this approach we found that in sporadic Lewy body diseases a highly modified, disease-associated 22-24kDa α-synuclein species is ubiquitinated. Conjugation of one, two and to a lesser extent three ubiquitins was detected. This 22-24kDa α-synuclein species represents partly phosphorylated protein. Furthermore, no generalized impairment of the proteolytic activity of the proteasome was detected in brain regions with Lewy body pathology. Since unmodified α-synuclein is degraded by the proteasome in an ubiquitin-independent manner, these data suggest that accumulation of modified 22-24kDa α-synuclein is a disease-specific event, which may overwhelm the proteolytic system leading to aberrant ubiquitination. Accordingly, carboxy-terminal truncated α-synuclein, presumably the result of aberrant proteolysis, is found only in association with α-synuclein aggregates.

Introduction:
Lewy bodies (LB) are intracytoplasmic eosinophilic inclusions, which ultrastructurally are made of a core of granular and filamentous material, surrounded by radially oriented filaments 10-15 nm in diameter (1). The element of the LB fibril remained unknown until genetic studies in early-onset autosomal dominant Parkinson’s disease (PD) led to the identification of two mutations in the α-synuclein gene (2, 3). This was followed by the identification of α-synuclein as the major component of the LB fibrils in sporadic PD and dementia with LB (DLB; 4, 5). LB pathology and α-synuclein aggregation in neurons may contribute to their dysfunction and degeneration. Formation of α-synuclein fibrils has been extensively studied in in vitro systems using recombinant...
Ubiquitination of modified α-synuclein in Lewy bodies

protein. However, the mechanism by which α-synuclein, a natively unfolded protein accumulates in neurons to form insoluble fibrils with amyloid characteristics is largely unknown. It is feasible that in vivo post-translational modifications interfere with the function and/or degradation of α-synuclein or alter its biophysical properties in a way to facilitate aggregation. Alternatively, protein modifications may occur in an attempt to prevent aberrant interactions and/or inhibit further aggregation. Therefore, detailed understanding of the extent to which disease-associated α-synuclein is modified may provide insights into cellular pathways that are activated during fibril formation.

In this study we used differential centrifugation and 2-dimensional gel electrophoresis (2-DE) to characterize LB-associated α-synuclein. This approach allows the separation of proteins on the basis of their solubility, charge and molecular weight. Using this method we show that in sporadic LB disorders, a disease-associated 22-24kDa α-synuclein species is a substrate for mono-, di- and tri- rather than poly-ubiquitination. This 22-24kDa species gives a characteristic pattern on 2-DE, which is consistent with a highly modified form of the protein and we identify phosphorylation as one of the modifications. Furthermore, we show that accumulation of ubiquitinated α-synuclein is not invariably associated with significant impairment of proteasome function. These data may help to unravel the role of the ubiquitin-proteasome pathway in LB formation.

**Experimental Procedures:**

**Human brain tissue:**

Brain tissue was obtained from the Neuropathology Department of the University of Indiana School of Medicine. The diagnosis of PD (n=3, age of death=70+/-8 years, duration of disease=6.3+/-3 years), DLB (n=3, age of death=75+/-1 years, duration of disease=7.7+/-2 years) and Alzheimer’s disease (AD; n=3, age of death=81+/-5 years, duration of disease=12+/-2 years) was established by standard neuropathological criteria. Immunohistochemistry using anti-α-synuclein and anti-ubiquitin antibodies was performed as described previously (5). Four cases with no neurological disease were used.
Ubiquitination of modified α-synuclein in Lewy bodies

as age-matched controls (age of death=65.8+/−9 years). Four brain regions were examined from each case: substantia nigra (SN), frontal, cingulate and occipital cortices.

