β-Synuclein Displays an Antiapoptotic p53-dependent Phenotype and Protects Neurons from 6-Hydroxydopamine-induced Caspase 3 Activation

CROSS-TALK WITH α-SYNUCLEIN AND IMPLICATION FOR PARKINSON’S DISEASE*

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We have established stable transfectants expressing β-synuclein in TSM1 neurons. We show that in basal and staurosporine-induced conditions the number of terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive β-synuclein-expressing neurons was drastically lower than in mock-transfected TSM1 cells. This was accompanied by a lower DNA fragmentation as evidenced by the reduction of propidium iodide incorporation measured by fluorescence-activated cell sorter analysis. β-Synuclein strongly reduces staurosporine-induced caspase 3 activity and immunoreactivity. We establish that β-synuclein triggers a drastic reduction of p53 expression and transcriptional activity. This was accompanied by increased Mdm2 immunoreactivity while p38 expression appeared enhanced, indicating that β-synuclein-induced p53 down-regulation likely occurs at a post-transcriptional level. We showed previously that α-synuclein displays an antiapoptotic function that was abolished by the dopaminergic derived toxin 6-hydroxydopamine (6OHDA). Interestingly, β-synuclein retains its ability to protect TSM1 neurons even after 6OHDA treatment. Furthermore, β-synuclein restores the antiapoptotic function of α-synuclein in 6OHDA-treated neurons. Altogether, our data document for the first time that β-synuclein protects neurons from staurosporine and 6OHDA-stimulated caspase activation in a p53-dependent manner. Our observation that β-synuclein contributes to restoration of the α-synuclein antiapoptotic function abolished by 6OHDA may have direct implications for Parkinson’s disease pathology. In this context, the cross-talk between these two parent proteins is discussed.

Parkinson’s disease (PD)† is one of the most common and devastating diseases in the elderly (1, 2). This pathology is characterized by intracellular aggregates, called Lewy bodies (LB) (3, 4) that are thought to be responsible for final dementia occurring not only in PD but also in Lewy body diseases. The main component of LB was identified as α-synuclein (5), a 140-amino acid-long synaptic protein (6) that accumulates within these neuropathological hallmarks (7–9). The central role of α-synuclein in PD pathology has been emphasized by the observation that familial forms of PD were due to two mutations borne by α-synuclein (10, 11). Interestingly, these mutations trigger alterations of the biophysical properties of α-synuclein, leading to an exacerbation of misfolding and aggregation (12). Therefore, as with many other neurodegenerative diseases such as Alzheimer’s and prion diseases, among others (13–15), PD can be documented as a disease associated with protein misfolding. The fact that such aggregates of proteins were recently shown to exhibit an intrinsic toxic potential (16) could lead to a reunifying theory linking misfolding and neurodegeneration.

Interestingly, we have shown that α-synuclein displays an antiapoptotic phenotype that was abolished by PD-related mutations (17). This antiapoptotic function was also prevented when neuronal cells were exposed to 6-hydroxydopamine (6-OHDA), a toxin derived from dopamine (18). This was associated with an increased expression of α-synuclein, and we postulated that the protein exerted a physiological antiapoptotic phenotype that was abolished in conditions of drastic overexpression and aggregation (18). This hypothesis was in line with the observation that overexpression of α-synuclein and its aggregation could lead to a PD-like phenotype in transgenic mice (19) and Drosophila (20).

β-Synuclein is another member of the synuclein family (21, 22) that lacks the non-amyloidogenic component (NAC) domain that appears to be responsible for the aggregating properties of α-synuclein (23). β-Synuclein is therefore considered to be a non-amyloidogenic homolog of α-synuclein. Interestingly, it was demonstrated that the ratio of β-synuclein to α-synuclein appeared altered in LB disease (24). Because β-synuclein can not seed α-synuclein aggregation (25), it was postulated that β-synuclein could act as a physiological inhibitor of α-synuclein aggregation (19). Indeed, it was reported that β-synuclein-derived peptides behave as anti-aggregating agents (26). That this β-synuclein property could lead to a therapeutic strategy was validated by the observation that β-synuclein inhibited α-synuclein aggregation in transgenic models of PD pathology (19). Of the greatest importance was the demonstration that
this was accompanied by an amelioration of motor deficits and neurodegenerative alterations (19).

Nothing is known concerning the cellular function of β-synuclein and, more particularly, whether, as is the case for α-synuclein, β-synuclein could control neuronal cell death. We establish here that β-synuclein is antiapoptotic in TSM1 neurons and that this function is linked to a drastic down-regulation of p53 expression and activity. We show that β-synuclein still protects neurons from 6OHDA insult. Finally, we demonstrate that β-synuclein contributes to the α-synuclein function and, more particularly, restores its antiapoptotic phenotype in 6OHDA-treated cells, an experimental condition that normally abolishes α-synuclein antiapoptotic function.

