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An Unfolded Putative Transmembrane Polypeptide, which Can Lead to Endoplasmic Reticulum Stress, Is a Substrate of Parkin

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Summary

A putative G protein-coupled transmembrane polypeptide, named Pael receptor, was identified as an interacting protein with Parkin, a gene product responsible for autosomal recessive juvenile Parkinsonism (AR-JP). When overexpressed in cells, this receptor tends to become unfolded, insoluble, and ubiquitinated in vivo. The insoluble Pael receptor leads to unfolded protein-induced cell death. Parkin specifically ubiquitinates this receptor in the presence of ubiquitin-conjugating enzymes resident in the endoplasmic reticulum and promotes the degradation of insoluble Pael receptor, resulting in suppression of the cell death induced by Pael receptor overexpression. Moreover, the insoluble form of Pael receptor accumulates in the brains of AR-JP patients. Here, we show that the unfolded Pael receptor is a substrate of Parkin, the accumulation of which may cause selective neuronal death in AR-JP.

Introduction

The second most common neurodegenerative disease, Parkinson's disease (PD), is a movement disorder pathologically characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. The etiology of PD is still unknown, but the recent identification of mutations in familial cases of PD has advanced understanding of the molecular mechanisms of this neurological disease.

Two rare missense mutations in the α-synuclein (α-SYN) gene (A53T and A30P) cause autosomal dominant familial PD (Kruger et al., 1998; Polymeropoulos et al., 1997). α-SYN is a presynaptic protein and a major component of heavily ubiquitinated cytoplasmic aggregates called Lewy bodies (LBs) that are pathological hallmarks of both sporadic and a subgroup of familial PD (Trojanowski et al., 1998). LBs frequently observed in degenerating neurons, including dopaminergic neurons in the substantia nigra, are currently strongly suggested to be involved in the pathogenesis of PD (Baba et al., 1998; Goldberg and Lansbury, 2000; Spillantini et al., 1998).

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An autosomal recessive form of PD (AR-JP), which is the major cause of juvenile PD, results from mutations of the Parkin gene (Kitada et al., 1998). In AR-JP patients, loss of the dopaminergic neurons and consequently, parkinsonian symptoms, can occur without LB formation (Mizuno et al., 1998).

Parkin is one of the largest genes of the human genome (1.5 Mb). It comprises twelve exons encoding a 465 amino acid protein with a molecular mass of 52 kDa (Kitada et al., 1998; Shimura et al., 1999). The NH_2-terminal 76 amino acids of Parkin are 62% homologous to ubiquitin. The COOH-terminal half of Parkin contains two RING fingers flanking a cysteine-rich domain, termed in between RING fingers (IBR) (Morett and Bork, 1999). Several studies have recently revealed that numerous proteins with RING finger motifs have ubiquitin-protein ligase (E3) activity (Jackson et al., 2000; Joazeiro and Weissman, 2000). We and others have demonstrated that Parkin is an E3 and that AR-JP-linked Parkin mutants are defective in E3 activity (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000).

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 (Hershko and Ciechanover, 1998; Hochstrasser, 1996). The exquisite specificity for the proteins to be ubiquitinated is usually determined by a diverse family of E3s with a specific E2. Because AR-JP is caused by a loss of functional Parkin, the accumulation of Parkin substrate(s) should lead to dopaminergic neuronal death. We found that overexpression of Parkin confers resistance to endoplasmic reticulum (ER) stress induced by the unfolded protein in dopaminergic neuroblastoma cells, suggesting that the substrate(s) of Parkin is an unfolded ER protein (Imai et al., 2000).

Here, we show that a putative G protein-coupled integral membrane polypeptide, named Pael receptor, is a substrate of Parkin, and that the accumulation of Pael-R might lead to dopaminergic neuronal death in AR-JP.

Results

Identification of Parkin-Interacting Protein

To identify a substrate(s) of Parkin, we performed yeast two-hybrid screening of human adult brain cDNA libraries using full-length Parkin as bait. A BLAST search revealed that one of the isolated clones from 8.5×10^6 library transformants encoded a partial clone (position 314 to termination) of a homolog of endothelin receptor type B, renamed Pael (*P*arkin-*a*ssociated *e*ndothelin *r*eceptor-*l*ike) receptor (Donohue et al., 1998; Marazziti et al., 1997; Zeng et al., 1997).

To examine the interaction of Pael receptor (Pael-R) with Parkin in mammalian cells, expression plasmids for C-terminal FLAG-tagged Pael-R (Pael-R-FLAG), or its related polypeptides (endothelin receptor type A and B [ETA- and ETB-R]) with a FLAG tag were transiently

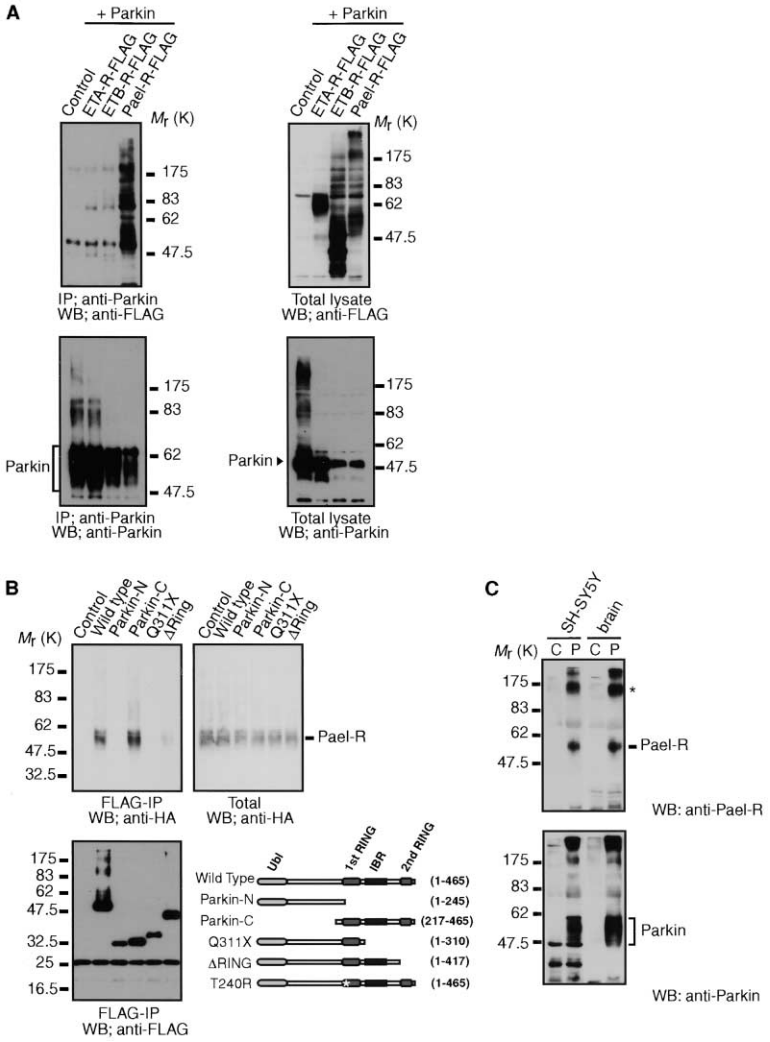

Figure 1. Parkin Associates with Pael Receptor (Pael-R) but Not Its Mutants

(A) Interaction of Parkin with Pael-R. Lysates from 293T cells transfected with an empty vector (Control), FLAG-tagged Pael-R (Pael-R-FLAG), FLAG-tagged endothelin receptor type A or B (ETA-R- or ETB-R-FLAG), and Parkin were immunoprecipitated (IP) with anti-Parkin polyclonal Abs. Immunoprecipitates (IP) and total soluble lysates (Total lysate) were analyzed by Western blotting (WB).

