Proteasome dysfunction has been demonstrated in Parkinson disease (PD), and proteasome inhibitors have been shown to induce degeneration of dopaminergic neurons in vitro and in vivo. The mechanism whereby proteasome dysfunction leads to dopaminergic cell death, however, is unknown. In this study, we show that proteasome inhibition in both PC12 cells and dopaminergic neurons derived from embryonic stem cells is associated with mitochondrial membrane permeabilization, activation of caspase-3, and nuclear changes consistent with apoptosis. Prior to the emergence of apoptotic features, we found that proteasome inhibition induced increased levels of phosphorylated p53. Inhibition of p53 by pifithrin-α or by RNA interference prevented mitochondrial membrane permeabilization and cytotoxicity. There was no increase in p53 mRNA in proteasome-inhibited cells, suggesting that p53 was increased in a transcription-independent manner. Further, there was no increase in Puma or Bax mRNA and p53 co-immunoprecipitated with Bcl-xL and Mdm2. These findings suggest that p53 mediates cell death by way of a direct mitochondrial effect in this model. We also observed increased levels of phosphorylated p53 in dopamine neurons of the substantia nigra pars compacta of mice following systemic administration of a proteasome inhibitor. These changes preceded degeneration of dopaminergic neurons. Increased phosphorylated p53 was also demonstrated in the substantia nigra pars compacta of post-mortem PD brains. These results suggest that abnormalities in p53 signaling play a role in dopaminergic cell death induced by proteasome inhibition and may be relevant to neurodegeneration in PD.

The characteristic pathology of Parkinson disease (PD) is degeneration of dopaminergic neurons coupled with protein accumulation and the formation of Lewy body inclusions in the substantia nigra pars compacta (SNc) (1). The mechanism underlying dopaminergic cell death in PD has been extensively sought but has yet not been elucidated. A variety of cellular and molecular changes indicative of mitochondrial dysfunction, oxidative stress, and apoptosis have been detected (see review in Ref. 2), however, the relationship between these changes and their relevance to the neurodegenerative process remain unclear. More recently, defects in the ubiquitin proteasome system with a failure to clear unwanted proteins have been implicated in the etiopathogenesis of both familial and sporadic PD (3–6). Specifically, we and others have demonstrated defects in the structure and function of the 26/20 S proteasome in patients with sporadic PD (7–9). The importance of this observation is supported by the demonstration that proteasome inhibitors can induce a relatively selective degeneration of dopamine (DA) neurons and a model of PD in both in vitro and in vivo studies (10–15). For example, treatment of rat ventral mesencephalic cultures or PC12 cells with proteasome inhibitors leads to degeneration of dopaminergic neurons and the formation of α-synuclein/ubiquitin-containing intracytoplasmic inclusion bodies. Further, we and others have found that systemic administration of a proteasome inhibitor induces a model of PD in rats with behavioral, imaging, pathological, and biochemical features that resemble what is found in human PD brain (16–19). These findings suggest that defects in the ubiquitin proteasome system, and more specifically proteasomal dysfunction, might play a role in the loss of dopaminergic neurons in PD.

Recent studies have demonstrated that degeneration of dopaminergic cells induced by toxins thought to be relevant to PD is associated with increased levels of the tumor suppressor gene p53 (20–22). p53 is a sequence-specific transcription factor that increases dramatically in response to a variety of cellular stresses and activates the expression of several genes that promote cell death (see reviews in Refs. 23–25). p53 is also known to be able to cause cell death by directly inducing mitochondrial permeabilization and apoptosis, independent of the transcriptional up-regulation of pro-apoptotic genes (26, 27). The regulation of p53 levels in the cell is complex and not completely understood (28). However, the ubiquitin proteasome system is known to play an important role in maintaining low levels of p53 in the cell, because the protein is primarily cleared by Mdm2-mediated

Puma, p53-up-regulated modifier of apoptosis; DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride; PFT, pifithrin; ERK, extracellular signal-regulated kinase; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone; siRNA, small interfering RNA; E3, ubiquitin-protein isopeptide ligase.
ated ubiquitination and subsequent proteasomal degradation (16). Accordingly, we hypothesize that proteasomal dysfunction in PD and related models could lead to increased levels of p53 that contribute to the degeneration of dopaminergic cells.