**Fractionation of human brain tissue:**

Subcellular fractionation of human brain was performed based on a modification of a previously published protocol (6, 7) as follows: Approximately 0.5g of tissue was homogenized on ice in 10 volumes of TBS+ (50 mM Tris-HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 1 mM N-Ethyl-Maleimide plus Complete proteasome inhibitor cocktail; Roche Diagnostics, Mannheim, Germany). After 5 min of centrifugation at 1000g the supernatant was ultracentrifuged for 30 min at 120 000g at 4°C. The resulting supernatant represented the TBS+ soluble fraction. All subsequent centrifugation steps were performed at 120 000g for 20 min at 4°C. The pellet was rinsed twice with TBS+ and extracted sequentially with 500 µl of TBS+ containing 1% Triton X-100 and TBS+1M sucrose to remove myelin. The pellet was then extracted with 500 µl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS). The extensively washed detergent-insoluble pellet was solubilized either in 8 M Urea/5% SDS and termed the urea-soluble fraction or buffer A+ (10 mM Tris-HCl pH 8.8, 7 M Urea, 2 M Thiourea, 5 mM MgCl2, 2% ASB14) for two-dimensional gel electrophoresis (2-DE). The 1 000g pellet from the first step was subsequently extracted in the same way.

**Immunoblotting:**

Twenty-30 µg of extract from each fraction was loaded per lane and separated on 12% or 15% SDS polyacrylamide gel and transferred to nitrocellulose. The membranes were then incubated with either 3% BSA or 4% milk in TBS to block non-specific binding. After washing, the membranes were incubated overnight with appropriate primary antibodies. Monoclonal antibodies Syn-1 (1:600; Transduction Lab, Lexington, KY), LB509 (1:5000; Zymed, San Francisco, CA) and polyclonal antisera directed against ubiquitin (1:800; Dako, Cambridgeshire, UK), carboxy-terminus of α-synuclein (PER4; 1:1000) and phosphoserine 129 of α-synuclein (PS129, 1:1000; kind gift of Dr T. Iwatsubo) were used. Bound antibodies were visualised with peroxidase-conjugated
secondary antisera (1:2000; Dako) and enhanced chemiluminescence (NEN Life Science, Boston, MA).

**Deglycosylation Assay:**

Urea-soluble fractions from human cases with LB disease were dialysed overnight against 50 mM Tris-HCl pH 7.2 in order to remove urea. Thirty µl of dialysates were subjected to a deglycosylation assay using N-glycosidase, sialidase A and endo-O-glycosidase as per manufacturer’s instructions (ProZyme, San Leandro, CA). Bovine fetuin (ProZyme) was used as positive control. Deglycosylation in dialysate samples was monitored using 5 µg/ml of biotinylated concanavalin A (a lectin which recognizes glycosylated proteins), which was visualized with avidin-biotin peroxidase and 3,3’ diaminobenzidine (DAB) as substrate (Vector Laboratories, Burlingame, CA).

**Two-dimensional gel electrophoresis (2-DE):**

For 2-DE, RIPA-insoluble pellets were resuspended in 150 µl buffer A+. Thirty-40 µl were then reconstituted into a final volume of 250 µl with rehydration buffer (7 M urea, 2 M Thiourea, 2% ASB14, 13 mM DTT, 1% IPG, Amersham) and loaded onto 13cm pH 3-10 IPG strips (Amersham) by rehydration loading for 200V/h over 10 hours using the IPGphor (Amerhsam). Isoelectric focusing on the IPGphor was subsequently performed for 500V/h over 1 hour, 1000V/h over 1 hour and 40000V/h over 5 hours. After focusing, strips were equilibrated in SDS buffer (100 mM Tris-HCl, 30% glycerol, 8 M urea, 1% SDS) for 15 minutes, loaded onto Hoeffer SE 600 10% or 12% SDS PAGE gels, and run for 15 minutes at 20 mA per gel, followed by 2.25 hours at 40 mA per gel. Gels were then blotted onto nitrocellulose membrane and processed as for one-dimensionsal gel electrophoresis.