(B) Interaction of Pael-R with Parkin and its mutants. Lysates from 293T cells transfected with Hemagglutinin (HA)-tagged Pael-R and an empty vector (Control), FLAG-tagged Parkin (Wild-type), Parkin-N, Parkin-C, Q311X or ΔRING were immunoprecipitated with an anti-FLAG mAb (FLAG-IP), then Western blotted (WB). The lower panel on the right diagrammatically represents Parkin and the mutants used to determine the Pael-R binding domain. Numbers in parentheses indicate corresponding amino acid residues of Parkin. An asterisk ("*") indicates the site of a point mutation. Ubl, ubiquitin-like domain; RING, RING-finger motif; and IBR, in between RING finger.

(C) Endogenous interaction of Parkin with Pael-R in brain tissue and cultured cells. Human brain or neuroblastoma SH-SY5Y cells were lysed as described in Experimental Procedures, then the supernatant fractions were immunoprecipitated (IP) with preimmune sera (C) or anti-Parkin (P) polyclonal Abs. The coprecipitated Pael-R was detected by Western blotting (WB) using anti-Pael-R mAb. Asterisk indicates about 120 kDa of anti-Pael-R-immunoreactive band, which is often observed in vivo and thought to represent an uncharacterized state of Pael-R.

transfected with a plasmid for human Parkin in human embryonic kidney 293T cells. Pael-R-FLAG was specifically, and ETB-R-FLAG was marginally, coimmunoprecipitated with Parkin by anti-Parkin antibodies (Abs) (Figure 1A). When Pael-R or ETB-R, but not control or ETA-R was overexpressed in 293T cells, these proteins migrated as high molecular mass broad smears on Western blots, suggesting covalent modifications, including glycosylation and ubiquitination. In addition, Pael-R- and ETB-R-expressing cells were dead (40% and 20% of the transfected cells, respectively; data not shown), resulting in the low expression level of Parkin (Figure 1A, lower panels). Next, we examined which region of Parkin binds to Pael-R in mammalian cells (Figure 1B). A series of Parkin mutants and a wild-type protein with FLAG-tag was coexpressed and coimmunoprecipitated with Pael-R tagged with HA (hemagglutinin) at its C terminus in 293T cells. Only the C-terminal portion of Parkin (Parkin-C; 217–465 aa) and full-length Parkin, but not a variety of Parkin proteins harboring mutations in their C-terminal part, retained full binding activity to Pael-R, indicating that the complete C-terminal structure is indispensable for binding to Pael-R.

Finally, we tested physiological interactions between endogenous Parkin and Pael-R in dopaminergic neuroblastoma SH-SY5Y cells and human brain tissues, both of which express Pael-R mRNA (see Figure 7C). As shown in Figure 1C, Pael-R immunoreative protein was coimmunoprecipitaed with endogenous Parkin.

The observation that Pael-R expressed in cultured cells is liable to migrate slowly in Western blots (as shown in Figure 1A) prompted us to examine whether Pael-R can be covalently modified by ubiquitin. Overexpressed ubiquitin with HA-tag (HA-Ub) was coexpressed with FLAG-tagged Pael-R in 293T cells, then immunoprecipitated with an anti-FLAG mAb. The immunoprecipitated Pael-R was covalently modified by HA-Ub, and even in the absence of HA-Ub, higher molecular-shifted Pael-R band was detected by an anti-Ub mAb (Figure 2). These are likely to represent the difficulty of the normal folding of Pael-R polypeptide, and polypeptides with abnormal conformations may be degraded by ubiquitin/proteasome pathway.

Parkin Interacts with ER-Associated E2s

We reported that Parkin is upregulated by unfolded protein stress and that it suppresses cell death induced by unfolded protein stress (Imai et al., 2000). In response

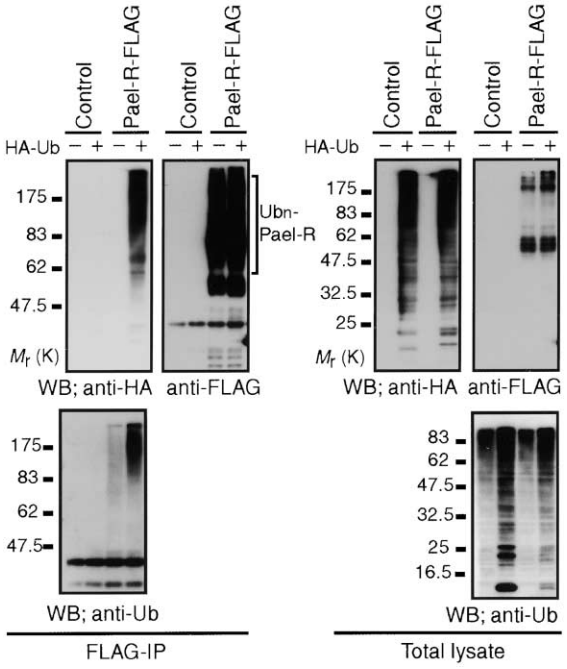

Figure 2. Pael-R Tends to Be Ubiquitinated

Vector plasmid (Control) or Pael-R-FLAG cDNA combined with empty vector (−) or HA-Ub construct (+) was transfected into SH-SY5Y cells. Immunoprecipitates with anti-FLAG mAb (FLAG-IP) and total soluble lysates (Total lysate) were analyzed by Western blotting using anti-FLAG, -HA or -Ub Ab.

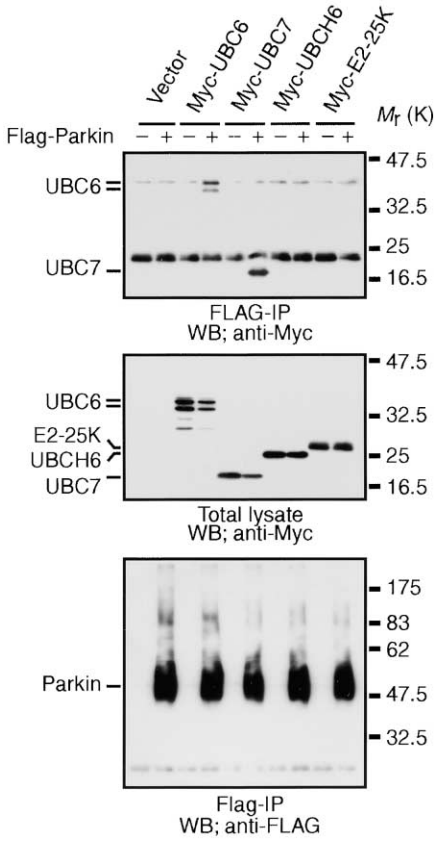

Figure 3. Parkin Associates with UBC6 and UBC7

Vector plasmid or FLAG-Parkin cDNA combined with an empty vector (−), constructs of Myc-tagged-UBC6, -UBC7, -UBCH6, or -E2-25K was transfected into 293T cells. Immunoprecipitates with anti-FLAG mAb (FLAG-IP) and soluble total lysates (Total lysate) were analyzed by Western blotting (WB) using anti-FLAG, or anti-Myc Ab.

to stress in the ER, the unfolded protein response (UPR) that regulates the expression of a series of genes, including ER-associated protein degradation (ERAD)-related genes, is induced (Travers et al., 2000). The ERAD system eliminates misfolded ER proteins including integral membrane and secretory proteins via degradation in the cytosol (Plemper and Wolf, 1999). ERAD substrates are retrotranslocated across the ER membrane into the cytosol, where they are ubiquitinated and degraded through the ubiquitin-proteasome pathway in cooperation with UBC6, UBC7, the proteasome complex, and other components (Biederer et al., 1997; Bordallo et al., 1998; Gilon et al., 2000; Wilhovsky et al., 2000). As our data has provided evidence that Parkin, an apparent RING-type E3 upregulated by UPR, could be involved in ERAD, we tested whether Parkin can bind to ER-associated E2s. Human 293T cells were transiently transfected with plasmids for various E2s with a Myc-tag in the presence or absence of FLAG-Parkin cDNA, then coimmunoprecipitated. The results revealed that Parkin interacts with ER-associated UBC6 and 7, but not with negative controls (UBCH6 and E2-25K) in vivo (Figure 3).

