Caspase-3-derived C-terminal Product of Synphilin-1 Displays Antiapoptotic Function via Modulation of the p53-dependent Cell Death Pathway*

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Parkinson disease is the second most frequent neurodegenerative disorder after Alzheimer disease. A subset of genetic forms of Parkinson disease has been attributed to α-synuclein, a synaptic protein with remarkable chaperone properties. Synphilin-1 is a cytoplasmic protein that has been identified as a partner of α-synuclein (Engelender, S., Kaminsky, Z., Guo, X., Sharp, A. H., Amaravi, R. K., Kleiderlein, J. J., Margolis, R. L., Troncoso, J. C., Lanahan, A. A., Worley, P. F., Dawson, V. L., Dawson, T. M., and Ross, C. A. (1999) Nat. Gen. 22, 110–114), but its function remains totally unknown. We show here for the first time that synphilin-1 displays an antiapoptotic function in the control of cell death. We have established translucent and stable transfectants overexpressing wild-type synphilin-1 in human embryonic kidney 293 cells, telecephalon-specific murine 1 neurons, and SH-SY5Y neuroblastoma cells, and we show that both cell systems display lower responsiveness to staurosporine and 6-hydroxydopamine. Thus, synphilin-1 reduces proapoptotic function in the control of cell death. Furthermore, we establish that synphilin-1 drastically reduces p53 transcriptional activity and expression and lowers p53 promoter transactivation and mRNA levels. Interestingly, we demonstrate that synphilin-1 catabolism is enhanced by staurosporine and blocked by caspase-3 inhibitors. Accordingly, we show by transcription/translation assay that recombinant caspase-3 and, to a lesser extent, caspase-6 but not caspase-7 hydrolyze synphilin-1. Furthermore, we demonstrate that mutated synphilin-1, in which a proapoptotic phenotype is no longer expressed, resists proteolysis by cellular and recombinant caspases and displays drastically reduced antiapoptotic phenotype. We further show that the caspase-3-derived C-terminal fragment of synphilin-1 was probably responsible for the antiapoptotic phenotype elicited by the parent wild-type protein. Altogether, our study is the first demonstration that synphilin-1 harbors a protective function that is controlled by the C-terminal fragment generated by its proteolysis by caspase-3.

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3 The abbreviations and trivial name used are: PD, Parkinson disease; LB, Lewy bodies; 6OH-DOPA, 6-hydroxydopamine; Ac-DEVD-CHO or DEVD, acetyl-Asp-Glu-Val-Asp-aldehyde; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; WT-synphilin-1, wild-type synphilin-1; STS, staurosporine; ALLN, N-acetyl-L-leucyl-L-norleucinal; E64, l-trans-epoxysuccinylleucylaminor-n-guanino]butanate.

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mutation analysis of the synphilin-1 gene in familial and sporadic German PD patients allowed the identification of the R621C mutation in two sporadic PD patients, suggesting a putative role of synphilin-1 as a genetic susceptibility factor for the disease (25). Due to the implication of synphilin-1 in PD and to the modulation of cell death by α-synuclein and parkin, two privileged binding partners of synphilin-1, we investigated the role of synphilin-1 in cell death control. We show that synphilin-1 lowers HEK293 cells, TSM1 neurons, and SH-SY5Y neuroblastoma responsiveness to staurosporine and 6OH-DOPA by decreasing caspase-3 activity and poly(ADP-ribose) polymerase and by down-regulating the p53-dependent proapoptotic pathway. In addition, in silico examination of the synphilin-1 sequence revealed a consensus site for a caspase-3 cleavage. Accordingly, we demonstrate the cleavage of synphilin-1 by cellular and purified caspase-3 and the abolishment of its antiapoptotic function by site-directed mutagenesis of the caspase-3 site in its sequence. Finally, we demonstrate that the C-terminal fragment of synphilin-1 generated by caspase-3 is indeed responsible for the antiapoptotic phenotype of synphilin-1.

EXPERIMENTAL PROCEDURES

Materials—Lactacystin, acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO; DEVD), pepstatin, L-trans-epoxyoxysuccinyl-leucil-amino-(n-guanino) butane (E64), N-acetyl-leucyl-leucyl-norleucinal (ALLN), α-phenanthroline, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, staurosporine, and 6OH-DOPA were purchased from Sigma.

