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 anionic 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

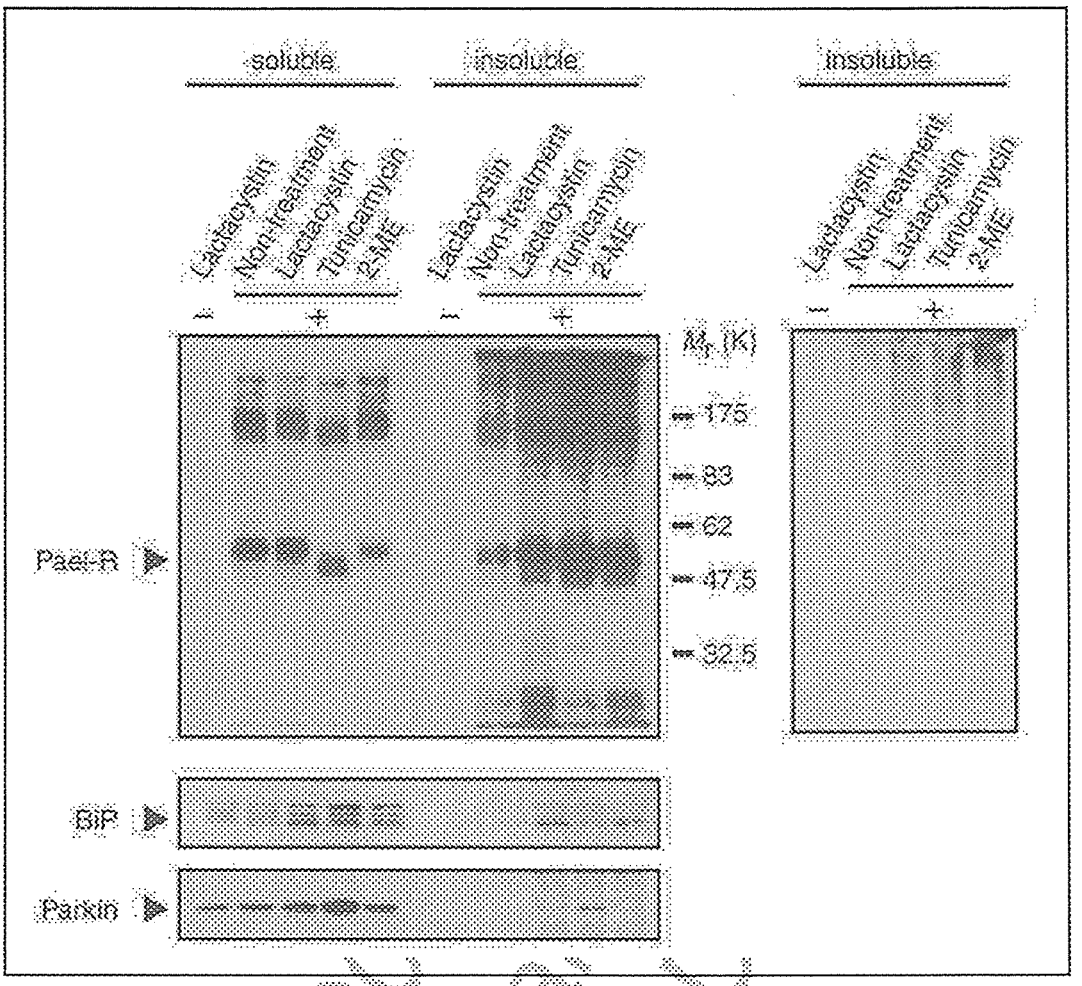

Figure 6. Pael-R tends to be unfolded and to induce ER stress. Pael-R is easily unfolded and rendered insoluble by ER stress. SH-SY5Y cells were transfected with empty vector (–) or Pael-R (+) for 20 hrs. Cells were then incubated with or without lactacystin (10 μM), tunicamycin (5 μg/ml) or 2-ME (1 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.

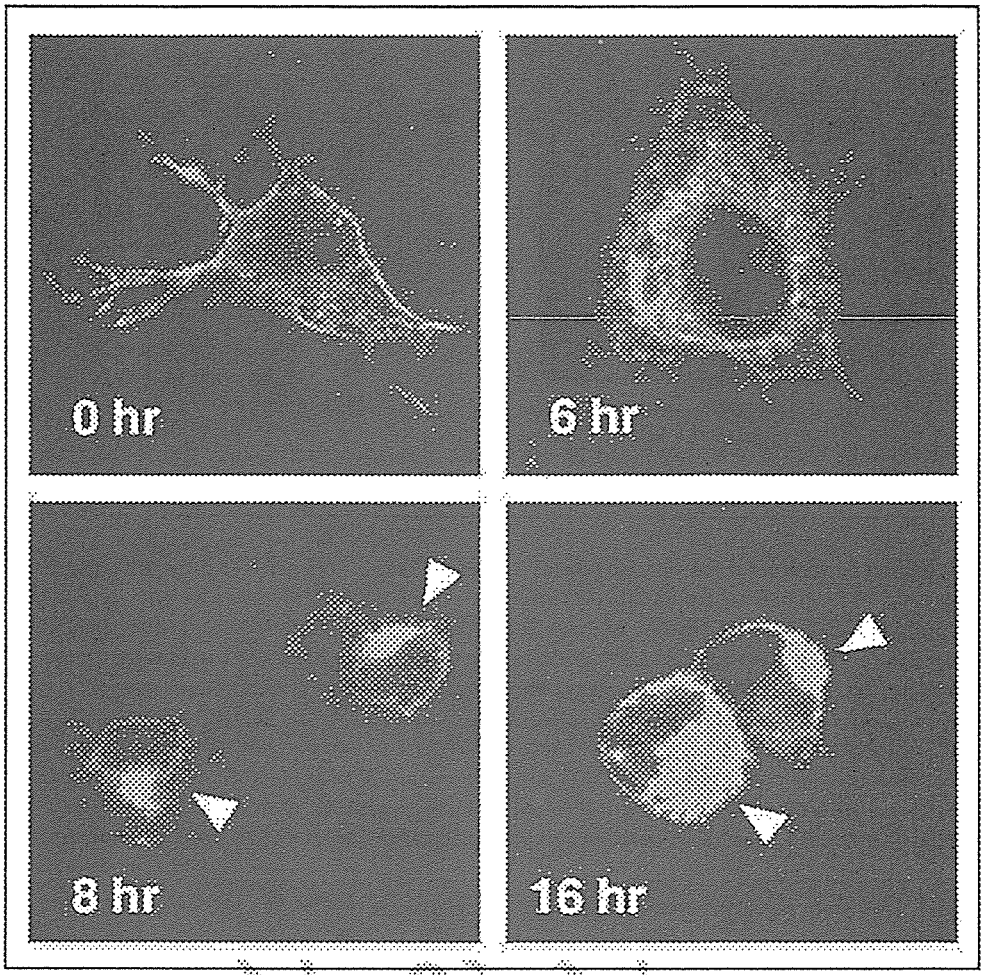

Figure 7. Accumulation of unfolded Pael-R causes neuronal death. SH-SY5Y cells transfected with Pael-R were treated with 20 μM lactacystin for the indicated periods. Cellular localization of Pael-R was visualized using an anti-Pael-R Ab (green). The perinuclear inclusion of Pael-R is indicated as arrowheads. 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.