Pael-R Is a Substrate of Parkin

Then, we evaluated whether Parkin ubiquitinates Pael-R with ER-associated E2s by an in vitro ubiquitination assay. To reconstitute the ubiquitin conjugation reaction of Pael-R, we added Parkin immunopurified from SH-SY5Y cells or whole-cell extract of SH-SY5Y cells to the reaction assay in the presence of purified Ub and E1.

We found that the fraction of Parkin-immunoprecipitates promoted the ubiquitination reaction of Pael-R much more remarkably than whole-cell extract alone (Figure 4A). It is noted that the fraction of Parkin-immunoprecipitates was sufficient for this reaction despite the absence of purified E2 (Figure 4A, lanes 6 and 7). These results imply that Parkin immunopurified from the cells mediates transfer of ubiquitin from cellular E2(s), which might form a complex with Parkin, to Pael-R. To examine this possibility, we used a recombinant protein consisting of Parkin with glutathione S-transferase (GST-Parkin) or of a disease-associated exon 4 deletion mutant of Parkin (GST-ΔE4) as an E3 ubiquitin-ligase. Because Pael-R may be an integral membrane polypeptide, and because de novo synthesized Pael-R with abnormal conformations may be subject to ERAD, the recombinant E2s we tested in this assay were UBC6 (or UBC6ΔC; 1–288 aa) and UBC7 in addition to UBCH7, which can function as an E2 for Parkin in vitro. The results presented in Figure 4B demonstrate that GST-Parkin substantially promoted the ubiquitination of Pael-R despite the absence of the E2s (Figure 4B, lane 4), suggesting that endogenous E2(s) interacting with the Pael-R produced in reticulocyte lysates was copurified. In contrast, a deletion mutant of recombinant Parkin, GST-ΔE4 failed to ubiquiti-

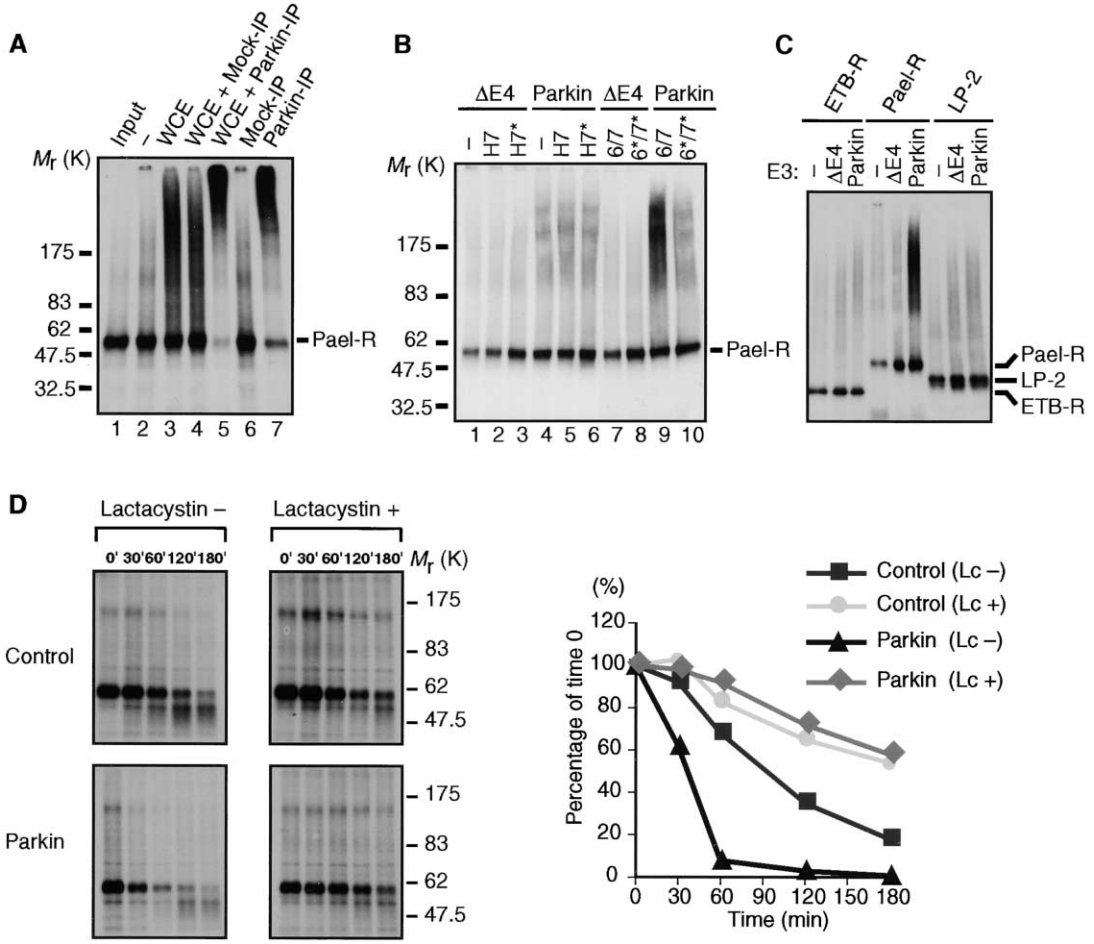

Figure 4. Pael-R Can Be a Substrate for Parkin

(A) In vitro ubiquitination assay of Pael-R using endogenous Parkin from cultured cells. ^{35}S-labeled Pael-R-FLAG was incubated in the presence of Ub, E1 with the following components: WCE, whole cell extracts of SH-SY5Y; Mock-IP, immunoprecipitates with preimmune sera from SH-SY5Y cells; Parkin-IP, immunoprecipitates with anti-Parkin Ab from SH-SY5Y cells. Input and "–" indicate Pael-R at time 0 and Pael-R incubated with Ub and E1 alone for 90 min, respectively.

(B) In vitro ubiquitination assay using recombinant Parkin and E2s. The labeled Pael-R as in (A) was reacted with Ub and E1 in the presence or absence (–) of the indicated recombinant E2 (H7, UBCH7; 6/7, UBC6ΔC and 7) together with GST-fused Parkin or GST-fused exon 4-deleted mutant of Parkin (ΔE4). Asterisks indicate that active cysteine of E2s was exchanged to serine residues.

(C) In vitro ubiquitination assay using Pael-R and its homolog as substrates. The labeled Pael-R, ETB-R, or ET_BR-LP-2 (LP-2) was reacted in the presence of UBC6ΔC and 7 as in (B).

(D) 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 (Lc +) or absence (Lc –) 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 the amount present at time 0 (right).

nate Pael-R even in the presence of the E2s (Figure 4B, lanes 1–3, 7, and 8). The combination of UBC6ΔC, UBC7, and GST-Parkin remarkably accelerated ubiquitination, whereas catalytic inactive mutants of both UBC6ΔC and 7 reduced ubiquitination to the basal level (Figure 4B, compare lanes 4, 9, and 10). The conjugation reaction by a combination of recombinant UBC6 (or UBC6ΔC)/UBC7 and GST-Parkin was highly specific for Pael-R, but not for ETB-R or ET_BR-LP-2, which are very closely related to Pael-R (Valdenaire et al., 1998) (Figure 4C). Together with the binding between Parkin and UBC6/7 (Figure 3), this in vitro ubiquitination assay suggests that UBC6 and UBC7 are involved along with Parkin in the Pael-R degradation pathway. To investigate whether Parkin accelerates Pael-R degradation via the ubiquitin/proteasome pathway, Pael-R turnover was analyzed in the presence or absence of Parkin by pulse-chase experiments (Figure 4D). Following a 180 min chase in the absence of lactacystin, 18% of de novo synthesized Pael-R remained in the control cells. Lactacystin decelerated the rate of Pael-R degradation in control cells and in those transfected with Parkin, resulting in 54% and 58% of protein remaining at 180 min of chase, respectively. In contrast, Pael-R degradation in the Parkin-transfected cells was considerably accelerated, such that 0.8% of the protein remained at 180 min. These results indicated that Parkin is involved in Pael-R degradation through the ubiquitin/proteasome pathway.