Mutagenesis—A putative consensus cleavage site for caspase-3 was identified in synphilin-1, in silico, by means of the peptide cutter ExPasy software. The D454A-synphilin-1 was obtained by oligonucleotide-directed mutagenesis from wild-type synphilin-1 V5-tagged cDNA by means of a QuickChange™ site-directed mutagenesis kit (Stratagene). The two primers 5’-GGCATCTGTTGGATGAGTACACAG-GATGGCAAC-3’ and 5’-GTTGCACCTGTGCTACTTCATC-CACAGGATGCC-3′ (Eurogentec) containing the D454A mutation were designed according to the manufacturer’s instructions. The cDNA encoding the V5-tagged caspase-3-derived C-terminal fragment of synphilin-1 was engineered by introducing an ATG codon in position 454 after the putative consensus cleavage site of caspase-3 (oligonucleotide 5’-TA-CCC-AAG-CTT-ATG-CAG-GAT-GGC-3′). An additional HindIII restriction site was also added, adjacent to the ATG codon, for further subcloning of the construction in pcDNA3.1/V5/His-TOPO.

Cell Systems and Transfections—TSM1 neurons (26), HEK293 human cells, and SH-SY5Y neuroblastoma were cultured as previously described (27, 28). Stable transfectants expressing empty vector (mock) and wild-type and mutated synphilin-1 in HEK293 cells were obtained after transfection with 2 μg of each CDNA (all in pcDNA3) by means of calcium phosphate precipitation. TSM1 neurons expressing empty vector (mock) and wild-type synphilin-1 were obtained after the transfection with 2 μg of each cDNA by means of Superfect reactive according to the manufacturer’s conditions. Positive clones were screened for their synphilin-1-like immunoreactivity as described below. Transient transfections were carried out by means of 2 μg of cDNA by calcium phosphate precipitation (HEK293 cells) or Lipofectamine (4 μl; TSM1 and SH-SY5Y).

Wild-type and Mutated Synphilin-1 Degradation—Wild-type and D454A-synphilin-1-overexpressing HEK293 and TSM1 cells were preincubated for 16 h in the absence or in the presence of various protease inhibitors at the following concentrations: Ac-DEVD-CHO (100 μM), pepstatin (10 μM), ALLN (100 μM), E64 (100 μM), α-phenanthroline (100 μM).

Then cells were lysed and analyzed for synphilin-1-like immunoreactivity by Western blot using anti-V5 antibodies as described below.

Western Blot Analysis—For the detection of wild-type and mutated synphilin-1, equal amounts of protein (50 μg) were separated on 8% gels and Western blotted with the anti-V5 mouse monoclonal antibodies (Invitrogen). For the detection of procaspase-3, human and mouse PARP, and β-tubulin immunoreactivities, equal amounts of protein (25 μg) were separated on 8 or 12% gels and Western blotted with anti-human procaspase-3 antibodies (Interchim) and anti-human (Euromedex) and anti-mouse (BD Biosciences) PARP antibodies. Anti-β-tubulin and anti-actin monoclonal antibodies were from Sigma. Immunological complexes were revealed as previously described (29).

Caspase-3 Activity Measurements—Stable transfectants were preincubated without or with staurosporine (0.5–2 μM) or 6OH-DOPA (0.03–0.3 mM) for various times, and then caspase-3-like activity was fluorimetrically measured as extensively detailed (12). Caspase-3-like activity is considered as the Ac-DEVD-CHO-sensitive Ac-DEVD-7-amino-4-methylcoumarin-hydrolyzing activity.

p53 Expression, Activity, and Promoter Transactivation—The activity of p53 was analyzed after transient transfection of the PG13-luciferase (PG13) cDNA designed and kindly provided by Dr. B. Vogelstein (Baltimore, MD) (30). The transcriptional activation of the human p53 promoter (hpps53) was measured after transfection of the cDNA coding for the human p53 promoter sequence in frame with luciferase (provided by Dr. M. Oren, Rehovot, Israel). All activities were measured after co-transfection of 0.5–1 μg of the above cDNAs and 0.25–0.5 μg of β-galactosidase cDNA, in order to normalise transfection efficiencies.

p53 immunoreactivity was analyzed by Western blot using an anti-p53 mouse monoclonal antibody (1:10,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in nuclear extracts prepared as previously described for cytochrome c translocation experiments (13).

