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Synphilin-1 and parkin show overlapping expression patterns in human brain and form aggresomes in response to proteasomal inhibition

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Lewy bodies (LBs) are the characteristic inclusions of Parkinson's disease brain but the mechanism responsible for their formation is obscure. Lewy bodies (LBs) are composed of a number of proteins of which alpha-synuclein (α-SYN) is a major constituent. In this study, we have investigated the distribution patterns of synphilin-1 and parkin proteins in control and sporadic PD brain tissue by immunohistochemistry (IH), immunoblotting, and immunoelectron microscopy (IEM). We demonstrate the presence of synphilin-1 and parkin in the central core of a majority of LBs using IH and IEM. Using IH, we show an overlapping distribution profile of the two proteins in central neurons. Additionally, we show sensitivity of both endogenous synphilin-1 and parkin to proteolytic dysfunction and their co-localization in aggresomes formed in response to the proteasome inhibitor MG-132. We confirm that synphilin-1 and parkin are components of majority of LBs in Parkinson's disease and that both proteins are susceptible to proteasomal degradation.
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Introduction

Parkinson's disease (PD) is a neurodegenerative disease of old age which affects about 2% of the population over 65 years (de Rijk et al., 1997). Most cases are sporadic and are of unknown etiology. However, recent identification of gene mutations in familial cases of PD has advanced our understanding of the molecular mechanisms that may underlie the sporadic disease (Dawson and Dawson, 2003; Hardy et al., 2003). The clinical signs of PD (bradykinesia, rigidity, rest tremor, and postural instability) are caused by a progressive and substantial loss of dopaminergic neurons in the substantia nigra leading to reduction of dopamine and its metabolites in the striatum (Zhang et al., 2000). The neuropathological hallmark of PD is the presence of intracytoplasmic, proteinaceous inclusions known as Lewy bodies (LBs) in the brain stem, midbrain, neocortex, and other structures. The presynaptic protein α-synuclein (α-SYN) is a major component of LBs (Spillantini et al., 1997, 1998). Although LBs are a prominent feature of PD, the underlying pathogenic mechanisms leading to their formation are unknown and it is also not clear whether LBs are a cause or a consequence of cellular degeneration (Olanow et al., 2004).

Synphilin-1, a 919 amino acid protein of unknown function, was identified as an alpha-synuclein interacting protein by yeast two-hybrid analysis (Engelender et al., 1999). The interaction between synphilin-1 and synuclein was confirmed by fluorescence resonance energy transfer technique (Kawamata et al., 2001) and also by using yeast two-hybrid β-galactosidase assay (Neystat et

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al., 2002). Synphilin-1 has been shown to promote the formation of eosinophilic, cytoplasmic inclusions similar to LBs when co-transfected with the non-amyloid component of α-SYN sequence in a small percentage of cultured cells (Chung et al., 2001; Engelender et al., 1999). Subsequently (Wakabayashi et al., 2000), it has been shown that synphilin-1 co-localizes with α-SYN in LBs, indicating that it may be involved in the pathophysiology of PD. In young rats, synphilin-1 is enriched in neuronal perikarya and localizes to nerve terminals in adulthood (Ribeiro et al., 2002). In this respect, its developmental profile is similar to that of α-SYN (Hsu et al., 1998; Petersen et al., 1999). Previous studies looking for synphilin-1 gene mutations in familial and sporadic PD failed to identify any coding changes (Bandopadhyay et al., 2001; Farrer et al., 2001; Maraganore et al., 2003) but, recently, a possible link between synphilin-1 and PD has been suggested with a novel C to T transition in position 1861 of the synphilin-1 gene leading to an arginine to cysteine substitution in two cases of PD (Marx et al., 2003). This mutation is probably uncommon, as it has not been identified in other populations.

Numerous mutations in the parkin gene are now known to be the commonest cause of early onset, autosomal recessive juvenile form of PD (AR-JP) and account for almost 50% of autosomal recessive PD cases in Europe (Lucking et al., 2003). Parkin is a RING-type ubiquitin–protein ligase (E3) and disease-associated mutations may nullify its E3 activity (Shimura et al., 2000; Zhang et al., 2000). Parkin is also able to protect midbrain, TH-positive neurons from the toxicity of α-SYN in culture (Petrucelli et al., 2002). LBs are generally absent from ARJP patients so far studied at postmortem, suggesting that intact parkin is vital for LB formation. Several recent reports have demonstrated the presence of parkin in LBs in sporadic PD (Murakami et al., 2004; Schlossmacher et al., 2002; Shimura et al., 2000) and in mitochondrial cybrids (Trimmer et al., 2004). A possible functional link between synphilin-1, parkin, and α-SYN in LB formation has been demonstrated in vitro by Chung and colleagues (Chung et al., 2001) who showed that synphilin-1 is ubiquitinated by parkin and when the three are co-transfected, ubiquitin-positive, LB-like cytosolic inclusions are formed in HEK cells. More recently, Lim and colleagues (Lim et al., 2005) have shown that parkin can mediate synphilin-1 degradation by both proteasomal-linked and proteasomal-independent manner in HEK cells overexpressing synphilin-1 and parkin and both these processes lead to LB-like inclusion formation.

