RESEARCH COMMUNICATION

α -Synuclein overexpression promotes aggregation of mutant huntingtin

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Protein aggregates are a neuropathological feature of Huntington's disease and Parkinson's disease. Mutant huntingtin exon 1 with 72 CAG repeats fused to enhanced green fluorescent protein (EGFP) forms hyperfluorescent inclusions in PC12 cells. Inclusion formation is enhanced in cells co-transfected with EGFP-huntingtin-(CAG)₇₂ and α -synuclein, a major component

of Parkinson's disease aggregates. However, α -synuclein does not form aggregates by itself, nor does it appear in huntingtin inclusions *in vitro*.

Key words: inclusion, Parkinson's disease, polyglutamine, trinucleotide.

INTRODUCTION

Huntington's disease is one of eight neurodegenerative diseases which are currently known to be caused by CAG trinucleotide repeat expansions. Mutant chromosomes in the Huntington's disease have 36 or more CAG repeats. The CAG repeats in Huntington's disease and related disease genes are translated into expanded polyglutamine tracts [1], which confer a deleterious gain-of-function on the mutant proteins.

The pathologies of many CAG/polyglutamine diseases, including Huntington's disease [2], spinobulbar muscular atrophy [3], dentatorubral-pallidolysian atrophy [4] and spinocerebellar ataxias type 1 [5], type 3 [6] and type 7 [7], are characterized by the development of intracellular protein aggregates (inclusions) in the nuclei and cytoplasm of vulnerable neurons. The formation of aggregates of abnormally folded proteins is also a feature of a number of other neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [1].

The Lewy body aggregates seen in Parkinson's disease and in Dementia with Lewy bodies, which contain α -synuclein [8], share many similarities with those seen in polyglutamine diseases. Like Huntington's disease inclusions, the Lewy body aggregates include ubiquitin and proteasomal subunits [8–10]. Both types of inclusion have β -sheet structures [1,11].

Recently, two rare mutations at codons 30 and 53 in the α -synuclein gene have been shown to segregate with autosomal dominant forms of Parkinson's disease [12,13]. Recombinant α -synuclein appears to aggregate *in vitro*, forming amyloid-like filaments [11,14–16]. The A30P (Ala³⁰→Pro) and A53T (Ala⁵³→Thr) mutant proteins aggregate more rapidly than the wild-type form when the recombinant proteins are 'aged' at 37 °C [11,14,16].

Engelender and colleagues have shown that wild-type α -synuclein does not aggregate when transfected into the human embryonal kidney cell line HEK293. However, aggregation occurred when a novel protein, synphilin-1 was transfected with a fragment of α -synuclein, the non-A β component of Alzheimer's disease amyloid [17]. We are not aware of any other studies

investigating if wild-type or mutant forms of α -synuclein can aggregate when transfected into 'neuronal' cell lines.

α -Synuclein appears to associate with other proteins that aggregate and is found in β -amyloid plaques and neuritic tangles in Alzheimer's disease [18–20]. In fact, α -synuclein was originally identified as the precursor of the major non-amyloid component of Alzheimer's disease plaques and may promote β -amyloid aggregation [20]. Since both huntingtin and α -synuclein are expressed in the cortex and striatum, the primary sites of Huntington's disease pathology, we hypothesized that α -synuclein may also contribute to huntingtin inclusion formation.

MATERIALS AND METHODS

Huntingtin constructs

pEGFP-C1-huntingtin constructs comprised enhanced green fluorescent protein (EGFP; ClonTech, Palo Alto, CA, U.S.A.) at the N-terminus fused to codons 8 to 57 inclusive of Huntington's disease exon 1 with 21 or 72 CAG repeats (numbering applies to huntingtin with 23 Q residues; equivalent to 21 uninterrupted CAG repeats). pHM6-huntingtin constructs were N-terminal haemagglutinin (HA)-tagged and C-terminal-His₆-tagged fragments comprising codons 8 to 57 inclusive of Huntington's disease exon 1 with 21 or 72 CAG repeats (see [21] for details of construction of these constructs).

