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Chapter 14

Parkinson's Disease and ER Stress

Yuzuru Imai, Ryosuke Takahashi

Abstract

Growing evidence indicates that neurodegenerative diseases including Parkinson's disease (PD) are caused by accumulation of various kinds of misfolded proteins. The presence of an abnormal neuronal inclusion, a Lewy body (LB), which is a neuropathological hallmark of common PD, strongly suggests that this disease indeed involves disturbances in the protein degradation system. Although the rare autosomal recessive juvenile Parkinsonism (AR-JP) is not usually accompanied by LBs in the affected regions, Parkin, the product of the PD gene, has turned out to be an enzyme involved in the ubiquitin proteasome system, i.e., a ubiquitin ligase. One of the substrates for Parkin is the integral membrane protein, Pael receptor (Pael-R). Pael-R has unique properties that allow it to be easily misfolded even under physiological conditions. When the degradation of Pael-R is blocked, the receptor accumulates in the endoplasmic reticulum (ER), resulting in ER stress-induced cell death. In this review, we will focus on the molecular mechanism of ER stress-induced neuronal death caused by misfolded Pael-R in AR-JP, and will further discuss the involvement of ER stress in common PD, as well as new therapeutic strategies for PD involving the control of ER stress.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by loss of dopaminergic neurons in the substantia nigra pars compacta. Although the etiology of PD, which usually occurs sporadically, is not well understood, recent identification of gene mutations in familial cases of PD has advanced the understanding of the molecular mechanisms underlying this neurological disease.

Two rare missense mutations in the α-synuclein (α-SYN) gene (A53T and A30P) cause autosomal dominant familial PD.[1,2] The function of α-SYN is unclear, but it is a small presynaptic protein that is a major component of Lewy bodies (LBs).[3] These bodies are frequent intracytoplasmic inclusions found in various regions of the brain of patients with typical PD, including the substantia nigra. In sporadic forms of PD, LBs also include aggregated α-SYN with ubiquitin-immunoreactivity, although how these α-SYN-ubiquitin aggregates are related to the selective loss of dopaminergic neurons remains unclear.[4-6] Another autosomal dominant familial form of PD is thought to be the result of a mutation in ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1/PGP9.5), in a German pedigree. The gene product is one of the poly-ubiquitin-processing enzymes and one of most abundant proteins in neurons.

An autosomal recessive form of juvenile parkinsonism, which is the major cause of juvenile PD, results from mutations of the *Parkin* gene.[7] In AR-JP patients, loss of the dopaminergic neurons and consequently, parkinsonian symptoms, can occur without LB formation.[8]

Parkin is one of the largest genes of the human genome (1.5 Mb), comprising 12 exons encoding a 465 amino acid protein with a molecular mass of 52 kDa.[7,9] The N-terminal 76 amino acids of Parkin are 62% homologous with ubiquitin. The C-terminal half of Parkin contains two RING fingers flanking a cysteine-rich domain, known as "in between RING

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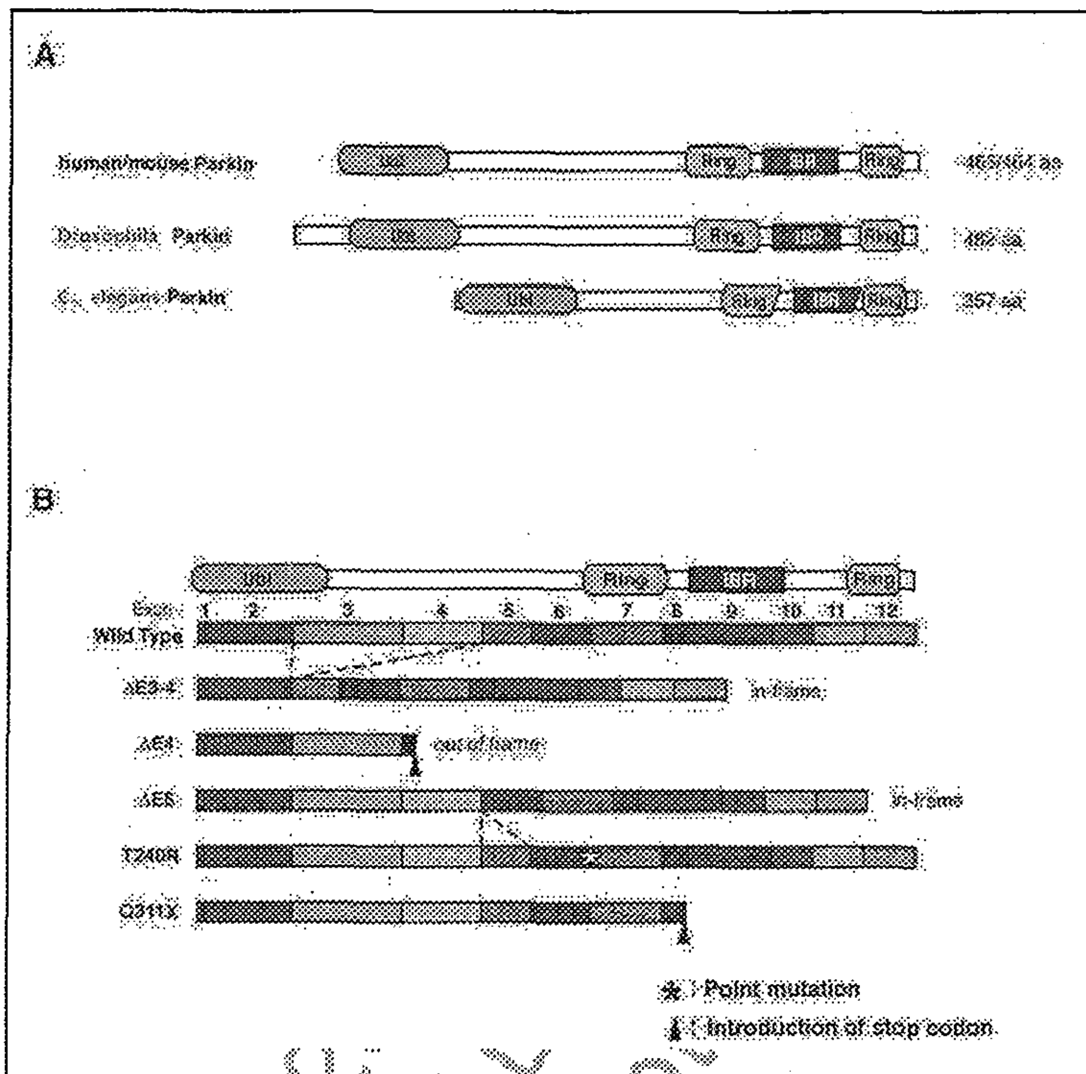

Figure 1. Parkin and representative mutants. A) Parkin protein is highly conserved from mammals to nematoda. Parkin has a ubiquitin-like domain (Ubl) at the N-terminus, and two RING-finger motifs and an IBR (in between RING-finger) at the C-terminus. The carboxyl terminus, including the RING-finger motifs and IBR, can recruit several E2 enzymes into the ubiquitination pathway. It is reported that Parkin binds *O*-glycosylated α-synuclein via Ubl, then ubiquitinates.[62] Some proteins with Ubl can recruit the proteasome components by Ubl.[63-66] B) Representative mutations that are associated with AR-JP. aa; amino acid, ΔE3-4; deletion of exons 3 and 4, ΔE4; deletion of exon 4, ΔE5; deletion of exon 5.

fingers" (IBR) (Fig. 1).[10] The *Parkin* gene is highly conserved, at least from nematodes to mammals. Although no evidence exists for the existence of Parkin in the yeast genome, several proteins with a RING-IBR-RING structure are found in yeast. Many studies have recently revealed that numerous proteins with RING finger motifs have ubiquitin-protein ligase (E3) activity.[11,12] Parkin has also been identified as an E3, and AR-JP-linked *Parkin* mutants are defective in E3 activity (Fig. 2).[13-15] Thus, disorders of the ubiquitination system appear to be closely associated with the pathogenesis of both the sporadic and familial forms of PD.

Proteins fated to degrade in the proteasomes are subject to covalent modification by ubiquitin as a small protein tag. Ubiquitination proceeds through a sequential enzymatic reaction composed of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and E3.[16,17] The exquisite specificity for the proteins to be ubiquitinated is determined directly by E2s or by a diverse family of E3s with a specific E2. Proteins conjugated over a tetra-ubiquitin chain