Unfolded Protein-Stress Promotes the Insolubility of Pael-R

When Pael-R was overexpressed in cultured cells even in the absence of the proteasome inhibitors, the Pael-R is heavily ubiquitinated (Figure 2). This finding is reminiscent of that of polyubiquitinated cystic fibrosis transmembrane conductance regulator (CFTR) or δ opioid receptors. The majority (75% and 60%, respectively) of de novo synthesized CFTR and δ opioid receptors failed to fold correctly (Jensen et al., 1995; Petaja-Repo et al., 2001; Ward et al., 1995). Such proteins are retrotransported from the ER to the cytosol via the translocon complex including Sec61 (a specific ER transport channel) (Plemper and Wolf, 1999). These are subsequently processed through ERAD that is dependent upon cytosolic ubiquitin-proteasome system.

As exposing cells to proteasome inhibitors causes accumulation of nonionic detergent-insoluble CFTR molecules, which are highly polyubiquitinated in vivo, we examined whether Pael-R exists in the detergent insoluble fraction in addition to detergent soluble fraction. Figure 5A shows that SH-SY5Y cells were transfected with or without Pael-R cDNA, and 1% Triton X-100-soluble and -insoluble fractions were subjected to Western blot analysis. The amount of Pael-R-immunoreactive material in the insoluble fraction of the control sample was comparable to that in the soluble fraction even with no treatment. Moreover, lactacystin and UPR-inducing reagents such as tunicamycin (an inhibitor of *N*-glycosylation) or 2-ME (a reducing agent) promoted the insolubility of this polypeptide with a high molecular-shifted modification. Tunicamycin caused an apparent low molecular-mass mobility shift of Pael-R in the soluble fraction, suggesting that *N*-glycosylation of Pael-R was inhibited. We recovered the insoluble Pael-R from insoluble fractions of the same samples, then analyzed them using anti-Ub polyclonal Abs (Figure 5A, right). In good correlation with the accumulation of Pael-R in the insoluble fractions, high molecular weight material reacted with the Ub Abs in the insoluble Pael-R-immunoprecipitated fractions. In contrast, little of such material was detected in the nontreated sample. We also noted that lactacystin and the UPR-inducing reagents added during Pael-R overexpression in cells also upregulated the ER-chaperone, BiP (GRP78) and endogenous Parkin in the soluble fractions, and that these proteins accumulated to some extent in the insoluble fractions (Figure 5A). These results are in agreement with our previous findings that UPR inducers upregulate Parkin and BiP (Imai et al., 2000).

Accumulation of Pael-R Induces Cell Death

As mentioned above, overexpression of Pael-R specifically induces cell death. One possible cause of cell death in the presence of overexpressed Pael-R is a disrupted intracellular signal transduction, including apoptotic pathways. Another possibility is cell death by unfolded protein stress. The latter is likely because Pael-R was often highly ubiquitinated (Figure 2), and because insoluble Pael-R accumulated when BiP was upregulated (Figure 5A). To test this hypothesis, we assessed the effect of proteasome inhibitor or UPR-inducing reagents on cell death induced by Pael-R-overexpression (Figure 5B). About 20% of transfected cells underwent cell death under these conditions (Figures 5B and 5C). Lactacystin (20 μM) for 16 hr did not affect SH-SY5Y cell death compared with nontreated mock-transfected cells, whereas the UPR-inducers, tunicamycin (5 μg/ml) and 2-ME (2 mM), induced death in about 40% of all mock-transfected cells. Under the same conditions, about 50% of Pael-R-overexpressing cells were dead in the presence of lactacystin (Figures 5B and 5C). Likewise, about 60% were killed by tunicamycin or 2-ME (Figure 5B). To further confirm the involvement of unfolded protein stress in cell death induced by overexpressed Pael-R, we examined the subcellular distribution of Pael-R in the presence or absence of lactacystin (Figure 5D). SH-SY5Y cells transfected with a plasmid encoding Pael-R were incubated with or without lactacystin, then stained with anti-Pael-R or anti-BiP Ab. Pael-R was mainly expressed on the cell surface of nontreated SH-SY5Y cells, as reported, and partially in the perinuclear positions (arrows) or the cytoplasm, suggesting that it is a plasma membrane protein. In contrast, incubation with lactacystin for 6 hr promoted Pael-R accumulation in the ER, inhibiting the expression on the cell surface. Pael-R localization in the ER was confirmed by costaining with anti-BiP, as well as with pDsRed-ER, which is a plasmid encoding a red fluorescent protein with an ER-targeting sequence (data not shown). Its specific localization in the ER but not the Golgi was further confirmed by costaining with a red fluorescent protein with a Golgi-targeting sequence (DsRed-Golgi) or rhodamine-labeled wheat germ agglutinin (data not shown). The results were the same in cells incubated with tunicamycin or 2-ME (data not shown). In fact, further incubation with these reagents for up to ~8–16 hr resulted in formation of aggresome-like inclusions at the juxtanuclear site (Kopito, 2000). At this stage, cell bodies were small and round, ready to undergo cell death (Figure 5E). Together with the results of the Western blotting analysis above, these immunocytochemical studies supported the notion that the accumulation in the ER of overexpressed Pael-R leads to unfolded protein-induced ER stress as indicated by BiP upregulation, finally causing cell death (Kozutsumi et al., 1988).

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

Because of the protective function of Parkin against unfolded protein-induced cell death, we anticipated that Parkin also rescues cells from overexpressed Pael-R-induced death. Thus, we transfected SH-SY5Y cells with vector plasmid or Pael-R cDNA combined with cDNAs for a series of FLAG-tagged Parkin and its mutants. Lysates from these transfected cells were separated into 1% Triton X-100-soluble and -insoluble fractions, immunoprecipitated with an anti-FLAG mAb, and then Western blotted (Figure 6A). Consistent with the observations in Figure 1, soluble Pael-R-immunoreactive bands were specifically detected in the immunoprecipitates of Parkin, Parkin-C and, partially, T240R point mutant from the soluble fraction. Another attempt to confirm the association in the insoluble fraction revealed that Parkin, Parkin-C and, partially, T240R still interacted with Pael-R in the insoluble fraction. The coprecipitated