Real Time Quantitative PCR—Total RNA from cells was extracted at the indicated times using the RNeasy kit following the instructions from the manufacturer (Qiagen). After treatment with DNase I, 2 μg of total RNA were reverse transcribed using oligo(DT) priming and avian myeloblastosis virus reverse transcriptase (Promega). Real time PCR was performed in an ABI PRISM 5700 Sequence Detector System (Applied Biosystems) using the SYBR Green detection protocol as outlined by the manufacturer. Gene-specific primers were designed using the Primer Express software (Applied Biosystems). Relative expression level of target genes was normalized for RNA concentrations with two different housekeeping genes (human glyceraldehyde-3-phosphate dehydrogenase, mouse γ-actin) according to the cell specificity.

In Vitro Transcription/Translation of Wild-type and Mutated Synphilin-1 and Cleavage by Caspase-3, -6, and -7 in a Cell-free System—Wild-type and D454A synphilin-1 were transcribed and translated using the Promega TNT coupled reticulocyte lysate system in the presence of [35S]methionine (ICN) as extensively described (31). Briefly, 2.5 μl of reticulocyte lysates were incubated in 50 μl of 25 mM HEPES, pH 7.5, 0.1% CHAPS, 5.0 mM dithiothreitol with 25 ng of recombinant caspase-3, -6, and -7 (Sigma) for 8 h at 37 °C. In some experiments, the effect of the caspase inhibitor benzoxycarbonyl-VAD (10 μM) was examined. Proteins were then electrophoresed on 11% polyacrylamide gels and autoradiographed using Amersham Biosciences hyperfilms.

Statistical Analysis—Statistical analysis was performed with PRISM software (Graphpad Software, San Diego, CA), by using the Newman-Keuls multiple comparison tests for one-way analysis of variance and Student’s t test.
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RESULTS

Wild-type Synphilin-1 (WT-synphilin-1) but Not D454A-synphilin-1 Undergoes Cellular Proteolysis by Ac-DEVD-CHO-sensitive Caspase-like Activity in HEK293 Human Cells and Is Cleaved by Purified Caspase-3 in Vitro—We have established stable transfectants overexpressing WT-synphilin-1 and mutated D454A-synphilin-1 in human embryonic kidney (HEK293) cells. The design of mutated D454A-synphilin-1 is based on an in silico study that identified a consensus cleavage site for caspase-3 (DEVD) on the WT-synphilin-1 sequence. Fig. 1A shows several of the wild-type and mutated stable transfectants obtained that overexpress a 120-kDa protein, a molecular mass corresponding to that expected for the V5-tagged synphilin-1 (1). Clones 10 and 11 (Fig. 1B), which display similar levels of wild-type and mutated synphilin-1 protein expression, were selected for the follow-up of our study.

Fig. 2 illustrates the susceptibility of WT-synphilin-1 to various protease inhibitors. Peptatin (acidic protease inhibitor), ALLN (calpain inhibitor), and o-phenanthroline (metalloprotease inhibitor) were unable to affect WT-synphilin-1 expression (Fig. 2, A and B). Ac-DEVD-CHO (caspase-3, -6, and -7 inhibitor) significantly increased WT-synphilin-1 immunoreactivity (Fig. 2, A and B) in a time-dependent manner (Fig. 2C), suggesting a processing of this protein by caspases. It is interesting to note that E64 (cysteine/serine protease inhibitor) also slightly but significantly potentiated WT-synphilin-1 expression (Fig. 2, A and B), in agreement with the fact that caspases activities belong to the class of cysteine proteases (32, 33). Interestingly, D454A-synphilin-1 remained completely insensitive to both E64 and capase inhibitor (Fig. 2, A and C). These data first confirm that the D454A mutation renders synphilin-1 resistant to proteolysis in HEK293 cells and indicates that caspase-like activities mainly contributed to synphilin-1 catabolism in HEK293 cells.