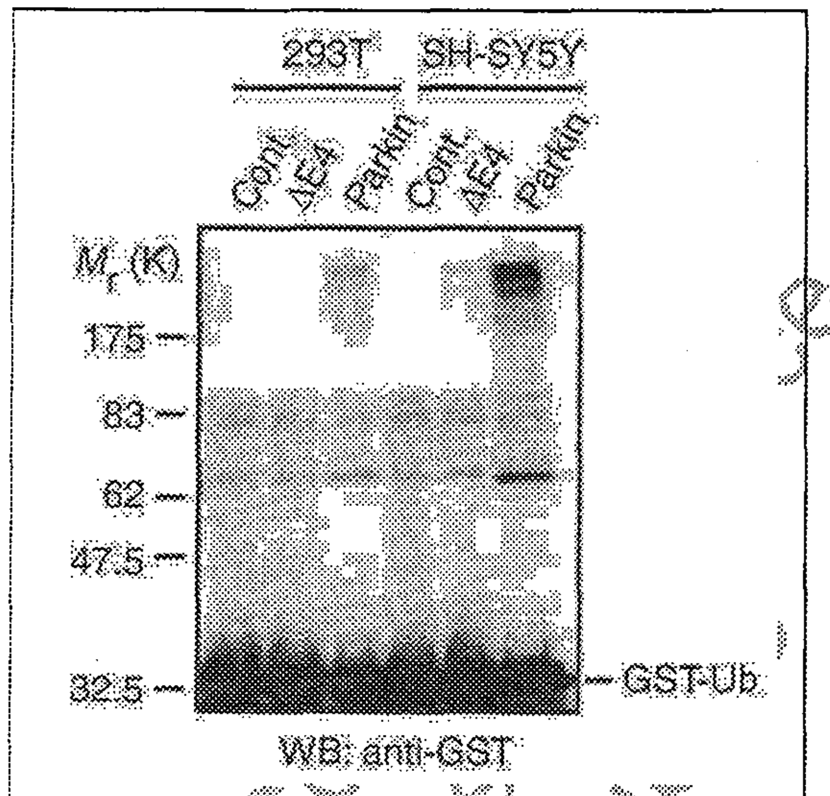

Figure 2. Parkin has E3 activity. Wild-type and a mutant form of Parkin were immunopurified from human embryonic kidney 293T or neuroblastoma SH-SY5Y cells transfected with vector (Cont.), FLAG-Parkin (Parkin) and a deletion mutant of exon 4 with FLAG-tag, (ΔE4). An in vitro ubiquitin-ligase assay was carried out by adding yeast E1 (0.4 pmol) and recombinant 6×His-tagged UbcH7 (3 pmol) and GST-Ub (167 pmol) to the immunopurified wild-type and mutant Parkin proteins in reaction buffer (50 mM Tris-HCl, pH7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol and 2 mM adenosine 5'-triphosphate) at 30°C for 90 min. Reactions were terminated with 3 x SDS sample buffer containing 280 mM 2-mercaptoethanol and samples resolved by SDS-PAGE, after which Western Blotting (WB) with anti-GST was performed.

are recognized and degraded by the 26S proteasome. Recently, a polyubiquitination assembly factor, E4, was detected participating in a ubiquitination reaction, which elongates the ubiquitin chains conjugated to proteins. The first reported E4 has a modified RING finger motif—U box, suggesting that E4 is a variant of E3. Because most of the ubiquitination reaction appears to proceed to sufficient extent to be recognized by a proteasome complex without E4, the physiological function of E4 remains an enigma.

Because AR-JP is likely to be caused by a loss of function in Parkin E3 activity, the accumulation of Parkin substrate(s) may lead to dopaminergic neuronal death. The mRNA for *parkin* is known to be ubiquitously expressed.[7] Therefore, the selective vulnerability of this brain region may be due to Parkin substrate(s) that might be specifically expressed and/or particularly toxic to dopaminergic neurons in the substantia nigra.

Pael-R—The Target of Parkin

A putative G-protein coupled integral membrane polypeptide (known as Pael receptor) has been cloned as a Parkin-binding protein using the yeast two-hybrid technique.[18] Pael receptor (Pael-R) interacts with Parkin through its C-terminal part, both in vitro and in vivo (Fig. 3A and B).

Recently, the SCF^{Fbx2} ubiquitin ligase complex was found to recognize specific sugar chains of ER-associated proteins.[19] Because Pael-R is apparently an *N*-glycosylated protein, Parkin is

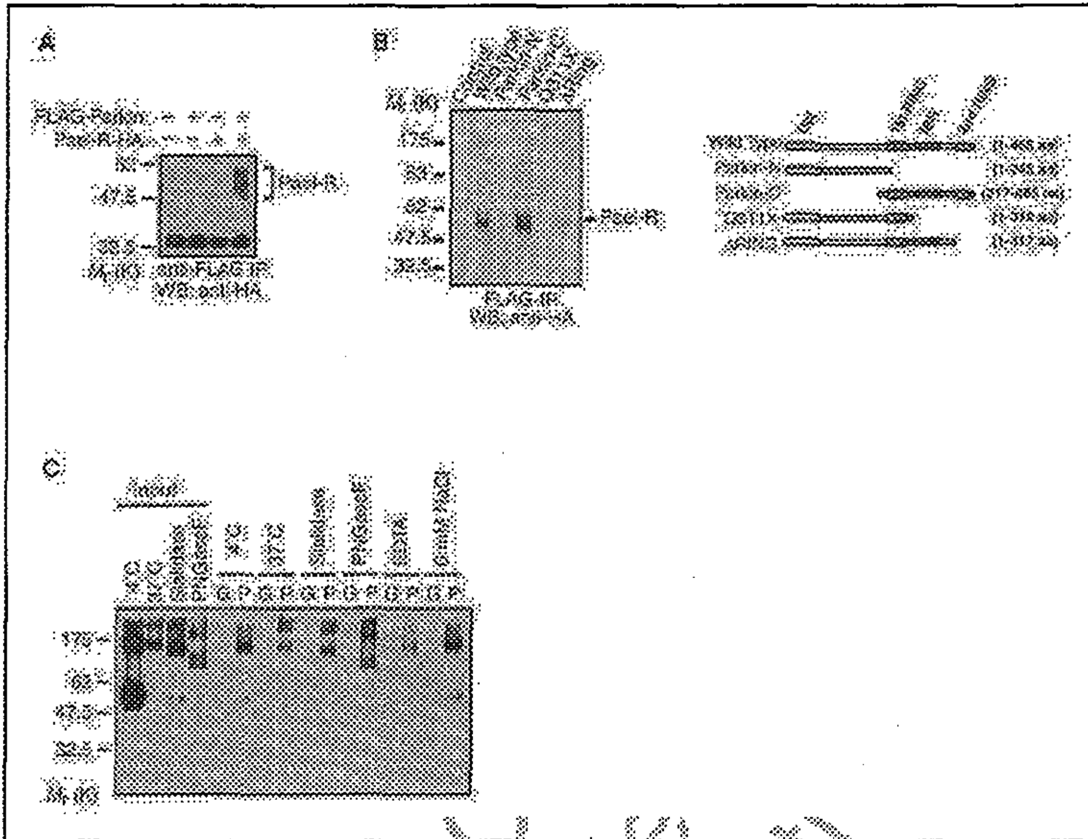

Figure 3. Parkin associates with Pael-R. A) Interaction of Parkin with Pael-R. Lysates from 293T cells transfected with an expression vector plasmid, plasmids encoding HA-tagged Pael-R (Pael-R-HA) and/or FLAG-tagged Parkin (FLAG-Parkin) were immunoprecipitated (IP) with anti-FLAG Ab. Immunoprecipitates were analyzed by Western blotting (WB) using anti-HA Ab. B) Lysates from SH-SY5Y cells transfected with an expression vector (Control), FLAG-tagged Parkin or its mutants, and HA-tagged Pael-R (Pael-R-HA) were analyzed as described in (A). C) Pael-R-FLAG immunopurified from cells, participated in enzymatic digestion of the sugar chains of Pael-R using the indicated enzymes (Sialidase and PNGaseF), or were incubated at different temperatures (4°C and 37°C). The interaction between the treated Pael-Rs and recombinant GST (G) or GST-Parkin (P) was analyzed in IP buffer (Tris-HCl, pH 7.3, 1% Triton-X100, 5 mM $MgCl_2$ and 120 mM NaCl). The interaction between the Pael-R incubated at 4°C and recombinant proteins was also analyzed in 10 mM EDTA plus or NaCl-free IP buffer.

also likely to be a sugar chain-recognizing ubiquitin ligase. However, Parkin interacts with Pael-R even without sugar chains (Fig. 3C). In contrast, addition of a divalent cations chelator EDTA abolishes the interaction (Fig. 3C). These results suggest that Parkin at least recognizes a peptide motif(s) unrelated to sugar chain modification and requires a divalent cation (probably Zn^{2+}) in order to bind.

Pael-R is abundantly expressed in the affected cells in PD, particularly in dopaminergic neurons in the substantia nigra (Fig. 4). Although Pael-R is widely expressed in the brain, most Pael-R-positive cells are CNPase-immunoreactive, suggesting that oligodendrocytes in the brain express Pael-R. On the other hand, most neuronal nuclei (NeuN)-positive cells are Pael-R-negative or only weakly positive.