**Proteasome assays in human brain:**

Four brain regions were examined: SN, frontal, cingulate and occipital cortices. The samples were carefully dissected on dry ice with dry-ice cooled blade. From each of the 4 brain regions examined, 3 different samples were taken for each patient, weighed and homogenised in 3 volumes of 50 mM Tris-HCl buffer pH 7.4. The crude extract was
centrifuged at 15 000 rpm for 5 min at 4°C. Fifty µg of each sample was assayed for the chymotrypsin-like activity of the proteasome as previously described (8) using 20 µM of Suc-Leu-Leu-Val-Tyr-amc (Affiniti) as substrate. The enzymatic activities were determined from the linear reaction rates and expressed as fluorescence units (FU)/30 min per mg of protein.

**Results:**

**Immunohistochemistry:**

PD and DLB cases with mild to moderate cell loss were selected to avoid artefacts related to extensive cell death. Immunohistochemistry with anti-α-synuclein antibodies showed several LB, pale bodies and Lewy neurites in both DLB and PD (Fig. 1A and B). In serial sections anti-ubiquitin antibodies stained only some of the α-synuclein positive inclusions (Fig. 1C).

**Fractionation of α-synuclein from human brain:**

To investigate the extent to which LB-associated α-synuclein is post-translationally modified, we used a method that is a modification of a previously reported protocol (6,7) and allows the separation of all forms of aggregated α-synuclein from soluble fractions. This is based on differential fractionation under conditions which do not solubilize the aggregates but simply separate them from the bulk of cytoplasmic and membrane proteins. The main purpose of this approach was to identify conditions, which maximise the recovery of all LB-associated forms α-synuclein (both filamentous and non-filamentous). The different fractions were analysed by conventional immunoblotting. Similar α-synuclein reactivity was detected in cytoplasmic and membrane fractions from SN, frontal and cingulate cortex of extracts from PD, DLB and control brains as shown in Fig. 2A. In these fractions, α-synuclein was detected using Syn-1 antibody as a single band of 19kDa in accordance with the apparent molecular weight of full-length protein in both disease cases and controls (5,9). No higher molecular weight α-synuclein bands were detected.
In contrast to control brains, in which urea-soluble extracts were virtually devoid of α-synuclein, strong immunoreactivity was found in urea-soluble extracts from PD and DLB (Fig. 2B). Oligomeric and higher molecular weight aggregates were the predominant species in this fraction. At lower molecular weight, when compared to recombinant protein, three distinct bands were detected with Syn-1 antibody, which recognizes the first 120 residues of the protein: One corresponding to monomeric α-synuclein (19kDa), a novel 22-24kDa band as well as truncated α-synuclein (15kDa). These bands were examined using a panel of antibodies raised against distinct epitopes (Fig. 2C and D). The 19kDa and 22-24kDa bands but not the 15kDa truncated protein were detected with LB509 antibody and PER4 antiserum, both directed against the carboxy-terminus of the protein (10). These results indicated that the 15kDa band is a carboxy-terminal truncated protein whilst the 19kDa and 22-24kDa are comprised of full-length protein.

Since, a glycosylated form of α-synuclein with a molecular weight of 22-24kDa was identified in cases of juvenile onset parkinsonism with mutations in the parkin gene (11), we investigated whether the 22-24kDa α-synuclein is glycosylated by treating our extracts from sporadic LB disease with the same deglycosylating enzymes as those used by Shimura et al. (11). We found that these enzymes, at the conditions used by Shimura et al., did not alter the electrophoretic mobility of the 22-24kDa α-synuclein band to the 19kDa position as detected with LB509 (Fig. 3A). In parallel control experiments, a significant reduction of glycosylated proteins was detected with concanavalin A (Fig. 3B). Deglycosylation of bovine fetuin used as positive control was detected by Coomassie stain as a shift in the electrophoretic mobility of the protein from 75kDa to 50kDa (data not shown).