α -Synuclein constructs

Wild-type α -synuclein was cloned into the *Hind*III and *Eco*RI sites of EGFP-C1 (ClonTech) or pHM6 (Roche Diagnostics). The α -synuclein coding sequence was PCR amplified from the IMAGE clone 49811 using gene-specific primers modified to include the appropriate restriction sites at their 5' end. For cloning into EGFP-C1 the primers G-Synuc-ATG, 5' GCA TAA GCT TCG ATG GAT GTA TTC ATG AAA GGA CTT TC 3' and G-Synuc-*Eco*RI TAA, 5' GCA TGA ATT CTT AGG CTT CAG GTT CGT AGT CTT G 3' were used. For cloning into

Abbreviations used: EGFP, enhanced green fluorescent protein; HA, haemagglutinin.

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pHM6 the primers used were: H-Synuc-ATG, 5' GCA TAA GCT TGA TGG ATG TAT TCA TGA AAG GAC TTT C 3' and H-Synuc-*EcoRI*GCC, 5' GCA TGA ATT CTC GGC TTC AGG TTC GTA GTC TTG ATA 3'. Purified PCR products were digested with *HindIII*, and partially digested with *EcoRI*, before ligation into a *HindIII*/*EcoRI*-digested vector.

α -Synuclein A30P and A53T mutants

α -Synuclein mutants A30P (base 140 of non-amyloid- β -component precursor, Accession number L08850, G to C) and A53T (base 209 of NACP, Accession number L08850, G to A) were cloned into pHM6 by PCR amplification of α -synuclein using primers containing the mutations. Initially, 2 separate PCRs were performed. In the first PCR a 5' (ATG) α -synuclein forward primer was used with a reverse primer containing the α -synuclein mutation. The second PCR used a forward primer containing the α -synuclein mutation with a reverse α -synuclein primer at the 3' end of the gene. The primers used for the α -synuclein A30P mutant were: H-Synuc-ATG with Synuc117RM, 5' TGT CTT TCC TGG TGC TTC TGC 3'; and Synuc97FM, 5' GCA GAA GCA CCA GGA AAG ACA 3' with H-Synuc-*EcoRI*GCC. Primers used for the α -synuclein Ala53Thr mutant were: H-Synuc-ATG with Synuc182RM, 5' ACT GTT GTC ACA CCA TGC AC 3'; and Synuc163FM, 5' GTG CAT GGT GTG ACA ACA GT 3' with H-Synuc-*EcoRI*GCC. Products from the first and second PCR were purified, pooled, and approx. 40 fmol of each was used as template for the second stage PCR employing the 5' forward primer, H-Synuc-ATG, and the reverse 3' primer, H-Synuc-*EcoRI*GCC. Purified PCR products were digested with *HindIII* and partially digested with *EcoRI* before ligation into pHM6.

α -Synuclein deletion constructs

α -Synuclein deletion constructs comprising amino acids 1–95, 29–95, 55–95, 61–95, 29–140, 96–140 or Δ 57–97 were cloned into pHM6 using the *HindIII* (at the 5' end of α -synuclein) and either the *EcoRI* or *NotI* sites. Specific α -synuclein primers with restriction sites added at the 5' end of each primer were used to amplify wild-type α -synuclein. PCR products were digested with the appropriate restriction enzymes and ligated into pHM6. The primers used were: for 1–95, H-Synuc-ATG and Synuc95V-*EcoRI*, 5' ACG TGA ATT CTC GAC AAA GCC AGT GGC TGC TG 3'; 29–95, Synuc29A-*Hind*, 5' GCA TAA GCT TGG CAG CAG GAA AGA CAA AAG AGG 3' and Synuc95V-*EcoRI*; 55–95, Synuc55V-*Hind*, 5' GCA TAA GCT TGG TGG CTG AGA AGA CCA AAG AGC 3' and Synuc95V-*EcoRI*; 61–95, Synuc61E-*Hind*, 5' ACG TAA GCT TGG AGC AAG TGA CAA ATG TTG GAG G 3' and Synuc95V-*EcoRI*; 29–140, Synuc29A-*Hind* and H-Synuc-NotGCC, 5' ACG TGC GGC CGC GAG GCT TCA GGT TCG TAG TCT TGA TA 3'; and for 96–140, Synuc96K-*Hind*, 5' ACG TAA GCT TGA AAA AGG ACC AGT TGG GCA AG 3' and H-Synuc-NotGCC. For the α -synuclein Δ 57–97 construct two PCR products were amplified using primers H-Synuc-ATG and Δ 1 (5' TCT TTG GTC TGA TCA GCC ACT G 3'); and Δ 2 (5' TGT CAA AAA TGA TCA GTT GGG C 3') with H-Synuc-NotGCC. PCR products were digested with *HindIII* plus *BclI* and *BclI* plus *NotI* respectively, to remove sequences coding for amino acids 57–97 and ligated into pHM6.