EXPERIMENTAL PROCEDURES

Cell Systems and Transfections—TSM1 neurons (27) were cultured as described previously (17). Stable transfectants expressing wild-type β-synuclein were obtained after transfection with 2 μg of β-synuclein cDNA (in pcDNA3) by means of DCA30 (Euregentec) according to the manufacturer’s recommendations. Positive transfectants were screened for their β-synuclein-like immunoreactivity as described below. Transient transfections were also performed in TSM1 neurons with DCA30 containing either 2 μg of cDNA encoding α-synuclein and β-synuclein, alone (28) or in combination. Cells were used 48 h after their transfection.

Western Blot Analyses—For the detection of α- and β-synucleins, equal amounts of protein (50 μg) were separated on Tris-tricine gels and Western blotted with the anti-α- and β-synuclein rabbit polyclonal antibodies (Affiniti Research Products). Active caspase 3, p53, Mdm2 phospho-p38, and β-tubulin immunoreactivities were analyzed by Western blot performed by means of anti-active caspase 3 (rabbit polyclonal; R&D Systems), anti-p53 (mouse monoclonal; Santa Cruz Biotechnology), anti-Mdm2 (mouse monoclonal; provided by Dr. R. Fahraeus), anti-phospho-p38 (rabbit polyclonal; Promega), and anti-β-tubulin (Sigma). Immunoreactive bands were visualized and quantitated on a charged image digital plate scanner (Umax Powerlook 2000). Protein bands were identified by their molecular masses.

RESULTS

Stably transfected TSM1 neurons overexpress a 19-kDa protein, a molecular mass corresponding to that expected for β-synuclein (Fig. 1A). The responsiveness of these cells to staurosporine, an apoptotic effector, has been examined. TSM1 cells display about 20% of TUNEL-positive neurons in basal conditions (Fig. 1B). This number increases drastically upon staurosporine treatment (Fig. 1, A and B). Interestingly, β-synuclein expression significantly reduces the number of TUNEL-positive cells under both basal and staurosporine-stimulated conditions (Fig. 1, B and C). This histological feature was reinforced by the observation that the propidium iodide incorporation measured upon staurosporine stimulation by flow cytometry revealed a lower DNA fragmentation in β-synuclein-expressing cells (data not shown). The β-synuclein-mediated antiapoptotic phenotype was associated with a drastic reduction of staurosporine-induced caspase 3 activation (Fig. 2A). This was accompanied by a lower active caspase 3 immunoreactivity (Fig. 2B). It should be noted that identical data were obtained with various independent β-synuclein-expressing clones (not shown).

p53-like immunoreactivity is lower in β-synuclein-expressing cells than in mock-transfected cells (Fig. 3, A and B). Interestingly, reduced p53 expression is associated with a statistically significant reduction of p53 transcriptional activity (Fig. 4). As p53 expression can be controlled at post-transcriptional levels by several proteins, we examined the influence of β-synuclein on the expression of Mdm2 and phosphorylated p38 (Fig. 5). Thus Mdm2 controls p53 expression mainly by regulating its ubiquitination/degradation rates (31). Conversely, phosphorylated p38 activates p53 by phosphorylation (32). In line with our observed decreased p53 expression, we found higher Mdm2-like immunoreactivity and reduced p38 expression in β-synuclein-expressing cells in both basal and staurosporine-stimulated conditions (Fig. 5). This suggests a possible control of p53 by β-synuclein at a post-transcriptional level. These data, however, do not totally rule out the possibility that β-synuclein could also control p53 expression at a transcriptional level.

β-Synuclein Protects Neurons from Caspase 3 Activation

This procedure has been extensively described (18). Briefly, cells were fixed for 20 min in 4% paraformaldehyde (in phosphate-buffered saline), rinsed in phosphate-buffered saline, left overnight in 70% ethanol, and then processed for the dUTP nick end labeling TUNEL technique as recommended (Roche Applied Science). Staining was assessed with a peroxidase-conjugated antibody, and labeling TUNEL technique was revealed with 3,3′-diaminobenzidine as black spots. All experiments were reinforced by the observation that the propidium iodide incorporation measured upon staurosporine stimulation by flow cytometry revealed a lower DNA fragmentation in β-synuclein-expressing cells (data not shown). The β-synuclein-mediated antiapoptotic phenotype was associated with a drastic reduction of staurosporine-induced caspase 3 activation (Fig. 2A). This was accompanied by a lower active caspase 3 immunoreactivity (Fig. 2B). It should be noted that identical data were obtained with various independent β-synuclein-expressing clones (not shown).

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Protein charge was monitored by black bars. 3-like activity was fluorometrically recorded in mock (black bars) and β-synuclein-expressing cells (β-SYN, white bars) in the absence (Basal) or presence of staurosporine (STS; 1 μM, 2h). Bars represent the results of eight determinations carried out in duplicate. Statistical analysis indicates that p < 0.001 by t test analysis for both basal and STS conditions when comparing mock- versus β-synuclein-stably transfected cells. B, active caspase-3-like immunoreactivity (Casp-3 act.) was analyzed in basal (−) and stimulated (+) conditions (STS; 1 μM, 2h) in the indicated cell lines as described under “Experimental Procedures.” Protein charge was monitored by β-tubulin analysis.