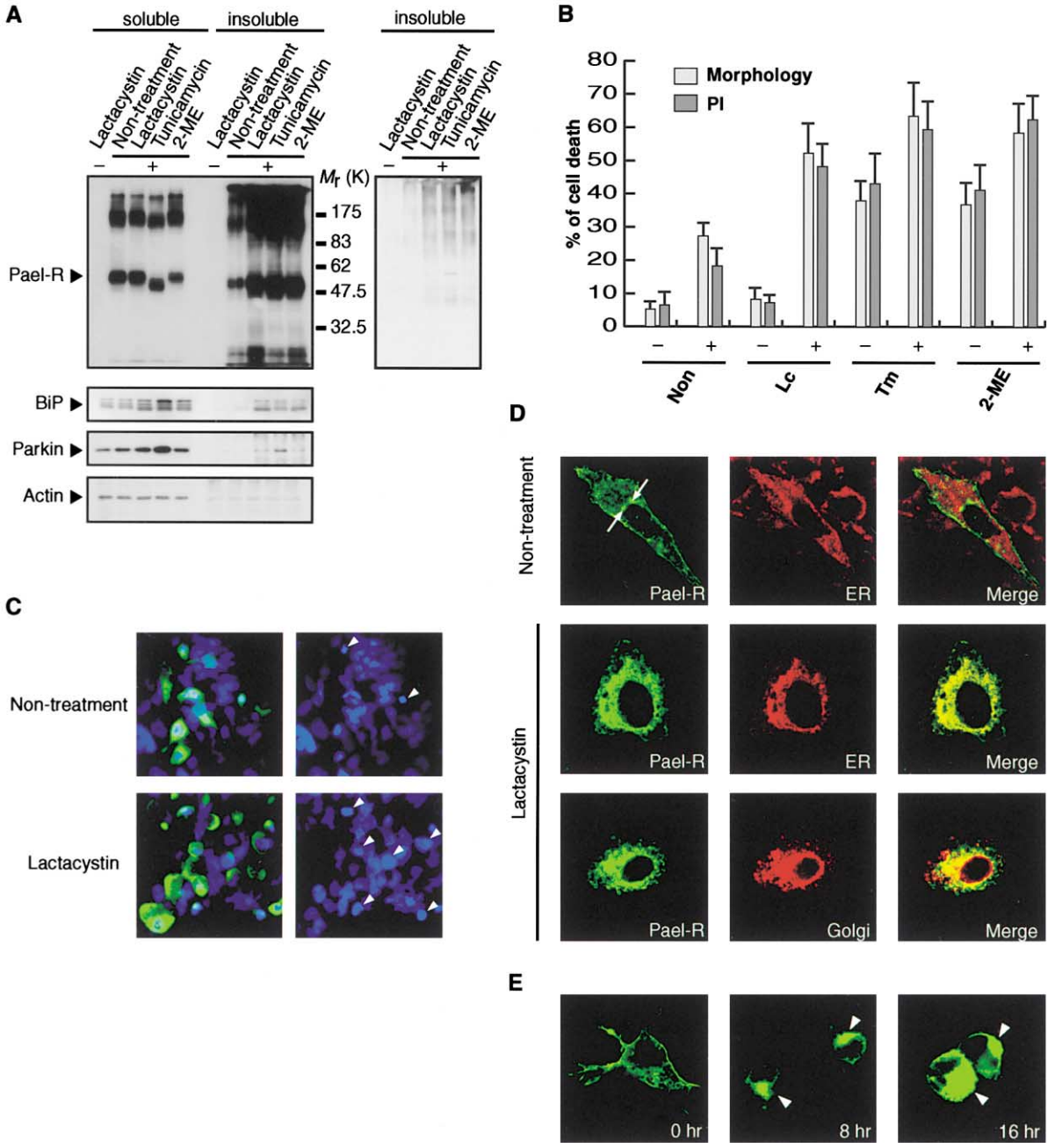

Figure 5. Pael-R-Induced Unfolded Protein Stress

(A) Pael-R is easily unfolded and rendered insoluble by ER stresses. SH-SY5Y cells were transfected with empty vector (−) or Pael-R (+) for 20 hr. Cells were then incubated with or without lactacystin (10 μM), tunicamycin (1 μg/ml), or 2-ME (1 mM) for 16 hr. The cells were lysed in 1% Triton X-100- containing buffer and fractionated as described (Ward et al., 1995). 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 in Experimental Procedures, and analyzed with anti-Ub polyclonal Abs (upper panel on the right).

(B) SH-SY5Y cells transfected with an empty vector (−) or Pael-R (+) together with a plasmid for enhanced GFP (EGFP) were incubated with or without (non), lactacystin (Lc, 20 μM), tunicamycin (Tm, 5 μg/ml), or 2-ME (2 mM) for 16 hr. The count of cell death was performed as described (Imai et al., 2000). Error bars represent standard deviation (SD) calculated from triplicate samples.

(C) Morphology of dead Pael-R-expressing cells. SH-SY5Y cells transfected with Pael-R were incubated with or without lactacystin as in (B). Cells expressing Pael-R were visualized using anti-Pael-R mAb (green) and by counterstaining the cell nucleus with 4′, 6-diamidino-2-phenylindole (DAPI, blue). Dying cells with a condensed nucleus and expressing Pael-R are indicated by arrowheads.

(D) Immunolocalization of overexpressed Pael-R in SH-SY5Y cells. SH-SY5Y cells transfected with Pael-R were incubated with (middle and lower panels) or without (upper panels) lactacystin (20 μM) for 6 hr. Pael-R-expressing cells were visualized with anti-Pael-R mAb (green), and by counterstaining ER and Golgi (red) with anti-BiP and DsRed-Golgi, respectively. Colocalization of Pael-R with ER, but not Golgi shows yellow field with a complete match. Arrows indicate the perinuclear accumulation of Pael-R.

(E) Cytoplasmic inclusion of Pael-R. 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 anti-Pael-R Ab (green). The perinuclear inclusion of Pael-R is indicated with arrowheads.

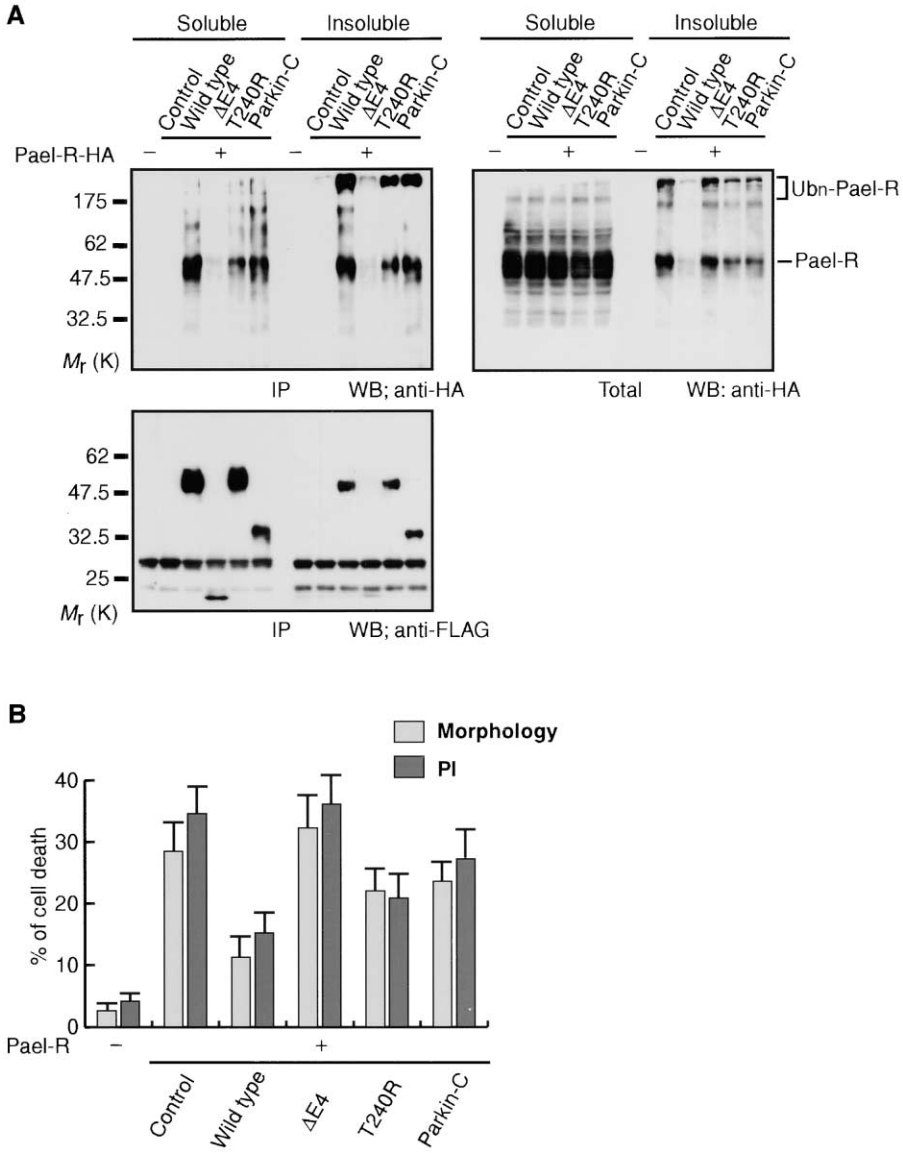

Figure 6. Parkin Suppresses Unfolded Pael-R-Induced Cell Death

(A) Parkin suppresses accumulation of insoluble Pael-R in the cells. SH-SY5Y cells transfected with vector plasmid (−) or construct for Pael-R-HA (+) combined with construct for mock (Control), FLAG-Parkin (Wild-type), -ΔE4, -T240R, or -Parkin-C, were lysed and separated into 1% Triton X-100-soluble (S) or -insoluble (I) fractions, then immunoprecipitated with anti-FLAG mAb (IP). Coprecipitated Pael-R was detected by Western blotting (WB) with anti-HA mAb. Polyubiquitinated Pael-R, which was confirmed by Western blotting on the same membranes with anti-Ub mAb (data not shown), indicates Ub_n-Pael-R.