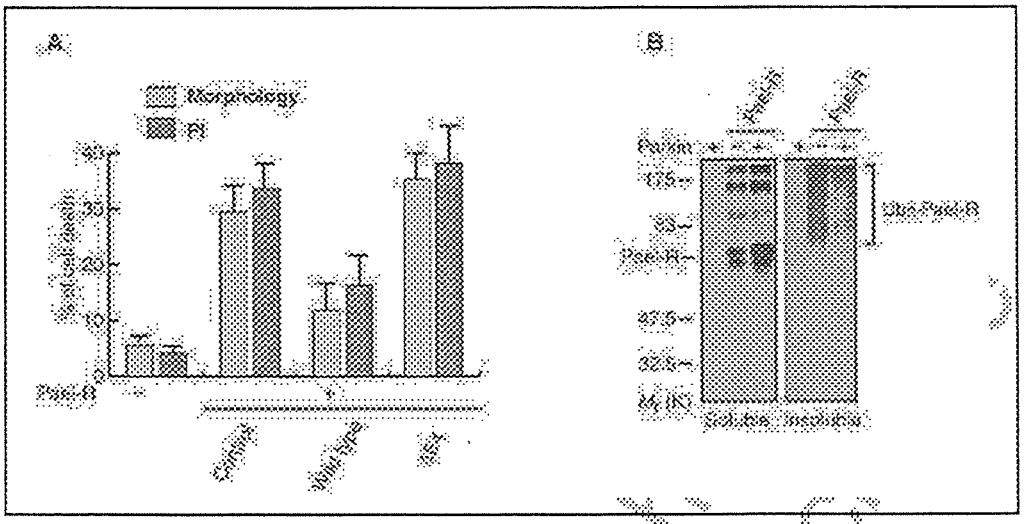

Figure 8. Parkin suppresses the accumulation of unfolded Pael-R and cell death by unfolded Pael-R. A) Vector plasmid (-) or construct for Pael-R (+) combined with construct for mock (Control), FLAG-Parkin (Wild type), or -ΔE4, were cotransfected into SH-SY5Y cells together with a plasmid for EGFP, as an indicator gene, for 48 hrs. About 300 GFP-positive cells were counted and the percentage of dead cells was determined by cell morphology (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).

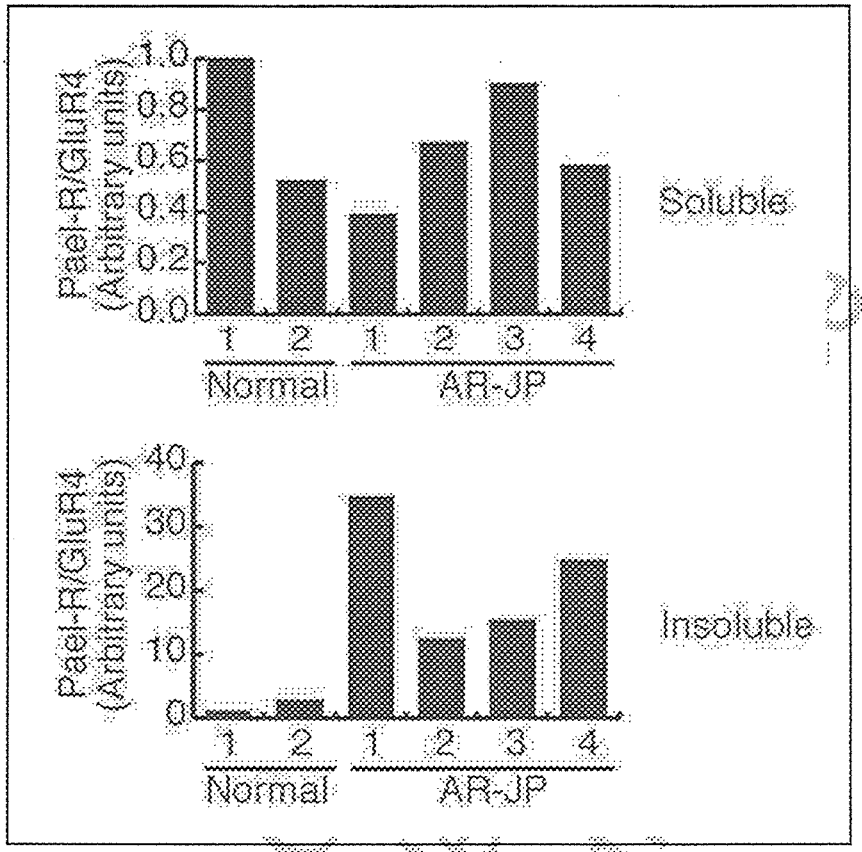

Figure 9. Accumulation of Pael-R in AR-JP brain. Human tissues of frontal lobe cortex from normal or AR-JP brains was separated into 1% Triton X100-soluble or -insoluble fractions as described,[18] then immunoprecipitated with an anti-Pael-R mAb. Subsequently, each fraction was immunoprecipitated with 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.[22-24] 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

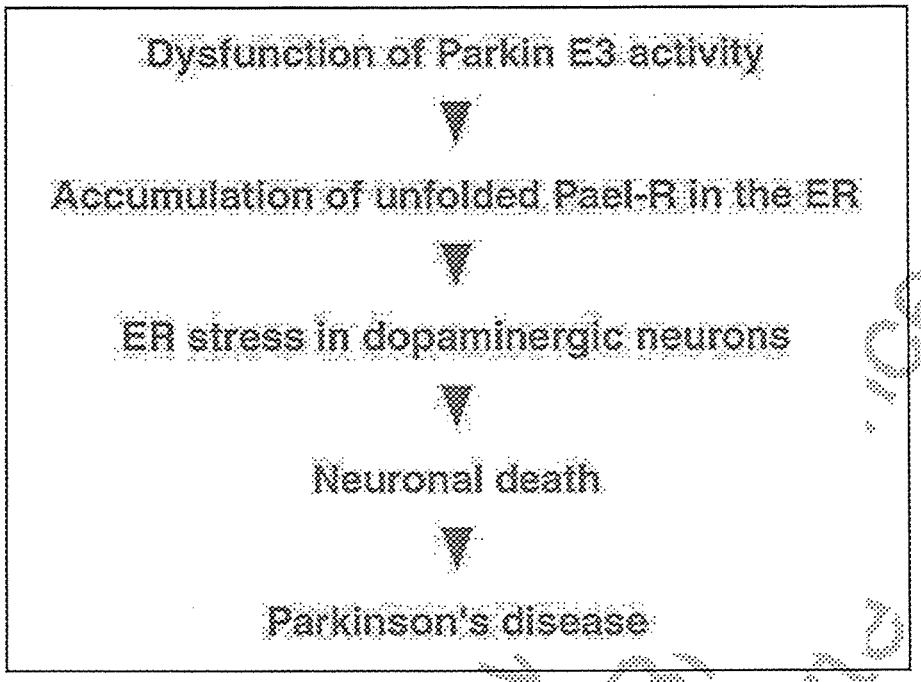

Figure 10. Hypothesis of the pathogenesis of AR-JP. Genetic mutation or certain circumstances cause the attenuation of Parkin's ubiquitin ligase activity, which leads to the accumulation of Parkin's substrate, Pael-R in the ER of the dopaminergic neurons. Chronic accumulation of Pael-R results in unfolded 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

