FIG. 5. Tissue distribution of parkin and Glup protein. Mouse various tissue samples were analyzed with Western blot for parkin, Glup, and Hsc70.

.dominant one is a small neuroblast-like cell and the other is a large hyaline fibroblast-like cell (31). GOTO-P3 has been established in tissue culture from a human neuroblastoma arising from the adrenal grand. The cells of GOTO-P3 are small and fibroblast-like (26). SH-SY5Y is a thrice-cloned subline of bone marrow biopsy-derived line SK-N-SH, both epithelial-like and neuroblast-like morphologies have been reported (2, 3, 13). In our hands, SH-SY5Y (H) cells are epithelial-like, whereas SH-SY5Y (J) cells demonstrate small neuroblast-like. Neuro2a is a mouse neuroblastoma line established from the spontaneous tumor of albino mouse strain. The cells are neuronal and ameboid-like (24).

The presence of conflicting data regarding different parkin alteration upon ER stress in the "same" SH-SY5Y cells prompted us to further characterize two SH-SY5Y cell lines, which we obtained from two different laboratories. Actually, these two cell lines demonstrated different properties among manipulation. SH-SY5Y (H) but not SH-SY5Y (J) responded to ER stress by upregulation of parkin. On the other hand, SH-SY5Y (H) was more refractory to induced differentiation compared with SH-SY5Y (J), strongly suggesting that SH-SY5Y (H) and SH-SY5Y (J) are different in their properties. It is thus conceivable that conflicting data observed previously in SH-SY5Y cells might be due to different properties of SH-SY5Y cells, possibly acquired in the process of maintenance in individual laboratories.

It remains unclear how ER stress causes upregulation of parkin in a subset of these neuroblastomas with neuronal properties. It is noted that upregulation of parkin was not observed in IMR32 as well as in SH-SY5Y (J) cells. Like SH-SY5Y (J) cells, IMR 32 has been demonstrated readily to differentiate into dopaminergic neuronal phenotype, since TH proteins were already detectable upon addition of TPA for only 3 days (19). Morphologically, parkin appeared to be upregulated in predominantly fibroblast-like SH-SY5Y (H), GOTO-P3, and Neuro2a cells, but not in predominantly neuroblast-like SH-SY5Y (J) and IMR32 cells. Consistent with the data from Ledesma et al. (18), our current experiments suggest that upregulation of parkin may not occur in response to ER stress in neurons.

It is likely that certain cell-type specific ER stress related molecules affect parkin expression. Recently it has been reported that OASIS (old astrocyte specifically induced substance) is a novel ER stress transducer that specifically regulates UPR signaling in astrocytes (7, 17). Although we examined whether OASIS is responsible for the cell type-

specific upregulation of parkin upon ER stress, only negative results were obtained (data not shown). Although parkin and Glup share the same promoter, the tissue distribution patterns of these proteins are different, suggesting the presence of cell type- or tissue-specific regulators for parkin and Glup protein expression (Fig. 5). Further work is necessary to examine the mechanisms whereby the upregulation of parkin occurs specifically in certain types of cells upon ER stress.

ACKNOWLEDGMENTS

ABBREVIATIONS

AR-JP, autosomal recessive juvenile parkinsonism; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER associated degradation; FCS, fetal calf serum; Glup, gene adjacent to parkin; 2-ME, 2-mercaptoethanol; Mn, manganese; OASIS, old astrocyte specifically induced substance; PACRG, parkin coregulated gene; PCR, polymerase chain reaction; PD, Parkinson's disease; RT, reverse transcript; TH, tyrosine hydroxylase; TPA, 12-O-tetradecanoylphorbol 13-acetate; UPR, unfolded protein response.

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Forum Review

Expanding Insights on the Involvement of Endoplasmic Reticulum Stress in Parkinson's Disease

HUA–QIN WANG and RYOSUKE TAKAHASHI

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies. The pathogenesis of PD remains incompletely understood. Environmental factors, oxidative damage, misfolded protein aggregates, ubiquitin–proteasome system impairment, and mitochondrial dysfunction might all be involved. Recent studies point to activation of endoplasmic reticulum (ER) stress-mediated cell death linked to PD. Accumulation of unfolded and/or misfolded proteins in the ER lumen induces ER stress. To withstand such potentially lethal conditions, intracellular signaling pathways collectively termed the unfolded protein responses (UPR) are activated. The UPR include translational attenuation, induction of ER resident chaperones, and degradation of misfolded proteins through the ER-associated degradation. In case of severe and/or prolonged ER stress, cellular signals leading to cell death are activated. Accumulating evidence suggests that ER stress induced by aberrant protein degradation is implicated in PD. Here the authors review the emerging role of ER stress in PD and related disorders, and highlight current knowledge in this field that may reveal novel insight into disease mechanisms and help to provide novel avenues to potential therapies. *Antioxid. Redox Signal.* 9, 553–561.

INTRODUCTION

PARKINSON'S DISEASE (PD) is the most common neurode-generative movement disorder among elderly people. The classical symptoms of the disease include rigidity, resting tremor, bradykinesia, and postural instability. The pathological hallmarks underlying the clinical phenotypes are characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), together with the presence of intraneuronal inclusions termed Lewy bodies (7). Although the molecular mechanisms underlying neurodegeneration remain elusive, its pathogenesis begins to be considered as a multifactorial cascade of deleterious factors. Mitochondrial dysfunction, protein aggregation, impairment of the ubiquitin–proteasome system (UPS), and activation of the stress kinase signaling pathways have been supposed to be involved in the pathogenesis of PD. Recently, emerging lines of evidence from familial forms of PD, coupled with those findings from toxin-induced PD

models, raise the possibility of widespread involvement of unfolded protein responses [UPR, also known as endoplasmic reticulum (ER) stress responses], the term given to an imbalance between the cellular demand for ER function and ER capacity (2, 43, 44), in the pathogenesis of this disease.

Neuronal loss in both familial and sporadic forms of neurodegenerative disorders is often accompanied by formation of inclusion bodies and aggregation of misfolded proteins (45). Upregulation of ER stress markers has been observed in postmortem brain tissues and cell culture models of many neurodegenerative diseases including PD, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and expanded polyglutamine diseases such as Huntington's disease and spinocerebellar ataxias (4, 26). Several chaperones ameliorate the accumulation of misfolded proteins triggered by oxidative or nitrosative stress, or of mutated gene products (26, 40, 58). The hypothesis that ER dysfunction plays an important role in the development of dopaminergic neuronal

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loss in PD has recently been put forward by observations that parkin has been associated with ER stress-induced cell death. Mutations in the *PARK2* gene coding for parkin cause autosomal recessive juvenile Parkinsonism (AR-JP), the most common form of familial PD. This review summarizes new observations implying that impairment of ER functioning is a common denominator of neuronal death in PD.

ER STRESS AND ER STRESS RESPONSES

Besides calcium storage and signaling, a central function of the ER is quality control for membrane or secretory proteins, which comprise nearly one-third of all cellular proteins (29). The importance of the ER for normal cell function is highlighted by the observation that blocking of the protein folding or processing reactions can be lethal for cells. Indeed, in various cases such as depletion of ER calcium stores, blocking the proteasome that is required for degradation of unfolded proteins, or genetic mutations resulting in proteins that cannot be properly folded, the ER functions are impaired

and unfolded proteins accumulate in the ER. Accumulation of unfolded proteins in the ER is a severe form of stress that will induce apoptosis if ER function cannot be restored. To cope with conditions associated with impairment of ER function, cells activate highly conserved stress response, the UPR (2, 43, 44). The main purpose of UPR is to remove aberrant substrates and restore the ER to an efficiently operating maturation compartment. The UPR pathway functions as a tripartite signal that comprises (i) inhibition of general translation to attenuate the load of proteins to the ER, (ii) transcriptional activation of ER chaperones to increase protein folding and processing capacity; (iii) activation of ER-associated degradation (ERAD) to promote degradation of terminally misfolded proteins. However, when the ER stress is severe or prolonged, the cells eventually activate apoptotic signals, leading to cell death (5, 29) (Fig. 1).