**Lewy body-associated α-synuclein immunoreactivity using 2-DE:**

In an attempt to investigate further the characteristics of disease-associated α-synuclein, urea-soluble fractions from disease cases and controls were subjected to further separation across a pH gradient of 3 to 10, before proteins were separated according to their molecular weight, i.e. 2-DE. To determine the electrophoretic mobility
Ubiquitination of modified α-synuclein in Lewy bodies

of monomeric full-length α-synuclein in 2-DE we loaded recombinant protein, which was found to run at the expected apparent molecular weight of 19kDa as in one-dimensional electrophoresis and pI of 4.4 (Fig. 4B). In brain regions with LB, α-synuclein immunoreactivity gave a characteristic pattern on 2-DE analysis which consisted of serial spots of similar molecular weight but different charge, as well as high molecular weight smears (Fig. 4A). This pattern was never detected in control cases.

When extracted α-synuclein was compared to the recombinant protein, we detected the full-length protein at 19kDa, a truncated form at a lower molecular weight around 15 kDa and a 22-24kDa species. The latter most likely represents a modified disease-associated form of α-synuclein because: (a) It was not detected in normal controls; (b) it consisted of groups of serial spots of similar or slightly increasing molecular weight separated across the pH gradient by an increase in negative charge. This was consistent with the finding of a 22-24kDa band using conventional immunoblotting analysis (Fig. 2B-C and Fig. 4A).

Modified 22-24kDa α-synuclein is ubiquitinated:

Although, the majority of LB are stained with antibodies to ubiquitin, the nature of the ubiquitinated protein(s) in these inclusions is uncertain. Ligation of each molecule of ubiquitin increases the apparent molecular mass of a protein on SDS-PAGE by 6-8 kDa and shifts its pI toward that of ubiquitin (pI 6.8). Urea-soluble extracts from PD and DLB were therefore subjected to 2-D SDS-PAGE followed by immunoblotting with either ubiquitin antiserum or anti-α-synuclein antibodies. This showed that some of the α-synuclein-positive pH separated spots represent an ubiquitinated form of the protein (Fig. 5A-D). No ubiquitinated α-synuclein was detected in soluble fractions or control brain regions (data not shown). Three regions of ubiquitin-protein conjugates were noted on 2-DE immunoblots of a molecular weight of about 29kDa, 36kDa and to a lesser extent 43kDa with associated pI shift towards 7.0. This pattern of molecular weight and charge shift is consistent with the addition of a single ubiquitin, thus giving groups of mono-, di- and less abundant tri-ubiquitinated α-synuclein. On 2-D SDS-PAGE, monomeric α-synuclein has an apparent molecular weight of 19kDa, and is therefore
Ubiquitination of modified α-synuclein in Lewy bodies

unlikely to be the substrate for ubiquitination. On the other hand we noted that the pattern of ubiquitinated α-synuclein immunoreactivity was almost identical to that of the 22-24kDa species, which itself was not positive for ubiquitin (Fig. 5, compare panels A-B and C-D). When compared to mono-ubiquitinated α-synuclein, the 22-24kDa species had the appropriate size and charge shift suggesting that this is the α-synuclein substrate for oligo-ubiquitination. These data show that in sporadic LB disease, a modified α-synuclein species of about 22-24kDa is a substrate for oligo-ubiquitination.

22-24kDa α-Synuclein is hyperphosphorylated on serine residues:

The pattern of α-synuclein-immunoreactive spots, which separated across the pH gradient but did not differ in their molecular weight, suggested that these could represent hyperphosphorylated species. Phosphorylation of α-synuclein on serine 129 was previously shown to strongly label LB-associated α-synuclein with little staining of normal protein (12). Anti-PSer129 antiserum showed that although monomeric protein was strongly labeled (Fig 6C, arrowhead) only some of the 22-24kDa species and associated ubiquitinated protein were positive (Fig. 6).