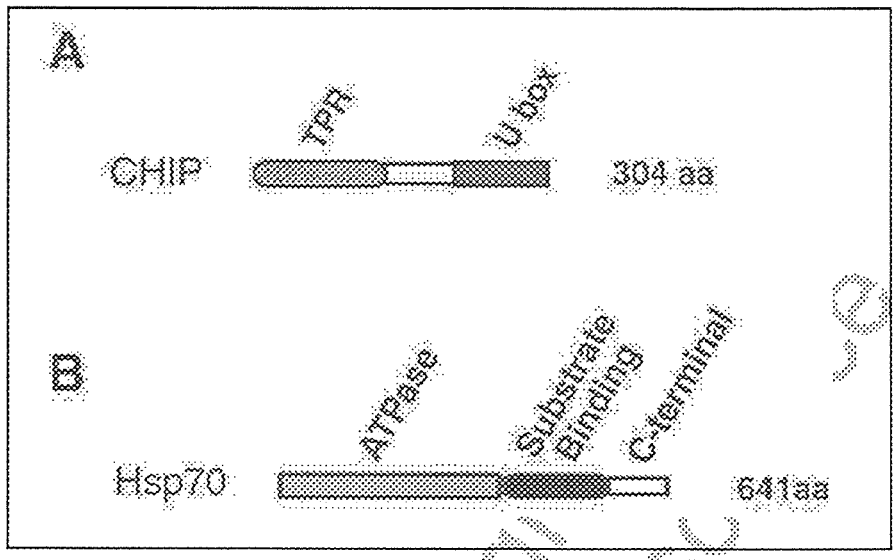

Figure 11. Parkin cofactors, CHIP and Hsp70. A) CHIP has a tetratricopeptide repeat (TPR) and a Ubox at the N- and C-terminus, respectively. CHIP binds to the C-terminal part of Hsp/Hsc70 via TPR and inhibits the ATPase activity of Hsp70. The Ubox is a RING-finger related motif, and has an E3 activity 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.[4] 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).

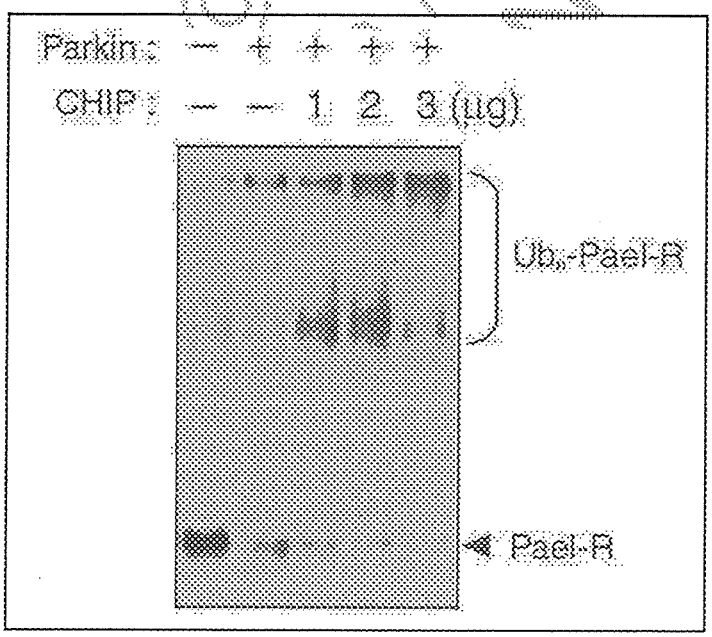

Figure 12. CHIP promotes Parkin-mediated Pael-R ubiquitination. In vitro ubiquitination assay of Pael-R using recombinant CHIP, Parkin and E2s (Ubc4, Ubc6 and Ubc7). Pael-R-FLAG generated in rabbit reticulocyte lysate was immobilized on anti-FLAG affinity gel. Pael-R on the gel was incubated at 30°C 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.

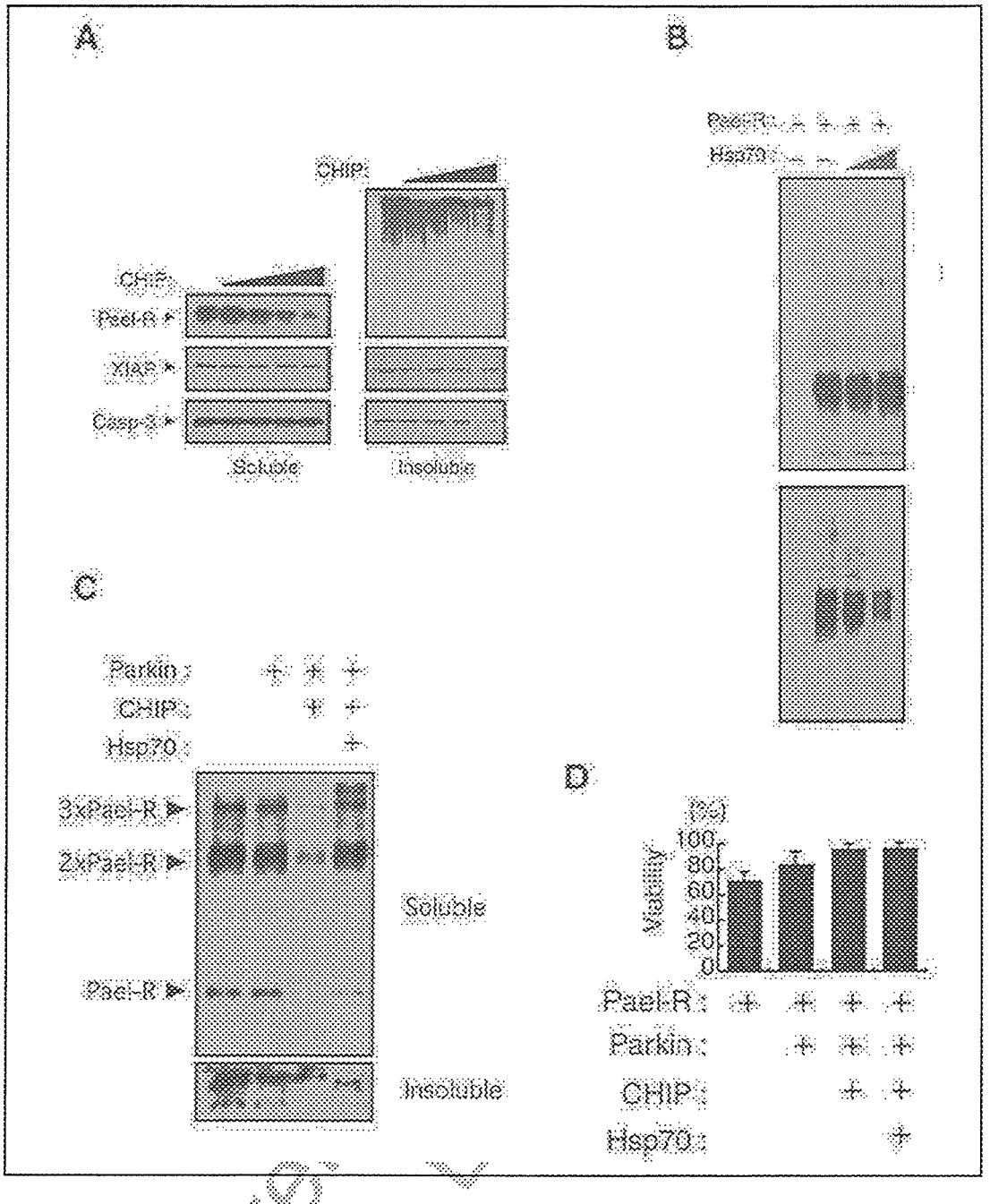

Figure 13. CHIP and Hsp70 cooperate with Parkin in removing unfolded Pael-R. A) Pael-R and increasing amounts of CHIP-transfected cells were lysed in fractionation buffer containing 1% Triton-X 100, and separated into soluble and insoluble fractions. Each fraction was subsequently Western blotted using 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 visibility 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.