Cells have developed two pathways for removing unfolded proteins from the lumen of the ER, increasing folding capacity through upregulation of ER chaperones (Fig. 2A) and promoting degradation of terminally misfolded proteins through activation of ERAD (Fig. 2B). The ERAD pathway is
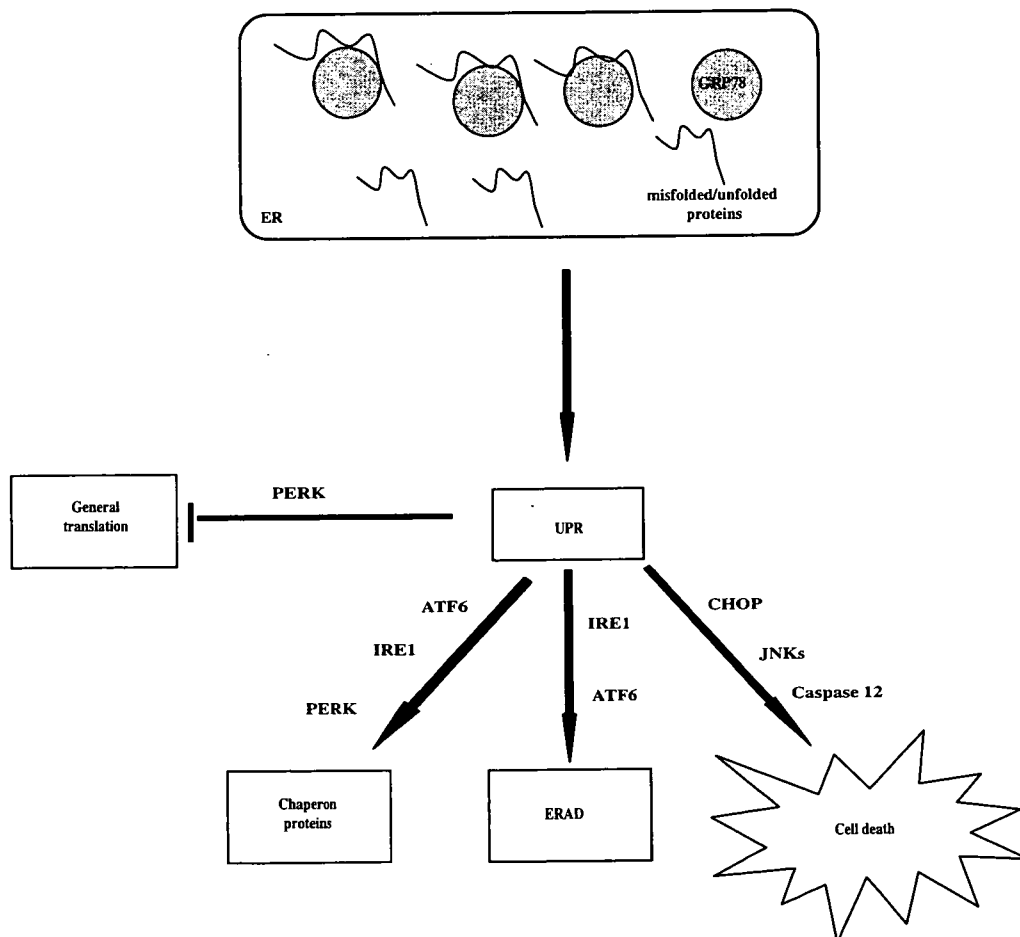
FIG. 1. The tripartite unfolded protein response. Three primary transducers of the unfolded protein response (UPR) signal, known as ATF6, IRE1, and PERK, seek to relieve ER stress through suppression of translational initiation, increased folding capacity of ER, and degradation of terminally misfolded proteins until the aberrations have been alleviated. However, severe or prolonged ER stress eventually activates apoptotic pathway.
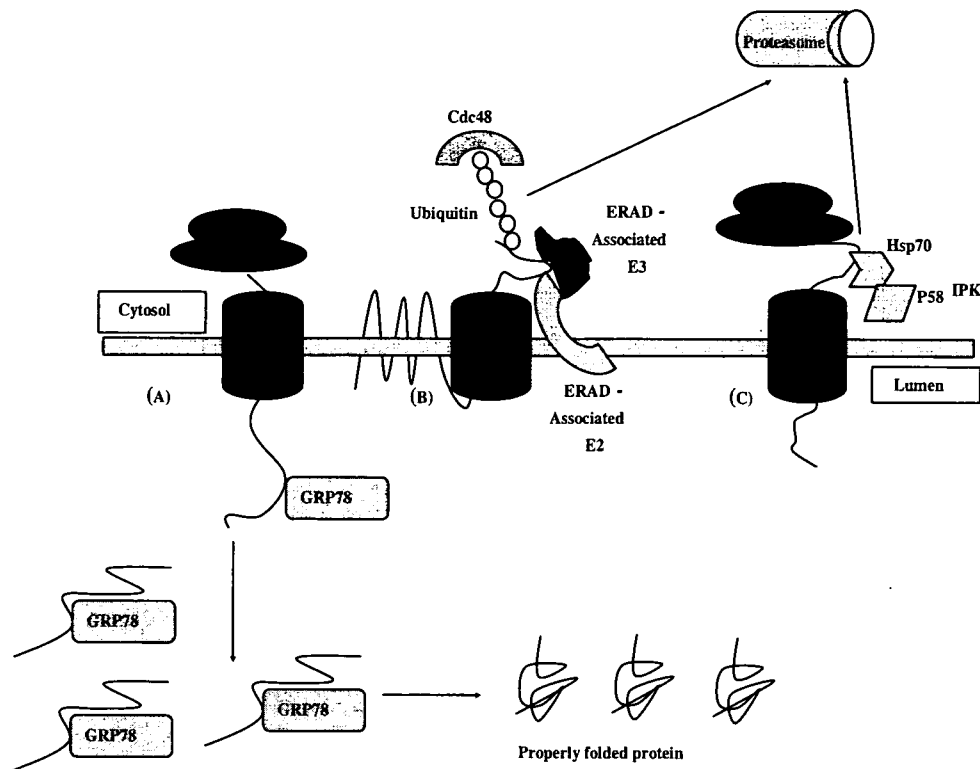
FIG. 2. The pathways related to clearance of unfolded proteins upon ER stress. Under ER stress conditions, cells develop three pathways to clear unfolded proteins in the ER. (A) ER chaperones such as GRP78 are upregulated to facilitate proper substrate folding. (B) Activation of ER associated degradation (ERAD)-mediated degradation of terminally unfolded proteins. (C) Cotranslational degradation of newly synthesized proteins trapped in the Sec61 translocon to decrease load burden to the ER.

characterized by the polyubiquitination and subsequent degradation of misfolded proteins (36–38). With the aid of the cytosolic AAA-ATPase p97/Cdc48, the misfolded ER protein is extruded through the ER membrane conduit Sec61, where it is then polyubiquitinated and delivered to the proteasome for degradation in the cytosol (Fig. 2B).

A recent study has revealed a new layer in the UPR pathway that permits the cotranslocational degradation of secretory proteins involving P58^{IPK}/DNAJC3, which collaborates with cytosolic chaperone networks and appears to assist in the co-translational/translocational degradation of nascent polypeptide chains that are stalled in ER translocons (Fig. 2C). This function diminishes the biosynthetic burden on the ER by degrading proteins at a stage earlier than previously envisioned. This protective effect might reflect a reduction in protein flux into the stressed ER's lumen. Alternatively, intervention early in the protein biogenesis by P58^{IPK}/DNAJC3 might allow the maturation and quality control machinery to focus its attenuation on the pre-existing improperly folded proteins that triggered the initial UPR signal (34).

ER STRESS IN FAMILIAL FORMS OF PD

As in most cases of PD, the degeneration is idiopathic, the etiology of the disease remains unknown. The recent identification of genetic mutations in familial cases of PD has advanced

our understanding of the molecular mechanisms that cause the neurodegeneration. So far, six PD-associated genes have been identified.

Autosomal dominant forms of PD

Three rare missense mutations in the α-*synuclein* gene (A30P, E46K, and A53T) cause autosomal dominant familial PD (23, 39). Although the function of α-synuclein is still unclear, the discoveries that α-synuclein is a main component of Lewy bodies (48) and that its overexpression and gene triplication can cause neurodegeneration (1, 46) suggest that abnormalities of α-synuclein might be crucial for the pathogenesis of both familial and sporadic forms of PD. α-Synuclein transgenic mouse or *Drosophila* at least partially recapitulated PD phenotype including α-synuclein positive aggregate formation, although no obvious dopaminergic neuronal loss was observed in transgenic mice (12, 49). Lentivirus-mediated overproduction of α-synuclein in rat substantia led to significant cell death (27). Leucine-rich repeat kinase 2 (LRRK2) has recently been added to the list of genes that are implicated in autosomal dominant PD (35, 59). LRRK2 is a GTP/GDP-regulated protein kinase, and increased kinase activity appears to be implicated in neurodegeneration (47). Another gene, ubiquitin carboxyl-terminal esterase L1 (UCHL1), has been associated with the dominantly inherited disease, but the genetic evidence for its pathogenicity is not established since only a single mutation with low penetrance has been identified in one family (39).

Autosomal recessive forms of PD

Three recessive forms of parkinsonism have been identified, including mutations in the genes that encode parkin, DJ1, and PTEN-induced kinase 1 (PINK1).

Mutations in the *parkin* gene were originally discovered from the linkage study of Japanese AR-JP families, the most frequent type of familial PD (20). Thereafter its mutations have been found worldwide. Parkin is a 465 amino acid protein characterized by a ubiquitin-like domain at its NH2-terminus, as well as two RING (really interesting new gene) finger domains flanking a domain known as the IBR (in-between RING) at its COOH-terminus (RING–IBR–RING). Like many other proteins containing a RING domain, parkin has been found to function as an ubiquitin ligase (E3) (Fig. 3). E3s are part of the cellular machinery that tags proteins with ubiquitin, thereby targeting them for degradation by the proteasome. The UPS plays a major role in many vital cellular processes, and its dysfunction has been implicated in the pathogenesis of neurodegenerative disorders including sporadic PD. Parkin mutants associated with AR-JP reduce or abolish its E3 activity. Therefore, the most straightforward mechanism by which the dysfunction of parkin would cause neurodegeneration is accumulation of some neurotoxic substrate protein(s), which leads to dysfunction and eventually the death of susceptible neurons.