Staurosporine and 6OH-DOPA have been shown to increase caspase-3 activity in various cell systems (13). We therefore examined whether treatment of WT-synphilin-1-expressing cells with these two proapoptotic effectors could enhance WT-synphilin-1 degradation. Indeed, Fig. 2D shows that staurosporine and 6OH-DOPA both decreased the expression of WT-synphilin-1, the levels of which appeared drastically increased upon Ac-DEVD-CHO treatment of the cells (Fig. 2D), in agreement with the above data suggesting an implication of caspases in the processing of synphilin-1. In order to identify the caspases involved in the cleavage of synphilin-1, we examined its susceptibility to proteolysis by recombinant caspase-3, -6, and -7 in vitro. Fig. 3A shows that WT-synphilin-1 is cleaved by recombinant caspases-3 and, to a much lesser extent, by caspase-6, whereas caspase-7 appeared unable to cleave WT-synphilin-1 (Fig. 3A). Ac-DEVD-CHO fully prevented caspase-3 and caspase-6-mediated hydrolysis of WT-synphilin-1 (Fig. 3A). Interestingly, D454A-synphilin-1 fully resisted proteolysis by recombinant caspase-3 (Fig. 3B). It should be noted that WT-synphilin-1 resisted proteolysis by recombinant and cellular overexpressed caspase-8 (not shown), in agreement with the fact that the site cleaved in synphilin-1 (DEVD) is also conserved in D454A-synphilin-1. These data suggest that D454A-synphilin-1 is not a caspase-8 substrate.

WT-synphilin-1 but Not D454A-synphilin-1 Reduces Staurosporine- and 6OH-DOPA-induced Caspase-3 Activation in HEK293 Cells and Lowers the p53-dependent Proapoptotic Pathway—The implication of caspases in the processing of synphilin-1 led us to investigate whether WT-synphilin-1 could control cell death and whether the caspase site mutation could influence such a phenotype. We analyzed the responsiveness of transiently or stably transfected WT-synphilin-1 and D454A-synphilin-1-expressing HEK293 cells to staurosporine (STS) and 6OH-DOPA and, more particularly, the levels of caspase-3. Staurosporine was used as a broad and non-specific proapoptotic inducer, whereas 6OH-DOPA is a natural dopaminergic toxin that triggers neurodegenerescence that mimics that observed in PD pathology (2). First, we confirmed that STS (Fig. 4, A, C, and G) and

FIGURE 1. Immunological analysis of WT-synphilin-1- and mutated D454A-synphilin-1-expressing HEK293 cells. HEK293 cells were stably transfected with empty pcDNA3 vector (Mock), wild-type-synphilin-1 (WTsynp), or D454A-synphilin-1 (D454Asynp) cDNA as described under “Experimental Procedures.” Synphilin-1-like immunoreactivities of wild-type (clones WT) and mutated synphilin-1 (clones D454A) were analyzed by electrophoresis on a 8% Tris-glycine gel, Western Blot, and incubation with anti-V5 primary antibodies as described under “Experimental Procedures.” Actin immunoreactivity was monitored as a control of protein charge (see “Experimental Procedures”). In B, the bars correspond to the densitometric analyses of the various clones normalized for actin expression.

FIGURE 2. Pharmacological analysis of wildtype and D454A-synphilin-1 degradation in HEK293 stable transfectants. Wild-type synphilin-1 (WTsynp) and mutated D454A-synphilin-1 (D454Asynp) expressing cells were incubated for 16 h (A) in the absence (C) or in the presence of the protease inhibitor Ac-DEVD-CHO (DEVD; 100 μM), pepstatin (PEP; 10 μM), ALLN (100 μM), E64 (100 μM), or o-phenanthroline (O-Phe; 100 μM) or for various time periods (C) with 100 μM Ac-DEVD-CHO, and then synphilin-1-like immunoreactivity was analyzed by Western blot with anti-V5 antibody as described under “Experimental Procedures.” Actin immunoreactivity was monitored as a control of protein charge (see “Experimental Procedures”). A, quantitative densitometric analysis of wild-type synphilin-1-like immunoreactivity recovered in A, D WT-synphilin-1 cells were preincubated for 16 h without (−) or with (+) Ac-DEVD-CHO (DEVD; 100 μM) and then treated with staurosporine (STS; 2 μM) or 6-hydroxydopamine (6OH-DOPA; 0.2 mM), and WT-synphilin-1 expression was analyzed as above. Bars are the means ± S.E. of four independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.005, compared with control untreated cells.
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6OH-DOPA (Fig. 4, B, D, and H) stimulate caspase-3 activity in a time- and dose-dependent manner. Interestingly, WT-synphilin-1 expression drastically reduced caspase-3 activity, whereas the D454A mutation drastically reverted this inhibitory control of caspase-3 activity (Fig. 4, A–D, G, and H). Accordingly, STS- and 6OH-DOPA-induced synphilin-1 catabolites were only observed in cells expressing the wild-type protein (Fig. 4I). Overall, these data indicate that caspase-resistant D454A-synphilin-1 was unable to modulate cell death in HEK293 cells and, therefore, that the antiapoptotic response elicited by synphilin-1 was controlled by its proteolysis by caspase-3.