Here, we report that proteasome inhibition induces degeneration of PC12 cells and dopaminergic neurons generated from mouse embryonic stem (DA-ES) cells. Dopaminergic cell death is accompanied by a nontranscriptional increase in phosphorylated (activated) p53, and p53 inhibition prevents cell death in these models. We also demonstrate that phosphorylated p53 accumulates in the SNC of mice treated with proteasome inhibitor as well as in patients with sporadic PD. These data suggest that p53 signaling plays a key role in cell death associated with proteasome inhibition and possibly in PD itself.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse embryonic stem cells (R1) in culture were propagated and differentiated into DA neurons (DA-ES) as described previously (20). All of the experiments using DA-ES neurons were conducted after 9 days of differentiation. PC12 cells, a dopaminergic cell line that expresses human D2 receptors (20, 29–31), were maintained in Dulbecco’s modified eagle medium (Invitrogen) supplemented with 500 μg/ml G418, 10% horse serum, and 5% fetal bovine serum and were propagated and differentiated into DA neurons (DA-ES) from mouse embryonic stem (DA-ES) cells. Dopaminergic cell death was quantified using a spectrofluorometer (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) as described (20). The data are highly fluorescent substrate by living cells was quantified using the conversion of nonfluorescent CellTiter-Blue reagent to a spectrofluorometer (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) as described (20). The data are

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Preparation of cDNA—Total cellular RNA was prepared using TRIzol reagent (Invitrogen). To determine the expression of target genes, reverse transcription PCR or quantitative real time PCR was carried out in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) using SYBR-Green assay, as previously described (20, 29). The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. The comparative cycle threshold method was used to analyze the data by generating relative values of the amount of target cDNA (29). The cycle threshold values are the means of triplicate measurements, and the experiments were repeated four times. Primer sequences were: p53 (5′CATCATCACGG-

TGGAGACTCT3′, 5′TTACAGCTCTCGGAACATCT3′, 176 bp); Puma (5′ATCTTCCTATGGAACATCT3′, 5′GCGAGTCGSTATATGCTACATG3′, 181 bp); Bax (5′GTGATGGC-

AACCTCAACTG3′, 5′GATGAGTCTGGACCTATTAG3′, 99 bp); Bcl2 (5′AGGCACAGAAGGAAGTCT3′, 5′TCTCC-

ACAAAAAGCATTCCCCAG3′, 120 bp); Mdm2 (5′CTATTTCC-

CAGCCGTGCAGCT3′, 5′TGCTGAGGATAGCGGAGAAA-

3′, 57 bp); c-fos (5′CAGCTGCACTATAGCAGGAT3′, 5′GTT-

GATGCACTGCTACATG3′, 196 bp); c-Jun (5′CACA-

GTGCGGAAAAGGAAGA3′, 5′GCTTACGATGTTGAGCA3′, 174 bp); egf1 (5′GGTTTACGGAGGAGCTT3′, 5′GAGT-

TTACCCAGGAGGCTT3′, 150 bp).

Luciferase Reporter Assay—The PC12 cells were plated in 12-well plates (1 × 10^5 cells/well) with complete medium. After 24 h, the cells were transfected with 0.5 μg of p53-Luc plasmid (Stratagene, La Jolla, CA), as well as with 50 ng of a Renilla luciferase plasmid (pRL-TK) (Promega) as a control, with the use of the TransIT-Neural transfection reagents (Mirus, Madison, WI) as described previously (31). The following day, the cells were treated with PSI (5 μM) for the time periods indicated, and the luciferase activity of the lysate was measured in
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FIGURE 1. Proteasome inhibition induces concentration-dependent cell death associated with accumulation of ubiquitinated proteins. A, dose-dependent cell death by proteasome inhibitor PSI in PC12 cells. PC12 cells were treated with indicated concentrations (Conc.) of PSI for 24 h. Cell viability was assessed by CellTiter-Blue fluorescence assay described under “Experimental Procedures.” The data represent the means ± S.E. of three independent experiments (n = 8). B, representative immunoblot showing the accumulation of ubiquitinated proteins in PC12 cells treated with PSI. PC12 cells were treated with 5 μM PSI for the indicated period of time, and immunoblotting was carried out using anti-ubiquitin antibody. C, PSI induces formation of ubiquitin-immunoreactive aggregates. PC12 cells were treated with 5 μM PSI for 16 h and stained with anti-ubiquitin antibody (red) and nuclei with DAPI (blue). Scale bar, 5 μm. D, PSI induces selective degeneration of dopaminergic neurons present in the DA-ES cell culture. Mouse DA-ES cells at day 9 of stage 5 were treated with the indicated concentrations of PSI for 24 h. Total cell loss was assessed by CellTiter-Blue fluorescence assay, and selective loss of dopaminergic neurons in DA-ES culture was determined by [3H] DA uptake. The data represent the means ± S.E. of three independent experiments (n = 8).