Proteasome function in LB disorders:

The 22-24kDa α-synuclein was present in all brain regions containing LB. Therefore we sought to determine whether accumulation of modified 22-24kDa α-synuclein in disease is associated with proteasome dysfunction. For this purpose we measured proteasome activity in 4 regions from brain specimens from PD, DLB, Alzheimer’s disease (AD) and age-matched controls: SN, which is most vulnerable to LB pathology in both PD and DLB, the cingulate, frontal cortices, which are affected in DLB and occipital cortex, which is devoid of LB. We found a 55% significant reduction in proteasome function in the SN of PD and DLB when compared to control cases (Fig. 7D). However, no significant difference was detected in cingulate, frontal and occipital cortices of PD, DLB and control cases (Fig. 7A-C). We investigated further the proteolytic activity of the proteasome in cingulate and frontal areas by comparing tissue extracts from DLB and AD cases, since in both diseases there is diffuse cortical
Ubiquitination of modified α-synuclein in Lewy bodies

involvement. We found that although there was a reduction in proteasome activity in brain regions with AD pathology, no significant decline was detected in DLB (Fig. 7E and F).

**Discussion:**

In this study we have shown the first analysis of LB-associated α-synuclein based on solubility, charge and molecular weight. Using this approach it is shown that in diseased tissue, a modified 22-24kDa α-synuclein is a substrate for oligo-ubiquitination. Furthermore, the proteolytic activity of the proteasome in areas with LB-associated 22-24kDa α-synuclein was not invariably impaired.

A number of studies have shown that ubiquitin and enzymes of the ubiquitin system are found in LB, but the nature of the substrate for ubiquitination remains unclear. Although we could detect poly-ubiquitinated proteins in all sub-cellular fractions examined, ubiquitinated α-synuclein was extracted biochemically only with disease-associated insoluble aggregates and not in soluble fractions. In 2-DE analysis, ubiquitinated α-synuclein gave a characteristic pattern of serial spots ranging from about 29 kDa to 43 kDa, separated by about 7 kDa and shifted towards a pI of 6.8 as expected by the addition of a free ubiquitin molecule. Our data clearly show that mainly one or two and to a lesser extent three ubiquitin moieties are conjugated on LB-associated α-synuclein. This pattern is remarkably similar to that of tau in paired helical filaments of neurofibrillary tangles, which is mainly mono-ubiquitinated (13). It is well established that for ubiquitin-dependent degradation to occur conjugation of at least 4 ubiquitins on a protein is necessary (14). We and others have previously shown that ubiquitination of unmodified α-synuclein does not occur following inhibition of the proteasome in transfected cells (8, 15-18). This is in agreement with the present data, which show that the 19kDa monomeric α-synuclein is not ubiquitinated. Taken together, these data support the notion that, ubiquitin-dependent degradation is unlikely to be a major physiological mechanism for α-synuclein degradation. Rather, ubiquitination of LB-
associated α-synuclein most likely represents a disease-specific pathway. In this respect, there are two obvious possibilities: ubiquitination could represent an unsuccessful “last-ditch stand” of cells in their attempt to unfold and/or degrade misfolded proteins either through the proteasome, which requires poly-ubiquitination or the lysosome which requires mono-ubiquitination (19). Alternatively it could represent a cytoprotective response to preclude interactions of inappropriately exposed domains (20). Which one of these possibilities is more likely remains to be seen.