In order to further confirm the influence of WT-synphilin-1 on caspase-3 modulation, we analyzed the immunoreactivities of the inactive procaspase-3 in control and STS- and 6OH-DOPA-stimulated conditions. Procaspase-3 is the inactive precursor of caspase-3 that is catalytically activated by caspase-8 and caspase-9 during apoptosis. Thus, a reduction of its immunoreactivity reflects an activation of cell death processes. As expected, STS (Fig. 4E) and 6OH-DOPA (Fig. 4F) treatment of mock-transfected cells drastically lowers procaspase-3 expression (Fig. 4, E and F). It should be noted that the extent of proteolytic maturation of procaspase-3 by 6OH-DOPA was more important than the one triggered by STS, in agreement with caspase-3 activity measurements (see Fig. 4, A–D, G, and H). WT-synphilin-1 elicited a reduction of procaspase-3 cleavage in stimulated conditions (Fig. 4, E and F), whereas D454A-synphilin-1-expressing cells still displayed procaspase-3 reduction (not shown). It should be noted that WT-synphilin-1 reverted procaspase-3 immunoreactivity to near control levels (Fig. 4, E and F). This suggests that the bulk of WT-synphilin-1-induced effects observed on "caspase-like" activities as well as its protection by the Ac-DEVD-CHO indeed reflects a functional link between WT-synphilin-1 and genuine caspase-3 rather than another caspase-like activity.

PARP is an enzyme implicated in the repairation of DNA that is proteolytically inactivated by caspase-3 during apoptosis. Thus, an augmentation of its 89-kDa cleavage product or a lowering of the precursor versus product ratio reflects an increase of caspase-3 activity and subsequent caspase-3-dependent apoptotic process. As expected, STS or 6OH-DOPA treatment of mock-transfected cells drastically augments the recovery of PARP product with concomitant virtual abolishment of PARP precursor immunoreactivity (Fig. 4, E and F). In both STS- and 6OH-DOPA-stimulated conditions, WT-synphilin-1 expression enhances PARP precursor immunoreactivity (Fig. 4, E and F), thereby leading to an augmentation of precursor versus product ratio. Altogether, our data demonstrate by both enzymatic and immunological approaches that WT-synphilin-1 triggers and antiapoptotic response by controlling caspase-3 activity and that this phenotype is fully reverted by site-directed mutagenesis of the synphilin-1 caspase-3 cleavage consensus site.

In order to further delineate the cellular intermediates involved in the WT-synphilin-1 antiapoptotic phenotype, we examined the influence of WT-synphilin-1 on the p53-dependent pathway. Fig. 5 shows that WT-synphilin-1-expressing HEK293 cells display drastically reduced p53 transcriptional activity (Fig. 5A) and nuclear expression (Fig. 5B). Furthermore, WT-synphilin-1 lowers the transactivation of the p53 promoter (Fig. 5C), in very good agreement with the reduced p53 mRNA levels established by real time PCR (Fig. 5D). Of most interest is...
our observation that the down-regulation of the p53 pathway was not observed in cells expressing mutated D454A-synphilin-1 (Fig. 5, A–D).