Mutations in *PINK1* were initially identified in three large consanguineous families with autosomal recessive forms of PD (52). Mutations in *PINK1* have differential effects on protein stability, localization, and kinase activity (3). As the kinase domain is the hot spot of mutations, disruption of the kinase activity is the most probable disease mechanism. Although functional data are limited, wild-type PINK1 protected neurons from mitochondrial dysfunction and apoptosis induced by oxidative stress (11), supporting an involvement of mitochondria in the pathogenesis.
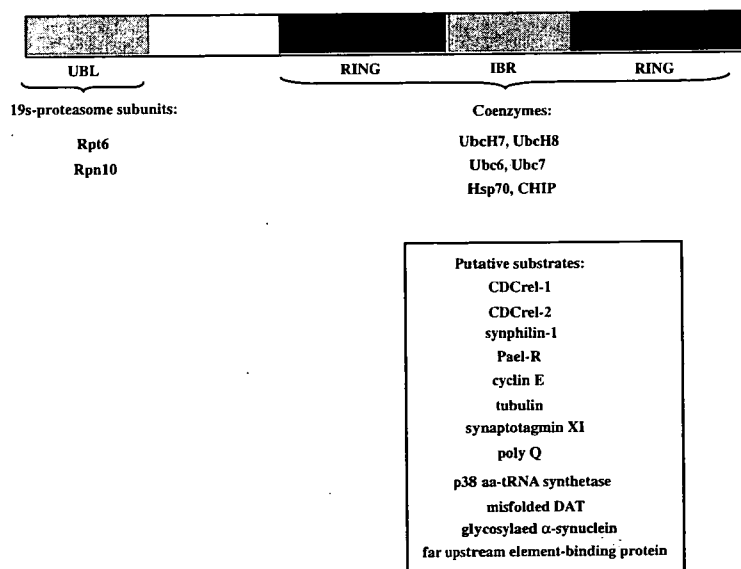
A third gene linked to recessively inherited albeit rare PD is *DJ-1* (6). DJ1 has been assigned various functions, but perhaps the most relevant function in terms of the pathogenesis of PD is its potential role in oxidative stress response, either as a redox sensor or antioxidant protein (8).

In this review, rather than attempting to overview the entire picture, we focus on potential involvement of ER stress in this disease according to published data.

ER stress in α-synuclein-associated PD

In a *Drosophila* model of PD engineered to express wild-type and mutant α-synuclein, expression of molecular chaperone heat shock protein 70 (Hsp70) prevented dopaminergic cell loss mediated by accumulation of α-synuclein (1). However, Hsp70 is not directly activated in the UPR. A recent study provided direct evidence indicating the implication of ER stress in α-synuclein-mediated cell death (47). In a mammalian cell culture model, induction of the expression of A53T α-synuclein induced ER stress, as evidenced by the elevation in expression of CHOP and GRP78, increased phosphorylation of eIF2α, and activation of caspase-12. Furthermore, decrease of eukaryotic initiation factor 2α (eIF2α) phosphorylation by inhibitor, or knockdown of caspase-12 levels by RNA interference partially protected against cell death (47), indicating that ER stress at least partially contribute to A53T α-synuclein-induced cell death. Overexpression of mutant forms of α-synuclein in cultured neuronal cells leads to decrease in proteasome activity (51). The mechanism underlying mutant α-synuclein-induced impairment of proteasome activity remains to be identified. α-Synuclein is reported to be degraded through several different pathways including macroautophagy, chaperone-mediated autophagy, and proteasome (10, 50, 53). Since α-synuclein interacts with a subunit of proteasome regulatory complexes (15), it is possible that mutant α-synuclein directly affects the proteasome complex. It is of interest that an important means of removing misfolded proteins from the ER is their degradation by proteasomes. In addition, it has been reported that partial inhibition of the proteasome activity by poly-Q was sufficient to cause ER stress in primary neurons (33). Therefore, ER stress observed in overexpression of mutant forms

UBL		RING	IBR	RING	

19s-proteasome subunits:	Coenzymes:
Rpt6	UbcH7, UbcH8
Rpn10	Ubc6, Ubc7
	Hsp70, CHIP

Putative substrates:
CDCrel-1
CDCrel-2
synphilin-1
Pael-R
cyclin E
tubulin
synaptotagmin XI
poly Q
p38 aa-tRNA synthetase
misfolded DAT
glycosylaed α-synuclein
far upstream element-binding protein

FIG. 3. Modular structure of parkin. Parkin has a modular structure, containing a ubiquitin-like (UBL) domain at the amino-terminus and two real interesting new gene (RING) fingers at its carboxy-terminus. In addition, an in-between RING (IBR) domain is inserted in the middle portion between two RING finger motifs. The two RINGs and IBR are named as a RING box. Furthermore, the linker region is located between UBL and RING box. The UBL binds to 19S proteasome subunits, and the RING–IBR–RING domain binds to specific co-enzymes and substrates (except for glycosylated α-synuclein, which binds to the UBL domain).
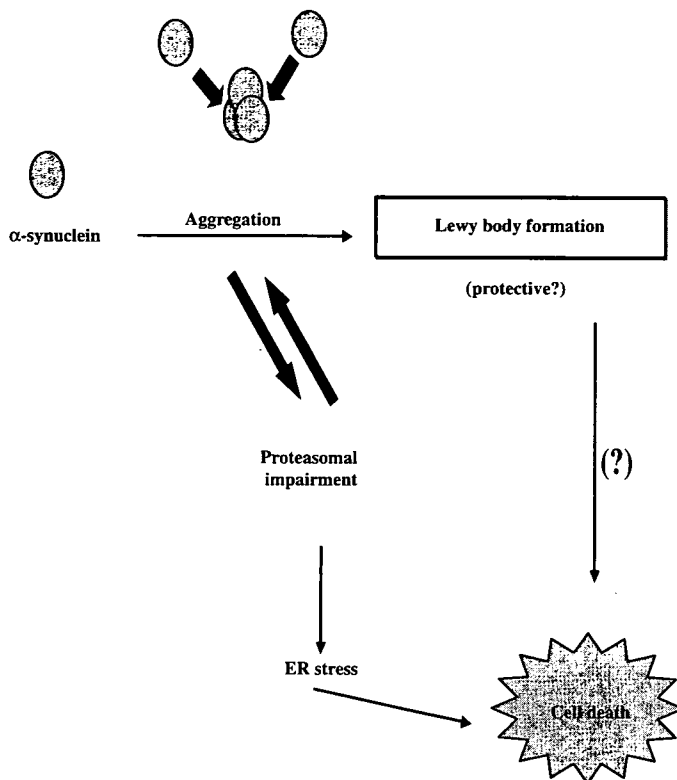
FIG. 4. Hypothetical mechanism of ER stress-mediated cell death induced by α-synuclein. The insidious loop feedback between α-synuclein aggregation and proteasomal impairment induces ER stress, which at least in part contributes to α-synuclein-mediated cell death.

of α-synuclein in cultured neuronal cells possibly derive from disruption of proteasome activity (Fig. 4).

## ER stress in parkin-associated PD

Evidence supporting the involvement of ER stress in parkin mutations-induced cell death is more potent. Parkin is an E3 enzyme that interacts with Hsp70 and CHIP and plays a general role in protein degradation during ER stress (17–19). In line with this notion, cognate E2 (ubiquitin conjugating enzyme) partners of parkin include Ubc6 and Ubc7, which are ER-associated E2s involved in ERAD, indicating parkin is a component of ERAD machinery. It is easily conceivable that disruption of parkin function directly leads to ER stress, since ERAD and ER stress are coordinately regulated and deletion of ERAD components results in ER stress (13).

Given that accumulation of the substrate of parkin might play a key role in the neurodegenerative process, identification of parkin substrates has therefore been a major focus of many laboratories working on parkin. Typically, one expects an E3 to be highly specific for one or possibly a small number of substrates. Unexpectedly, a large number of putative parkin substrates have been reported (Fig. 3). Interestingly, several parkin substrates are misfolded or aggregation-prone proteins and are components of Lewy bodies. Considering that misfolded proteins, associated molecular chaperones, and proteasomal subunits are accumulated in Lewy bodies, the substrates of parkin may represent a subset of misfolded proteins. The C-terminus of Hsc70 interaction protein

(CHIP), a U-box containing E3, has been shown to recognize misfolded protein through the heat-shock protein Hsp70 and is proposed to be a "quality control E3" that is contributed to the clearance of misfolded proteins (31). Given that parkin also binds to Hsp70, parkin may have a similar function to CHIP in dealing with misfolded proteins.

Among these substrates of parkin, one of the best-characterized parkin substrates, Pael-R underscores the ER stress-mediated cell death in the pathogenesis of AR-JP (18). Pael-R is a multipass G protein-coupled transmembrane protein with homology to the endothelin receptor type B, the function of which is unknown. Folding of Pael-R is a formidable challenge to cells. When overexpressed in cultured cells, Pael-R tends to become unfolded and insoluble; at the early stage of Pael-R accumulation, ER chaperones showed transcriptional upregulation, indicating that accumulation of Pael-R actually induced ER stress. Interestingly, CHIP serves as a cofactor of parkin. When Pael-R misfolding exceeds the cellular chaperone capacity, CHIP is upregulated, which sequesters Hsp70 and facilitates parkin-mediated ubiquitination of Pael-R (17). Under these conditions, parkin apparently acts as part of the ERAD machinery, utilizing the ER associated E2 enzymes Ubc6 and Ubc7 as the collaborating partners.