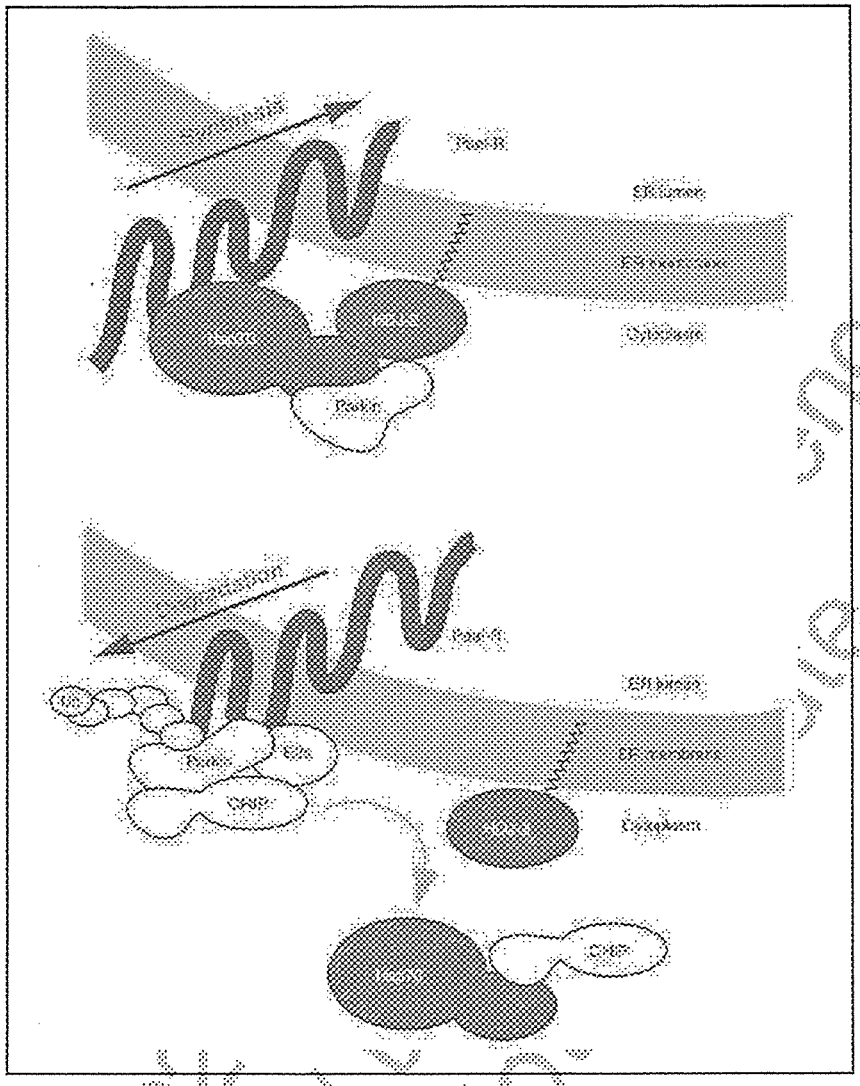

Figure 14. Hypothesis for quality control of Pael-R. Upper, newly synthesized Pael-R is assisted in entering into the ER by chaperones (Hsp70 and Hdj-2). Hsp70 may also bind to Parkin to protect Pael-R from ubiquitination by Parkin. Lower, when unfolded Pael-R is generated in the ER, unfolded Pael-R 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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トピックス編

■2■

ユビキチンとアポトーシス

—IAPを中心に—

高橋 良輔

アポトーシスは発生時期から成熟後を通じて不要，有害な細胞を除去する合目的的な細胞死の一形態である．アポトーシスの実行因子はカスパーゼと呼ばれるシステンプロテアーゼで，何百という基質タンパク質を分解することによってアポトーシスを引き起こす．一方アポトーシスを抑制するタンパク質も生体は用意しているが，その中にアポトーシス阻害タンパク質（IAP）という分子ファミリーが知られている．IAPはカスパーゼに直接結合し，その活性を阻害することによってアポトーシスを阻害する内因性のカスパーゼ阻害因子であるが，最近になってIAPの一部にユビキチンリガーゼ（E3）活性をもつものがあることがわかってきた．ショウジョウバエと哺乳類での最近の知見をもとに，IAPの機能におけるE3活性の意義について述べる．

【キーワード&略語】
IAP，XIAP，DIAP1，Dronc，N-end rule，RHGタンパク質
IAP：inhibitor of apoptosis protein
（アポトーシス阻害タンパク質）
XIAP：X-linked Inhibitor of Apoptosis Protein
（X連鎖アポトーシス阻害タンパク質）
DIAP1：Drosophila IAP1
（ショウジョウバエアポトーシス阻害タンパク質1）
IBM：IAP binding motif