DNA concentrations were determined spectrophotometrically and confirmed by estimation on an ethidium bromide-stained agarose gel using a low mass DNA ladder (Gibco BRL) as a reference.

Cell culture and transfection experiments

For transfection, cells were grown to 60–80% confluency on coverslips for 24 h, exposed to a mixture of 10 μ l lipofectamine reagent (Life Technologies) and 2 μ g of plasmid DNA for 5 h in serum-free medium, after which culture medium was added and the cells grown for 24, 48 or 72 h.

Immunocytochemistry

At the appropriate time post-transfection, cells were washed with PBS and fixed with ice-cold methanol for 5–10 min. Alternatively, cells were fixed with 4% paraformaldehyde for 20 min, washed twice and permeabilized (0.2% Triton X-100 in 1 $\times$ PBS) for 15 min. After 3 wash steps, cells were immersed in blocking buffer (5% fetal bovine serum) for 30 min at room temperature, and incubated with primary antibody for 1 h at room temperature. The antibodies used were: mouse monoclonal anti-HA.11 (BabCO, Richmond, CA, U.S.A.), mouse monoclonal anti-His₆ (Invitrogen, Groningen, The Netherlands), or rabbit anti- α -synuclein, amino acids 111–131 (Chemicon International, Temecula, CA, U.S.A.), each diluted 1:200. Cells were washed 3 times and incubated for 1 h in the dark at room temperature with either goat anti-mouse IgG-Texas Red conjugated, or goat anti-rabbit IgG-Texas Red conjugated antibodies (Molecular Probes, Eugene, OR, USA), each diluted 1:1000. Cells were finally washed 3 times, and then dried and mounted in antifadent (Citifluor, London, U.K.) supplemented with 4,6-diamidino-2-phenylindole (3 μ g/ml).

Statistical analysis

We counted about 220 EGFP- or HA-expressing cells per slide (blinded) in multiple randomly chosen visual fields and determined the numbers of cells containing one or more inclusions. The proportion of huntingtin-expressing cells with inclusions was used as a measure for inclusion formation. Pooled estimates for the changes in inclusion formation resulting from perturbations assessed in multiple experiments were calculated as odds ratios with 95% confidence limits. Odds ratios and *P* values were determined by unconditional-logistical-regression analysis, using the general loglinear analysis option of SPSS v6.1 software (SPSS Inc., Chicago, IL, U.S.A.). Experiments using α -synuclein deletion constructs were corrected for multiple testing.

RESULTS AND DISCUSSION

Over-expression of α -synuclein increases aggregation of a mutant huntingtin fragment

In order to study the biology of Huntington's disease in cultured cells we have developed a panel of constructs containing part of huntingtin exon 1 with 21, 41, 51, 62 or 72 CAG repeats. Huntington's disease exon 1 constructs were fused to the C-terminus of EGFP [21]. We have used an exon 1 fragment of huntingtin for these studies, since large fragments do not show inclusion formation or cell death in cell lines [22–24]. Only a small N-terminal polyglutamine-containing part(s) of huntingtin is found in inclusions *in vivo* and the exact nature of this fragment(s) is unclear. While exon 1 models may not be specific for Huntington's disease, they are powerful tools to study inclusion formation in relation to cell death/dysfunction in polyglutamine diseases and have been extensively studied in transgenic mice and cell models [22–26].