Wild-type Parkin specifically ubiquitinated Pael-R but not its homologue, endothelin type B receptor, in the presence of ER-resident E2s Ubc6 and Ubc7 in an in vitro ubiquitination assay (Fig. 5A). Another familial Parkinson's disease-related gene product, α-SYN, which can be ubiquitinated in cultured cells, is a candidate substrate of Parkin because the over-expressed N-terminal portion of Parkin and over-expressed α-SYN weakly interact in cultured cells (data

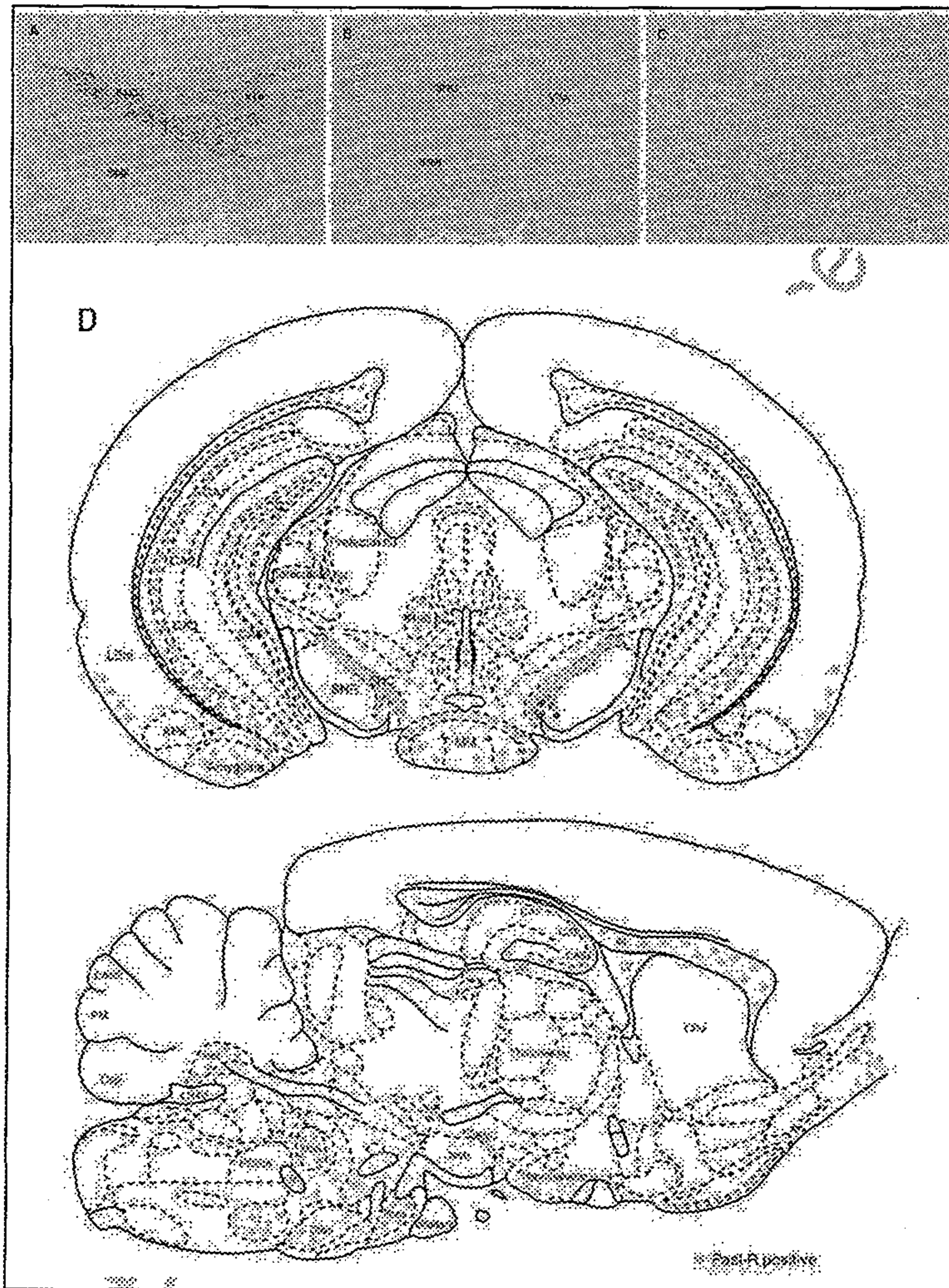

Figure 4. Pael-R is highly expressed in the substantia nigra of the brain. Immunolocalization of tyrosine hydroxylase (A, brown) and Pael-R (B and C, brown) in a coronal section of the murine brain. Pael-R is mainly expressed in the dopaminergic neurons in the substantia nigra per compacta (SNC) and ventral tegmental area (VTA) in the midbrain. C) High power magnification of (B) in the SNC. Original magnification, x 40 (A and B) or x 200 (C). D) The distribution of Pael-R-immunoreactive neurons and other cells was determined in paraffin-embedded sections. The strongly stained regions in the brain are shown as dots. Upper and lower schemata indicate brain coronal and sagittal sections including the substantia nigra, respectively. A color version of this figure can be viewed at www.Eurekah.com.

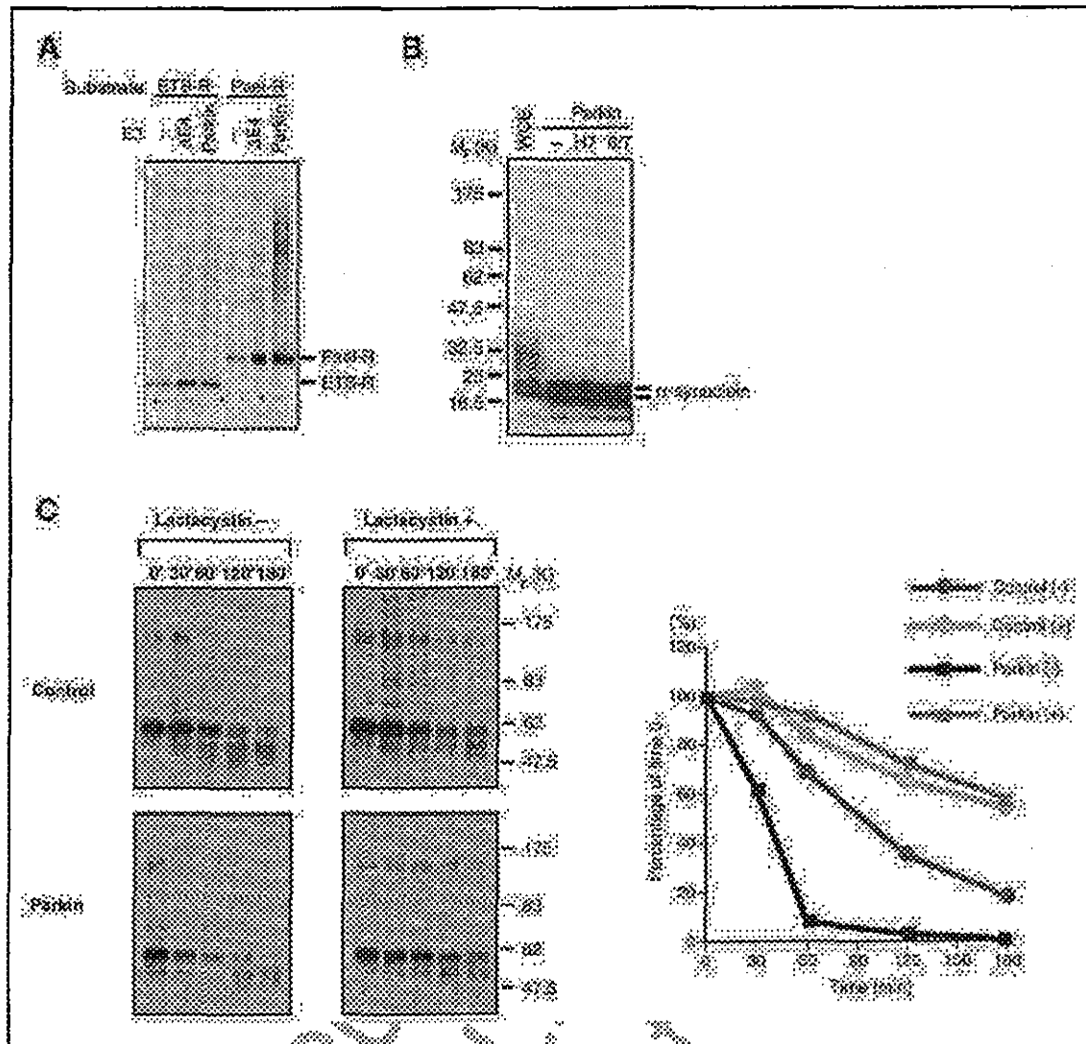

Figure 5. Parkin is involved in Pael-R degradation. A) In vitro ubiquitination assay of Pael-R and its homologue using recombinant Parkin. ^{35}S-labeled Pael-R-FLAG (Pael-R) or endothelin receptor type B-FLAG (ETB-R) were generated in TNT rabbit reticulocyte lysates, then immunopurified using anti-FLAG affinity gel. These receptors were reacted with Ub and E1 in the presence of the recombinant E2s (Ubc6 and 7) together with or without GST fused Parkin or GST fused exon 4-deleted mutant of Parkin (ΔE4). B) In vitro reconstitution assay of α-SYN as a substrate of Parkin in the presence of UbcH7 (H7) or Ubc6 and Ubc7 (6/7) did not significantly modify α-SYN with a polyubiquitin chain, although whole neuronal cell extracts (WCE) activated ubiquitin-conjugation. C) SH-SY5Y cells transfected with a construct for Pael-R-FLAG combined with an empty vector (Control) or a plasmid for Parkin were incubated with or without 10 μM lactacystin, then pulse-labeled with ^{35}S-methionine/cysteine and chased for the indicated periods in the presence (+) or absence (-) of 10 μM lactacystin. ^{35}S-labeled Pael-R was immunoprecipitated, detected by autoradiography (left), then quantified by phosphorimaging. Levels of labeled Pael-R are plotted relative to amount present at time 0 (right).

not shown).[13] The in vitro reconstitution assay of α-SYN as a substrate of Parkin in the presence of UbcH7, UbcH8 or Ubc6 and Ubc7 does not significantly modify α-SYN with a polyubiquitin chain, although whole cell extracts promote polyubiquitination (Fig. 5B and data not shown). Thus, Parkin E3 appears to have a certain specificity for target proteins. In an in vivo analysis of Pael-R degradation, the half-life of transiently transfected Pael receptor in cultured neuroblastoma SH-SY5Y cells was dramatically shortened from one hour to less than

thirty minutes by Parkin coexpression (Fig. 5C). Furthermore, a proteasome inhibitor, lactacystin, significantly decelerated the rate of Pael-R degradation even in the Parkin-over-expressing cells (Fig. 5C). These results suggest that Parkin is involved in Pael-R degradation through the proteasome pathway.