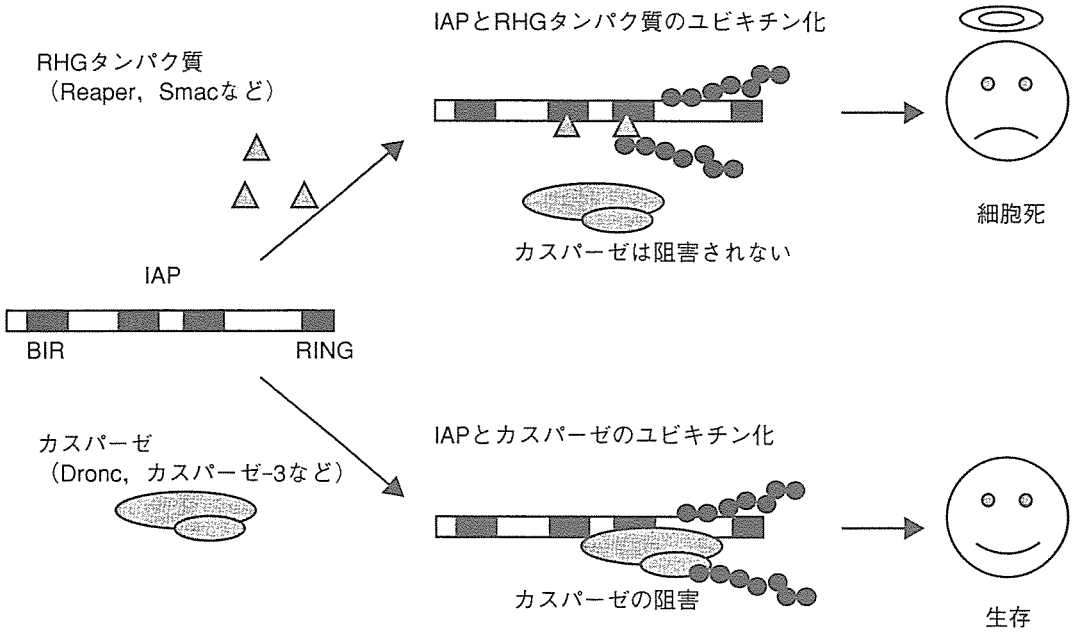
1 アポトーシス阻害タンパク質（IAP）とは

アポトーシスは核の断片化やアポトーシス小体の形成のような特異な形態で定義される細胞死であるが，しばしば「細胞の自殺」に例えられるように，個体の恒常性を維持するために細胞の能動的なシグナル伝達で引き起こされる細胞死でもある．アポトーシスの実行因子はカスパーゼ*と呼ばれるタンパク質分解酵素であり，少なくとも200種類以上の基質タンパク質を分解することによって，アポトーシスを引き起こす．一方，アポトーシスを制御するために，アポトーシスを抑制するタンパク質もいくつか知られている．アポトーシス阻害タンパク質（inhibitor of apoptosis protein：XIAP）は最初，バキュロウイルスでみつかったアポトーシス抑制因子であるが，その後，ショウジョウバエ，哺乳類でも存在が明らかになり，ショウジョウバエでは4種類，ヒトでは8種類のIAPファミリー分子が存在する．IAPは，BIR*ドメインと呼ばれるモチーフをもつことが構造上の特徴である[1)2)]．

IAPのアポトーシス抑制のメカニズムに関して，ヒトのXIAPがカスパーゼ-3と-7に直接結合して阻害する作用のあることが発見されたことを皮切りに，ほかのほとんどのヒトIAP，さらにはショウジョウバ

＊カスパーゼ
システインプロテアーゼで配列特異的にアスパラギン酸のC末側で限定分解する性質をもつ．ヒトでは14種類が知られ，アポトーシスに関与するものと炎症性サイトカインの産生に関わるものに分類される．

＊BIR
約70アミノ酸から成り，システインとヒスチジンが規則正しく配列され，亜鉛結合能をもつ．BIRを含むタンパク質の中にはアポトーシス抑制以外にサバイビンのように主として細胞質分裂に関与する分子もある．



■概略図■　IAP の E3 活性と細胞死

IAP は内因性のカスパーゼ阻害因子だが，RHG タンパク質が BIR ドメインと結合すると，カスパーゼと結合できなくなり，細胞は死の方向に進む．一方カスパーゼと結合すると，カスパーゼの活性を阻害し，しかも E3 活性でユビキチン化することによって細胞は生存の方向に向かう．両方のケースで IAP の自己ユビキチン化が起こると思われるが，その意義は明らかではない

エの IAP もカスパーゼ阻害作用のあることが示され，IAP が種を超えて内因性のカスパーゼ阻害因子である事実が確立された[1]．興味深いことにヒト，ショウジョウバエでそれぞれ最も強いアポトーシス阻害作用をもち，よく解析されている XIAP と DIAP1 は複数の BIR をアミノ末端側にもつ以外に，カルボキシ末端に多くのユビキチンリガーゼ（E3）を特徴づける RING フィンガーモチーフを有する（図 1）[2]．事実，この 2 種類の IAP は E3 活性を有することが判明し，それがアポトーシス制御のしくみにかなり違いのあるショウジョウバエとヒトの 2 つの種で共通して IAP の生理機能に深く関与していることがわかってきた．

2 ショウジョウバエにおける IAP とその E3 活性

ショウジョウバエでは IAP はアポトーシス制御の中心的役割を担っている[3]．DIAP1 の機能喪失型変異のホモ接合体は原腸形成直後に広汎なアポトーシスを起こして死んでしまう．またショウジョウバエ由来の S2 細胞では DIAP1 の発現を RNAi で抑制するとそれだけでアポトーシスが起こる．これは発生過程の組織でも培養細胞でも常にカスパーゼが活性化しており，それを DIAP1 が抑え込んでいることを反映している．いっぽうショウジョウバエでは細胞死誘導作用をもち，染色体の H99 という領域に局在する Reaper，Hid，Grim という一群の因子が知られていた（Grim Reaper は死神の意，以下これらを RHG タンパク質と省略する）[2][3]．これらによる細胞死を DIAP1 が抑制し，しかもこれらの因子と DIAP1 が直接結合することから，DIAP1 にはカスパーゼ以外に RHG タンパク質による未知の細胞死誘導経路を抑制する作用があるものと最初は考えられていた．ところが，逆に DIAP1 とカスパーゼの結合を RHG タンパク質が阻害するために活性化型カスパーゼの抑制が解かれ，アポトーシスが誘導されることが遺伝学的実験から明白になった．RHG タンパク質は N 末端に IAP 統合モチーフと呼ばれる共通配列をもち（IAP binding motif : IBM*），N 末端で DIAP1 の BIR ドメインに強固に結合するこ

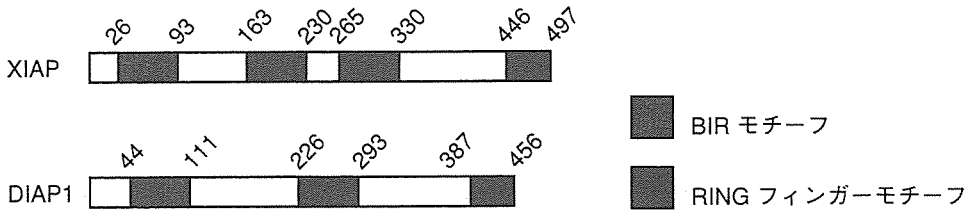


図1 ◆ヒト XIAP とショウジョウバエ DIAP1 の構造比較

とによって，DIAP1 とカスパーゼの結合を妨げる[2]．

アポトーシスに関わるカスパーゼはアポトーシスの引き金を引き下流のカスパーゼを限定分解により活性化する役割をもつ上流のイニシエイターカスパーゼと，さまざまなタンパク質を分解して直接アポトーシス特有の形態変化を引き起こす下流のエフェクターカスパーゼの2種類に分類される．DIAP1 はイニシエイター，エフェクター両方のカスパーゼを阻害する広い特異性をもつが，生理的に特に重要なのはイニシエイターカスパーゼに属する Dronc の阻害作用である．Dronc は生理的条件下で Dapaf-1/DARK というアダプター分子と結合して **apoptosome** *という複合体を形成し，下流のカスパーゼを活性化してアポトーシスを引き起こすが，DIAP1 は Dronc に直接結合して，これを不活性化する．この不活性化が DIAP1 の E3 活性による Dronc のユビキチン化によって行われるのである[4]．DIAP1 は RING 領域を介して UbcD1 というユビキチン結合酵素（E2）および E2 のモチーフはもつが，活性中心が変異している E2 様分子 Morgue と結合する．おそらくこれらの分子，あるいは類似の E2 と協調して Dronc の活性化型およびその前駆体のユビキチン化を促進するものと考えられる[3]．遺伝学的にも DIAP1 の E3 活性による Dronc のユビキチン化が不活性化に必須であることが示された[4]．面白いことにユビキチン化された Dronc はプロテアソームによる分解は受けないらしい．ユビキチン化はプロテアソームによる分解のシグナル以外にも転写制御やエンドサイトーシスなどさまざまな生物学的現象に関与しているが，この場合はカスパーゼの不活性化に働くようである．