WT-synphilin-1 Reduces STS- and 6OH-DOPA-induced Cell Death in TSM1 Neurons and in SH-SY5Y Neuroblastoma Cells—In order to rule out a problem of cell specificity, we have analyzed the ability of synphilin-1 to modulate cell death in TSM1 neurons and in SH-SY5Y, a cell model particularly relevant to study Parkinson disease (34–37). We have established TSM1 stable transfectants overexpressing WT-synphilin-1 (Fig. 6A). As shown in Fig. 6A, the immunoreactivity of synphilin-1 is drastically augmented after treatment with Ac-DEVD-CHO, confirming the susceptibility of synphilin-1 to cleavage by caspases in a neuronal cell line. Fig. 6B shows that WT-synphilin-1 significantly reduced Ac-DEVD-CHO-sensitive caspase-3 activity in basal conditions. This phenotype was further exacerbated in both STS-stimulated (Fig. 6B, left) and 6OH-DOPA-stimulated (Fig. 6B, right) conditions. Fig. 6C illustrates the immunological profile of PARP cleavage in mock-transfected and WT-synphilin-1 expressing TSM1 neurons. As expected, STS and 6OH-DOPA treatment of mock-transfected cells led to decreased PARP precursor expression and concomitant detection of a related product, the formation of which was fully prevented by Ac-DEVD-CHO, confirming the implication of caspase-3 on the processing of PARP precursor (Fig. 6C). Overexpression of WT-synphilin-1 blocks 70–100% of PARP product formation in STS- and 6OH-DOPA-stimulated conditions, respectively (Fig. 6C). Comparative transient transfection analyses (Fig. 6D) show that, unlike WT-synphilin-1, D454A-synphilin-1 did not protect TSM1 neurons from STS- and 6OH-DOPA-induced caspase-3 activation (Fig. 6D). The latter data were fully confirmed in SH-SY5Y (Fig. 7). Thus, transient transfection of WT-synphilin-1 but not D454A-synphilin-1 coding cDNA (Fig. 7B) lowered SH-SY5Y responsiveness to STS (Fig. 7A, left) and 6OH-DOPA (Fig. 7A, right). Altogether, these data confirm the susceptibility of WT-synphilin-1 to caspase-3 proteolysis and the ability of this protein, but not its casapse-resistant mutated counterpart, to down-regulate STS- and 6OH-DOPA-stimulated caspase-3 activation in TSM1 neurons and SH-SY5Y neuroblastoma cells.

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The Caspase-3-derived C-terminal Fragment of Synphilin-1 Lowers HEK293 and TSM1 Responsiveness to STS- and 6OH-DOPA-induced Caspase-3 Activation—The fact that WT-synphilin-1 undergoes caspase-3-mediated proteolysis together with the observation that the mutation that renders WT-synphilin-1 resistant to this cleavage also abolished its antiapoptotic phenotype strongly suggested that the C-terminal fragment of WT-synphilin-1 (synphilin-1-CTF) generated by caspase-3 could indeed be responsible for the WT-synphilin-1-associated protective phenotype. In order to directly examine this possibility, we have designed the V5-tagged synphilin-1-CTF (Fig. 8), and we have assessed its influence after transient transfection in HEK293 cells and TSM1 neurons. Synphilin-1-CTF lowers the STS-induced (Fig. 8, A and C) and...
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DISCUSSION

PD-affected brains exhibit selective loss of substantia nigra pars compacta neurons and are invaded at late stages by cytoplasmic inclusions called Lewy bodies (LB) (38–40). Dopaminergic neuron cell death is apparently linked to exacerbated oxidative stress and p53-dependent apoptosis (41–44) that could be the consequence of the accumulation and aggregation of misfolded proteins. Thus, it has been demonstrated that aggregated proteins display inherent toxicity (45) and harbor the ability to inhibit the proteasome (46). In this context, when the cellular capacity of refolding, recovery, and degradation are saturated, misfolded proteins accumulate, aggregate (47), and ultimately kill the cells. LB reflect such an accumulation process in PD. These structures are mainly composed of ubiquitin, a number of elements of the proteasomal machinery and aggregated proteins among which α-synuclein is the main component (48). α-Synuclein, one of the key proteins implicated in familial PD (10, 49, 50), has a high propensity to aggregate in vitro and in vivo, and several studies showed that α-synuclein aggregation can be exacerbated by pathogenic mutations and by different factors, including the dopaminergic derivative prooxidant toxin 6OH-DOPA (for reviews, see Refs. 51–53). Interestingly, α-synuclein aggregation impairs its function. Thus, the A53T familial-associated PD mutation and 6OH-DOPA both trigger α-synuclein aggregation and abolish its antiapoptotic function (13).