(B) Expression constructs as in (A) were cotransfected into SH-SY5Y cells together with a plasmid for EGFP as an indicator gene for 48 hr. The count of cell death was performed as described in the legend for Figure 5B.

Pael-R-immunoreactive material with FLAG-Parkin and its mutants from the insoluble fraction included high molecular-shifted smeared bands, indicating modification by polyubiquitination (Figure 6A). The amount of insoluble Pael-R in the total insoluble fraction was significantly reduced in the presence (compared with the absence) of Parkin. In contrast, the disappearance of insoluble Pael-R was partially or completely suppressed in the presence T240R and Parkin-C, or ΔE4, respectively. In agreement with these data on insoluble Pael-R, cell death induced by overexpressed Pael-R was significantly and partially alleviated by Parkin and T240R or Parkin-C coexpression, respectively, whereas the cell death was not suppressed by ΔE4 (Figure 6B). These findings indicated that intact Parkin, but not its mutants, promotes proteasomal degradation of Pael-R before the receptor accumulates in the insoluble fractions.

Accumulation of Pael-R in AR-JP Brain

Based on the results described above, specific dopaminergic neuronal death in AR-JP is most likely caused by the accumulation of the Parkin substrate, Pael-R. To obtain evidence supporting this idea, we assessed the amount of endogenous Pael-R in the human brain. The

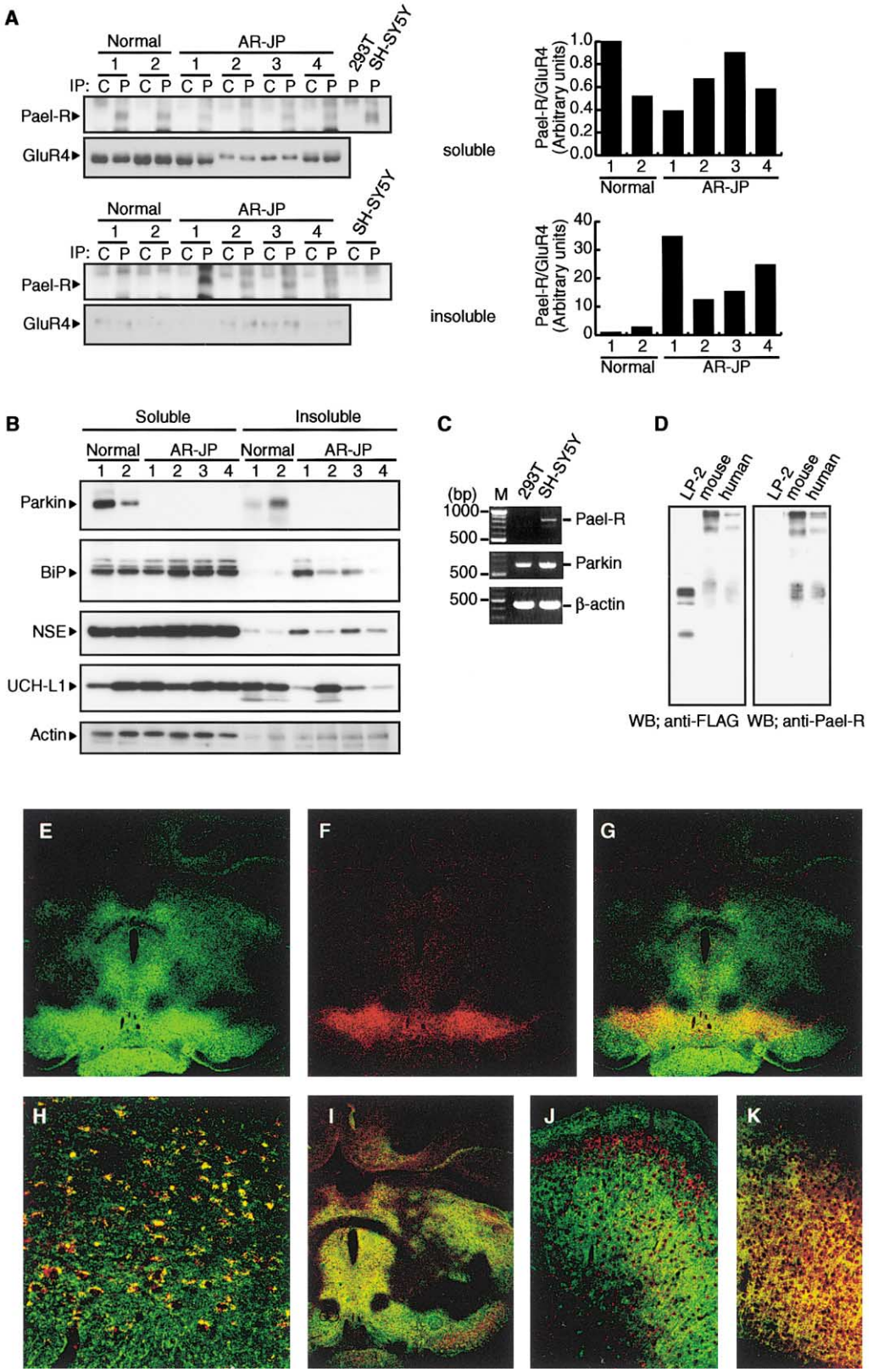

Figure 7. Pael-R in Brains of Patients with AR-JP

(A) Human tissues of frontal lobe cortex from normal or AR-JP brains, SH-SY5Y, or 293T cells were separated into 1% Triton X-100-soluble or -insoluble fractions, then immunoprecipitated (IP) with isotype-matched control IgG (mouse IgG_{2b}, C) or anti-Pael-R mAb (clone 1, P). Each fraction was, subsequently, immunoprecipitated with anti-GluR4 mAb. Precipitates were examined by Western blotting with another anti-

frontal lobe cortices from 4 patients with AR-JP and 2 control individuals were homogenized and separated into 1% Triton X-100-soluble or -insoluble fractions. The fractions were then immunoprecipitated using a Pael-R mAb or a control mAb. Then, the amount of immunoprecipited Pael-R was normalized by that of glutamate receptor 4 (GluR4) (Figure 7A). Pael-R was immunoprecipitated from the control soluble fractions, and from the soluble AR-JP samples, with an insignificant variance between 0.4- to 1.0-fold compared to a control sample (Normal 1). In contrast, 12- to 35-fold Pael-R-immunoreactive material was detected in the insoluble faction of AR-JP compared to Normal 1. Both fractions prepared from SH-SY5Y cells and 293T cells, in which the expression of Parkin mRNA but not Pael-R mRNA was detected (Figure 7C), were likewise analyzed. The same band was detected in Pael-R-immunoprecipitates of the soluble fraction from SH-SY5Y cells, but not from those of 293T cells, confirming the specificity of anti-Pael-R mAb. We could hardly recover Pael-R-immunoreactive material from the insoluble fraction of SH-SY5Y cells as well as from non-AR-JP human brain, suggesting that the normal expression level of Pael-R does not lead to the insoluble accumulation of Pael-R. Furthermore, in both normal and AR-JP brain tissues, ubiquitinated Pael-R was undetectable by Pael-R-immunoprecipitation followed by Western blotting with anti-Ub Ab (data not shown).