As we showed previously, 6OHDAs, a dopaminergic toxin, triggers drastic caspase 3 activation (Fig. 6). β-synuclein fully prevents 6OHDAs-induced caspase activation, with the activity remaining close to the control value (Fig. 6). Furthermore, β-synuclein fully prevents the 6OHDAs-induced increase in caspase 3 immunoreactivity observed in mock-transfected cells (Fig. 7C). Therefore, β-synuclein retains its antiapoptotic potential with distinct caspase activators, namely staurosporine and 6OHDAs. This is not the case for α-synuclein. Thus, although α-synuclein displays a clear protective effect toward staurosporine in neurons (18), our data on caspase activity (Fig. 7A) and immunoreactivity (Fig. 7C) indicate that 6OHDAs abolishes the α-synuclein-induced antiapoptotic phenotype in agreement with our previous study (18). However, of the most interest is our observation that β-synuclein restores the α-synuclein-like antiapoptotic phenotype when both proteins are co-expressed (Fig. 7A). Thus, caspase activity (Fig. 7B) and immunoreactivity (Fig. 7C) returned the values observed with β-synuclein alone. It should be noted that in both basal and 6OHDAs-treated neuronal cells the two proteins do not trigger additive protection (Fig. 7B), suggesting that they were likely using the same molecular pathways and that their expression could be redundant, at least for this effect.

**DISCUSSION**

The synucleins constitute a family of small proteins, including α-, β-, and γ-synucleins (for review, see Ref. 33). Recently, α-synuclein draw particular attention because of the demonstration that this presynaptic protein (34) coexists with the amyloid β-peptide as the main non-amyloidogenic component of the senile plaques in Alzheimer’s disease (6, 35, 36). More puzzling was the observation that α-synuclein was also the main component of Lewy bodies (5) (37), the intracellular inclusions that accumulate in Parkinson’s disease and other dementia-related diseases (38). That this protein played a central role in PD was suggested by the observation that a subset of familial PD was indeed due to autosomal dominant mutations borne by α-synuclein (10, 11). Indeed, these mutations abolish various physiological functions elicited by the protein (for review, see Ref. 33), and this was thought to be due to an exacerbation of its aggregation properties (39, 40) triggered by the
Fig. 5. β-Synuclein expression increases Mdm2 immunoreactivity and lowers p38 expression. Mdm2 and active p38-like immunoreactivities in β-synuclein-expressing neurons were monitored as described under “Experimental Procedures.” Thresholds were set to one of three representative densitometric analysis of the Mdm2 and p38-active (p38act) immunoreactivities. Protein charge is indicated by β-tubulin (Tub.) analysis. Ct, control; STS, staurosporine.

The density of Lewy bodies (19) and prevent the behavioral properties of β-synuclein not only can resist aggregation but can also interbe interference with the aggregating process of β-synuclein because it reduced the neuronal responsiveness to staurosporine. Therefore, one can envision that α-synuclein could complement α-synuclein deficiency at least in the first stages of PD pathophysiology. Furthermore, α-synuclein remains anti-apoptotic in the absence of β-synuclein in 6OHDA-treated neurons. Therefore, one can consider that β-synuclein could also act as a neuroprotective factor via the preservation of α-synuclein antiapoptotic properties. In this context, it is noteworthy that the ratio of β-synuclein over α-synuclein is altered in Lewy bodies (24). As α- and β-synucleins have been shown to physically interact (19), it is tempting to hypothesize that lowered levels of β-synuclein in PD could result first in an alteration of intrinsic β-synuclein-mediated control of cell death and, second, in a defective α-synuclein anti-apoptotic phenotype normally controlled by β-synuclein.

The above data have clear implications for PD and strengthen the track of searching for β-synuclein derivatives able to protect α-synuclein from aggregation, thereby preserving its beneficial antiapoptotic properties. This strategy could perhaps be extrapolated to other neurodegenerative diseases. Thus, we previously established that presenilin 2, its mutated counterpart (48), and its C-terminal fragment (49) trigger a pro-apoptotic phenotype that could, to some extent, account for some of the neuropathological stigmata observed in Alzheimer’s disease (50, 51). Amazingly, in prion-related diseases we also recently showed that overexpression of PrP+ led to a drastic Mdm2-regulated, p53-dependent caspase 3 activation (29). Therefore, p53 can be seen as a key factor acting as a common denominator involved in various neurodegenerative diseases.
Targeting p53 can be seen as a therapeutic strategy. Thus, p53 inhibitors were recently shown to protect dopaminergic neurons and improve motor deficits observed in an experimental animal model of Parkinsonism (52). Furthermore, p53 deletion inhibitors were recently shown to protect dopaminergic neurons from caspase-3 activation (54) should be considered cautiously with respect to putative animal model of Parkinsonism (52). Furthermore, p53 deletion inhibitors were recently shown to protect dopaminergic neurons from caspase-3 activation (54) should be considered cautiously with respect to putative animal model of Parkinsonism (52).

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