The UPR induces upregulation of parkin mRNA per se, and cells overexpressing parkin, but not mutant parkins found in AR-JP patients, are particularly resistant to unfolded protein-induced cell death (19). Furthermore, when astrocytes and neurons were exposed to conditions associated with ER stress, parkin protein levels were upregulated in astrocytes
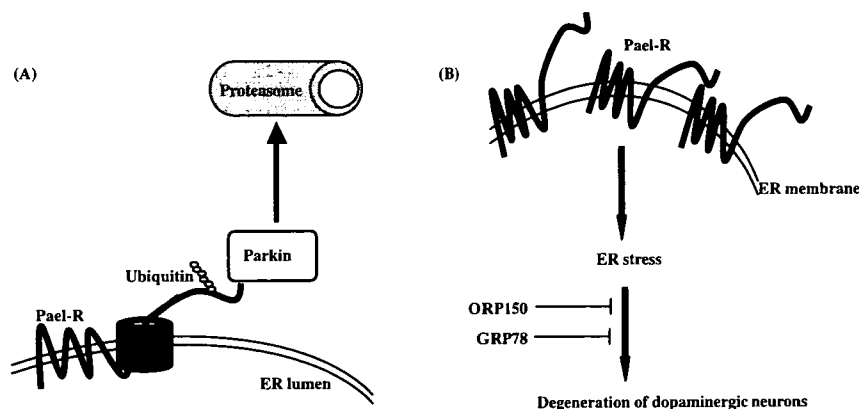
FIG. 5. Implication of ER stress in Pael-R-induced neuronal degeneration. (A) Pael-R is a difficult protein to be folded, and parkin ubiquitinates misfolded Pael-R and facilitates its degradation by UPS. (B) When dysfunction of parkin or overexpression of Pael-R, unfolded Pael-R accumulates in the ER and causes ER stress. ER chaperones, GRP78 and ORP150, suppress Pael-R-induced neuronal degeneration, possibly through enhancing the folding capacity of the ER.

but not in neurons (25). In the brain, Pael-R is primarily expressed in oligodendrocytes and shows little expression in neurons, except for a few distinct subpopulations of neurons, including hippocampal neurons and dopaminergic neurons in the SNpc. This implies that dopaminergic neurons of patients suffering from AR-JP are less well protected from neurotoxicity arising under conditions of Pael-R accumulation-induced ER stress. Thus, the inability of neurons to respond to ER stress by activating the expression of parkin and distributional pattern of Pael-R may contribute to the high vulnerability of dopaminergic neuronal cells. Using a transgenic Drosophila expressing human Pael-R, Yang et al. (55) found that this fly model revealed the age-dependent selective degeneration of dopaminergic neurons in spite of pan-neuronal expression of Pael-R. This Pael-R mediated dopaminergic neuronal loss was suppressed by the coexpression of human parkin and exacerbated by knockdown of endogenous parkin in the Drosophila by RNA interference. Recent in vivo observations in mice further highlights the important role of ER stress in Pael-R-mediated toxicity (21). Adenovirus-mediated overexpression of Pael-R in dopaminergic neurons induced ER stress and degeneration. This Pael-R-mediated neuronal death was suppressed by increased GRP78 or oxygen regulated protein 150 (ORP150), whereas cell death was exacerbated by downregulation of parkin or ORP150 (Fig. 5). Furthermore, a complicated interplay between ER stress and dopamine toxicity might present a mechanism underlying Pael-R-induced selective dopaminergic neuronal death, as evidenced by a neuroprotective effect of a tyrosine hydroxylase (TH) inhibitor (21).

CHOP, was dramatically upregulated by these reagents, as well as numerous markers of UPR including GRP78, splicing of XBP1, PERK, and the JNKs pathway. The assumption that ER dysfunction may play a role in the pathological process resulting in PD is corroborated by the observation that exposing cells to 6-OHDA, rotenone, or MPP$^+$, which are cellular models mimicking pathological disturbances associated with PD, induces a striking increase in transcripts associated with UPR (16, 42, 54). A number of reports have shown that both proteasome inhibition and ROS can trigger ER stress-mediated cell death pathways. One possible mechanism of ER stress induced by these mitochondria toxins is that accumulation of damaged oxidized proteins by the effects of these reagents on mitochondrial respiration causes ER stress (13). Alternatively, oxidative stress can directly compromise proteasomal components (41). However, the oxidative stress caused by the effects of these agents on mitochondrial respiration may not be totally attributable since a nonselective oxidant does not trigger ER stress. In addition, neurons lacking expression of PERK are defective in ER response and are significantly more sensitive to the death-promoting effects of PD mimetics (42). Thus, not only do mitochondria toxins provoke ER stress, but neurons lacking the capacity to deal with this by inducing an appropriate UPR are at greater risk of death, suggesting that ER stress is likely to play a causative role in neuronal cell death induced by these mitochondria toxins. Coupled with evidence from familial forms of PD, the induction of UPR and ER stress in these generally used neurotoxin models raise the possibility of widespread involvement of ER stress-mediated cell death in the pathogenesis of PD and other related disorders (Fig 6).

## ER STRESS IN TOXIN-INDUCED PD MODELS

Mitochondria toxins, 6-OHDA, rotenone, and MPP$^+$, are believed to contribute to dopaminergic neuronal death. These reagents can promote the generation of reactive oxygen species (ROS) via the inhibition of mitochondrial complex I or their oxidative function (14). Using functional genomics approaches to identify transcriptional alterations, numerous changes in genes associated with UPR were identified (42). Notably, a major target of the UPR pathway, the transcription factor

CROSSTALK OF ER STRESS WITH OXIDATIVE STRESS IN PD

PD has been closely associated with oxidative stress and mitochondrial dysfunction. In addition, dopaminergic neurons are particularly subjected to increased oxidative stress due to production of free radicals during dopamine autooxidation and dopamine metabolism (24). ER stress is intricately connected to oxidative stress. As described above, oxidative stress can directly or indirectly induce ER stress (13, 41). Evidence is also accumulating for a converse
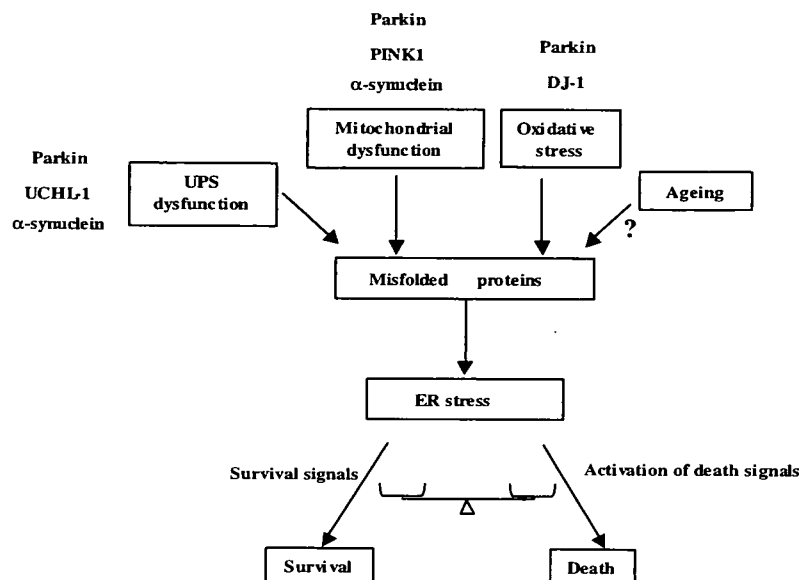
FIG. 6. Widespread involvement of ER stress in the pathogenesis of Parkinson's disease. In Parkinson's disease, proteasome dysfunction, oxidative stress, mitochondrial dysfunction, and possibly aging could directly or indirectly cause accumulation of misfolded proteins in the ER, thus induce ER stress. Cells counteract ER stress by activation of unfolded protein responses (UPR), which activates protective signals to eliminate misfolded proteins. However, when UPR fails to eliminate misfolded proteins, cells undergo apoptosis.

mechanism, whereby ER stress can result in secondary oxidative damage. One target of CHOP is ER oxidoreductase ERO1α, which participates in protein disulfide bond formation during protein refolding in the ER to help relieve ER stress, but in doing so also promotes production of ROS (28). The interplay between ER stress and oxidative stress might be mediated in part by parkin. Parkin is itself sensitive to oxidative stress, and is inactivated by nitric oxide-mediated nitrosylation or dopamine, which could lead to a simultaneous ER stress and oxidative damage (9, 24, 56). Inactivation of parkin is likely to create a feed-forward amplification loop, rendering dopaminergic cells more susceptible to oxidative and ER stress.

CONCLUSIONS

Evidence has been presented in various experimental studies that impairment of ER function may be involved in the neuronal cell death in PD. Environmental toxins, oxidative damage by dopamine itself, and mitochondrial abnormalities are all believed to play a role in sporadic PD (30). All these affect protein folding in the cytoplasm and lead to ER stress by compromising the process of ERAD (4). Alternatively, a genetic defect such as parkin mutation could impair the ability of cells to adapt to ER stress through impairment of its E3 activity. Since some novel components of the canonical UPR are expressed in a cell type-specific fashion, different types of cells may have unique responses for adaptation to ER stress (22, 32, 57). It is noted that parkin is upregulated in astrocytes, but not in neurons upon ER stress (25), suggesting parkin may represent as another unique response for adaptation to ER stress. Further investigation of parkin regulators will improve our chances of identifying novel targets for designing effective therapeutic strategies to impede the pathological processes.

