Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Neurology 197 (2006) 515–520
Experimental Neurology
www.elsevier.com/locate/yexnr

Regular Article

The alpha-synuclein mutation E46K promotes aggregation in cultured cells

Neeraj Pandey [a,b], Robert E. Schmidt [c], James E. Galvin [a,b,d,*]

[a] Department of Neurology, Washington University School of Medicine, St. Louis, MO 63108, USA
[b] Alzheimer Disease Research Center, Washington University School of Medicine, 4488 Forest Park, Suite 130, St. Louis, MO 63108, USA
[c] Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63108, USA
[d] Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63108, USA

Received 3 August 2005; revised 10 October 2005; accepted 18 October 2005
Available online 1 December 2005

Abstract

Parkinson's disease (PD) is characterized by the polymerization of wild-type (WT) or mutant alpha-synuclein (AS) into aggregates and fibrils, which are observed as Lewy bodies (LBs) and Lewy neurites (LNs) in PD patients. However, inability to demonstrate aggregation in many cell culture systems is a major drawback for effective in vitro modeling of AS aggregation. Utilizing PCR-based cloning approach, we generated A30P, A53T, and the recently reported E46K encoding mutation in the KTKEGV repeat region of AS gene. While cloning E46K mutant, a glycine deletion mutation (E46KΔG) adjacent to the intended lysine mutation was serendipitously generated. Expression of mutant constructs and green fluorescent protein (GFP)-tagged mutant constructs in catecholaminergic SH-SY5Y (5Y) cells revealed 40% of AS-E46KΔG and 18% of AS-E46K transfected cells formed aggregates as compared to 12% in AS-A53T, 6% in AS-WT, and 2% in AS-A30P transfected cells. Western blot analysis demonstrated the formation of high molecular weight AS aggregates. Electron microscopic analysis of 5Y cells expressing the E46K and E46KΔG mutants demonstrated two distinct kinds of inclusions: Type I, which showed dense granular profile; and Type II, which were largely membranous vacuolar inclusions without granular material. These two inclusions are reminiscent of Lewy bodies and pale bodies observed in PD postmortem brain samples. Our results demonstrate that mutations in 4th KTKEGV repeat lead to higher propensity of aggregation of AS compared to other mutants.

Keywords: Alpha-synuclein; Lewy bodies; Parkinson's disease; Aggregation

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting 1–2% of the population older than 65 years and 4–5% of those aged over 85 years (Eriksen et al., 2003). To date, more than 10 genes and loci have been implicated in PD (Dekker et al., 2003); however, most sporadic and familial forms of PD are linked to the aggregation of α-synuclein (AS). Symptoms of PD are largely attributed to the loss of dopaminergic neurons in substantia nigra pars compacta. The pathology of PD is characterized by the accumulation and aggregation of AS in neuronal cell bodies as Lewy bodies (LBs), and in neuronal processes as Lewy neuritis (LNs) or spheroids (Forno, 1996). Deposition of AS is common to several other neurodegenerative diseases such as Dementia with Lewy bodies (DLB), the Lewy body variant of Alzheimer's disease (LBVAD), multiple systems atrophy (MSA), and neurodegeneration with brain iron accumulation type-1 (Galvin, 2003; Galvin et al., 2000).

We hypothesize that PD is a disease of protein misfolding due to the structural perturbations and dynamic instability of protein–protein interactions of AS protein with its interaction partners. Structure prediction algorithms and structural data demonstrate AS to be unstructured protein (Dunker et al., 2001; Uversky et al., 2001; Uversky, 2002) having extensive interactions with other proteins and co-factors (Peri et al., 2003; Uversky, 2003). Structural perturbation of the protein due to point mutations may lead to aberrant interactions initiating a cascade which ultimately leads to aggregation and fibrillization of AS into LBs and LNs. However, it has been difficult to model AS aggregation in cell culture models.

* Corresponding author. Alzheimer Disease Research Center, Washington University School of Medicine, 4488 Forest Park, Suite 130, St. Louis, MO 63108, USA. Fax: +1 314 286 2763.
E-mail address: galvinj@neuro.wustl.edu (J.E. Galvin).

0014-4886/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2005.10.019

Model systems using non-neuronal lines such as human embryonic kidney cells (HEK293) or adrenal pheochromocytoma cells lines (PC12) (Stefanis et al., 2001) have inconsistently demonstrated punctate aggregates without fibrillar forms, other investigators have been entirely unable to demonstrate aggregates (Iwata et al., 2001). Utilizing the catecholaminergic, neuroblastoma-derived cell line SH-SY5Y (5Y cells) as a model system, we describe the effect of three known familial AS mutations and one novel mutation on the aggregation properties of AS, and document the presence of two distinct kinds of inclusions in cultured cells expressing mutant forms of AS protein.