Pael-R Unfolding and Accumulation

The ubiquitin-proteasome pathway plays an important role in the degradation of membrane or secretory proteins at the level of the endoplasmic reticulum (ER). About one-third of newly synthesized proteins in cells enter the ER, where chaperones such as BiP/GRP78 bind and ensure the proper folding of these proteins. Properly folded proteins are, then, allowed to enter the normal secretory pathway composed of the Golgi apparatus, plasma membrane and various intermediate transport compartments.[20] On the other hand, the unfolded proteins that fail to be correctly folded are retrotranslocated to the cytosol, where they are constitutively degraded. For example, most de novo synthesized polypeptides of the chloride channel CFTR and δ opioid receptors, (about 75 and 60%, respectively) fail to fold correctly.[21-23] Such proteins are retrotransported from the ER to the cytosol via the translocon complex including Sec61 (a specific ER transport channel).[24] These are subsequently processed through the cytosolic ubiquitin-proteasome system. This process is known as ER-associated degradation (ERAD).[24] The ER luminal or ER transmembrane proteins are generally good substrates for ERAD.

Parkin binds to Ubc6 and Ubc7, the ER-resident E2s involved in ERAD, and the membrane protein Pael-R is ubiquitinated in vitro in the presence of both Parkin and Ubc6/7, suggesting that Parkin is an E3 involved in ERAD and Pael-R is a substrate for ERAD.

When Pael-R-over-expressing cells are lysed with nonionic detergent such as Triton X-100, unfolded aggregated proteins fall into the detergent-insoluble fractions (Fig. 6). Interestingly, a significant amount (up to 50%) of highly ubiquitinated Pael-R is detected in the insoluble fraction when Pael-R is transiently transfected without any additional treatment, suggesting that Pael-R is prone to be ubiquitinated and form aggregates. Treatment with inhibitors of protein folding such as tunicamycin and 2-mercaptoethanol increases the level of insoluble Pael-R and decreases the ratio of soluble Pael-R, indicating that unfolded Pael-R easily becomes insoluble. Moreover, treatment with lactacystin also increases the level of insoluble Pael-R, with the level of soluble Pael-R unaffected. This finding indicates that inhibition of ERAD by lactacystin results in the accumulation of unfolded, insoluble Pael-R and provides strong evidence that Pael-R is a substrate for ERAD.

ER Stress and Subsequent Neuronal Death by Pael-R Accumulation

Abnormal accumulation of unfolded proteins in the ER may result from various stimuli in the cellular environment. This condition is known as unfolded protein stress (UPS)—a type of ER stress. Suffering cells make attempts to overcome UPS through various pathways including transcriptional upregulation of ER chaperones such as GRP78/BiP and components of ERAD such as Ubc7 and general translational suppression. These cellular responses are known as the unfolded protein response (UPR).[20] An excessive amount of accumulated unfolded protein, or chronic or excessive unfolded protein stress (UPS) might result in cell death accompanied by JNK and caspase activation.[25,26] UPS in the cells could be monitored by the upregulation of unfolded protein response-regulated genes such as *chop, ubc7, hsp70* and others. An ER chaperone, BiP/GRP78, is also one of the UPS-inducible genes, the transcripts of which are markedly upregulated by UPS.[27] Although the upregulation of BiP protein during UPS is not so great as its transcript, BiP is a good UPS reporter at both the mRNA and protein levels.

When Pael-R over-expressing cells are treated with lactacystin or UPS-inducing reagents, BiP upregulation is observed with good correlation to the amount of detergent-insoluble Pael-R, indicating that accumulation of the unfolded Pael-R in cells can stimulate substantial UPS. Moreover, prolonged incubation with lactacystin or UPS-inducing reagents leads to neuronal

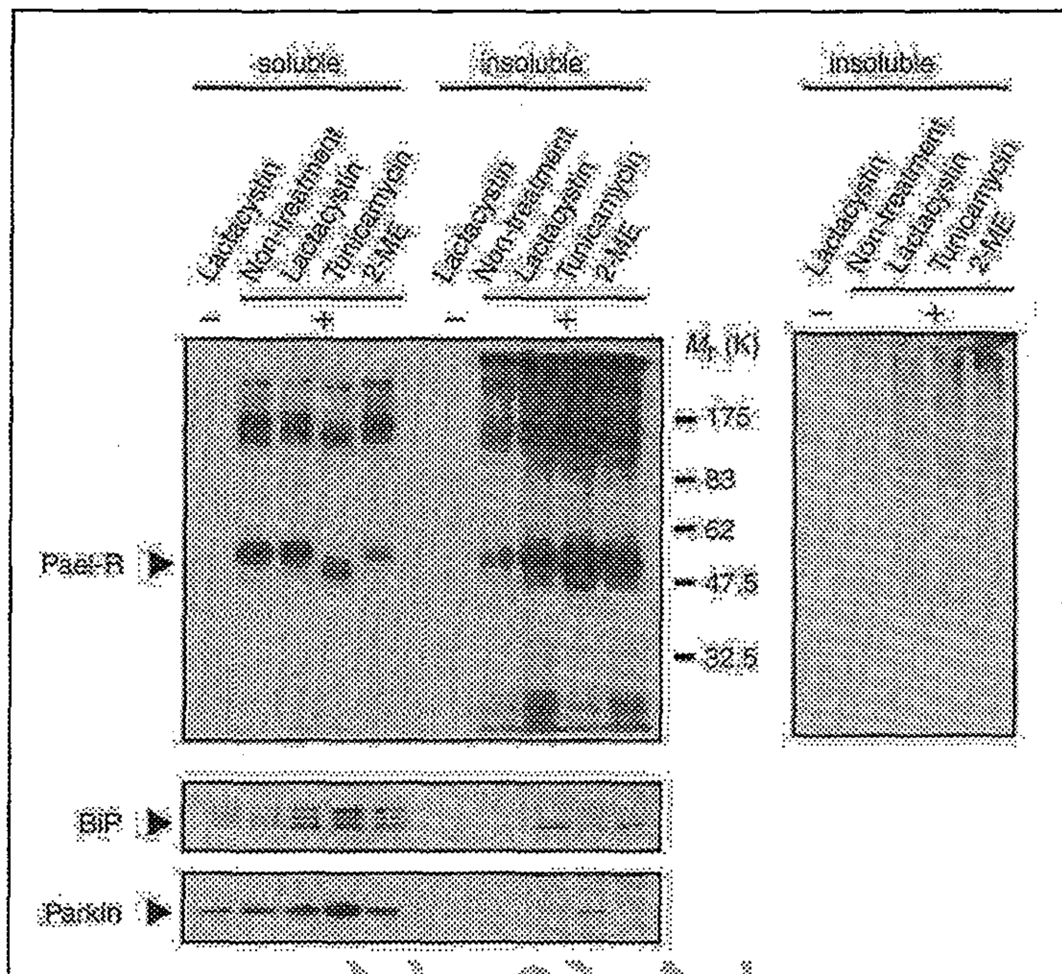

Figure 6. Pael-R tends to be unfolded and to induce ER stress. Pael-R is easily unfolded and rendered insoluble by ER stress. SH-SY5Y cells were transfected with empty vector (-) or Pael-R (+) for 20 hrs. Cells were then incubated with or without lactacystin (10 μM), tunicamycin (1 μg/ml) or 2-ME (1 mM) for 16 hrs. The cells were lysed in fractionation buffer (10 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 1% Triton X-100 with protease inhibitors), then fractionated by centrifugation at 15,000 x g for 30 min. The supernatants and the insoluble pellet washed four times with the cell fractionation buffer were dissolved in SDS-sample buffer containing 280 mM 2-ME. Each fraction was subsequently Western blotted using antibodies against the indicated proteins. Insoluble Pael-R was immunoprecipitated from insoluble fractions of the same samples as described,[18] and analyzed with anti-Ub polyclonal Abs (upper panel on the right).

death (Fig. 7). Immunocytochemical studies support the notion that the accumulation of over-expressed Pael-R in the ER leads to unfolded protein-induced ER-stress, as indicated by BiP upregulation, finally causing cell death (Fig. 7). At the early stage (~6 hr) of treatment with lactacystin, Pael-R accumulates in the ER. Recent findings have led to models of ER-membrane dislocation coupled to the ubiquitin-proteasome pathway.[24] Pael-R accumulation in the ER at the early stage of the proteasome blockade is in agreement with some reports suggesting that proteasomes function in driving the extraction of retrograde proteins from the ER during ERAD.[28,29] Further treatment for up to 8-16 hrs results in the formation of inclusions at the juxtanuclear site of the cytosol—the so-called aggresome,[30] which suggests that unfolded Pael-R is quickly retrotranslocated from the ER to the cytosol. At this stage, cell bodies become shrunken and round, showing apoptotic morphology (Fig. 7). These observations clearly indicate that the accumulation of unfolded Pael-R is highly toxic for neuronal cells.