triplect with a dual luciferase reporter assay system (Promega) using the Sirius luminometer (Berthold Technologies). The luciferase activities of the p53-luciferase vector were normalized based on Renilla luciferase activity of the co-transfected vector. At a minimum, transfections were done in triplicate and on two independent occasions.

RNA Interference—Custom SMARTpool plus small interfering RNA (siRNA) to target rat p53 (29) was designed and synthesized by Dharmacon (Lafayette, CO) (product number Q5-004997-00-70). siRNA (50 pmol) was transfected into PC12 cells using TransIT-TKO transfection reagent (Mirus, Madison, WI) as described previously (29, 31). After 48 h upon transfection, the cells were treated with PSI (5 μM) or vehicle for 24 h, and cell viability was assessed by CellTiter-Blue assay. A nonspecific RNA duplex (Dharmacon; catalog number D-001206-09-05) was used in control experiments.

Animals and Proteasome Inhibitor Treatments—All of the experiments were performed in accordance with the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine and National Institutes of Health guidelines on the ethical use of animals. Male 129Sv mice (purchased from Taconic Farms, Germantown, NY; weight, 25–30 g) were housed in plastic cages and provided free access to food and water. The animals were maintained in temperature- and humidity-controlled rooms with a 12-h light-dark cycle (light from 6:00 a.m. to 6:00 p.m.). The mice were randomly divided into two treatment groups. In the control group, the animals were administered vehicle (70% ethanol). In the active treatment group, the mice were treated (subcutaneously) with 3.0 mg/kg PSI in 70% ethanol. The animals received six subcutaneous injections spaced over a 2-week period as previously described (16). During and for up to 6 weeks after the end of the injections, the animals were assessed for alterations in body weights and monitored for evidence of systemic toxicity. At 1 and 6 weeks after the cessation of treatment, the mice were sacrificed, and brain histopathological analyses were performed.

Immunohistochemistry—For immunohistochemistry, the mice were anesthetized with pentobarbital (10 mg/kg intraperitoneal) and fixed by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The brains were post-fixed in the same solution overnight and then cryoprotected in 30% sucrose/phosphate-buffered saline. For immunostaining, the brains were cut coronally in 25-μm sections with a sliding microtome equipped with a freezing stage, and two successive slices of vehicle- and PSI-treated animals were arranged serially on the same slide. The sections were either stained for TH using a Vector ABC immunostaining procedure with DAB chromogen or a double immunofluorescence for TH and phospho-p53 or phospho-ERK with donkey anti-sheep (Alexa 488; 1:400; Molecular Probes, Eugene, OR), and goat anti-rabbit (Alexa 568; 1:400) was used as a secondary antibody to detect TH and phospho-p53 or phospho-ERK, respectively. The nuclei were stained with the fluorescent DNA dye DAPI, and the sections were subsequently mounted in Vectashield. The sections were examined, and the images were acquired with an Olympus BX65 microscope equipped with a high resolution digital camera. The number of neuronal cell bodies in specific brain areas was counted manually as we described previously (16).