Figure 1

(A) Fluorescent microscopy and immunocytochemistry of a PC12 cell transfected with EGFP-huntingtin-(CAG)₇₂ (green) and HA/His6-wild-type α -synuclein (red) constructs. The HA tag was detected with mouse anti-HA and Texas Red-conjugated antibodies to mouse IgG. (B) Fluorescent microscopy of a PC12 cell transfected with EGFP-wild-type α -synuclein. (C) and (D) Fluorescent microscopy of a PC12 cell co-transfected with EGFP-huntingtin-(CAG)₇₂ and HA/His6-huntingtin-(CAG)₂₁. (C) The EGFP-huntingtin-(CAG)₇₂ inclusions (with a FITC filter), which sequester HA/His6-huntingtin-(CAG)₂₁, are detected in (D) using mouse antibodies to HA and Texas Red-conjugated antibodies to mouse IgG (through a Texas Red Filter).

Inclusions are formed by constructs with CAG repeats of 41 or more [21] and appear as very bright hyperfluorescent EGFP aggregates under fluorescent microscopy (Figure 1) [21]. The proportion of transfected (EGFP-positive) cells with inclusions increases as a function of time post-transfection (Figure 2), expression level and CAG-repeat length [21]. Huntingtin constructs with 21 CAG repeats show no aggregation [21].

In order to investigate whether α -synuclein could modulate the rate of huntingtin exon 1 inclusion formation, we initially co-transfected the rat pheochromocytoma cell line PC12 with an EGFP-huntingtin-(CAG)₇₂ construct and wild-type α -synuclein cDNA tagged with N-terminal HA and C-terminal His₆ epitopes (see Figure 1). In all experiments, we used a 4:1 ratio of α -synuclein construct or empty pHM6 vector to EGFP-huntingtin-(CAG)₇₂ construct. This approach ensures that practically all EGFP-huntingtin-(CAG)₇₂ expressing cells also expressed the α -synuclein construct or contained the control vector. We have confirmed the validity of this approach by detecting HA-tagged α -synuclein with mouse anti-HA and Texas Red-conjugated antibodies to mouse IgG in EGFP-huntingtin-(CAG)₇₂-expressing

cells (e.g. Figure 1A). Indeed, we have reported previously that even a 1:1 ratio of EGFP-huntingtin-(CAG)₂₁ to HA-tagged-huntingtin-(CAG)₇₂ resulted in co-localization of both constructs to inclusions in all inclusion-containing cells studied [21]. Since a 4:1 α -synuclein (or empty vector)/EGFP-huntingtin ratio will ensure that virtually all EGFP-expressing cells will express both constructs, we have scored inclusion formation in EGFP-expressing cells. Overexpression of α -synuclein induced an increase in the proportion of EGFP-expressing cells with inclusions at both 24 and 48 h post-transfection (Figure 2). Since the greatest relative increase was observed at 24 h, subsequent experiments were performed at this time point.

Co-expression of full-length wild-type or mutant (A30P or A53T) α -synuclein constructs with huntingtin-(CAG)₇₂ constructs increased inclusion formation (Table 1), but did not cause inclusions to be formed with EGFP-huntingtin-(CAG)₂₁ (results not shown). An increase was observed irrespective of whether the α -synuclein constructs were EGFP- or HA/His₆-tagged. α -Synuclein overexpression resulted in significantly greater huntingtin inclusion formation compared to cells overexpressing

Figure 2

Proportion of EGFP-expressing cells with huntingtin inclusions, 24 and 48 h following co-transfection of EGFP-huntingtin-(CAG)₇₂ with α -synuclein (filled bars) or pHM6 empty vector (open bars) in 4 independent experiments. Each column represents data from a single slide.