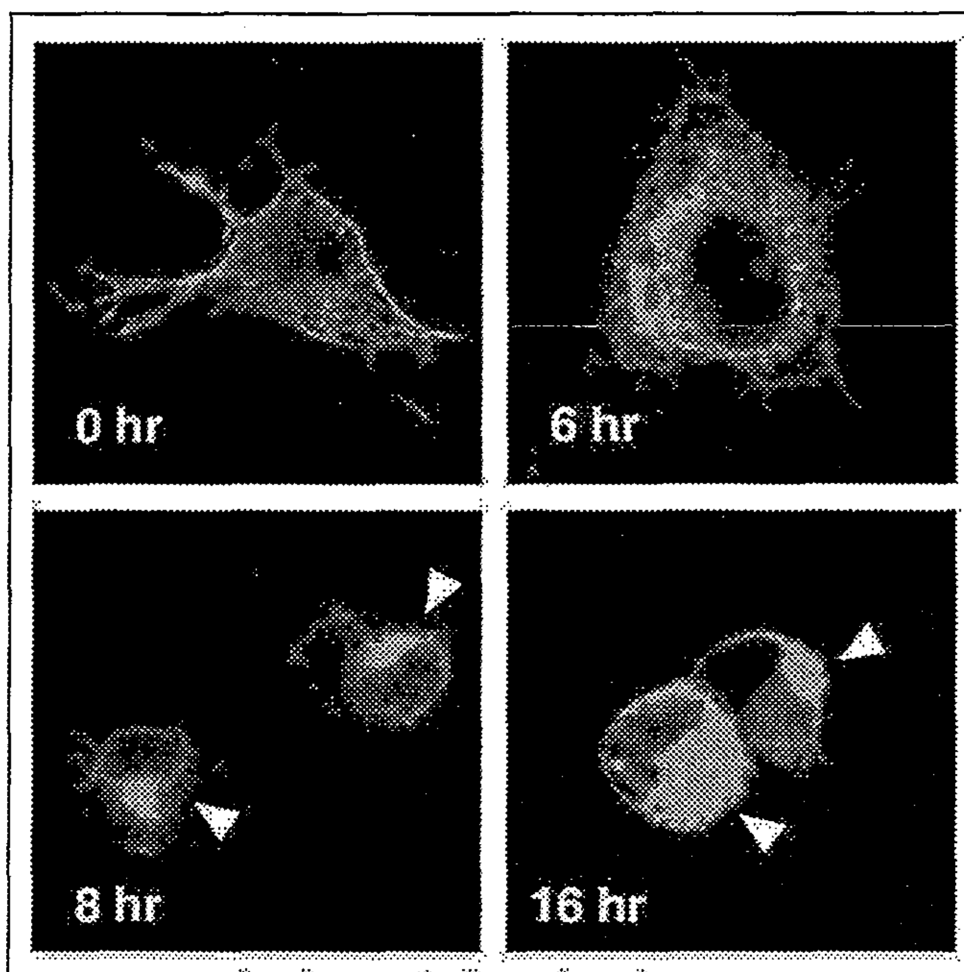

Figure 7. Accumulation of unfolded Pael-R causes neuronal death. SH-SY5Y cells transfected with Pael-R were treated with 20 μM lactacystin for the indicated periods. Cellular localization of Pael-R was visualized using an anti-Pael-R Ab (green). The perinuclear inclusion of Pael-R is indicated as arrowheads. Six hrs inhibition of proteasome activity led to the accumulation of Pael-R in the ER. Subsequent inhibition of 16 hrs caused most of the cells to die. A color version of this figure can be viewed at www.Eurekah.com.

Parkin Suppresses the Accumulation of Pael-R and Subsequent Cell Death

As dysfunction of Parkin is thought to lead to selective neuronal cell death, it is assumed that accumulation of unfolded Pael-R would be observed in degenerating neurons. In contrast, upregulation of Parkin mRNA has been observed during UPS, and unfolded Pael-R is degraded through a Parkin-mediated proteolytic pathway. Consistent with these results, cell death induced by over-expressed Pael-R is significantly alleviated by Parkin coexpression (Fig. 8A). The amount of insoluble Pael-R in the total insoluble fraction is reduced in the presence, compared with in the absence, of Parkin (Fig. 8B). These findings imply that Parkin functions not only in conjugation of ubiquitin to Pael-R but also in the promotion of proteasomal degradation before the accumulation of insoluble Pael-R, which has toxic consequences for the cells.

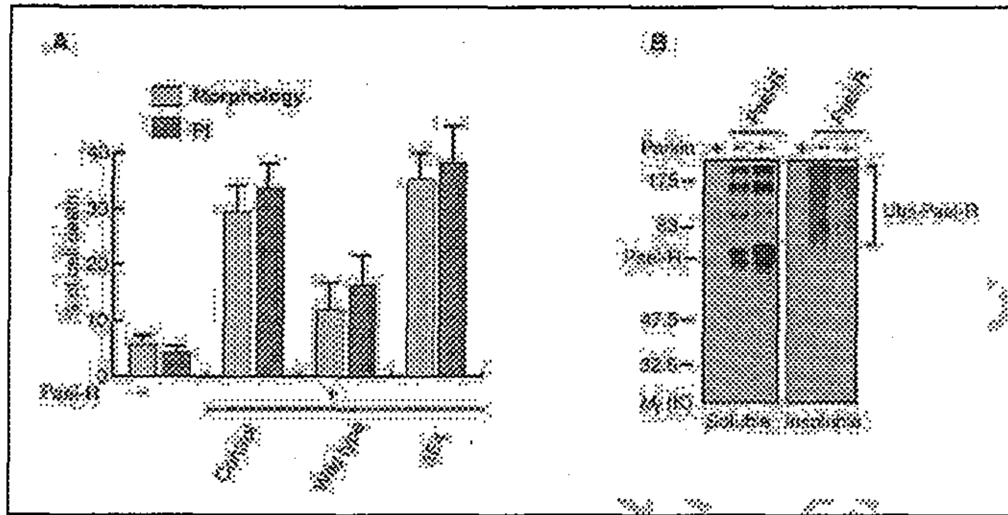

Figure 8. Parkin suppresses the accumulation of unfolded Pael-R and cell death by unfolded Pael-R. A) Vector plasmid (-) or construct for Pael-R (+) combined with construct for mock (Control), FLAG-Parkin (Wild type), or -ΔE4, were cotransfected into SH-SY5Y cells together with a plasmid for EGFP, as an indicator gene, for 48 hrs. About 300 GFP-positive cells were counted and the percentage of dead cells was determined by cell morphplogy (Morphology) and propidium iodide dye exclusion assay (PI). Error bars represent standard deviation (S.D.) calculated from triplicate samples. B) Parkin suppresses accumulation of insoluble Pael-R in the cells. SH-SY5Y cells transfected with vector plasmid or construct for Pael-R-HA (Pael-R) combined with construct for mock (-) or FLAG-Parkin (Parkin) (+), were lysed and separated into 1% Triton X 100-soluble (Soluble) or –insoluble (Insoluble) fractions, then detected by Western blotting with an anti-HA mAb. Ub_n-Pael-R indicates polyubiquitinated Pael-R in the insoluble fraction.

Clinical Evidence of Involvement of Pael-R in AR-JP

Based on these findings, accumulation of Pael-R is the most likely pathogenetic mechanism underlying AR-JP. Supporting this idea, the protein level of 1% Triton X-100 -insoluble, but not -soluble Pael-R was 10-30 fold higher in AR-JP brains than in nonAR-JP brains (Fig. 9). In three out of four available AR-JP brains examined, an increased level of BiP was observed, suggesting that AR-JP brains are also under UPS caused by the accumulation of unfolded Pael-R. Given that neurodegeneration in AR-JP is caused by UPS-induced cell death, cytoplasmic inclusions composed of unfolded Pael-R may not be formed before cellular demise, providing reasonable explanation for the absence of Lewy bodies or other types of neuronal inclusion bodies in AR-JP.

Hypothesis for Molecular Mechanism of AR-JP

As Parkin is involved in protein degradation of the ubiquitin pathway, the substrate(s) of Parkin could theoretically accumulate in the substantia nigra of the brain. Although the accumulation of protein(s) is believed to cause the degeneration of dopaminergic or other neurons, LBs do not accompany the degenerating neurons in AR-JP. The fact that the pathological findings of AR-JP differ from that of common PD suggests that the mechanism of AR-JP neurodegeneration is also different from that of common PD.

A putative seventh transmembrane protein, Pael-R, which interacts with Parkin, is ubiquitinated through a Parkin-mediated ubiquitination pathway. Parkin is likely to be involved in ERAD since it is upregulated along with BiP during UPR and it specifically binds to the ER-resident E2s, Ubc6 and Ubc7. An immunocytochemical and ultrastructural study showing close association of Parkin with the ER also supports this notion,[31] although Parkin is still localized in other parts of the cell such as the cytoplasm or presynapse (data not shown).