In keeping with ubiquitination being a disease-specific pathway is the finding that it is almost invariably associated with further post-translational modifications. Since ligation of each molecule of ubiquitin increases the apparent molecular mass of a protein by 6-8 kDa and shifts its pI towards that of ubiquitin (pI 6.8), electrophoretic resolution of ubiquitin-protein conjugates by both charge and mass can provide insight into the identity of ubiquitinated substrates. Using 2-DE, we show here for the first time that in sporadic LB diseases, a 22-24kDa α-synuclein species and not monomeric protein is the substrate for oligo-ubiquitination. This is in contrast to a recent study, which reported that monomeric α-synuclein is oligo-ubiquitinated (21). This difference could be explained by the fact that Hasewaga et al. analysed samples, which were prepared using a sarkosyl-based method and therefore are enriched mainly in filamentous α-synuclein. In contrast, our samples contained both filamentous and non-filamentous α-synuclein from early stages. Furthermore, we and others (22-24) find that monomeric full-length α-synuclein runs with an apparent molecular weight of 19kDa whereas in Hasewaga et al., this is shown at 15 kDa. This discrepancy makes it difficult to predict whether our 22-24kDa non-ubiquitinated α-synuclein band is the same as what they describe as monomeric ubiquitinated α-synuclein. However, it is possible that monomeric α-synuclein becomes ubiquitinated at later stages of disease and is detected in filament preparations. We found that some of the 22-24kDa α-synuclein is phosphorylated on serine residues whilst only a few higher molecular weight forms were positive with anti-phosphotyrosine antibodies (personal observations). Which modifications other than phosphorylation could be responsible for producing a change in pI with a little mass increment is currently unclear but acetylation, methylation or amino terminal clipping are possible. Interestingly, a
small shift in pI was also detected in recombinant protein. Our data may also help to explain the absence of extensive ubiquitinated α-synuclein inclusions in transgenic models since ubiquitin-conjugation on α-synuclein appears to occur only in the presence of further post-translational modifications. Therefore, this complex cellular response may represent a disease-specific pathway, which is not fully activated in transgenic models. In this respect, identification of the relevant ubiquitin-ligase(s) may provide insights into the cellular response to fibril formation. For example, a 22kDa glycosylated form of α-synuclein has been reported to be poly-ubiquitinated \textit{in vitro} by parkin, an ubiquitin-protein ligase (11). Contrary to the findings of Shimura et al. (11) we did not detect glycosylation of disease-associated α-synuclein in extracts from sporadic LB disorders. It remains possible that a different form of glycosylation occurs in LB-associated α-synuclein. Further purification of the 22-24kDa form of α-synuclein will allow the characterisation of this species in more detail. More recently, α-synuclein has been shown to be a substrate for ubiquitination by UCH-L1, which contrary to the conventional understanding of the ubiquitin system acts in an ATP-independent manner (25).

Whether α-synuclein is a substrate for the proteasome has been a subject of debate (15, 16). We subsequently showed that unmodified full-length α-synuclein is degraded directly by the 20S proteasome in an ubiquitin-independent manner (8). Since α-synuclein is a natively unfolded protein, it is feasible that \textit{in vivo}, unfolded α-synuclein could bypass the need for ubiquitination and unfolding and enter the 20S proteasome directly. The above is in accordance with the finding that other small relatively unfolded proteins can be degraded by the proteasome in an ubiquitin-independent manner (26, 27). An earlier report has shown that proteasome activity is reduced in the SN of PD patients (28). It is therefore feasible that accumulation of α-synuclein is associated with proteasome dysfunction. However, LB-associated α-synuclein is also found outside the SN and therefore all regions should be investigated. We found significant reduction in the proteolytic activity of the proteasome in the SN from PD and DLB cases but not in other regions with LB such as the cingulate and frontal cortices of DLB cases. Furthermore, in the cerebral cortex, AD but not LB pathology was associated with significant reduction in
Ubiquitination of modified α-synuclein in Lewy bodies

proteasome function. One possible explanation for the findings in the SN is that this brain region is more uniformly involved in α-synucleinopathies. On the other hand, in cortex the ratio of normal to affected cells is greater thus possibly masking the reduction of proteasome function in cells with LB. However, the above is unlikely to be the sole explanation since the tissue was taken from early stages of the disease when with mild to moderate cell loss. Since the protease-active sites are buried deep in the core of the proteasome, it is possible that in vivo regulatory regions on the outside ring, which cannot be detected with peptidase assays, are primarily damaged. For example, oxidative stress, can lead to covalent modification of proteasome subunits (29). These alterations may prevent the assembly and stabilisation of the multi-subunit complex or directly impair the binding of substrates to the 20S proteasome rather than their degradation. On the other hand, abnormal modification of substrates per se may impair their binding and/or recognition by the 20S. In this respect, it is feasible that accumulation of modified and presumably undegradable α-synuclein might overload the proteolytic system leading to aberrant ubiquitination. The latter could in turn interfere with the processing of monomeric α−synuclein thus initiating a vicious circle that could lead to further protein accumulation and inclusion formation.