Statistical Analysis—The data were analyzed using GraphPad Prism data analysis program (GraphPad Software, San Diego, CA). For the comparison of statistical significance between two groups, Student’s t tests for paired and unpaired data were used. For multiple comparisons, one-way analysis of variance followed by post hoc comparisons of the group means according to the method of Tukey was used.
RESULTS

Proteasomal Inhibition Causes Dopaminergic Cell Loss and Protein Aggregation in Vitro—We examined the cytotoxic effects of proteasome inhibitors on dopaminergic cells in culture. A number of previous investigations have demonstrated that the peptidyl aldehyde PSI is a specific inhibitor of proteasome chymotrypsin-related activity (16, 32). Treatment of PC12 cells with PSI at 1–10 μM induced a concentration-dependent cell death. At 1 and 5 μM, there was ~20 and 50% cell loss after 24 h of treatment (Fig. 1A). Consistent with reported studies (33) proteasome inhibition caused a time-dependent increase in ubiquitinated proteins (increased by ~2-fold at 16 h) as determined by Western immunoblotting (Fig. 1B). Immunocytochemistry following PSI treatment similarly showed a marked increase in cytoplasmic and nuclear staining for ubiquitinated proteins along with the formation of aggregates resembling inclusions bodies (Fig. 1C). We also examined the effects of PSI in cultures containing dopaminergic neurons derived from ES cells (DA-ES) because this system provides a relatively high density of dopaminergic neurons (~25% of total cells) in which cell death mechanisms can be studied. PSI also induced dose-dependent degeneration of these neurons. At 1 and 10 μM, total cell death was ~20 and 30%, respectively, after 24 h of incubation (Fig. 1D). To determine the specificity of PSI toxicity for dopaminergic neurons, we measured [3H]DA uptake. The percentage of total cell death observed with PSI at 10 μM was 28.6 ± 1.1, whereas [3H]DA uptake showed an almost complete loss of DA uptake (95.6 ± 7.1) (Fig. 1D). These findings confirm previous reports that proteasome inhibitors can induce a relatively selective dose-dependent degeneration of dopaminergic cells with protein accumulation and inclusions.

Proteasome Inhibition Causes Mitochondrial Membrane Permeabilization and Caspase-3 Activation—Proteasome inhibition is known to be associated with apoptotic cell death (18). In our studies, PSI treatment induced morphological features of apoptosis such as fragmented nuclei in PC12 cells (Fig. 2A) and retracted processes in DA-ES cells (Fig. 2B). To determine whether this process involves a mitochondrially mediated mechanism, we examined mitochondrial membrane permeabilization and caspase-3 activation following PSI exposure. In PC12 cells, mitochondrial membrane permeabilization, as measured by MitoTracker Red CMXRos uptake, was significantly reduced in dopaminergic cells treated after 6 h of incubation with PSI (Fig. 3A, upper panels). This was associated with reduced focal (presumably mitochondrial) staining for cytochrome c at 16–24 h (Fig. 3A, lower panels), consistent with the diffusion of the protein from mitochondria to the cytoplasm. At 24 h, immunostaining for activated caspase-3 was observed in TH-positive DA-ES cells and in PC12 cells (Fig. 2, A and B). Moreover, caspase-3 activation was exclusively present in cells that failed to take up MitoTracker Red CMXRos (Fig. 3B), further indicating that mitochondrial membrane permeabilization precedes caspase-3 activation.

To determine whether caspase-3 mediates proteasome inhibitor-induced death, DA-ES and PC12 cells were pretreated with the caspase-3 inhibitor Z-DEVD-fmk before being treated with 1 or 5 μM of PSI, respectively. As shown in Fig. 2 (C and D), 10 μM Z-DEVD-fmk treatments significantly prevented the loss of DA-ES and PC12 cells induced by PSI. These results suggest that PSI induces cell death via a caspase-3-dependent mechanism in these in vitro models.

Proteasomal Inhibition Leads to Accumulation of Phosphorylated p53 in Dopaminergic Cells—Because the degradation and clearance of p53 is controlled by the ubiquitin-proteasome system (34), we examined the effects of proteasome inhibition with PSI on the accumulation of p53 in PC12 cells. Treatment with 5 μM PSI was associated with an increase in the level of p53 protein of 4–5-fold after 16 and 24 h of exposure (Fig. 4, A and B). Accumulated p53 was highly phosphorylated at serine 15 (Fig. 4, A and B), which is known to increase p53 stability (accumulation) (35). Similarly, in DA-ES cell cultures, p53 protein level and phosphorylation were markedly increased after treatment with 1 μM PSI for 16 h (Fig. 4C).