EGFP (265 amino acids), mutant Hsp40 (Δ 450, 373 amino acids) [27] and a C-terminal deletion mutant form of emerin fused to EGFP (EGFP-emerin 1–220, 488 amino acids) [28] (Table 1). The latter localizes to the nucleoplasm of transfected cells [28]. Each of these test and control constructs was driven by the human cytomegalovirus immediate early promoter. These control proteins are comparable in size to the pHM6 and EGFP α -synuclein fusion proteins, which contain 167 or 389 amino acids respectively. Furthermore, overexpression of huntingtin constructs with 21 repeats with huntingtin with 72 repeats does not increase inclusion formation relative to overexpression of empty EGFP vector [21].

Results in Table 1 are expressed as odds ratios, which summarize a series of independent experiments comparing the proportions of EGFP-expressing cells with inclusions when co-transfected with α -synuclein, to the proportions observed when co-transfected with control vectors. Odds ratios were considered to be the most appropriate summary statistic, since the percentage of cells with inclusions under specified conditions varies between experiments on different days, whereas the relative change in the proportion of cells with inclusions induced by an experimental

perturbation is expected to be more constant. For example, line 1 of Table 1 summarizes 5 experiments, where co-transfection of HA/His₆-tagged wild-type α -synuclein with EGFP-huntingtin-(CAG)₇₂ resulted in a 1.9-fold the increase in the proportion of cells with inclusions versus huntingtin-expressing cells without inclusions, compared to cells co-transfected with EGFP-huntingtin-(CAG)₇₂ and the empty pHM6 vector (odds ratio = 1.9, $P < 0.001$).

In an attempt to define a region(s) of the α -synuclein protein that promoted huntingtin aggregation, a series of deletion constructs were prepared. Each HA/His₆-tagged construct was co-transfected with EGFP-huntingtin-(CAG)₇₂ into PC12 cells in triplicate experiments. α -Synuclein constructs containing amino acids 55–95, 1–120, 29–120, 55–120 and 55–140 each enhanced EGFP-huntingtin-(CAG)₇₂ inclusion formation ($P < 0.001$); constructs 1–95, 29–95, 96–140, Δ 57–97 and 29–140 did not significantly affect inclusion formation, while construct 61–95 significantly reduced the number of inclusions obtained with co-transfected EGFP-huntingtin-(CAG)₇₂ ($P < 0.001$). These results do not consistently identify a domain(s) of α -synuclein, which enhances huntingtin inclusion formation. For example, although the α -synuclein 55–95 amino acid construct increased huntingtin aggregation, this result was observed with some (e.g. 29–120 and 55–140) but not all (e.g. 29–95 and 29–140) constructs containing this domain. In solution, wild-type α -synuclein is unfolded in the native state [29], but its conformation in a cellular environment is unknown. With the exception of the Δ 57–97 construct, all the constructs used had N-terminal and/or C-terminal deletions. The results with deletion mutants may have been confounded by some of these over-expressed proteins adopting inappropriate conformations.

Mutant or wild-type α -synuclein constructs do not form visible aggregates in PC12 cells

In order to investigate the possibility of α -synuclein aggregation in PC12 cells, we transiently transfected these cells with full-length α -synuclein constructs coding for either the wild-type protein, or the A53T and A30P mutations. α -Synuclein was uniformly distributed in the cytoplasm and nucleus of PC12 cells, when it was EGFP-tagged and assessed directly by fluorescent microscopy (Figure 1), or when it was HA/His₆-tagged and localized with antibodies to HA, His₆ or α -synuclein (results not shown). None of the full-length constructs formed inclusions visible by fluorescent microscopy within 48 h of transfection. In order to

Table 1 Co-transfection of PC-12 cells with EGFP-huntingtin-(CAG)₇₂ and α -synuclein cDNA or control constructs

The increase in inclusion formation between α -synuclein and control constructs from multiple independent experiments (n) is indicated as an odds ratio (number of GFP-expressing cells with inclusions/number of GFP-expressing cells without inclusions) in cells transfected with α -synuclein construct / (number of GFP-expressing cells with inclusions/number of GFP-expressing cells without inclusions) in cells transfected with control construct, with 95% confidence intervals (95% CI).