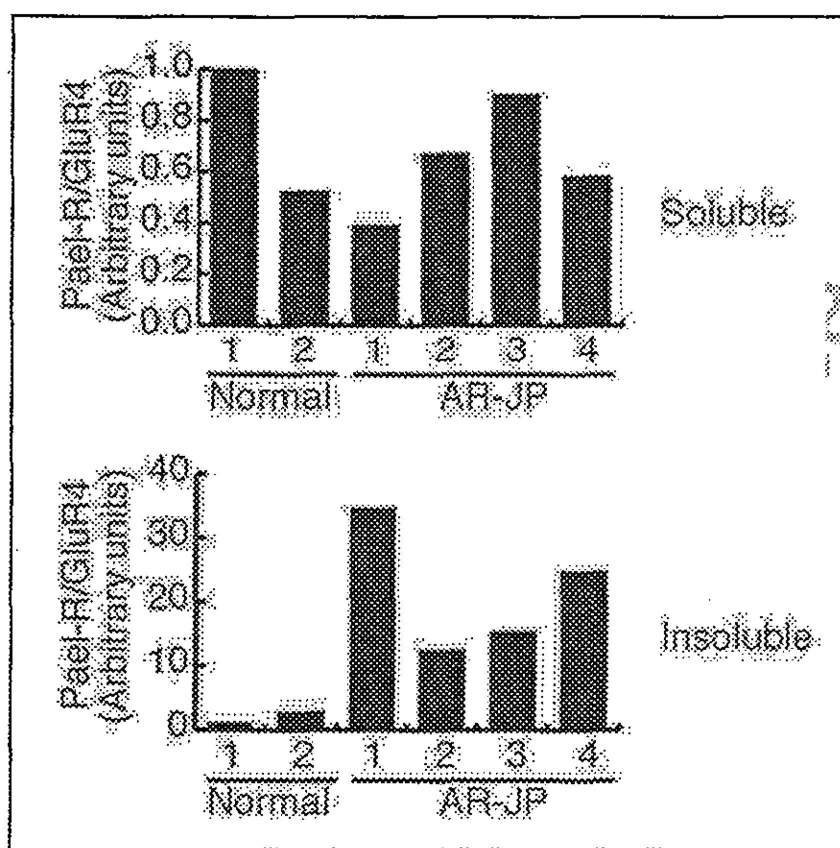

Figure 9. Accumulation of Pael-R in AR-JP brain. Human tissue of frontal lobe cortex from normal or AR-JP brains was separated into 1% Triton X100-soluble or -insoluble fractions as described,[18] then immunoprecipitated with an anti-Pael-R mAb. Subsequently, each fraction was immunoprecipitated with an anti-GluR4 mAb. Precipitates were examined by Western blotting with another anti-Pael-R or anti-GluR4 mAb. The anti-Pael-R-precipitated material was quantified, and normalized against anti-GluR4-immunoreactive material for each sample.

If Parkin's function is attenuated by genetic mutation or specific circumstances, Pael-R may accumulate in the substantia nigra due to its high expression in the dopaminergic neurons. Although Pael-R is expressed in both neurons and nonneuronal cells (most likely glial cells), the neuronal expression of Pael-R is restricted to dopaminergic neurons in the substantia nigra and some other neuronal populations. The fact that postmitotic neuronal cells are more vulnerable than renewable glial cells might partially explain the selective degeneration of dopaminergic neurons, which cannot replicate (unlike glial cells).

A number of de novo synthesized Pael-Rs are liable to be unfolded even under physiological conditions, causing them to be constitutively degraded through the ERAD system. If the amount of accumulated unfolded Pael-R in the ER becomes too large to control, the cells respond by UPR that transactivates multiple genes, including molecular chaperones and ERAD-associated molecules.[32-34] Beyond the capacity of UPR, the cells will die due to the unfolded Pael-R-induced ER stress.[25,26] The morphology of most cells is apoptotic, being round, shriveled and forming cytoplasmic aggregations at the late stage of unfolded Pael-R accumulation (Fig. 7). Auto-protection of neurons from the accumulation of abnormal polypeptides that will disturb normal cellular function necessarily involves highly complex systems.[35] The capacity of the ER to deal with protein aggregates might be rather small, and the ER appears to have specific apoptotic pathways against ER stress induced by various stimuli, such as the disruption of internal calcium homeostasis or accumulation of unfolded ER-proteins. Therefore, ER

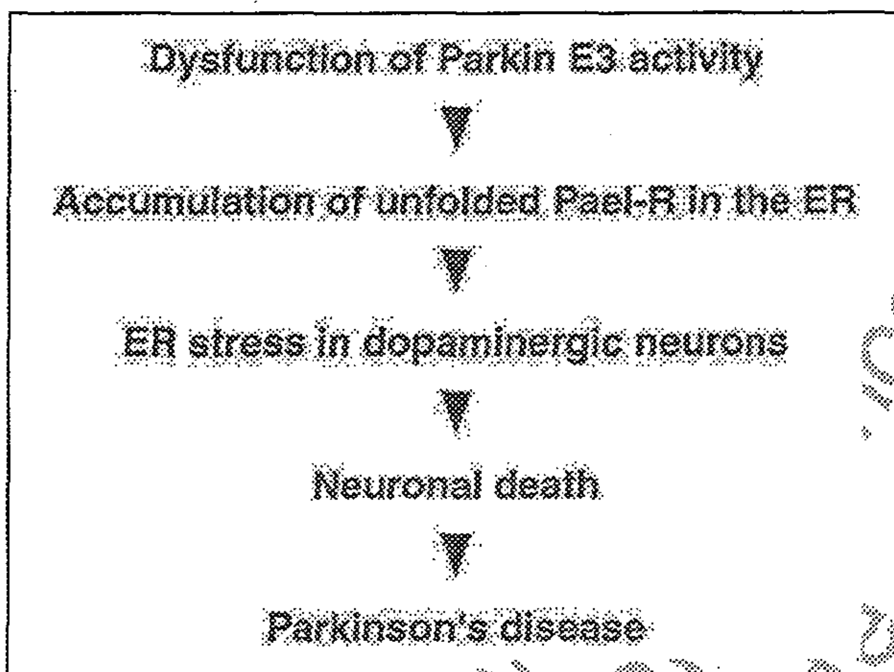

Figure 10. Hypothesis of the pathogenesis of AR-JP. Genetic mutation or certain circumstances cause the attenuation of Parkin's ubiquitin ligase activity, which leads to the accumulation of Parkin's substrate, Pael-R in the ER of the dopaminergic neurons. Chronic accumulation of Pael-R results in unfolded protein stress (a type of ER stress). Transcriptional suppression, and upregulation of the chaperone system and proteasomal degradation system, known as the unfolded protein response, repel the accumulation of Pael-R in the ER. When neuronal cells face insurmountable unfolded protein stress, cell death is initiated, which may be mediated by a specific apoptotic pathway in the ER. Finally, the ablation of dopaminergic neurons to a large extent leads to Parkinson's disease.

stress-induced neuronal death would occur very rapidly without aggregation, and individuals afflicted with AR-JP might develop the disease when Pael-R accumulates in the ER through Parkin dysfunction. In addition, most of the Pael-R-positive cells, including dopaminergic neurons, in the substantia nigra might be especially vulnerable to unfolded protein-induced ER stress and undergo ER stress-induced cell death before aggregate formation. This scenario well explains the neuropathological findings of AR-JP characterized by selective nigral degeneration without LBs (Fig. 10).

Cofactors of Parkin

The Parkin-dependent ubiquitin-conjugating reaction of Pael-R can be reproduced in vitro. The reaction using Parkin immunopurified from a neuroblastoma or brain tissue, is much more efficient than that using recombinant GST-fusion Parkin. The former system ubiquitinates Pael-R more heavily than the latter. These observations suggest that ubiquitination by Parkin in vivo under physiological conditions requires an additional component(s) that might be copurified with Parkin from the cells.

Two Parkin cofactors have been purified biochemically to explain the differences described above.[36] One of them is the U box protein, CHIP (carboxy-terminus of Hsp70-interacting protein) (Fig. 11A). A U box, which was originally identified in the yeast E4 Ufd2, is a variant of the RING-finger motif. Although a U box motif appears to lack the important residues for metal-chelating, the RING-finger fold is thought to be maintained structurally and the RING-finger-like structure is believed to have the common function of protein ubiquitination.[37] CHIP was previously identified as a negative regulator for chaperone ATPase activity,[38] and has a U box-dependent E3 activity.[39-41] Furthermore, it has been shown that CHIP ubiquitinates

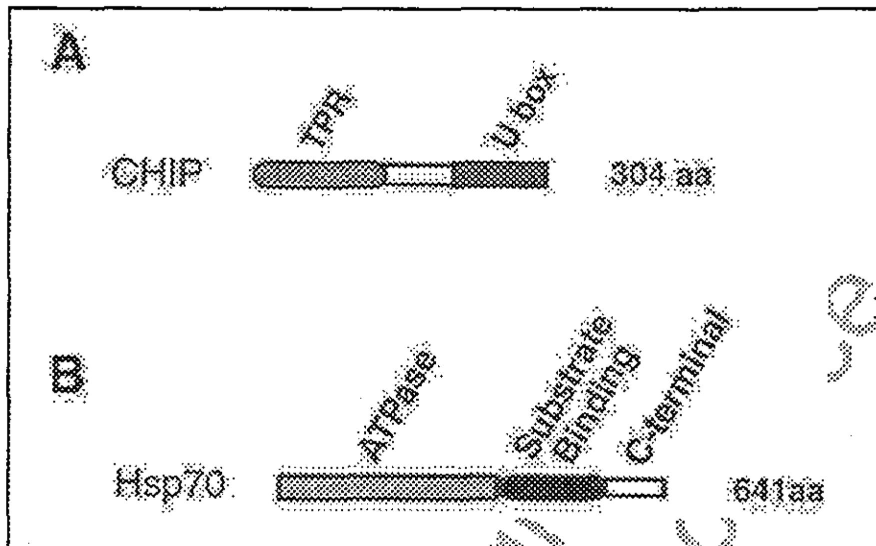

Figure 11. Parkin cofactors, CHIP and Hsp70. A) CHIP has a tetratricopeptide repeat (TPR) and a U box at the N- and C-terminus, respectively. CHIP binds to the C-terminal part of Hsp/Hsc70 via TPR and inhibits the ATPase activity of Hsp70. The U box is a RING-finger related motif, and has an E3 activity by recruiting specific E2s (Ubc4 and UbcH5). Thus, CHIP links the chaperone system to the ubiquitin-proteasome system. B) Hsp70 is a stress-inducible molecular chaperone with ATPase activity. A closely related chaperone, Hsc70 is constitutively expressed in cells. Although their functional difference remains unknown, they are thought to assist the folding of newly synthesized or damaged proteins, and to be involved in proteasomal degradation. ATPase; ATPase domain, Substrate Binding; substrate binding domain.

unfolded proteins only when they are first captured by chaperones, suggesting that CHIP is an important link between the ubiquitin-proteasome and chaperone systems, both of which deal with misfolded proteins.[41] CHIP dramatically enhances the Parkin-mediated ubiquitination of Pael-R (Fig. 12).[36] The addition of CHIP, in the presence of its specific partner E2, Ubc4, facilitates longer polyubiquitination-chain formation of Pael-R. Consistent with in vitro observations, over-expression of CHIP strongly degrades Pael-R in vivo, resulting in a striking reduction in the steady state level of Pael-R protein (Fig. 13A).