We further examined ATM kinase (which phosphorylates p53 at the Ser15 position) and Mdm2 (an E3 ligase that ubiquiti-
were treated with 5 μM PSI for the indicated period of time and mitochondrial membrane permeability was assessed using MitoTracker Red CMXRos (upper panels, red) and cytochrome c immunostaining (lower panels, green). Decreased MitoTracker uptake and cytochrome c staining was observed after 6 h of PSI exposure. B, PC12 cells were treated with 5 μM PSI for 24 h and then subjected to MitoTracker uptake (red) and caspase-3 immunostaining (green). In PSI-treated cells, caspase-3 activation was found exclusively in cells with decreased MitoTracker uptake and fragmented nuclei (blue). Similar results were observed in four independent experiments. Scale bar, 5 μm.

Changes in phospho-p53 accumulation were first observed at 6 h, prior to alteration in staining for mitochondrial membrane permeability, caspase activation, or nuclear change indicative of apoptosis. Further, p53 accumulation was no longer observed in cells at 24 h when markers of apoptosis were detected (data not shown). These observations suggest that changes in p53 accumulation occur early in the apoptotic process associated with proteasome inhibition.

p53 Accumulation Is Not Dependent on Transcriptional Activation—We used quantitative real time PCR analyses to determine whether the increase in the levels of p53 is associated with increased transcriptional activity. Treatment of PC12 cells with 5 μM PSI did not cause an increase in the mRNA levels of p53 or p53-dependent genes such as Puma and Bax. However, there was a significant increase in the mRNA levels for egr1, a downstream effector of the ERK pathway in PC12 cells (29), and Mdm2 (Fig. 5). These observations indicate that the increase in p53 levels is likely not transcriptionally induced.

p53 Co-immunoprecipitates with Mdm2 and Bcl-xL in a Nonubiquitinated Form—The level of p53 is regulated by proteasomal degradation following its ubiquitination, which is mediated by the E3 ubiquitin ligase Mdm2 (36). To determine the effects of proteasome inhibition on p53/Mdm2 interactions in dopaminergic cells, we treated PC12 cell cultures with PSI for 7 h, prepared cell lysates, and performed immunoprecipitation using a specific antibody to Mdm2. Analysis of immunoprecipitates found that p53 co-immunoprecipitated with Mdm2 as well as with the mitochondrial protein Bcl-xL in PSI-treated cells but not in untreated control cells (Fig. 6B). The p53-Mdm2-Bcl-xL complex in PSI-treated cells was not ubiquitinated (Fig. 6, C and D). Following treatment with proteasome inhibitors, immunocytochemistry showed that increased p53 levels occurred within the cytoplasm of PC12 and DA-ES cells (Fig. 7, A and B) and that phosphorylated p53 was predominantly found in TH-positive neurons. These results indicate that proteasome inhibition leads to an increase in cytoplasmic p53, which appears to result from impaired proteasomal degradation coupled with impaired Mdm2-mediated ubiquitination in dopaminergic cells.

p53 Transcriptional Activity Is Not Required for Cell Death Induced by Proteasome Inhibition—PC12 cells were transiently transfected with a luciferase reporter plasmid containing a p53-responsive element. As shown in Fig. 8A, there was activation of
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Inhibition of p53 Activity Prevents Mitochondrial Damage and Cell Death in Response to Proteasome Inhibition—PFT-α is known to inhibit p53 activity. To determine whether a p53-mediated pathway is involved in the death of DA neurons following exposure to proteasome inhibition, we pretreated these cells with PFT-α. Pretreatment for 1 h with varying concentrations of PFT-α significantly protected PC12 cells from the toxic effect of PSI in a dose-dependent manner (Fig. 9A). Significant neuroprotection was observed at concentrations of 5 μM and above, with the maximal response at a 10 μM concentration. To further determine whether p53 is involved in the degenerative process, we evaluated cytotoxicity following inhibition of p53 with RNA interference as described previously (29). PC12 cell death elicited by PSI was significantly prevented by p53 siRNA (Fig. 9B).