また DIAP1 は Dronc 以外に Dcp-1，drICE といったエフェクターカスパーゼに結合しその活性を阻害するが，Dcp-1 または drICE によって N 末端の 20 アミノ酸が切り落とされ，アスパラギン酸が N 末端に露出すると，ユビキチンプロテアソーム系による分解を受けやすくなる[5]．Varshavsky たちは酵母の解析を通じて N 末端のアミノ酸がある種のタンパク質の安定性を決定する要因になることを見出し，N-end rule（N 末端則）と名づけている．カスパーゼによって N 末端を切断された DIAP1 は多細胞生物で初の N-end rule によって分解される生理的基質になることがわかった[5]．意外なことにカスパーゼで切断されなくなった DIAP1 は，RING 変異の DIAP1 同様，アポトーシス抑制効果を失ってしまう．つまり，DIAP1 が分解されることがアポトーシス抑制に必要なのである．おそらく DIAP1 はカスパーゼを道連れにして分解されることによって結果としてアポトーシスを抑制するものと思われる（**図2**）．ショウジョウバエの強力な遺伝学的アプローチから導き出された「IAP の分解が IAP の抗アポトーシス作用に必須」というメッセージは「抗アポトーシス因子である IAP の分解は当然アポトーシス誘導の方向に働くはず」という従来支配的であったドグマを転換することになった．

＊ IAP binding motif（IBM）
アラニンから始まる RHG タンパク質に共通する N 末配列．例えば Reaper は AVAF という配列をもつ．BIR の構造上のポケットの中に入り込み，カスパーゼと IAP の結合を阻害する．

＊ apoptosome
ショウジョウバエでは Dronc と Dapaf-1，哺乳類ではそれぞれに対応するカスパーゼ-9 と Apaf-1，さらにシトクロム-c から成るタンパク質複合体で，哺乳類ではシトクロム-c が形成を促進する．

3 哺乳類における IAP とその E3 活性

哺乳類でもカスパーゼ，IAP，IAP 阻害因子の関係はショウジョウバエと似通っている．DIAP1 に対応する XIAP もイニシエイターカスパーゼ（カスパーゼ -9）とエフェクターカスパーゼ（カスパーゼ -3，-7）をともに阻害するうえ，哺乳類の RHG タンパク質である Smac，Omi/HtrA2 はアポトーシス時にミトコンドリアの膜間スペースから細胞質に放出されて XIAP に結合し，XIAP による阻害からカスパーゼを解き放つ[2]．しかしショウジョウバエとの大きな違いは IAP，IAP 阻害因子のノックアウトマウスが目立った表現型を示さないことである．これは分子のリダンダンシー（redundancy：冗長性）のためと思われるが，IAP の E3 活性の生理的意義の解明を困難にしている．これまでに IAP の E3 活性の意義については，自己ユビキチン化で分解されてアポトーシス進行を促進するという考えと，カスパーゼの分解を早めて，アポトーシスを抑制するという相反する 2 つの考え方が提出されている．前者はアポトーシス誘導時に胸腺細胞で XIAP と，同じく RING を有する c-IAP1 が自己ユビキチン化によって分解されるが，RING を欠いた IAP では自己分解が抑えられ，アポトーシスも抑制されるというデータによって支持されている[6]（ちなみに DIAP1 の自己ユビキチン化は RHG タンパク質である Reaper によって促進されるが[3]，哺乳類 IAP のアポトーシス時の自己ユビキチン化機構は不明である）．しかし自己ユビキチン化しなくなった変異 XIAP のアポトーシス抑制能が野性型と変わらないことがわかり，自己ユビキチン化の意義は再検討を迫られている[7]．一方，後者の考えは XIAP が，活性化型の構造をもつ改変カスパーゼ -3 をポリユビキチン化するうえ，RING に変異のある XIAP は Fas による細胞死抑制効果が野性型より弱くなるというわれわれの結果などに基づいている（図 3）[8]．さらにカスパーゼ -8，-10 の新規 E3，CARPs（caspase-8 and-10 associated RING protein）が報告されており，少なくともカスパーゼのユビキチン化と分解がアポトーシス制御に深く関わっていることは確からしい[9]．最近低分子化合物による IAP の阻害が報告され，癌治療に有効との期待がもたれているが[10]，今後の研究の進展によって E3 機能制御による IAP 阻害が可能になれば，新たな癌治療の手段を提供することになるかもしれない．

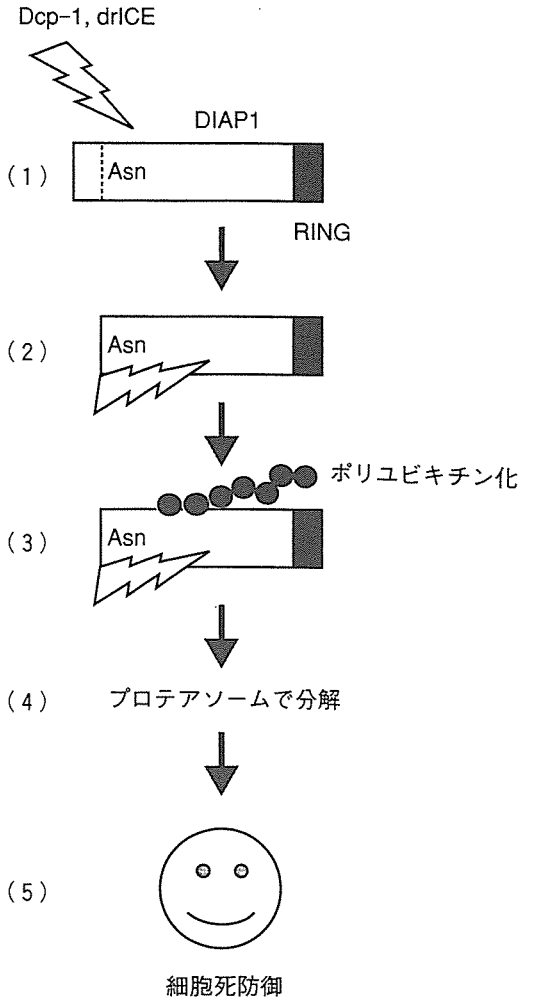


図 2 ◆ N-end rule による DIAP1 の分解
DIAP1 の N 末端の 20 アミノ酸が p35 によって阻害されるカスパーゼ（Dcp-1，drICE など）によって切り落とされると，アスパラギン酸が露出し，N-end rule によってユビキチンプロテアソーム系基質となって分解される．N 末端が切断されなくなる変異型の DIAP1 は細胞死抑制機能を失うことから，N-end rule による DIAP1 の分解はおそらくカスパーゼも同時に分解することによって細胞死防御の方向に働くものと思われる

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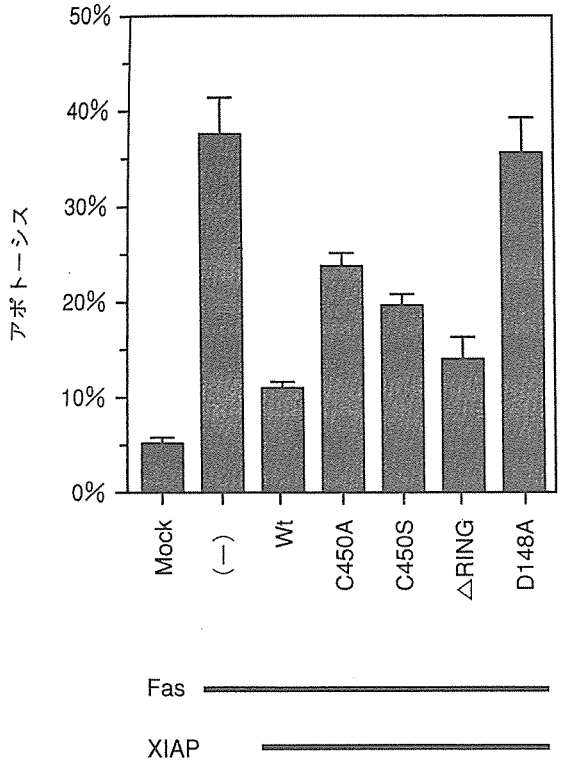