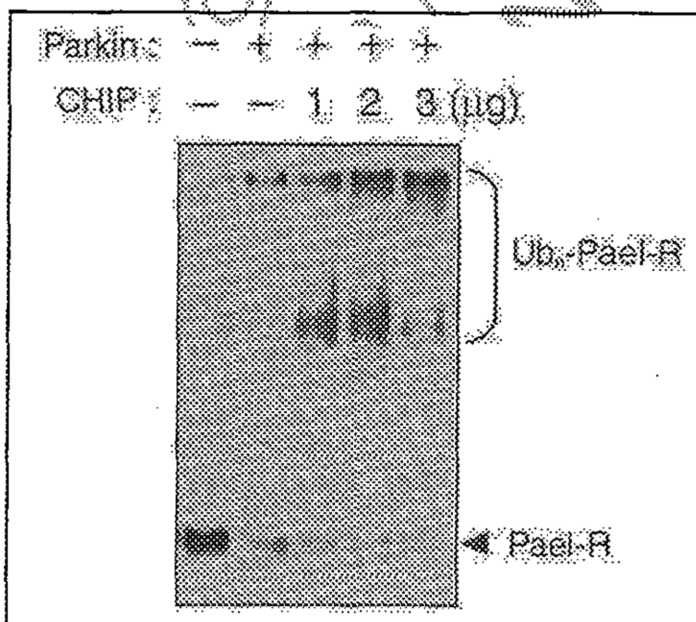

Figure 12. CHIP promotes Parkin-mediated Pael-R ubiquitination. In vitro ubiquitination assay of Pael-R using recombinant CHIP, Parkin and E2s (Ubc4, Ubc6 and Ubc7). Pael-R-FLAG generated in rabbit reticulocyte lysate was immobilized on anti-FLAG affinity gel. Pael-R on the gel was incubated at 30°C for 90 min with Ub, E1, E2s and/or increasing amounts of CHIP (1, 2 and 3 µg) together with GST or GST-fused Parkin. After the reaction, the gel was washed and subjected to Western blot analysis using anti-Pael-R Ab.

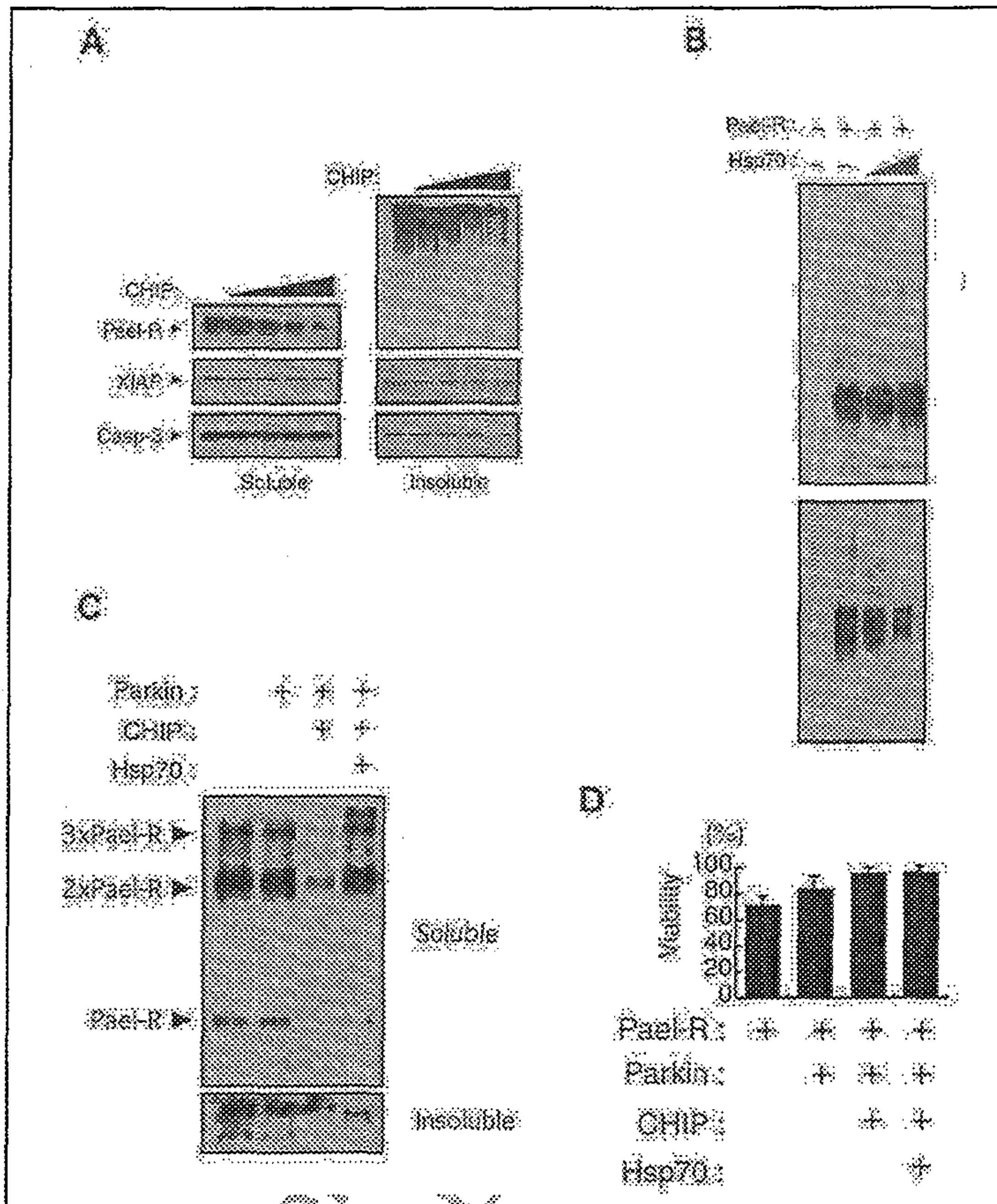

Figure 13. CHIP and Hsp70 cooperate with Parkin in removing unfolded Pael-R. A) Pael-R and increasing amounts of CHIP-transfected cells were lysed in fractionation buffer containing 1% Triton-X 100, and separated into soluble and insoluble fractions. Each fraction was subsequently Western blotted using anti-Pael-R. Endogenous XIAP and caspase-3 (Casp-3) were also detected with anti-XIAP or anti-Casp-3 Ab. B) Pael-R and increasing amount of Hsp70 were transfected into the cells. Detergent soluble and insoluble Pael-R was detected as in (A). C) Cells transfected with the cDNAs in the indicated combination, were lysed and separated into soluble and insoluble fractions. Each fraction was Western blotted as in (A). The ~120 kDa and 180 kDa forms of Pael-R (2 x Pael-R and 3 x Pael-R, respectively), which are most likely SDS-resistant dimer and trimer of Pael-R, are shown in the panel of the soluble fraction. Heavily ubiquitinated Pael-R, which could not migrate into the separating SDS-PAGE gel and stayed in the stacking gel, is shown in the insoluble fractions. D) The viability of SH-SY5Y cells after 36 hrs transfection with the indicated cDNAs was assessed by mitochondrial dehydrogenase activity using WST-1 reagent (Roche Diagnostics) and expressed as a percentage of the viability of the vector control. Data express mean ± S.D.

Another cofactor is the 70-kDa heat shock protein Hsp70 (Fig. 11B). In the fly model of PD, Hsp70 protects against neurotoxicity of over-expressed α-synuclein without obvious suppression of its inclusion formation.[42] In another neurodegeneration event caused by protein inclusion, Hsp70 or Hsp40 alleviates the toxicity of polyglutamine proteins despite no inhibitory effect on the formation of nuclear inclusions.[43-45]

Although Hsp70 appears to inhibit ubiquitination of Pael-R in vitro, Hsp70 effectively suppresses the insolubilization of Pael-R in vivo (Fig. 13B). The effect of over-expressed Hsp70 resembles that of over-expressed Parkin. Moreover, Hsp70 inhibits CHIP-mediated degradation of soluble (probably functional) Pael-R, so that only insoluble aggregated Pael-R is removed. Concomitantly, unfolded Pael-R-induced cell toxicity is suppressed in an additive manner (Figs. 13C and D).