ABBREVIATIONS

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AR-JP, autosomal recessive juvenile Parkinsonism; bZIP, basic leucine zipper; CHIP, C-terminus of Hsc70 interaction protein; CHOP, C/EBP homologous protein; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERO1α, ER oxidoreductase 1α; GRP78, glucose regulated protein 78; Hsp70, heat shock protein 70; IBR, in-between RING; JNK, c-Jun NH2-terminal kinase; Pael-R, parkin associated endothelin like-receptor; ORP150, oxygen regulated protein 150; PINK1, PTEN-induced kinase 1; PD, Parkinson's disease; RING, really interesting new gene; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; UPR, unfolded protein response; UPS, ubiquitin–proteasome system.

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ELSEVIER

Capillary beds are decreased in Alzheimer's disease, but not in Binswanger's disease

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Abstract

Morphological abnormalities of the cortical microvessels have been reported in Alzheimer's disease (AD), but not in Binswanger's disease (BD), a form of vascular dementia. Therefore, we compared the capillary beds in AD and BD brains, using a modified Gallyas silver impregnation method and immunohistochemistry for β amyloid. Eight autopsied brains with AD and seven with BD were compared with six control brains. The cortical microvessels in AD were frequently narrowed, and torn off, especially in close proximity to the senile plaques. The capillary densities in AD were significantly decreased as compared with the control brains. In contrast, there were no significant changes in the capillary densities and their morphologies in BD brains. Immunohistochemistry for β amyloid revealed numerous deposits in the vascular wall and perivascular neuropil exclusively in AD brains. Cortical microvascular changes in AD and their absence in BD may indicate a role of β amyloid for the microvessel pathology in AD.
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Keywords: Microvessel; Alzheimer's disease; Vascular dementia; Binswanger's disease; Silver impregnation; Amyloid protein

Alzheimer's disease (AD) and vascular dementia are major causes of dementia and disabilities in the elderly. These two conditions have been believed to have an independent pathoetiology. However, in recent studies, co-morbid factors have been revealed in AD and vascular dementia [10,12]. These factors include hypertension, diabetes mellitus, hyperlipidemia, apo E4 ε genotype, cholinergic deficits, and white matter lesions. In addition, patients with vascular lesions reportedly develop dementia more frequently than those without vascular lesions among those subjects with senile changes [18]. Taken together, this evidence has shed light on the interrelationship between AD and vascular dementia, and raised the hypothesis that vascular factors may have a role in the pathogenesis of AD. In concordance with this hypothesis, previous electron microscopic studies have reported thickening of the basement membrane, denervation of the perivascular nerves, and bulging or narrowing of the cortical microvessels in AD brains [14,17].

Binswanger's disease (BD) is a form of vascular dementia, featured by diffuse white matter lesions, lacunar infarcts and fibrohyaline thickening of the microvessel [13]. Fibrohyaline thickening of the microvessels is marked in BD, and significant but less severe in AD in the cerebral white matter [19]. However, with respect to the cortical microvessels, there are no studies in BD. Therefore, we aimed to compare the alterations of the cortical microvessels in AD and BD using a modified Gallyas silver impregnation method and immunohistochemistry for β amyloid, which enable us to examine the network of the brain capillaries, and senile plaques.

We examined 21 brains, including 8 from patients with AD (3 males), 7 from patients with BD (4 males), and 6 from patients who did not have any neuropsychiatric symptoms or brain lesions (3 males). The age was 79 ± 12 years (mean $\pm$ S.D.) in the AD, 74 ± 13 years in the BD and 73 ± 4 in the control groups, respectively, among which no significant differences were observed ($p < 0.05$). The brain weight was 1020 ± 111 g in the AD, 1093 ± 112 g in the BD, and 1244 ± 57 g in the control groups, respectively. The brain weight in the AD group was significantly lower than in the control and BD groups ($p < 0.05$). The patients with AD and BD, but not the control patients, met

the diagnostic criteria for dementia (diagnostic and statistical manual of mental disorders; DSM-IV) [1] in the occasion of diagnosis and, most of these patients suffered in a bed-ridden condition in their terminal stages.

The diagnosis of AD was made based on the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) diagnostic neuropathologic criteria [15] and excluded the brains with large cerebral infarctions. The diagnosis of BD was made clinico-pathologically, and retrospectively met the pathological inclusion criteria including (1) presence of diffuse white matter lesions, (2) lacunar infarctions in the perforator territory, (3) arteriolosclerosis such as fibrohyalinosis and fibrinoid necrosis and (4) absence of cortical infarctions, as well as the clinical criteria by Bennett et al. [3], and excluded the brains with significant pathologic hallmarks of AD. The control group included two patients with pneumonia, one patient each with renal cancer, lung cancer, chronic renal failure and pulmonary emphysema.

The tissue blocks were sectioned in a cryostat (30 or 100 μm-thick) and kept in 0.1 M phosphate buffer (pH 7.4). The tissue sections were processed according to the silver impregnation method described by Gallyas [8]. Briefly, the sections were incubated for 30 min in 5% periodic acid (HIO4) solution. The sections were then immersed in 4% sodium hydroxide solution for 30 min, and washed in 0.5% acetic acid solution in double-distilled water for 5 min. They were further incubated in a mixture (pH 13.0) of 90 ml of an ammoniated silver nitrate solution (AgNO3, 0.5 g and ammonium nitrate, 2.5 g in 900 ml double-distilled water), and 10 ml of 4% sodium dihydroxide in double-distilled water for 30 min at 20 °C. These sections were left in a physical developer solution, which was composed of a mixture of 10 ml of solution A, 5 ml of solution B and 5 ml of solution C at 25 °C. The composition of each solution was as follows; sodium carbonate, 50 g in 1000 ml of distilled water (solution A); ammonium nitrate 1.9 g, silver nitrate, 2.0 g; tungsto-silicic acid (SiO2·2WO3), 10 g in 1000 ml of distilled water (solution B); and ammonium nitrate, 1.9 g; silver nitrate, 2.0 g; tungsto-silicic acid (SiO2·2WO3), 10.0 g; and 6.1 ml of 40% formalin solution in 1000 ml of distilled water (solution C). The reaction was terminated in 0.5% acetic acid solution, and the extent of silver impregnation was monitored intermittently under light microscopy.

For immunohistochemistry, autoclaved paraffin sections were incubated with a mouse anti-amyloid β protein antibody (Dakopatts, diluted 1:200), biotinylated anti mouse IgG (Vector laboratories, diluted 1:200) and an avidin biotinylated peroxidase complex (Vector Laboratories, diluted 1:200). They were finally visualized with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H2O2 in 0.05 M Tris–HCl (pH 7.6). To test for the specificity of the immunohistochemical reaction, control sections were incubated with normal mouse IgG instead of the primary antibody.

The density of the capillary beds was determined by the test grid method [7], in which the number of vascular intersections were counted against 6 × 6 square test grids each with a 50 μm width. The average counts from five representative fields in the layers II–IV of the frontal and parietal cortices, respectively,

were used as the capillary densities in each patient. The data were expressed as means ± S.D. and the Mann–Whitney U-test was used to compare between the groups.

Using the modified Gallyas stains, the microvessels in the cerebral cortices appeared smooth and regular in diameter in the non-neurological control and BD brains (Fig. 1A and C, respectively). There were no senile plaques nor neurofibrillary tangles. In contrast, the brains with AD had numerous senile plaques and neurofibrillary tangles, which were intermingled by irregularly-shaped microvessels in both frontal and parietal cortices (Fig. 1B). The microvessels were frequently narrowed and irregular in diameter for a variable length of the vessel (Fig. 1D–F). These vessels often showed bulging of their walls. In close proximity to the senile plaques, the microvessels were blunted and torn off in the sections with a thickness of 100 μm (Fig. 1E). These microscopic changes were not observed in the non-neurological control and BD groups.

With immunohistochemistry, β amyloid-immunoreactivity was localized in senile plaques which accumulated numerously in the superficial layer, as well as perivascular deposits in the vascular wall itself and perivascular neuropil in the AD group (Fig. 1G–I). Beta amyloid-immunopositive fine texture fibrils were distributed in the neuropil with or without contact to the microvessels. In contrast, there was almost no deposit of β amyloid in the cerebral cortices of the non-neurological control and BD groups. In the semi-quantitative measures of the microvessels, the microvascular densities were significantly lower in the AD group as compared to the other two groups in both frontal and parietal cortices (Fig. 2).

The capillaries in AD have been shown to exhibit thickening of their basement membrane, atrophy, perivascular fibrosis and degeneration of the pericytes [5,6,16], which may correspond to the bulging of the microvessels observed here. In semi-quantitative measures, some authors have not observed any decrease in the capillary densities [2], while others showed a decrease in selected or non-selected regions of AD brains [4,7]. The present study underscored the morphological abnormalities of the capillaries, and further revealed their numerical decrease in AD and absence of capillary damages in BD. The actual reduction rate in the capillary density of AD brains may be more severe, because significant atrophy in this group should have ameliorated the reduction ratio. The fact that there were no β amyloid-deposits nor damages in the cortical microvessels in BD brains was not contradictory to the major site of the pathologic process, which involve subcortical white matter and perforator territory in BD. However, in previous studies, slight but significant neuronal dysfunction has been noted in the cerebral cortex, such as a decrease in the synaptic densities and neuronal viabilities [11,22].