Materials and methods

To study the phenomenon of aggregation and the effect of E46K mutation on aggregation in comparison with other known AS mutants, A30P, A53T, and E46K mutants were generated through polymerase chain reaction (PCR) and cloned into pcDNA 3.1 (Invitrogen) vector. Wild-type (WT) AS (Jakes et al., 1994), a gift from Michael Goedert was similarly sub-cloned into pcDNA 3.1 cloning vector. While cloning the E46K mutant, a glycine deletion mutation (E46KΔG) adjacent to intended lysine mutation was serendipitously generated. In addition, GFP fusion proteins of each of the constructs were generated by in-frame cloning of AS constructs in EGFPN-1 (Clontech) vector. The sequence of all the mutants was confirmed by Sanger's Dideoxy-mediated chain termination method.

Cell culture and immunofluorescence

5Y cells were grown in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, USA) supplemented with 10% Fetal bovine serum (Invitrogen, USA) and 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in 5% CO_2. Cells were transfected by Lipofectamine2000 (Invitrogen, USA) as per manufacturer's protocol; briefly, a day prior to transfection, cells were trypsinized and plated at 1 to 3 million cells per well on a 6-well plate or one fifth the above number for 4-well chamber slide (Nunc, USA). Subsequently, 5 μg of appropriate plasmid DNA (estimated by O.D) was diluted in 250 μl of OPTI-MEM medium (Invitrogen, USA) and mixed with equal volume of OPTI-MEM medium containing 5 μl Lipofectamine 2000. The mix was incubated for 30 min at room temperature and added directly to the cells in 6-well plate. For 4-well chamber slides, one fifth volumes were used and cells were transfected as described above. The cells were allowed to grow in OPTI-MEM medium for 6 h; afterwards, it was replaced with normal IMDM medium containing antibiotics. Cells were grown for a period of 48–72 h before either fixing them for immunofluorescence assays or harvesting them for immunoblot analysis. Aggregates were counted in 100 transfected cells in duplicate using different view fields. The cells were initially screened for aggregates at 20× magnification and aggregates were confirmed by examination at higher magnifications of 40× and 60×.

Immunofluorescence was done as per standard protocols described elsewhere (Jana et al., 2000). The AS antibody (Tu et al., 1998), syn202 (gift of John Trojanowski) was used at a dilution of 1:2000 for immunofluorescence assays. Appropriate secondary antibodies were used at a dilution of 1:2000 for immunofluorescence experiments and cells were observed under a confocal microscope (Zeiss LSM 5 PASCAL system).

Western blotting

Seventy-two hours posttransfection; the 5Y cells grown in monolayers were harvested, sonicated, and lysed. The samples were centrifuged at 12,000 × *g* for 10 min at 4°C and 25 μg of supernatant protein samples was electrophoresed on NuPAGE 4–12% Bis–Tris Gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Immunoblotting was done as per standard protocols described elsewhere (Jana et al., 2000). Syn202 antibody at a dilution of 1:4000 and LB509 (Baba et al., 1998) antibody at a dilution of 1:200 were utilized as primary antibodies. β-actin (Abcam, UK) antibody at a dilution of 1:1000 was utilized as loading control. Horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) was used at a dilution of 1:6000. The immune complexes were visualized with the use of the enhanced chemiluminescence (ECL) Plus or the ECL Advance kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol.

Electron microscopy

Cultures were grown on Permanox LabTek chamber slides (Nalge, Nunc). After transfection and treatments, cells were fixed for 4 h in 3% glutaraldehyde in 100 mM phosphate buffer, pH 7.4 containing 0.45 mM Ca^{2+} (Chang et al., 2003). Cultures were postfixed overnight in phosphate-buffered 2% OsO_4 containing 1.5% potassium ferricyanide, dehydrated in graded concentrations of alcohol, and embedded in Epon with propylene oxide as an intermediary solvent. One-micrometer-thick plastic sections were examined by light microscopy after staining with toluidine blue. Ultrathin sections of individual cultured cells were cut onto formvar-coated slot grids, and stained with uranyl acetate and lead citrate and examined with a JEOL 1200 electron microscope (JEOL, Peabody, MA). Cytoplasmic structures with or without limiting membranes that were at least half filled with electron-dense material were considered to be "Type I" inclusions. Membrane-limited, cytosolic structures that were less than half filled with electron-dense material were counted as "Type II" inclusions.

Results

Generation of AS mutants

A PCR-based approach was utilized to generate AS mutants. The clones were screened by restriction fragment length analysis and confirmed by dideoxy-mediated chain termination method. Serendipitously, during the generation of the E46K