α -Synuclein construct	Control construct	Odds ratio	95% CI	P	n
HA/His ₆ -wild-type	Empty pHM6	1.9	1.6–2.3	< 0.001	5
HA/His ₆ -A30P mutant	Empty pHM6	1.7	1.4–2.1	< 0.001	4
HA/His ₆ -A53T mutant	Empty pHM6	1.9	1.6–2.3	< 0.001	4
HA/His ₆ -wild-type	Hsp40 Δ 450 mutant	2.0	1.6–2.4	< 0.001	4
HA/His ₆ -A30P mutant	Hsp40 Δ 450 mutant	1.8	1.5–2.2	< 0.001	4
HA/His ₆ -A53T mutant	Hsp40 Δ 450 mutant	2.1	1.7–2.5	< 0.001	4
EGFP-wild-type	Empty pEGFP	5.2	3.9–7.0	< 0.001	4
EGFP-wild-type	EGFP-emerin 1–220 mutant	3.2	2.5–4.1	< 0.001	4

test whether aggregates may form after longer time periods we examined PC12 cells 144 h after transfection with EGFP-tagged wild-type α -synuclein but still observed no inclusions.

We considered the possibility that α -synuclein may need to be cleaved prior to subsequent aggregation. However, no aggregates were formed by α -synuclein deletion constructs comprising amino acids 1–95, 29–95, 55–95, 61–95, 29–140, 96–140 or Δ 57–97 in PC12 cells, which were analysed by immunocytochemistry using antibodies to the N-terminal HA-tag or C-terminal His₆-tag (wild-type and 55–95 constructs only, results not shown).

One possible explanation for our results was that α -synuclein acted as a seed for huntingtin aggregation. Analysis of PC12 cells transfected with huntingtin-(CAG)₇₂ and wild-type α -synuclein failed to show any co-localization of the latter with huntingtin inclusions (Figure 1). These experiments were performed by co-transfecting the EGFP-huntingtin-(CAG)₇₂ construct with the HA/His₆-wild-type- α -synuclein construct, or by co-transfecting HA/His₆-tagged huntingtin-(CAG)₇₂ with EGFP-wild-type- α -synuclein constructs. Similarly, deletion mutants of α -synuclein did not co-localize with huntingtin inclusions when cells were transfected with EGFP-huntingtin-(CAG)₇₂ and HA/His₆-tagged α -synuclein deletion constructs comprising amino acids 1–95, 29–95, 55–95, 61–95, 29–140, 96–140 or Δ 57–97 (results not shown). Experiments were analysed using fluorescent microscopy and immunocytochemistry using antibodies to HA (all constructs), His₆ (wild-type or 55–95 constructs only) or α -synuclein (wild-type construct only). As a positive control, HA/His₆-tagged huntingtin-(CAG)₂₁ co-localized with inclusions formed with EGFP-huntingtin-(CAG)₇₂ (Figure 1), as previously reported for COS-7 cells [21].

In summary, both the wild-type and the A30P and A53T mutant forms of α -synuclein increase the aggregation rate of a part of exon 1 of huntingtin containing expanded repeats in rat pheochromocytoma PC12 cells. However, α -synuclein does not form aggregates by itself, and does not appear in the huntingtin inclusions. We cannot formally exclude the possibility that α -synuclein is present in huntingtin aggregates but is undetectable due to the epitopes for each of the antibodies used (anti-HA, anti-His₆ and anti- α -synuclein 111–131) being inaccessible for antibody binding. However, no co-localization of EGFP- α -synuclein was observed in huntingtin inclusions formed by HA/His₆-tagged huntingtin-(CAG)₇₂, suggesting the absence of a direct physical interaction between α -synuclein and huntingtin. Thus the most likely explanation for our results is that α -synuclein overexpression enhances huntingtin aggregation as a consequence of secondary changes in cellular metabolism, rather than by a direct physical interaction. The mechanism for such an effect is unknown and difficult to predict, since α -synuclein overexpression may perturb many pathways, including those regulated by phospholipase D [30], the 14-3-3 proteins, a family of protein chaperones, and protein kinase C [31].

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