The reduction in the vascular densities and the spatial proximity of β amyloid deposits to the microvascular changes may suggest some vascular toxicity due to β amyloid. Indeed, preamyloid deposits were found in the extracellular space and extended directly into the capillaries [16]. Vinters and Farag [20] raised a neurovascular hypothesis, in which β amyolid accumulates on the outer side of the basement membrane and
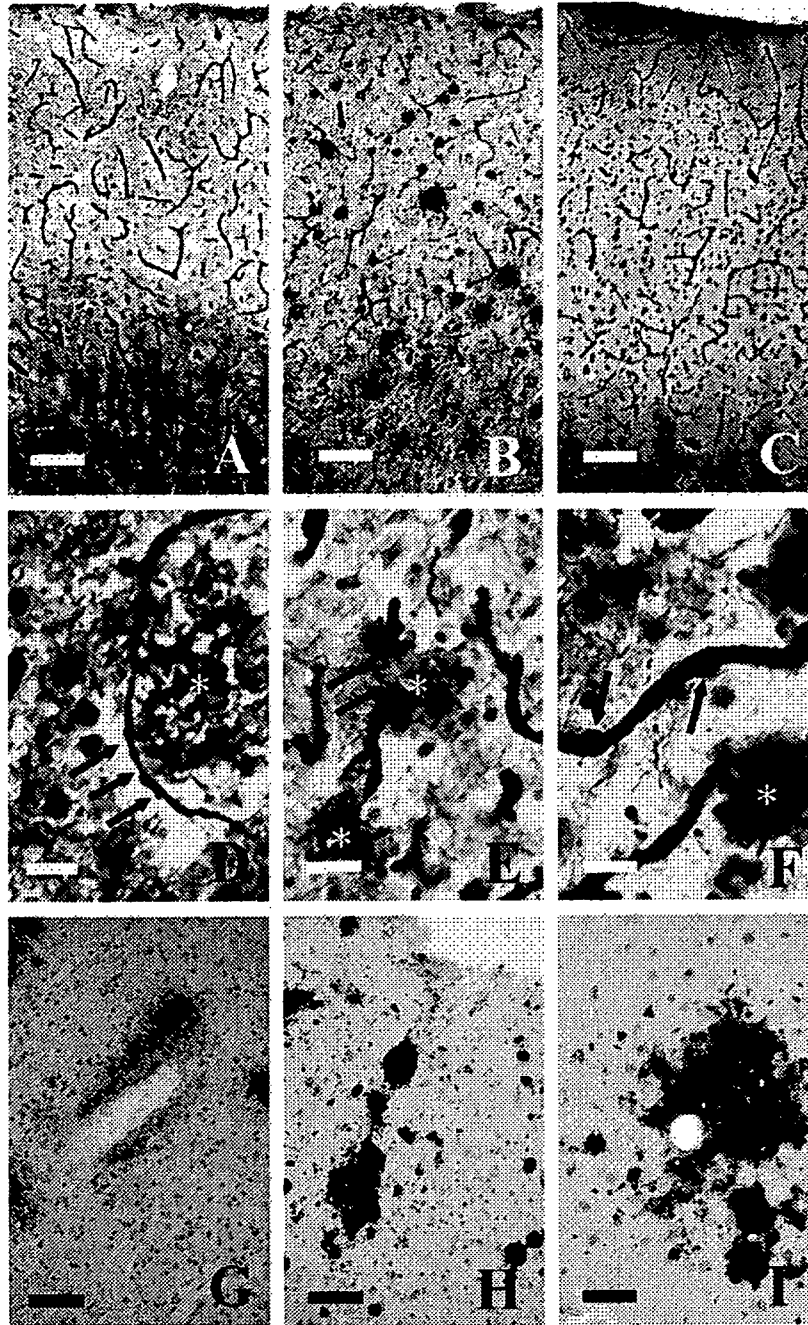
Fig. 1. Photomicrograph of Gallyas stains (A)–(F) and immunohistochemistry for β amyloid (G)–(I). The photographs were taken in the cerebral cortex of non-neurological (A); AD (B), (D)–(I); and BD patients (C). Numerous senile plaques (asterisks) were observed exclusively in AD brains. The capillary density appears less dense in AD as compared with the non-neurological and BD brains. The small vessels showed narrowing (arrows in D), tearing off (arrows in E) and scattered bulging (arrows in F). There were perivascular deposit in the vascular wall which extended into the neuropil diffusely (G). A heavy accumulation of perivascular β amyloid was also seen in the tangential (H) and axial planes (I), in which the perivascular deposits continued into the neuroglial deposits. Bars indicate 100 μm for (A)–(C), (I), 30 μm for (D)–(F) and 200 μm in (G), (H).

may promote local neurovascular inflammation. In support for this hypothesis, GAX, a gene encoding a homeodomain-transcription factor box gene related to vascular differentiation [9], is downregulated in AD brains. The downregulation of GAX activates a proapoptotic pathway, and may result in a decrease in the number of cerebral microvessels and cerebral blood flow (CBF), by way of activating the forkhead transcription factor, AFX-1. This activation may also downreg-ulate low density lipoprotein receptor-related prtoein-1 (LRP) which enhances efflux of β amyloid from the brain [21]. This impaired clearance of β amyloid may further increase soluble β amyloid and fibrillary β amyloid levels [23]. Finally, we hypothesized a vicious cycle in which β amyloid may cause microvascular regression, brain hypoperfusion and neurovascular inflammation, although this will be addressed in future studies.

(A)

(B)

Number of crossing points
in a 6x6 grid (0.16 mm²)
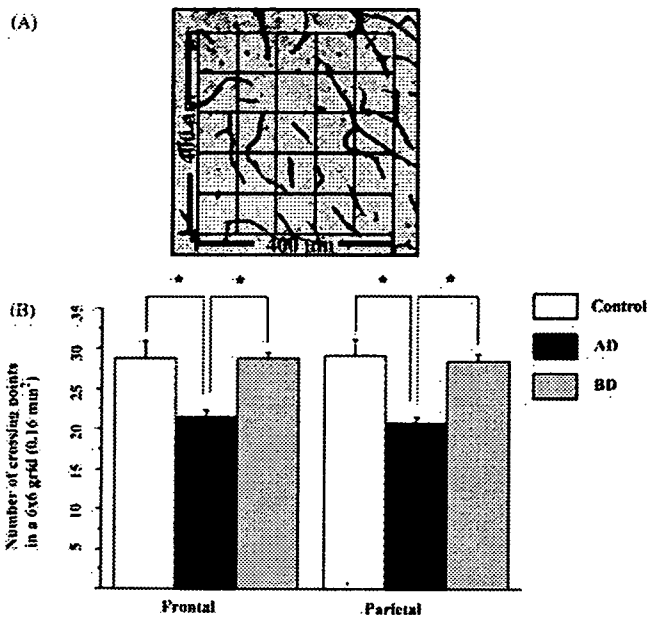
□ Control
■ AD
▨ BD

Frontal   Parietal

Fig. 2. Quantitative evaluation of capillary densities in the cerebral cortex. (A) indicates the grid applied for a non-neurological control brain; (B) indicates the capillary densities in the non-neurological, AD and BD brains. $*p < 0.05$ by Mann–Whitney U-test.

Acknowledgement

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Evolution of mitochondrial cell death pathway: Proapoptotic role of HtrA2/Omi in *Drosophila*

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Abstract

Despite the essential role of mitochondria in a variety of mammalian cell death processes, the involvement of mitochondrial pathway in *Drosophila* cell death has remained unclear. To address this, we cloned and characterized DmHtrA2, a *Drosophila* homolog of a mitochondrial serine protease HtrA2/Omi. We show that DmHtrA2 normally resides in mitochondria and is up-regulated by UV-irradiation. Upon receipt of apoptotic stimuli, DmHtrA2 is translocated to extramitochondrial compartment; however, unlike its mammalian counterpart, the extramitochondrial DmHtrA2 does not diffuse throughout the cytosol but stays near the mitochondria. RNAi-mediated knock-down of DmHtrA2 in larvae or adult flies results in a resistance to stress stimuli. DmHtrA2 specifically cleaves *Drosophila* inhibitor-of-apoptosis protein 1 (DIAP1), a cellular caspase inhibitor, and induces cell death both *in vitro* and *in vivo* as potent as other fly cell death proteins. Our observations suggest that DmHtrA2 promotes cell death through a cleavage of DIAP1 in the vicinity of mitochondria, which may represent a prototype of mitochondrial cell death pathway in evolution.
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Keywords: Apoptosis; Cell death; *Drosophila*; HtrA2/Omi; Mitochondria

Mitochondria play crucial roles in regulating cell death in mammals [1,2]. Upon receipt of apoptotic stimuli, the cell activates caspase protease cascade to execute cell death. The activation of caspases is largely regulated by mitochondrial proteins such as cytochrome *c* (cyt *c*), Smac/DIABLO, and HtrA2/Omi, which are released to cytosol following cell death stimuli [1,2]. cyt *c* directly activates the cytosolic caspase-activating protein Apaf-1, thereby triggering a cascade of caspase activations [1,3]. On the

other hand, Smac/DIABLO and HtrA2/Omi indirectly activate caspases by antagonizing inhibitor-of-apoptosis proteins (IAPs), a family of cellular caspase inhibitors [4–7]. HtrA2/Omi, as well as cyt *c*, has also been shown to be important for cell survival, as loss of HtrA2/Omi gene results in a neurodegeneration in mice [8,9].