If the proteolytic system is overwhelmed by modified α-synuclein species, then α-synuclein fragments, generated as a result of aberrant proteolysis could be found specifically in association with LB. Accordingly, we found that truncated α-synuclein is selectively recovered from fractions containing aggregated α-synuclein but not in controls. Characterization of these fragments shows that they represent carboxy-terminal truncated protein. Since in fibrils both the amino- and carboxyl-terminal portions of α-synuclein extend out in the periphery (30), the detection of only carboxy-terminal truncated protein is unlikely to be an artefact of extraction. Previous studies have reported the existence of truncated α-synuclein (6, 7, 31). In contrast to data presented here, truncated α-synuclein protein was found in both soluble and insoluble fractions and was equally present in soluble fractions from disease and control cases (6, 7). α-Synuclein is susceptible to nonenzymatic hydrolysis (32) and a number of proteolytic digestion fragments are readily generated during purification procedures that involve formic acid
Ubiquitination of modified α-synuclein in Lewy bodies

extraction (31) and sonication (6, 7). Therefore, the extraction method might confound previous suggestions that truncated α-synuclein is a normal soluble breakdown product. With our extraction procedure degradation products are unlikely to form, suggesting that aberrant proteolysis, either by the proteasome or other proteases could be a feature of neurodegeneration associated with LB formation. The finding that α-synuclein is degraded by the proteasome at internal peptide bonds without ubiquitination (33) suggests that the 20S proteasome could contribute to the formation of these fragments.

In summary, we have shown that in PD and DLB, a modified 22-24kDa α-synuclein is a substrate for oligo-ubiquitination, which occurs in the absence of a generalized significant impairment of the proteolytic activity of the proteasome. Since unmodified α-synuclein is degraded by the proteasome in an ubiquitin-independent manner (8) our data suggest that accumulation of modified 22-24kDa α-synuclein is a disease-specific event that may overwhelm the proteolytic system, presumably due to impaired recognition and/or flux through the 20S core of the proteasome leading to aberrant ubiquitination and inclusion formation. Finally, the spatial separation of α-synuclein immunoreactive spots on 2-DE may be helpful in providing insights into novel, yet unknown modifications and therefore cellular pathways that may be involved in LB formation.

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Ubiquitination of modified α-synuclein in Lewy bodies


Ubiquitination of modified α-synuclein in Lewy bodies


Ubiquitination of modified α-synuclein in Lewy bodies


**Figure Legends:**

**FIG 1:** Immunohistochemistry of sections from PD and DLB cases: (A) α-Synuclein staining of frontal cortex from a patient with DLB. (B) α-Synuclein and (C) ubiquitin staining in serial sections of SN from a patient with PD. LB (*arrows*), pale bodies (*arrowheads*) and some Lewy neurites are stained. Ubiquitin stained less deposits compared to α-synuclein. Scale bar in A, 180 µm; in B for B and C, 100µm.

**FIG 2:** Immunoblotting of α-synuclein extracted from PD, DLB and control brains: (A) α-Synuclein immunoblots with Syn-1 antibody from cytoplasmic (TBS) and membrane (T/T) fractions are shown from brains of PD, DLB and age-matched controls (CON). (B) Urea-soluble fractions from CON, DLB and PD were immunoblotted with Syn-1 antibody. Note the presence of high- and low-molecular weight aggregates in disease but not in control tissue. Truncated, full-length and 22-24kDa α-synuclein (*arrows*) were associated with high-molecular weight aggregates. (C) α-Synuclein in urea-soluble fractions was examined with a panel of anti-α-synuclein antibodies, which have been raised against distinct epitopes (panel D). Note that monoclonal antibody LB509 and polyclonal antiserum PER4 recognise the 22-24kDa species and full-length but not truncated protein (*arrows*).