Hsp70 appears to bind Parkin or CHIP directly, and ER-associated Hsp40, Hdj-2, is also found as a cofactor of Hsp70 in the Parkin-Pael-R immuno-complex. The presence of Hdj-2 in the Parkin complex strongly implies that part of Parkin is localized at the cytoplasmic surface of the ER toward the cytosol. Immunoelectron microscopic analysis also supports this notion.[36]

Roles of Hsp70 and CHIP during ERAD

Under physiological conditions, most nascent polypeptides associate with Hsp/Hsc70 and other cochaperones. The Hsp70 system plays an essential role in ensuring the proper folding and intracellular localization of newly synthesized polypeptides.[46] Under unfolded protein stress, Hsp70 is one of the most rapidly inducible proteins.[47,48] Hsp70 functions as a major cellular defense molecule against the accumulation and aggregation of damaged protein caused by a diverse array of stress conditions, by retarding protein degradation and promoting proper refolding.[49-51] In fact, Hsp 70 acts as an inhibitory factor that suppresses the ubiquitination of Pael-R mediated by Parkin in vitro, and enhances the efficiency of folding of over-expressed Pael-R in vivo. On the other hand, Hsp70 may play a role in the transfer of unrecoverable polypeptides to the degradation complex. Taking the results of our studies into consideration, steady state cell conditions might allow Hsp70 and cochaperone Hdj-2 to recruit newly synthesized Pael-R, thereby suppressing unwanted aggregation and promoting translocation into the ER (Fig. 14, upper). Even when Parkin is already associated with the Pael-R-chaperone complex, Hsp70 might inhibit its E3 activity. Upon unfolded protein stress, the cells might rapidly induce Hsp70, thereby suppressing the aggregation and accumulation of unfolded Pael-R dislocated from the ER. Subsequently, CHIP and Parkin will be induced. CHIP appears to associate with Hsp70, thus promoting dissociation of Hsp70 from Pael-R and activating Parkin and E2 activity (Fig. 14, lower). According to the hypothesis shown in (Fig. 14), an elevation in the CHIP level is a decisive event in promoting the transition from the substrate (nascent peptides)-chaperone complex to the substrate (unfolded peptides)-disassembly complex. In addition, Hsp70 has a dual function in that it transiently holds newly synthesized polypeptides at the ER surface or in the cytosol, and unfolded polypeptides from the ER or in the cytosol.

ER Stress and Neurodegeneration

The ER is an intracellular machine serving to control cellular calcium homeostasis and the folding or processing of membrane or secretory proteins. Disturbance in ER function must affect various organs. In the brain, neuronal cells need to produce various transmitters in the ER during normal activity. Therefore, it is no wonder that ER dysfunction causes various neuropathological processes. Recently, ER stress including UPS has been suggested to be involved in Alzheimer's disease.[26,52,53] Furthermore, neural injury that results from transient ischemia has been implicated in ER dysfunction.[54] Thus, emerging pathological and biochemical evidence indicate that ER function is disturbed in acute and chronic diseases of the brain.

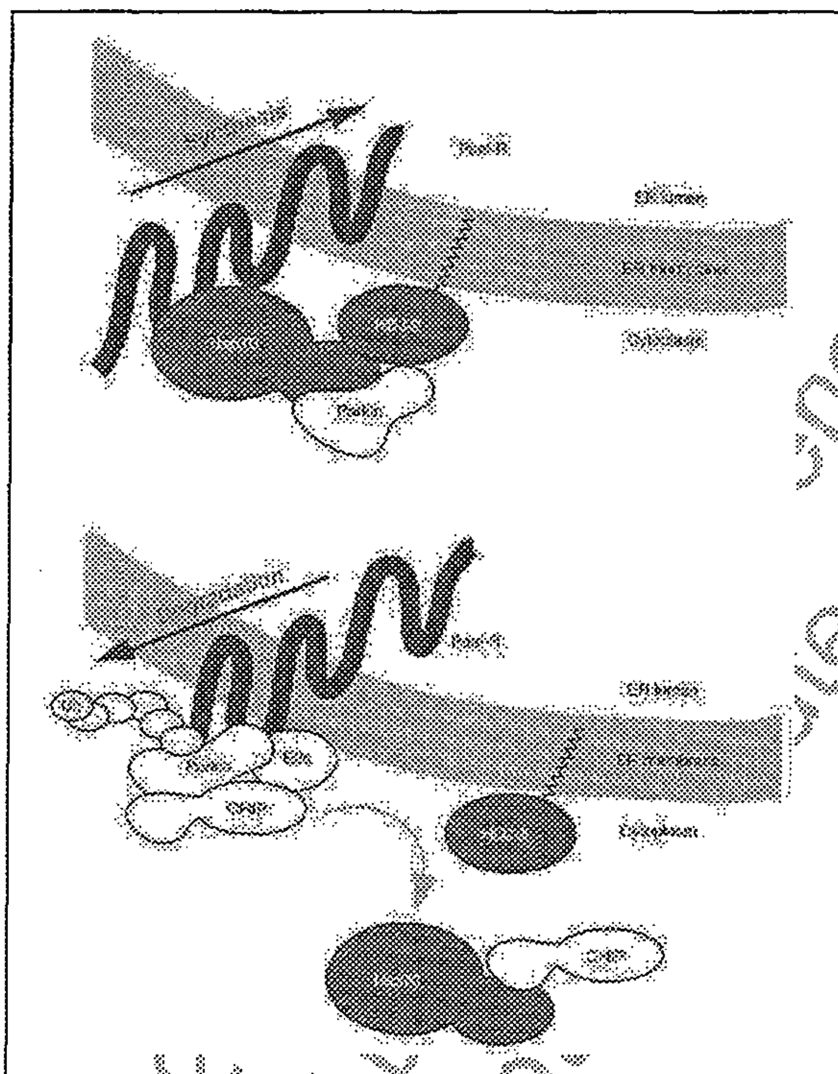

Figure 14. Hypothesis for quality control of Pael-R. Upper, newly synthesized Pael-R is assisted in entering into the ER by chaperones (Hsp70 and Hdj-2). Hsp70 may also bind to Parkin to protect Pael-R from ubiquitination by Parkin. Lower, when unfolded Pael-R is generated in the ER, unfolded Pael-R is retrotranslocated from the ER to the cytosol. Hsp70 and Hdj-2 transiently bind to retrotranslocated Pael-R to prevent the unfolded Pael-R from becoming insoluble. CHIP then binds to Hsp70, promoting the release of Hsp70 and Hdj-2 from Pael-R. On the other hand, CHIP, Parkin, and their partner E2s such as Ubc4, Ubc6 and Ubc7 form a complex on the ER surface to promote ubiquitination of Pael-R.

ER Stress Markers

Although ER stress has been implicated in human disease, confirmation of ER stress involvement by diagnostic testing is difficult. No appropriate marker for ER stress is available for histological examination of tissue sections. BiP, CHOP, phosphorylated IRE1 or phosphorylated PERK are often used as ER stress markers in Northern and Western blot analysis. However, the phosphorylation of IRE1 or PERK might be a transient event during ER stress.[55,56] In contrast, BiP and CHOP transcripts are dramatically upregulated when cells are exposed to acute ER stress. XBP-1 mRNA processed by activated (phosphorylated) IRE1 encodes a transcription factor with strong UPR activity. The detection of processed XBP1 mRNA or its product seems to be useful for analysis of ER stress, in particular unfolded protein stress. Thus, one might easily estimate ER stress response by the detection of activation of these genes,

whereas whether these genes are still being upregulated during chronic ER stress is unknown.[57] Moreover, in secretory tissue such as the pancreas, the UPR-related pathway is constitutively activated. Furthermore, the upregulation of some kinds of molecular chaperone appear to depend on glucose homeostasis or dietary condition,[58] which suggests that variation in daily food content causes the level of these chaperones to fluctuate. Assessing only the well-known ER stress-inducible genes may result in failure to correctly estimate the neuropathological status of each patient. In contrast, some part of UPS-related neurodegeneration may be attributed to the failure or suppression of UPR transduction, which may result from genetic mutations in UPS sensing molecules or environmental conditions.

Recently, Parkin was found in LBs in PD and dementia with LBs, implying that Parkin is sequestered in LBs of affected neurons,[59] leading to the possibility that the function of Parkin is impaired in sporadic PD. To obtain solid evidence of the involvement of ER stress in sporadic PD, appropriate diagnostic tests of ER stress need to be developed.

Therapeutic Strategies for AR-JP

AR-JP develops early in life, compared to general PD, suggesting that the etiology of AR-JP consists of the most direct mechanism of degeneration of dopaminergic neurons. Although introducing the gene for Parkin or CHIP into the affected region of the brain and invoking stable expression therein is relatively straightforward, difficulties have arisen in recently developed gene therapy techniques in individuals. Transient knock-down of Pael-R using antisense or siRNA techniques could provide a potentially effective therapy, although disturbance of the physiological functions of Pael-R might result in unpredictable side effects. Until now, several survival factors against unfolded Pael-R-induced cell death have been identified using the screening of cell death assay of unfolded Pael-R. Although an ER stress-induced apoptotic pathway has been proposed, general anti-apoptotic inhibitors tested so far do not seem to be powerful survival factors. On the other hand, BiP has the strongest protective effect on neuronal death by unfolded Pael-R, and Hsp70 also suppresses the insolubilization of Pael-R. Recently, a type of SCF-type E3 complex has been shown to recognize a specific status of sugar chains of ER protein.[19] This function seems to cover common glycosylated proteins. The finding raises a possibility that there remain several unknown E3s that monitor the quality of ER proteins. Given that such quality control E3s can compensate in part for the loss of Parkin function, it is conceivable that detoxification of unfolded Pael-R by chaperones is a key step in the prevention of neuronal death. Upregulation of molecular chaperones might be the most promising prophylactic therapy against UPS-associated diseases.[60] In light of this idea, the fact that dietary-restriction induces upregulation of chaperones provides clues to the future development of chaperone therapy.[58]

Conclusion

We have provided strong evidence that accumulation of unfolded Pael-R is causative of AR-JP. Recently, the accumulation of denatured proteins has been implicated in many neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and polyglutamine disease.[35,61] Evidence obtained from extensive analysis of AR-JP has elicited common pathogenic mechanisms of PD and other neurodegenerative disorders involving disturbances of the ubiquitin-proteasome pathway and molecular chaperones.

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