Despite abundant similarities in cell death mechanisms between vertebrates and flies, the involvement of mitochondria in *Drosophila* cell death machinery has remained unclear. *Drosophila* cell death is largely regulated by three killer proteins Reaper, Hid, and Grim. These proteins are thought to be functional counterparts of mammalian mitochondrial killer proteins Smac/DIABLO and HtrA2/Omi, as they bind to and antagonize IAPs through their

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conserved four-amino-acid sequences called RHG (Reaper, Hid, and Grim) motif or IBM (IAP-Binding Motif) [3,10]. Unlike mammalian counterparts, however, RHG normally reside in the cytoplasm and their activities are regulated either at transcriptional levels or by phosphorylation [11]. The presence of two Drosophila Bcl-2 family proteins that localize to mitochondria [12,13] indicates that mitochondrial cell death pathway may also exist in flies [14]. Here, in order to investigate the role of mitochondria in Drosophila cell death pathway, we cloned and characterized DmHtrA2, a Drosophila homolog of HtrA2/Omi. Our data suggest that DmHtrA2 promotes cell death through a cleavage of Drosophila IAP1 (DIAP1) in the vicinity of mitochondria, which may represent a prototype of mitochondrial cell death pathway in evolution.

Materials and methods

Stress resistance. For UV-resistance, third-instar larvae were irradiated with 5 mJ/cm^2 UV and allowed to develop at 25 °C. Paraquat resistance was tested essentially as described [15]. Adult flies (age 10–20 days) were starved for 6 h and transferred to vials containing two 2 cm × 2 cm filter squares wetted with 20 mM paraquat (Sigma) in 5% sucrose solution. Survival was scored at 18 or 24 h after the transfer. The ingestion rate was determined by dye intake by adding 10 mg/ml bromophenol blue instead of paraquat. Fly lysate was analyzed by monitoring the absorbance at 595 nm at 18 h.

Recombinant proteins and cleavage assay. The C-terminally His$_6$-tagged recombinant human HtrA2 protein (HsHtrA2ΔN133-His$_6$) was described previously [6]. The N-terminally GST-tagged recombinant DmHtrA2 protein (GST-DmHtrA2ΔN92) was produced in the Escherichia coli. The protein was purified by affinity chromatography using Glutathione Sepharose™ 4B (Amersham Bioscience). N-terminally FLAG-tagged DIAP1 or Hop was translated in vitro in the presence of [^{35}S]-methionine using a TNT T7 Quick Coupled Transcription/Translation System (Promega). [^{35}S]-labeled proteins were incubated with recombinant HtrA2 proteins (100 nM) in Tris buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT for 1 h at 37 °C. The reaction mixtures were subjected to SDS–PAGE and visualized by autoradiography.

Cell death assay. Cell death assay was performed as described previously [12]. In brief, S2 cells were transfected with pUAST-derived expression constructs with a driver plasmid pWAGAL4 (actin promoter-GAL4), together with a reporter plasmid pCaspeR-hs-lacZ that encodes β-galactosidase under the control of the hsp70 promoter. Twenty-four hours after transfection, the cells were heat-shocked at 37 °C and cultured for another 24 h. The cells were lysed at 48 h and assayed for β-galactosidase activity in a reaction mixture containing o-nitrophenyl-β-D-galactopyranoside.

For information regarding cloning, expression constructs, antibodies, cell culture, subcellular fractionation, Western blotting, and fly stocks, see Supplementary information.

Results and discussion

We cloned the DmHtrA2 cDNA (DDBJ/EMBL/GenBank, Accession No. AB112473) from the total RNA of wild-type fly embryo. DmHtrA2 encoded a protein of 422 amino acids with an N-terminal transmembrane (TM) domain, a central trypsin-like serine protease domain, and a C-terminal PDZ domain, as well as an IBM-like (ASKM) sequence that locates adjacent to the TM (Supplemental Fig. 1). RT-PCR analysis revealed that DmHtrA2

mRNA was expressed at all stages of Drosophila development (Supplemental Fig. 1).

To investigate the role of DmHtrA2 in cell death, we induced cell death in Drosophila S2 cells by UV-irradiation. Four hours after irradiation, the cells began to exhibit apoptotic morphological changes (Fig. 1A and B). We found that the protein level of DmHtrA2 was significantly up-regulated in the irradiated cells in a time-dependent
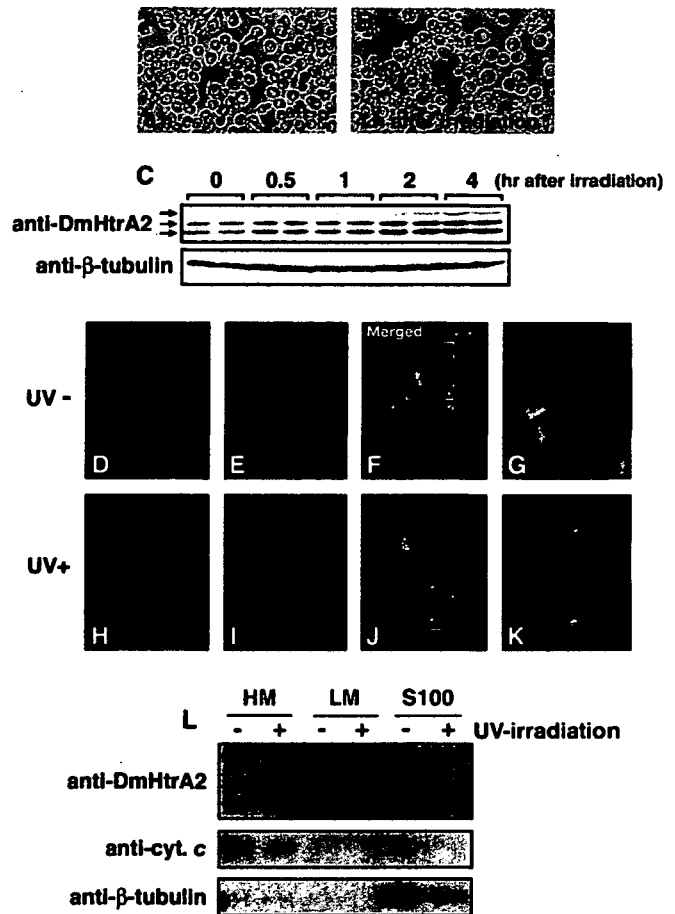

Fig. 1. Apoptotic stimulus up-regulates DmHtrA2 and induces its translocation from mitochondria to extramitochondrial compartment. (A,B) S2 cells were irradiated with UV (200 mJ/cm^2) and cultured for another 4 h. (C) Endogenous DmHtrA2 levels were assessed by Western blotting using an anti-DmHtrA2 antibody before and after UV-irradiation. An anti-β-tubulin antibody was used for a loading control. The anti-DmHtrA2 antibody recognized three bands; the highest band corresponded to the size of full-length form, and the lowest band corresponded to the size of putative mitochondrial mature (ΔN) form (C, upper panel). The experiments were performed using duplicate dishes and were repeated three times. (D–K) Confocal images of S2 cells co-stained with Mitotracker (magenta) and an anti-DmHtrA2 antibody (green). Mitochondria and the endogenous DmHtrA2 protein were visualized before (D–G) or 4 h after (H–K) UV-irradiation (200 mJ/cm^2). G and K show magnified images from F and J, respectively. (L) S2 cells were fractionated before (−) or 4 h after (+) UV-irradiation (200 mJ/cm^2), and the cell lysate was subjected to Western blot analysis using anti-DmHtrA2, anti-cytochrome c, and anti-β-tubulin antibodies. For DmHtrA2 protein, the total amount of protein from each fraction was adjusted to 2.5 μg. For cytochrome c and β-tubulin, the mitochondrial and cytosolic markers, respectively, an equal volume (10 μl) from each fraction was used for Western analysis.

manner (Fig. 1C). This up-regulation was observed even at 2 h after irradiation, preceding the morphological changes (Fig. 1C). Immunostaining of DmHtrA2 revealed that it exclusively localized to mitochondria under the normal condition (Fig. 1D–G). Four hours after UV-irradiation, the anti-DmHtrA2 staining still showed a punctate pattern with a higher signal intensity, but it no longer merged with the Mitotracker-labeled mitochondria (Fig. 1H–K). This suggests that DmHtrA2 is translocated to extramitochondrial conpartment in response to UV-irradiation. We further analyzed the subcellular localization of DmHtrA2 by fractionating S2 cell lysates before and after irradiation. DmHtrA2 protein was detected in the heavy membrane (HM) fraction, as was cyt c, but not in either the light membrane (LM) or cytosolic (S100) fraction (Fig. 1L). We found that UV-irradiation did not alter this distribution (Fig. 1L), suggesting that DmHtrA2 is released to the extramitochondrial compartment but stays in the vicinity of the mitochondria. Since no difference was observed in the mitochondrial membrane potential between control and UV-irradiated S2 cells (Fig. 1D and H; [16]), it is unlikely that the complementary staining of anti-DmHtrA2 and Mitotracker in irradiated cells was due to a loss of membrane potential in a subset of mitochondria.