**FIG 3:** Deglycosylation does not produce a shift in the α-synuclein immunoreactive bands: (A) Urea-soluble fractions were dialysed overnight to remove urea, treated with...
Ubiquitination of modified α-synuclein in Lewy bodies

\[ N\text{-glycosidase, endo-}O\text{-glycosidase and sialidase A and immunoblotted with LB509. No change in the electrophoretic mobility of 22k-24Da } \alpha\text{-synuclein } (\text{arrow}) \text{ was detected. (B) Samples treated as in (A) were stained using biotinylated concanavalin A and visualized with avidin peroxidase and DAB as substrate. Note the reduction in the amount of glycosylated proteins.} \]

**FIG 4: Immunoblotting of LB-associated α-synuclein using 2-DE:** (A) Urea-soluble fractions from PD and DLB cases were subjected to separation across a pH gradient before separating the proteins according to their molecular weight. A representative example of the results is shown from frontal cortex of DLB using Syn-1 antibody. Full-length α-synuclein is indicated with an *arrow* and truncated protein with an *arrowhead*. Note the characteristic α-synuclein immunoreactive spots of similar molecular weight but different charge (marked with *asterisks*), which show a progressive increase in molecular weight of about 6-8 kDa and a shift towards pI of 7.0. Higher molecular weight aggregates are indicated in *bracket*. (B) The electrophoretic mobility of recombinant α-synuclein is shown for comparison. This was found to run with an apparent molecular weight of 19kDa as in one-dimensional SDS-PAGE and the expected pI of 4.4.

**FIG 5: ubiquitination of LB-associated α-synuclein:** 2-DE immunoblot analysis with either Syn-1 monoclonal antibody or ubiquitin polyclonal antiserum on urea-soluble fractions from frontal cortex (*A* vs *B*) and cingulate cortex (*C* vs *D*) from DLB cases. Note the characteristic pattern of spots (*asterisks*) immunoreactive for both α-synuclein and ubiquitin at about 29, 36 and to a lesser extent 43kDa separated by a charge shift and a size shift corresponding to the addition of a single ubiquitin. The modified 22-24kDa α-synuclein spots (*arrow*) is not positive for ubiquitin but when compared to mono-ubiquitinated α-synuclein show the appropriate decrease in size and charge shift that corresponds to the putative substrate for ubiquitination. Full-length α-synuclein has an apparent molecular weight of 19kDa (*arrowhead*).
FIG 6: Ubiquitinated α-synuclein is phosphorylated on serine residues: 2-DE analysis of urea-soluble fraction using Syn-1 (panel A), anti-ubiquitin (panel B) and PS129 (panel C). Note that most of the monomeric form of α-synuclein (arrowhead) is phosphorylated, whereas 22-24kDa α-synuclein (arrow) and higher molecular weight ubiquitinated α-synuclein (asterisks) is partly phosphorylated on serine residues.

FIG 7: Proteasome function in Lewy body disorders and AD: Tissue extracts from 4 different brain regions, frontal (panel A), cingulate (panel B), occipital (panel C) cortices and substantia nigra (panel D) were analysed for proteasomal chymotryptine-like activity. Significant decrease in activity was only detected in the SN of patients with PD and DLB when compared to controls. Comparison of proteasomal function in cortical areas of cases with AD and LB pathology shows that proteasomal activity is decreased in frontal (panel E) and cingulate (panel F) cortices in AD but not in DLB cases. Data are mean +/- SEM values. * p<0.05, ** p<0.005
FIGURE 3
FIGURE 6
Ubiquitination of alpha-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function
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