Because p53 can mediate mitochondrial dysfunction, we explored a role for p53 in proteasome-mediated mitochondrial dysfunction. Pretreatment of the PSI-exposed PC12 cells with 10 μM of PFT-α prevents permeabilization of mitochondria, indicating that p53 mediates the mitochondrial abnormality (Fig. 9C). These results suggest that inhibition of p53 alone is sufficient to prevent the PSI-mediated toxic-
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We previously demonstrated that accumulation of p53 in proteasome inhibitor-induced cell death. Proteasomal Inhibition Causes Dopaminergic Cell Loss with Accumulation of p53 in Mice—We previously demonstrated that systemic exposure of rats to PSI induces degeneration with ubiquitin- and α-synuclein-positive inclusions in the SNc and other brain areas as in PD (16, 19). To determine whether activation of a p53-related pathway mediates neurodegeneration in vivo as we have observed in vitro, we treated mice with 3.0 mg/kg (subcutaneously) PSI six times over 2 weeks (16). The animals did not exhibit evidence of systemic toxicity or paralysis and did not lose weight, and all of them survived. Quantitative assessment of behavioral activity showed that PSI-treated animals demonstrated a progressive decline in motor activity, similar to what we had described in PSI-treated rats (16). Six weeks after the end of PSI treatment, TH staining of the midbrain from animals treated with PSI showed a marked reduction in dopaminergic cell bodies and terminals in the SNc and ventral tegmental area as compared with vehicle-treated controls (Fig. 10 and supplemental Fig. S1). Quantitative assessment using manual counting methods showed an approximate 45% reduction in dopamine neurons in the SNc in PSI-treated animals compared with controls (Fig. 10B).

We used immunoblotting and fluorescent immunostaining to examine the levels of phosphorylated p53 in the midbrain of vehicle and PSI-treated animals at 1 week following the cessation of treatment (Fig 11). There was a marked increase in phosphorylated p53 predominantly in TH-positive neurons in the SNc, but not elsewhere in the brains of PSI-treated mice compared with controls (Fig. 11, B and D, and supplemental Fig. S2). In contrast, there was increased immunostaining for phosphorylated ERK in the substantia nigra reticulata (SNr) and in non-TH-staining neurons in the SNc of PSI-treated mice (Fig. 11, C and E). As evidenced from the TH immunostaining, increased phospho-p53 accumulation in the SNc occurred prior to the degeneration of DA neurons in the midbrain. These results suggest that activation of p53 in TH-positive neurons mediates degeneration of dopaminergic neurons in response to proteasome inhibition in vivo. Further, up-regulation of an ERK-mediated pathway, which is cytoprotective (29), could underlie the sparing of non-TH neurons in the SNc and SNr found in PSI-treated animals (Fig. 11, C and E, and supplemental Fig. S3).
The in vitro and in vivo demonstration that up-regulation of a p53 signaling pathway mediates cell death following proteasomal inhibition prompted us to examine p53 in the SNc of PD patients. We used quantitative Western blot to measure the levels of phospho-p53 in the SNc and cerebellum of five clini-


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In the present study, we show that proteasome inhibition induces degeneration of cultured PC12 cells and dopaminergic neurons derived from embryonic stem cells. Neuronal death in these models occurred at least in part by way of apoptosis as evidenced by increased mitochondrial permeability, activation of caspase-3, and nuclear condensation. Prior to the emergence of these changes, we noted the early accumulation of Ser15-phosphorylated p53 primarily in the cytoplasm. Increased p53 was not associated with an increase in mRNA, suggesting that the accumulation was secondary to impaired proteasomal degradation and not transcriptional up-regulation. Further there was no increase in mRNA for Puma or Bax, and co-immunoprecipitation studies show that accumulated p53 was bound to Bcl-xL, a mitochondrial permeabilization and apoptosis (26, 27). In this study, proteasome inhibition-induced cell death in PC12 cells was associated with an increase in phosphorylated p53 levels, and cell death could be prevented by p53 inhibitors such as PFT-α or RNA interference. These findings suggest that p53 plays a central role in the cell death process. However, we did not find evidence of an increase in p53 mRNA, suggesting that increased levels of p53 in this model occur through a nontranscriptional mechanism. Indeed, phosphorylated p53 was primarily localized to the cytoplasm and not the nucleus. Further, co-immunoprecipitation studies demonstrated that p53 was associated with Mdm2, whereas p53 must be dissociated from Mdm2 to be stabilized and to translocate to the nucleus to induce p53-dependent gene transcription. It is interesting that p53 was not ubiquitinated even though it was associated with Mdm2, and ubiquitin levels in the cells were significantly increased. This could reflect post-translational changes to p53 and/or Mdm2 or a complex variety of protein interactions that might have occurred as a consequence of proteasomal dysfunction and that interfered with normal ubiquitination. We propose that p53 accumulation occurred because of proteasome inhibition and the inability to clear the protein rather than by transcriptional up-regulation.