α-Synuclein displays remarkable chaperone properties (53), and recently, synphilin-1 has been characterized as one of its binding partners (1, 19). Interestingly, synphilin-1 accumulates in LB (19), and the co-overexpression of α-synuclein and synphilin-1 favors the formation of eosinophil cytoplasmic inclusions that resemble LB (1, 21). Therefore, the possible implication of synphilin-1 in the formation of the LB and its possible functional link with α-synuclein led us to study the role of synphilin-1 in cell death.

We have established that wild-type synphilin-1 has a protective phenotype in human HEK293 cells, TSM1 neurons, and SH-SYSY neuroblastoma cells. Thus, synphilin-1 reduces STS- and 6OH-DOPA-induced caspase-3 activation and PARP cleavage. In agreement with its protective function, WT-synphilin-1 also drastically down-regulated the proapoptotic p53 pathway. Interestingly, synphilin-1 function appears regulated by its proteolysis. Thus, we show that cellular synphilin-1 degradation is enhanced by the proapoptotic effectors STS and 6OH-DOPA and reduced by caspase-3 inhibitor. In agreement, we found that synphilin-1 is cleaved preferentially by caspase-3 in vitro. Interestingly, D454A-synphilin-1, a mutant in which a consensus cleavage site for caspase-3 had been abolished, fully resisted proteolysis by recombinant caspase-3.

What is the molecular influence of caspase-3 cleavage on synphilin-1 function? At least two theoretical hypotheses could stand. First, synphilin-1 holoprotein itself would be responsible for the protective phenotype, and caspase-3 cleavage could be seen as an inactivating process. Second, synphilin-1-associated antiapoptotic phenotype would be associated with one of its caspase-3-derived proteolytic products. Our data strongly argue in favor of the latter view. Thus, synphilin-1-induced antiapoptotic phenotype is drastically reduced when synphilin-1 is rendered resistant to caspase-3 proteolysis by mutagenesis of a caspase-3 cleavage site consensus sequence. This observation strongly suggested a role of caspase-3 in the generation of a synphilin-1-derived product with
antiapoptotic properties. Indeed, we have shown that the caspase-3-derived C-terminal fragment of synphilin-1 lowered staurosporine- and 6-OH-DOPA-induced caspase-3 activation. In this context, one could envision that cellular stress or environmental factors trigger caspase-3 activation and associated cell death but also provide a means to downregulate apoptosis by concomitantly increasing the production of caspase-3-derived synphilin-1 proteolytic fragment. It should be noted that this type of regulation has already been documented for other proteins. Thus, presenilins (54, 55) and β-amyloid precursor protein (56–60) undergo caspase-derived cleavages, generating proteolytic fragments controlling cell death. More related to PD, parkin, another binding partner of synphilin-1 (18, 61) displaying an antiapoptotic phenotype (62, 63), is also cleaved by caspases, but unlike for synphilin-1, this endoproteolysis leads to a loss of function of this protein (64).

It is worth noting that although both α-synuclein and synphilin-1 protect human cells and neurons from STS stimulation (12, 13), only synphilin-1 keeps its protective function in the presence of 6-OH-DOPA (this work). This phenotype is reminiscent of the one associated with β-synuclein, the homologue of α-synuclein. Thus, both synphilin-1 and β-synuclein remain protective toward 6-OH-DOPA (55) and lower the p53 pathway. Furthermore, β-synuclein restores the protective activity of α-synuclein, even in the presence of 6-OH-DOPA (55). Whether synphilin-1 restores the antiapoptotic potential of α-synuclein in the presence of the dopaminergic derivative remains to be established. However, it should be noted that α-synuclein and synphilin-1 co-localize in LB at the late stages of the pathology and that aggresomes formed by α-synuclein and synphilin-1 are cytoprotective (65). These observations together with the present demonstration of a protective function of synphilin-1 argue in favor of a caspase-3-regulated protective role of synphilin-1 and for a functional cross-talk between α-synuclein and synphilin-1 within LB.

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