図 3 ◆ XIAP の E3 活性は細胞死防御の方向に働く
XIAP による Fas 誘導性細胞死の抑制効果は野性型（Wt）に比べ，RING 点変異（C450A，C450S）または RING 欠損型（Δ RING）で減弱している．D148A はカスパーゼ-3 に結合しない XIAP の点変異（文献 8 より改変）

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  ≫≫ IAP のユビキチンリガーゼ機能についてややくわしく概説．

PARK6-linked autosomal recessive early-onset parkinsonism in Asian populations

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Abstract—The authors performed linkage analysis in 39 families with autosomal recessive early-onset PD (AR-EOPD) negative for *parkin* and *DJ-1* mutations. Eight families including three Japanese, two Taiwanese, one Turkish, one Israeli, and one Philippine showed evidence of linkage with PARK6 with multipoint log of the odds (lod) score of 9.88 at D1S2732. The results indicate worldwide distribution of PARK6-linked parkinsonism.

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The *parkin* gene, responsible for autosomal recessive juvenile parkinsonism (ARJP), was identified in 1998.[1] Mutations of this gene have been detected in approximately 50% of cases with autosomal recessive early-onset Parkinson disease (AR-EOPD), indicating that this form is the most frequent among patients with familial PD.[2] Recently, *DJ-1* mutations responsible for PARK7 were reported to cause another type of AR-EOPD.[3] In contrast to PARK2, this gene is unlikely to be of numerical significance in clinical practice.[4] Thus, it is possible that other loci are responsible for the remaining patients with AR-EOPD. Based on this view, it is important to identify the PARK6 gene and to screen AR-EOPD families with no *parkin* or *DJ-1* mutations. The locus for PARK6 was identified on human chromosome 1p35-p36 in several European families.[5,6] The clinical features of PARK6-linked AR-EOPD are similar to those of PARK2- or PARK7-linked AR-EOPD. However, PARK6-linked families frequently lack dystonia at onset.[6,7] Thus, the lack of dystonia at onset might be a distinct sign for differentiating this form from PARK2- or PARK7-linked AR-EOPD. To further narrow the critical region for PARK6-linked AR-EOPD and to define the genotype–phenotype correlations, we performed a linkage study in AR-EOPD families without *parkin* and *DJ-1* mutations.

Patients and methods. *Patients and DNA preparation.* Blood samples and clinical information on patients were obtained from their neurologists in several countries. Diagnosis of AR-EOPD was adopted by the participating neurologists. We investigated 39 AR-EOPD families from seven countries, including 26 Japanese families, 3 Taiwanese, 3 Israeli, 3 Turkish, 2 Moroccan, 1 Philippine, and 1 Brazilian. In the present study, the subjects were from families of consanguineous marriages or at least two affected siblings in the same generation. The study was approved by the ethics review committee of Juntendo University. Blood samples for genetic analysis were collected after obtaining an informed consent from 60 patients and 24 unaffected relatives. DNA was prepared using standard methods. None had mutations in *parkin* or *DJ-1* gene. We analyzed *parkin* mutations by direct sequencing

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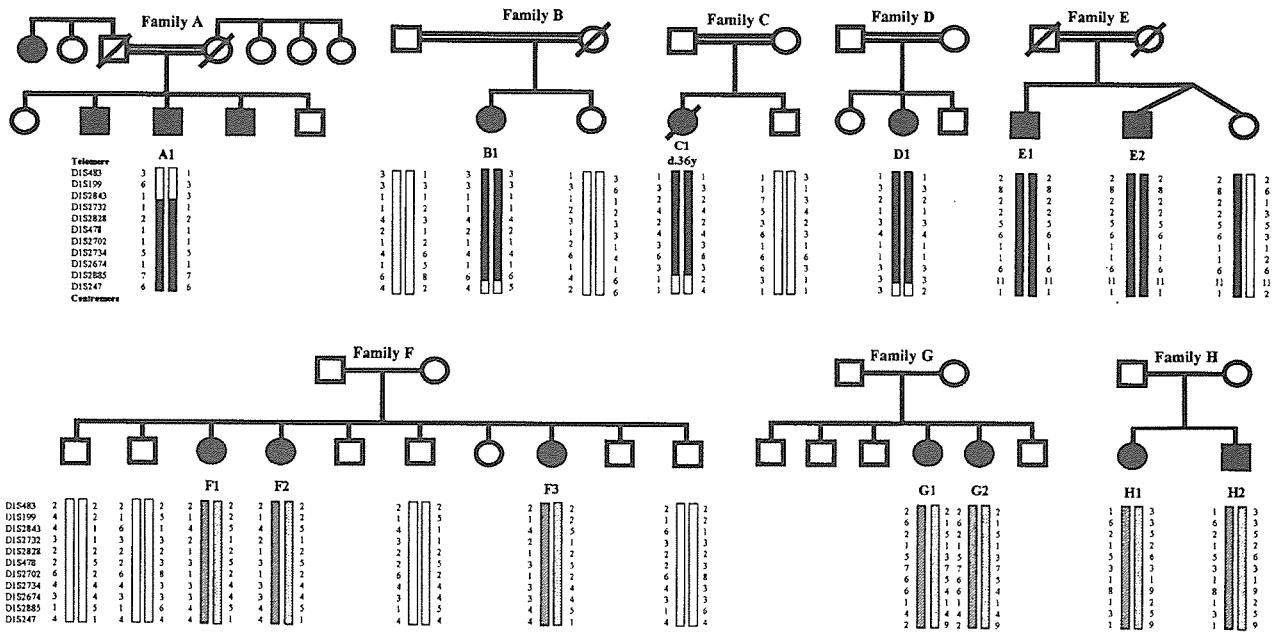

Figure 1. Pedigree structure and haplotype analysis of the families. The affected individuals studied were born to consanguineous parents in five families (Families A, B, C, D, and E) and to non-consanguineous parents in three families (Families F, G, and H). The parents were first cousins in Families A, B, C, D, and E. Haplotypes for 11 polymorphic markers on 1p35–36 are shown. Telomeric to centromeric markers are arranged from top to bottom. Disease-linked homozygous haplotypes are boxed in black. Heterozygous haplotypes shared by affected members of each family are marked in dotted and diagonally striped boxes. The patient, C1, died in a traffic accident.

of the coding regions including exon-intron boundaries using previously described primers.[1] *DJ-1* screening was also performed.[3]

Linkage analysis. We performed linkage analysis in 84 individuals. We used the polymorphic DNA markers D1S483, D1S199, D1S2843, D1S2732, D1S2828, D1S478, D1S2702, D1S2734, D1S2674, D1S2885, and D1S247 selected from the Center for Medical Genetics, Marshfield Medical Research Foundation (http://research.marshfieldclinic.org/genetics/). The primers were labeled by fluorescence and the sequence GTTTCTT was placed on the 5' end of reverse primers for definite genotyping. Pooled PCR products were separated by electrophoresis on 5% polyacrylamide gels on an ABI 377 DNA sequencer (Applied Biosystems). Alleles were sized by GENESCAN and scored with GENOTYPER software. We used the GENEHUNTER program for multipoint parametric log of the odds (lod) scores to find the maximum lod score for each marker. We assumed an autosomal recessive model with complete penetrance in both sexes and a frequency of 0.001 for the disease allele. Since the allele frequencies of the markers were not known, lod scores were calculated by assuming equal allele frequencies.