To examine the physiological role of DmHtrA2, we generated DmHtrA2 knock-down flies using an RNAi construct of DmHtrA2 and a ubiquitous driver da-GAL4. In the knock-down larvae and adults, DmHtrA2 protein level was greatly reduced (Fig. 2A). The knock-down flies were viable and fertile with no detectable morphological abnormalities. We found that the DmHtrA2 knock-down larvae were more resistant to UV-induced lethality compared to control larvae (Fig. 2B). Furthermore, the DmHtrA2 knock-down adult flies were more resistant to dietary paraquat, a superoxide stress agent (Fig. 2C). The ingestion rate of the knock-down flies was not affected (data not shown). These observations suggest that DmHtrA2 is involved in stress-induced toxicity *in vivo*.

Mammalian HtrA2/Omi induces cell death by cleaving and thereby inactivating IAPs through its serine protease activity [4–7]. We therefore assumed that the stress-induced toxicity mediated by DmHtrA2 is through a cleavage of DIAP1. We performed an *in vitro* cleavage assay using recombinant DmHtrA2 and DIAP1 proteins, and found that both *Drosophila* and human HtrA2 proteins specifically cleaved DIAP1 (Fig. 3A). These HtrA2 proteins did not cleave a control protein FLAG-tagged Hop (Hsp70/Hsp90-organizing protein) (Fig. 3A). Thus, the specific serine protease activity of HtrA2/Omi is conserved in *Drosophila*.

Finally, we examined whether DmHtrA2 can kill the cell. Overexpression of DmHtrA2 in S2 cells significantly reduced their viability as potent as other *Drosophila* killer proteins such as Reaper (Fig. 3B and data not shown). This cell death could not be blocked by caspase inhibitors such as DIAP1, p35, or p49, similar to the one caused by human HtrA2/Omi [4,6]. We further examined the toxicity of DmHtrA2 *in vivo*. Overexpression of DmHtrA2 in developing *Drosophila* eye resulted in "no eye" phenotype (Fig. 3C), suggestive of an extensive cell death during development. This phenotype was also resistant to caspase inhibitors (Fig. 3C). Together, these data suggest that DmHtrA2 potently induces cell death through a cleavage of DIAP1.

Our observations suggest that stress stimuli such as UV-irradiation cause translocation of DmHtrA2 from mito-
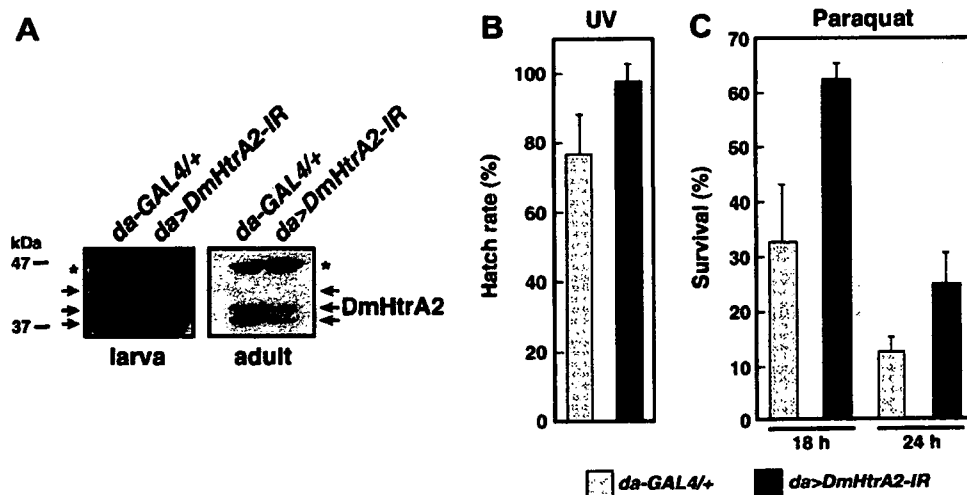

Fig. 2. DmHtrA2 mediates stress stimuli *in vivo*. (A) An RNAi construct (inverted-repeat; IR) for DmHtrA2 (UAS-DmHtrA2-IR) was driven by ubiquitous expression driver da-GAL4 in third-instar larvae or adult flies. Eight animals were homogenized with 96 µl of conventional SDS loading buffer and subjected to SDS–PAGE (4 µl/lane) followed by an anti-DmHtrA2 blotting. The non-specific bands detected by the anti-DmHtrA2 antibody (asterisks) show equal loadings of the protein. (B) Control (da-GAL4) or knock-down (da-GAL4, UAS-DmHtrA2-IR) third-instar larvae were irradiated with UV (5 mJ/cm²), and the resistance was assessed by the number of adult flies that hatched. (C) Control or knock-down adult flies were starved and subjected to dietary paraquat. Survival was scored at 18 or 24 h after the beginning of ingestion.
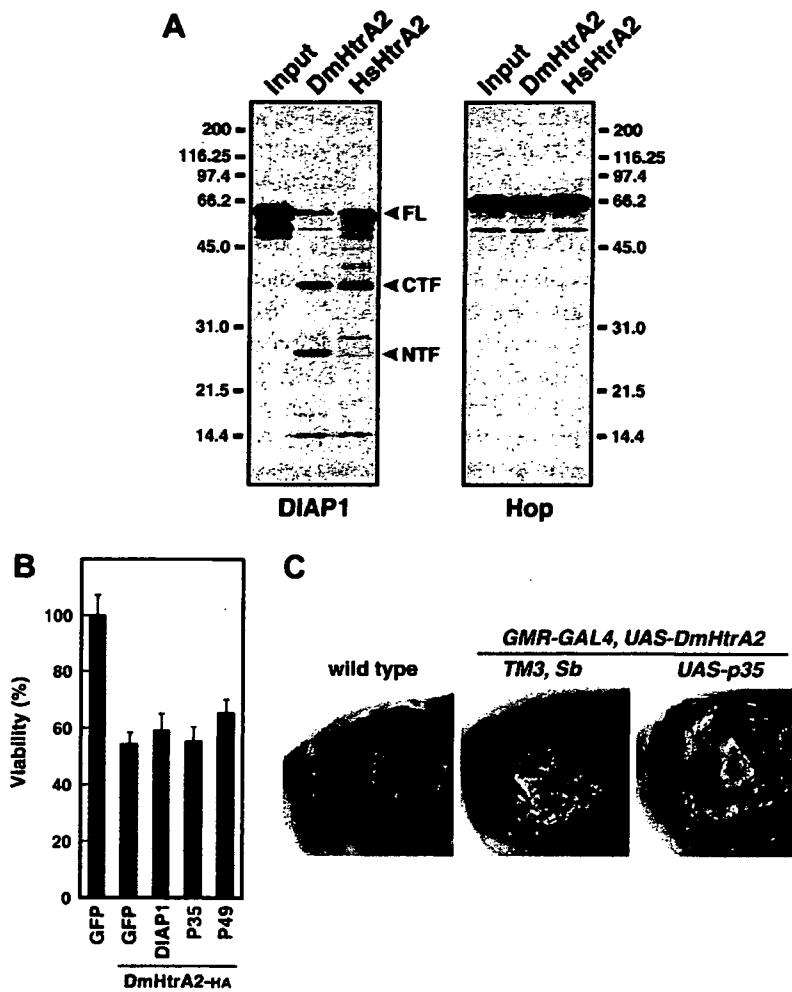
Fig. 3. DmHtrA2 is a potent cell death inducer that cleaves DIAP1. (A) [^{35}S]-labeled DIAP1 or Hop was incubated with *Drosophila* or human HtrA2 recombinant protein, subjected to SDS–PAGE, and visualized by autoradiography. The bands of C-terminal fragment (CTF) and N-terminal fragment (NTF) were predicted by Western blotting of S2 cell lysate co-expressing DmHtrA2 and FLAG-tagged DIAP1 (data not shown). (B) S2 cells were transfected with expression constructs for the indicated proteins and subjected to Cell Death assay. (C) DmHtrA2 was overexpressed in developing *Drosophila* eye using the GMR-GAL4 driver. The eyes at late pupal stage of wild-type, GMR-GAL4/UAS-DmHtrA2[2 6]; *TM3, Sb*/+, and GMR-GAL4/UAS-DmHtrA2[2 6]; UAS-p35/+ are shown.

chondria to extramitochondrial compartment, which in turn promotes cell death through inactivation of DIAP1. Indeed, a significant proportion of the *Drosophila* caspase DRONC, a fly counterpart of caspase-9, has been shown to localize near mitochondria [17]. Intriguingly, overexpression of DmHtrA2 caused caspase-independent cell death both *in vitro* and *in vivo*. This is consistent with the previous report that down-regulation of DIAP1 triggers a caspase activity-independent cell death pathway that is mediated by DRONC [18]. Our findings suggest that the mitochondrial regulation of cell death machinery could be conserved in *Drosophila*, and that the diverse roles of mitochondria in mammalian systems may have been coopted through the evolution of cell death mechanisms.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007. 03.079.

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