The molecular changes underlying the initiation and progression of sporadic PD disease remain unclear, but accumulating evidence implicates abnormal processing of a variety of cellular proteins via the ubiquitin/26S proteasomal system in the development of PD pathology. Reports demonstrating the presence of proteasome subunits (Ii et al., 1997) and UCH-L1 (Lowe et al., 1990) within LBs in sporadic cases support this hypothesis. Mutations in parkin and UCH-L1, two enzymes involved in ubiquitin–proteasomal (UPS) pathway, are responsible for some cases of familial PD (Kitada et al., 1998; Leroy et al., 1998) and triplication and missense mutations in the α-SYN gene are also associated with the disease (Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003). Altered protein handling and proteolytic stress are prominent features in postmortem brain in PD (Jenner, 2003). Furthermore, systemic exposure to naturally occurring and synthetic protease inhibitors in rats caused progressive parkinsonism which was improved by apomorphine treatment (McNaught, 2004). At postmortem, these animals exhibited many of the pathological features of PD, including the formation of LB-like inclusions. Failure of UPS-mediated proteolysis could be a common feature of both familial and sporadic PD.

Previous studies of overexpression of either parkin or synphilin-1 demonstrated formation of intracellular inclusions with or without proteasomal inhibition in cultured cells (Junn et al., 2002; Lee et al., 2002; O'Farrell et al., 2001), suggesting that these proteins are vulnerable to proteolytic stress and have the ability to form aggregates or aggresomes. Elucidation of the mechanisms of the genesis of LB formation could thus provide clues about potential therapeutic targets in the human disease.

More recently, mutations in other genes, *DJ-1* (PARK6) (Bonifati et al., 2003), *PINK1* (PARK7) (Valente et al., 2004), and *LRRK2* (PARK8) (Paisan-Ruiz et al., 2004; Zimprich et al., 2004), have been found in familial cases of PD. Findings from studies at this laboratory indicate that DJ-1 is not a major component of LBs or LNs (Bandopadhyay et al., 2004), while the presence of either PINK1 or dardarin (the protein product of LRRK2 gene) in these inclusions has yet to be elucidated. Wild-type DJ-1 is not degraded by the ubiquitin–proteasome (UPS) pathway, and no evidence is available as to whether dardarin or PINK1 is degraded by the same pathway. However, some of the proteins implicated in familial PD could be substrates for phosphorylation by PINK1 or dardarin as these proteins are putative kinases.

Although the presence of both synphilin-1 and parkin immunoreactivity has been shown in LBs in separate studies, controversy exists in the literature with regard to their prevalence (Murray et al., 2003; Wakabayashi et al., 2000). Moreover, given that synphilin-1 may be an important substrate of parkin-mediated ubiquitination (Chung et al., 2001; Lim et al., 2005), it is important to examine the distribution of both proteins in the same group of cases. We have therefore examined the expression of synphilin-1 and parkin in human postmortem brain particularly in regions with a predilection for LB formation. Frontal cortex and brain stem regions were studied by immunohistochemistry (IH), immunoelectron microscopy (IEM), and immunoblotting. Brains from neurologically normal controls and neuropathologically verified idiopathic PD cases were used. To examine interactions of synphilin-1 with other proteins implicated in familial PD, we used the yeast two-hybrid system. We also examined the properties of synphilin-1 and parkin in SH-SY5Y cells in conditions of proteolytic stress to determine whether these two proteins co-localized within aggregates formed in response to proteasome inhibition. This is particularly important in the light of proteasomal inhibition being proposed as a mechanism of LB formation (McNaught and Olanow, 2003).

Materials and methods

Cases

Brain tissue was obtained from the Queen Square Brain Bank, Institute of Neurology, Queen Square, London, UK. Human tissue was collected with informed consent of next of kin and with ethical approval from the National Hospital for Neurology and Neurosurgery and Institute of Neurology Joint Research Ethics Committee. Perimortem details of the brains used in this study are listed in Table 1.

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