Results. Haplotypes were constructed using 11 microsatellite markers in the PARK6 region to a 12.5 cM interval. Results are shown in figure 1. Families A, B, C, D, and E showed homozygosity while compound heterozygosity was suggested in Families F, G, and H, who shared the same haplotypes with other affected siblings. Three families were Japanese, two Taiwanese, and one each from Israel, Turkey, and the Philippines. No common haplotype could be detected in the families tested, thus excluding possible single founder effect. Multipoint linkage analysis using the full set of 11 markers, shown in figure 2, indicated a maximum lod score of 9.882 at D1S2732. Multipoint lod scores were >9.7 for five markers spanning ~6.4 cM, with markers D1S2732 and D1S2734 defining telomeric and centromeric boundaries.

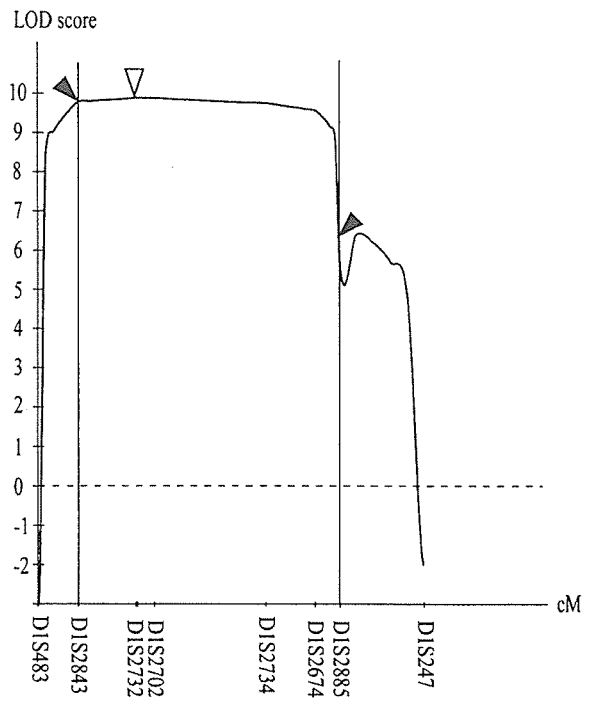

Figure 2. Multipoint linkage analysis of a 12.5 cM region on 1p35–36. The maximum multipoint lod score of 9.882 was obtained at D1S2732 (open arrowhead). We mapped the disease locus to be within an 8.5 cM region extending between markers D1S2843 and D1S2885 (solid arrowheads) by homozygosity mapping.

Table *Clinical features of the patients*

Characteristics	Family A (Japan), A1	Family B (Japan), B1	Family C (Turkey), C1	Family D (Japan), D1	Family E (Israel)		Family F (the Philippines)			Family G (Taiwan)		Family H (Taiwan)	
					E1	E2	F1	F2	F3	G1	G2	H1	H2
Age at onset, y	30	23	28	33	33	25	32	27	27	19	18	56	48
Disease duration, y	17	15	8	8	17	21	19	23	18	24	22	16	19
Sex	M	F	F	F	M	M	F	F	F	F	F	F	M
Clinical presentation													
Resting tremor	−	+	+	PT	−	−	+	+	+	+	+	−	+
Rigidity	+	+	+	+	+	+	+	−	+	+	+	+	+
Bradykinesia	+	+	+	+	+	+	+	+	+	+	+	+	+
Postural instability	+	+	−	+	−	−	+	+	+	−	−	−	+
Frozen gait	−	−	−	−	+	+	−	+	−	−	−	−	−
Wearing off	+	+	−	−	+	+	+	+	+	+	+	+	+
On/off	−	−	−	+	+	+	−	−	−	−	−	−	−
Asymmetry at onset	+	+	+	+	−	−	−	+	+	+	−	−	+
Levodopa-induced dyskinesia	+	−	−	+	+	+	−	+	+	+	+	−	+
Sleep benefit	−	+	+	−	+	+	−	−	−	+	+	−	−
Dystonia at onset	−	−	−	+	−	−	−	−	−	−	+	−	−
Hyperreflexia	+	+	+	+	−	+	−	−	−	−	−	−	−
Psychosis	−	HA	−	−	DE	SC	−	−	−	−	−	−	HA
Dementia	−	−	−	−	+	−	−	−	−	−	−	−	−
Other special comment							FE	DBS					
UPDRS III (on/off)	17/?	20/?	5/34	7/13	29/71	12/16	9/50	25/66	7/59	16/33	8/24	30/44	36/51

All subjects had a good clinical response to levodopa.

PT = postural tremor; HA = hallucination; DE = depression; SC = like schizophrenia; FE = festination; DBS = deep brain stimulation; UPDRS = Unified Parkinson's Disease Rating Scale; ? = information could not be obtained.

The table summarizes the clinical characteristics of the eight families (13 patients, mean ± SD age at onset 30 ± 10.7 years, range 18 to 56 years). Every family presented with at least two cardinal features out of resting tremor, bradykinesia, rigidity, and postural abnormality. In addition to the clinical features of parkinsonism, some of the affected members had various neurologic signs and symptoms such as levodopa-induced dyskinesia, sleep benefit, dystonia at onset, and hyperreflexia. No other clinical signs (gaze palsy, ataxia, rapid progression of disease, and pathologic reflex) were found.

Discussion. Our results indicate that PARK6-linked AR-EOPD is not only distributed in Europe but also in Asia. Five of 39 families tested showed evidence of linkage with PARK6 as homozygous. Families F, G, and H did not show homozygosity, indicating that they may be compound heterozygotes or not linked to this region.

PARK6-linked AR-EOPD was first reported in nine European families.[5-7] The characteristics of PARK6-linked AR-EOPD included wide range age at onset, slow progression, lack of dystonia at onset, and lack of sleep benefit, resembling late-onset PD.[5,6] Families A, F, and H also showed slow progression, lack of dystonia at onset, and sleep benefit, indicating similarity to the European families reported previously.[5-7] With regard to the PARK6-linked AR-EOPD in Japanese families (Families A, B, and D), they showed little similarities to Japanese patients with ARJP. These families were rather similar to the phenotypes of PARK6-linked AR-EOPD in European families described in the original report.[5]

The affected individual in Family C was heterozygous at marker D1S2885, thus defining the centromeric limit of the disease gene interval at this marker. In addition, a recombination event for the telomeric border was observed at D1S2843 in Family A, indicating a reduction of the critical region to 8.5 cM flanked by markers D1S2843 and D1S2885. Moreover, this region is smaller than that described in the original report.[5]

Before identification of *DJ-1* mutations, some families were found to share the haplotypes at two regions of PARK6 and PARK7 due to the proximity of