Given that the lack of Parkin led to Pael-R accumulation, we investigated the relationship between the accumulation of Pael-R and the resultant UPR (Figure 7B). The total soluble and insoluble fractions of the control and AR-JP brain tissues were Western blotted using anti-BiP polyclonal Abs. BiP was upregulated in the soluble fraction of most AR-JP brain tissues compared with controls. Moreover, BiP was also detected in the insoluble fraction from most AR-JP brain tissues, indicating that UPR is induced in the AR-JP brain and that the insoluble fraction contains unfolded ER proteins with which BiP may associate. Western blotting using anti-neuron-specific enolase (NSE), anti-UCH-L1 and anti-actin Abs showed that the amount of protein derived from neurons and other glial cells in the brain tissues was similar.

We confirmed whether or not Pael-R is expressed in dopaminergic neurons in the zona compacta of the substantia nigra, which selectively degenerate in PD. We prepared an anti-Pael-R Ab, which recognized mouse and human Pael-R, but not its homolog LP-2 (Figure7D), ETA-R, or ETB-R (data not shown). Immunohistochemical studies showed that Pael-R was widely expressed in the brain, including the substantia nigra (Figure 7E). Most of the Pael-R-positive cells were 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-immunoreactive oligodendrocytes (Figures 7I and K). On the other hand, most of neuronal nuclei (NeuN)-positive cells were Pael-R-negative or weak positive (Figure 7J). However, tyrosine hydroxylase-immunoreactive cells were largely Pael-R-positive, indicating that dopaminergic neurons in the zona compacta of the substantia nigra belong to an exceptional subpopulation of neurons expressing Pael-R (Figures 7E–7H).

Discussion

In this study, we identified and characterized a putative seventh transmembrane protein, termed Pael-R, which interacts with Parkin. We concluded that this protein is an authentic in vivo substrate of Parkin from the following evidence: First, 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. Second, Pael-R is specifically ubiquitinated through the Parkin-dependent ubiquitination pathway in vitro in the presence of UBC6 and UBC7. Third, the degradation of Pael-R is promoted when it is cooverexpressed with Parkin in cells. Finally, Pael-R protein is accumulated in the detergent-insoluble fraction of AR-JP brain.

Recent findings have led to models of ER-membrane dislocation coupled to the ubiquitin-proteasome pathway (Plemper and Wolf, 1999). Pael-R accumulated in the ER at the early stage of the proteasome blockade in agreement with some reports suggesting that proteasomes function in driving the extraction of retrograde proteins from the ER during ERAD (Figure 5D) (Mayer et al., 1998; Yu and Kopito, 1999). In contrast, at the late stage of inhibition, the morphology of most cells was apoptotic, being round, shriveled, and forming cytoplasmic aggregations (Figure 5E). Taking into account that ubiquitination and the proteasomal degradation of ERAD are cytoplasmic events for which dislocation of unfolded proteins from the ER is prerequisite, the cytoplasmic aggregation of Pael-R would correspond to the

Pael-R (clone 7) or anti-GluR4 mAb. The anti-Pael-R-precipitated materials were quantified using Quantity One software (BioRAD), and normalized against anti-GluR4-immunoreactive materials for each lane.

(B) Twenty and ten μg of the total protein from soluble and insoluble fractions, respectively, were analyzed by Western blotting. The ER stress response was estimated by the BiP expression level, and total cellular protein concentrations in each sample were confirmed by comparison with amounts of neuron-specific enolase (NSE), UCH-L1, and actin.

(C) Reverse transcription (RT)-PCR of total RNA isolated from SH-SY5Y and 293T cells. First strand cDNA was synthesized by RT, amplified by PCR using various specific primers, and analyzed on a 1% agarose gel. M, 100 bp ladder marker.

(D) Specificity of anti-Pael-R Ab. The extracts of mouse (mouse), human Pael-R with FLAG-tag (human) or ETB-LP-2 with FLAG-tag (LP-2) overexpessing 293T cells were Western blotted with anti-FLAG (left) or anti-Pael-R (right).

(E–H) Immunolocalization of Pael-R (E, green) and tyrosine hydroxylase (F, red) in a coronal section of the murine brain. Yellow indicates the expression of Pael-R (green) in tyrosine hydroxylase-positive neurons (red) in the zona compacta of the substantia nigra (G and H). Original magnification, ×40 (E–G) or ×400 (H).

(I–K) Pael-R is mainly expressed in the oligodendrocytes in the brain. (I and K) Yellow indicates the expression of Pael-R (green) in CNPase-positive cells (red) in a serial section in (E)–(H). (J and K) Immunolocalization of Pael-R (green) and NeuN (red in J) or CNPase (red in K) in the cerebral cortex. Original magnification, ×40 (I) or ×100 (J and K).

ubiquitinated insoluble form of Pael-R detected by Western blotting (Figure 5A). Based on the results obtained from cultured cell experiments, Parkin seems to prevent the cytoplasmic aggregation of dislocated Pael-R and subsequent cell death, participating in the degradation pathway. Moreover, Parkin may promote the retrotranslocation of Pael-R from the ER to suppress ER stress in cooperation with translocon complex and proteasome complex.

One of the pathological hallmarks of AR-JP is the absence of Lewy bodies, which is well explained by the hypothesis that accumulation of Pael-R is causative in AR-JP. The putative membrane polypeptide Pael-R is apparently retained in the ER by a proteasome inhibitor, lactacystin, or by UPR-inducible reagents, suggesting that folding of Pael-R is inefficient. When the amount of unfolded proteins in the ER becomes too large to control, the cells respond by UPR that transactivates multiple genes including molecular chaperones and ERAD-associated molecules, at least in yeast (Casagrande et al., 2000; Friedlander et al., 2000; Travers et al., 2000). The capacity of the ER to deal with unfolded proteins, however, might be rather small and the ER seems to have specific apoptotic pathways against ER stresses such as the disruption of intraluminal calcium homeostasis or accumulation of unfolded ER-proteins (Nakagawa et al., 2000; Urano et al., 2000). Therefore, ER stress-induced neuronal death would occur without aggregates. Similarly, individuals afflicted with AR-JP might develop disease when accumulation of Pael-R in the ER induces cell death through ER-specific apoptotic pathway without cytoplasmic aggregate formation.

The tissue distribution of Pael-R is also consistent with the dopaminergic neuron-selective degeneration observed in AR-JP. Messenger RNA for Pael-R is highly expressed in the central nervous system. Within the brain, Pael-R mRNA is particularly abundant in the corpus callosum and the substantia nigra (Zeng et al., 1997; Donohue et al., 1998). In this study, we have examined immunohistochemical localization of Pael-R in mouse brain using antiserum that very specifically recognizes Pael-R (Figure 7D). Most of the Pael-R immunoreactive cells were CNPase-positive, oligodendrocytes of fiber tracts. On the other hand, Pael-R also localized to a limited subpopulation of neurons including the dopaminergic neurons in the substantia nigra, hippocampal neurons in the CA3 region, and cerebellar Purkinje cells (Figure 7 and data not shown). Assuming that neurons are more vulnerable to ER stress than oligodendrocytes, selective degeneration of dopaminergic neurons in AR-JP can be partly explained by this unique tissue expression of Pael-R. Although the reason why dopaminergic neurons are specifically affected among the Pael-R-expression neurons is not clear, it could be that the expression level of Pael-R in neurons of the substantia nigra is significantly high, as implicated by Northern analyses (Zeng et al., 1997; Donohue et al., 1998). Very recently, Zuscik et al. have reported that the substantia nigra as well as other areas in the brains of transgenic mice overexpressing α_{1B}-adrenergic receptor (α_{1B}AR), which is also a G protein-coupled receptor, degenerate, showing PD-like symptoms (Zuscik et al., 2000). A blockade of α_1AR signal transduction partially rescues the symptoms. The insufficient rescue of the PD-like symptoms, however, might suggest that unfolded protein-induced ER stress contributes to the degeneration of the substantia nigra in the mice. This tissue seems especially sensitive to the unfolded protein stress when the ERAD or ER quality control system is disturbed.