Features of mitochondrial permeability, reduced levels of cytochrome c, activation of caspase-3, and condensed nuclei indicate that cell death induced by proteasome inhibition occurred, at least in part, by way of apoptosis. However, there was no increase in mRNA expression of pro-apoptotic genes such as Puma or Bax, suggesting that cell death in this model was not mediated through transcriptional up-regulation of p53-dependent genes. Consistent with the up-regulation of Mdm2 RNA, proteasome inhibition caused transcriptional activation of p53-luciferase reporter construct. However, the capacity for PSI to induce cell death was unaffected by inhibition of transcription. Although PSI caused p53 to activate transcription, p53 did not activate pro-apoptotic transcripts such as Puma and Bax. A previous study has suggested that PFT-α protects cells from apoptosis by suppressing the transcriptional effects of p53 (39). Our results indicate that PFT-α is also protective against a transcriptionally independent p53-mediated apoptosis. These findings suggest that PFT-α may also suppress nontranscriptional pro-apoptotic activity of p53, possibly acting through other signal transduction pathways besides p53 (40).

p53 has also been reported to cause non-neuronal cell death in a nontranscriptional manner by directly binding to mitochondrial membrane proteins and promoting mitochondrial permeabilization with apoptosis (26, 27). In this context, it is noteworthy that accumulated p53 was primarily localized to the cytoplasm and bound to Bcl-xL, a mitochondria...
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...this is of interest that nontranscriptional p53-mediated cytotoxicity has not previously been described in dopaminergic cells.

In these studies, we also demonstrated that the proteasome inhibitor PSI induced degeneration of dopaminergic neurons in the SNc of mice. These findings are consistent with our previous reports indicating that proteasome inhibitors induce a progressive model of PD in rats (16, 19). Although some laboratories have failed to reproduce the model (41–43), others have succeeded in developing at least some characteristics of the model (17, 18). In the rat model, neuronal death caused by proteasome inhibition occurred by apoptosis as evidenced by an increase in caspases-3 activation and the appearance of nuclear condensation in dopaminergic neurons in the SNc. In the present study, we observed that proteasome inhibition was associated with increased levels of phosphorylated p53 that was primarily localized to the cell body and neuronal processes of dopaminergic neurons and appeared prior to evidence of dopaminergic cell loss. Further, we observed increased staining for phosphorylated ERK in non-TH staining cells of the SNC and SNR. The ERK pathway has been linked to a protective response (29, 44) and could relate to why these cells are relatively spared in the proteasome inhibition model and in PD (1, 16, 45). In contrast, activated p53 primarily accumulates in TH-positive cells, which are prone to degenerate following exposure to proteasome inhibitors and in PD.

The mechanism leading to neuronal death in PD is unknown and remains the subject of intense investigation. In this study, we demonstrate increased levels of phosphorylated p53 in the SNc and suggest that p53-mediated apoptosis may play a role in the cell death process. There are several reasons that could account for increased levels of p53 in PD. We have previously shown that oxidative stress can induce a transcriptional increase in p53 (20), and there is evidence of oxidative stress in the SNc in PD (46). Additionally, α-synuclein aggregation is associated with increased levels of p53, although the mechanism whereby this occurs is not understood (47). It is also possible that p53 accumulation in PD could result from proteasomal dysfunction, as we have observed in vitro and in vivo. Based on these experiments, we speculate that failure of the ubiquitin proteasome system to clear p53 may lead to its nontranscriptional accumulation and consequent mitochondrial damage and apoptotic cell death. This concept has implications for PD and other neurodegenerative disorders and suggests novel targets for neuroprotective therapies.

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