Taken together, we provided strong evidence that accumulation of unfolded Pael-R is causative in AR-JP. Recently, the accumulation of denatured proteins is implicated in many neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, and polyglutamine disease (Julien, 2001; Sherman and Goldberg, 2001). Emerging pathological and biochemical evidence obtained from the studies of these diseases has elicited common pathogenic mechanisms involving disturbances of the ubiquitin-proteasome pathway and molecular chaperones. AR-JP can now be added to this list of the neurodegenerative diseases associated with accumulation of unfolded proteins.

Experimental Procedures

Plasmids, Antibodies, Proteins, and Cultured Cells

Expression plasmids for human Parkin, its mutants, UBCH6, UBCH7, and Ub are described elsewhere (Imai et al., 2000). Full-length cDNAs of human and mouse Pael-R, human ET_BR-LP-2, human UBC6 and 7, and E2-25K were cloned by reverse transcription and polymerase chain reaction (RT-PCR). ETA-R and ETB-R cDNAs were obtained from the RIKEN Gene Bank. Recombinant GST-Parkin, GST-ΔEx 4, His_6-tagged UBC6 and UBC7 were produced in the *E. coli* strain BL21(DE3)pLysS (Novagen). Site-directed mutagenesis of the active cysteine of UBC6 and 7 was achieved by PCR. The deletion form of the putative membrane integrated region of UBC6 (UBC6ΔC; 1–288 aa) was used in the ubiquitination assay in vitro, because UBC6ΔC gave better expression in bacteria and was functionally superior. Anti-Parkin mAbs were raised against recombinant His_6-tagged human Parkin protein produced in bacteria. Anti-human Pael R mAbs were raised against 293T cells overexpressing human Pael-R. Anti-Myc (9E10), anti-HA (Y-11), anti-Ub (FL-76), anti-BiP (N-20), and anti-actin (C-2) Abs were purchased from Santa Cruz Biotech. Anti-FLAG (M2), anti-HA (3F10), anti-Ub (1B3), anti-GluR4 (AB1508), and anti-NSE (BBS/NC/VI-H14) were obtained from Sigma, Roche Diagnostics, MBL (Nagoya, Japan), Chemicon, and Dako, respectively. Anti-UCH-L1 Ab was a kind gift from K. Wada (National Institute of Neuroscience, Japan). Human embryonic kidney 293T and neuroblastoma SH-SY5Y cells were transfected, and used for immunoprecipitation, Western blots, immunocytochemistry, and the cell death assay as described elsewhere (Imai et al., 2000).

Yeast Two-Hybrid Screening

Plasmid pGBT9 (Clontech) harboring cDNA for full-length human Parkin was generated as a bait for library screening. Yeast two-hybrid screening was performed against a mixture of cDNA libraries of human adult whole brain in pACT2 (purchased from Clontech) and human substantia nigra in pGAD424 (prepared in our laboratory) using a Matchmaker Two-Hybrid System kit according to the manufacturer's protocol (Clontech).

Immunopurification and Western Blot Analysis

Cells were suspended in lysis buffer (20 mM HEPES [pH 7.4], containing 120 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% Triton X-100 with complete protease inhibitors [Roche Diagnostics]), and lysed for 30 min at 4°C. Human brain tissues of the frontal cortex were suspended in lysis buffer and then disrupted using a Dounce homogenizer. The detergent-soluble/insoluble fractionation and the subsequent immunoprecipitation were modified from the methods described (Ward et al., 1995). The suspensions were fractionated by centrifugation at 15,000 × g for 30 min. The supernatants from the 1% Triton X-100-soluble fraction were directly immunoprecipitated.

The insoluble pellet fractions were washed four times with ice-cold lysis buffer, and then solubilized in SDS (final concentration 1%) for 1 hr at 60°C. Ten volumes of lysis buffer with 10 mM $MgCl_2$ and 25 μg/ml of DNase I were added, then the samples were incubated for 10 min at 37°C. After centrifugation at 15,000 × g for 30 min, the supernatants from 1% Triton X-100-insoluble fractions were immunoprecipitated with various Abs and protein G-coupled or protein A/G (50/50%)-coupled Sepharose beads (Amersham-Pharmacia), then washed four times in lysis buffer without protease inhibitors. Immunoprecipitates or total cell extracts from 1% Triton X-100-soluble or -insoluble fractions were Western blotted using ECL detection reagents (Amersham-Pharmacia). Protein was quantified using the Coomassie protein assay reagent (Pierce).

Immunochemical Studies

For immunocytochemistry, SH-SY5Y cells on 8-well chamber slides were transfected with 1 μg/well of vector for human Pael-R with or without 0.3 μg/well of expression vector for red fluorescent protein (pDsRed1-N1, Clontech) with an ER-targeting sequence of calreticulin or a Golgi-targeting sequence from human β1, 4-galactosyltransferase. After a 20 hr incubation, the cells were incubated with or without 20 μM lactacystin for 16 hr. The cells were washed with PBS, fixed with 0.2% glutaraldehyde and 2% formaldehyde, then stained with anti-human Pael-R or anti-BiP Ab. For immunohistochemistry, the C-terminal part of mouse Pael-R (541–600 aa) was produced as GST-fusion protein in *E. coli*. Anti-Pael-R polyclonal Ab was raised against the GST-removed recombinant protein. The 14 μm frozen sections of the C57BL/6 mouse brains were stained using anti-Pael-R (1:200 dilution), anti-TH mAb (Chemicon, 1:100), anti-NeuN (Chemicon, 1:200), or anti-CNPase (Sigma, 1:100). Primary antibodies were localized by secondary Abs conjugated to Alexa 488 or 546 (Molecular Probes). Stained cells or sections were mounted in SlowFade (Molecular Probes), then analyzed with a laser scanning confocal microscope system (Fluoview, Olympus).

Pulse-Chase Experiment

SH-SY5Y cells were transfected with vector expressing the Pael-R with a C-terminal FLAG-tag (Pael-R-FLAG) with or without plasmid for Parkin, and 36 hr later, the cells were starved for 1 hr in methionine/cysteine-free DMEM (M/C-free DMEM) containing 5% fetal calf serum (FCS) with or without 10 μM lactacystin, then labeled for 30 min at 37°C with 200 μCi/ml ^{35}S-methionine/cysteine in M/C-free DMEM with 5% FCS with or without 10 μM lactacystin. Cells were then washed and incubated with DMEM plus 10% FCS with or without 10 μM lactacystin for the indicated periods. At each time point of the chase, cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma), resolved by SDS-PAGE, and visualized using an imaging analyzer (BAS-5000; Fujifilm). Metabolically labeled Pael-R was quantified using Image Gauge software (Fujifilm).

In Vitro Ubiquitination Assay

^{35}S-labeled Pael-R-FLAG was produced by TNT quick-coupled transcription/translation systems (Promega) with or without canine pancreatic microsomal membranes (Promega), then immunopurified using anti-FLAG affinity gel. While both Pael-Rs produced in the presence or the absence of the microsomal membranes work indistinguishably in ubiquitination assays in vitro, Pael-R produced without the microsomal membranes was used in most assays. In vitro ubiquitination assay was performed as described elsewhere except for Ub (Sigma, 167 pmol) (Imai et al., 2000).

RT-PCR

Total RNA was isolated using ISOGEN reagent (Nippon Gene, Japan Toyama). First strand cDNA was synthesized using 5 μg of total RNA by a SuperScript First Strand Synthesis kit (GIBCO-BRL). The oligo-dT primed cDNA was then amplified by PCR using the following primers: Pael-R forward primer, 5′-CCTCCAGCTCTTCCTTC AGA-3′; Pael-R reverse primer, 5′-TTTCTGCCGGAGCTCGGCCA-3′; Parkin forward primer, 5′-GGAGGCGACGACCCCAGAAAC-3′; Parkin reverse primer, 5′-GGGACAGCCAGCCACACAAGG-3′; β-actin forward primer, 5′-CAAGGCCAACCGCGAGAAGA-3′; and β-actin reverse primer, 5′-GGAAGGCTGGAAGAGTGCCT-3′. The quality of RNA was confirmed by comparison with mRNA of the housekeeping β-actin gene.

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