Molecular Cell, Vol. 10, 55–67, July, 2002, Copyright ©2002 by Cell Press

CHIP Is Associated with Parkin, a Gene Responsible for Familial Parkinson's Disease, and Enhances Its Ubiquitin Ligase Activity

Yuzuru Imai,[1] Mariko Soda,[1] Shigetsugu Hatakeyama,[3,4] Takumi Akagi,[2] Tsutomu Hashikawa,[2] Kei-Ichi Nakayama,[3,4] and Ryosuke Takahashi[1,5]

[1]Laboratory for Motor System Neurodegeneration
[2]Laboratory for Neural Architecture
RIKEN Brain Science Institute
Saitama 351-0198
[3]Department of Molecular and Cellular Biology
Medical Institute of Bioregulation
Kyushu University
3-1-1 Maidashi, Higashi-ku
Fukuoka 812-8582
[4]CREST
Japan Science and Technology Corporation
4-1-8 Honcho, Kawaguchi
Saitama 332-0012
Japan

Summary

Unfolded Pael receptor (Pael-R) is a substrate of the E3 ubiquitin ligase Parkin. Accumulation of Pael-R in the endoplasmic reticulum (ER) of dopaminergic neurons induces ER stress leading to neurodegeneration. Here, we show that CHIP, Hsp70, Parkin, and Pael-R formed a complex in vitro and in vivo. The amount of CHIP in the complex was increased during ER stress. CHIP promoted the dissociation of Hsp70 from Parkin and Pael-R, thus facilitating Parkin-mediated Pael-R ubiquitination. Moreover, CHIP enhanced Parkin-mediated in vitro ubiquitination of Pael-R in the absence of Hsp70. Furthermore, CHIP enhanced the ability of Parkin to inhibit cell death induced by Pael-R. Taken together, these results indicate that CHIP is a mammalian E4-like molecule that positively regulates Parkin E3 activity.

Introduction

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

Missense mutations within the α-synuclein (α-SYN) gene are known to cause a rare form of autosomal dominant familial PD (Krüger et al., 1998; Polymeropoulos et al., 1997). α-SYN, a presynaptic protein, tends to aggregate, leading to the formation of cytoplasmic inclusions called Lewy bodies (LBs), which are pathological hallmarks of sporadic PD as well as some forms of familial PD.

[5]Correspondence: ryosuke@brain.riken.go.jp

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

Parkin is a 465 amino acid protein with a molecular mass of 52 kDa. It has a ubiquitin-like domain at its amino terminus and two RING finger motifs flanking a cysteine-rich region that is known as the in between RING fingers (IBR) motif (Morett and Bork, 1999). Recent studies have revealed that numerous RING finger-containing proteins have ubiquitin-protein ligase (E3) activity (Jackson et al., 2000; Joazeiro and Weissman, 2000). Along with other researchers, we have demonstrated that Parkin has E3 activity and that AR-JP-linked Parkin mutants are defective in E3 activity (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). We found that the membrane protein Pael-R is a substrate for Parkin (Imai et al., 2001). Accumulation of Pael-R in the ER results in ER stress-induced neuronal death (Imai et al., 2001).

Unfolded or misfolded protein generated under diverse conditions must be either refolded by molecular chaperones such as Hsc/Hsp70 and Hsp40 or eliminated by the ubiquitin-proteasome protein degradation system. It is thought that ubiquitination occurs via a sequential enzymatic reaction involving the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) (Hershko et al., 2000). Moreover, a new ubiquitination enzyme, yeast Ufd2, has been shown to be involved in a multiubiquitin chain assembly reaction termed E4 (Koegl et al., 1999). Ufd2 has a conserved motif called a U box, which is structurally similar to the RING finger motif. Very recent reports showed that CHIP (carboxyl terminus of the Hsc70-interacting protein), previously identified as a negative regulator for chaperone ATPase activity (Ballinger et al., 1999), also contains the U box motif and has U box-dependent E3 activity (Hatakeyama et al., 2001; Jiang et al., 2001; Murata et al., 2001). Furthermore, it has been shown that CHIP ubiquitinates unfolded proteins only when they are first captured by chaperones, suggesting that CHIP is an important link between the degradation and chaperone systems (Murata et al., 2001).

Here, we report that CHIP and Hsp70 are binding partners of Parkin. We found that Hsp70 transiently associates with unfolded Pael-R and inhibits the E3 activity of Parkin, whereas CHIP enhances the E3 activity of Parkin through promotion of the dissociation of Hsp70 from Parkin-Pael-R complexes. Moreover, we demonstrate that CHIP is an E4-like molecule that positively regulates Parkin E3 activity.

Results

Identification of Proteins Interacting with Parkin

Two-dimensional (2D) electrophoresis of coimmunoprecipitates of FLAG-tagged Parkin expressed in SH-

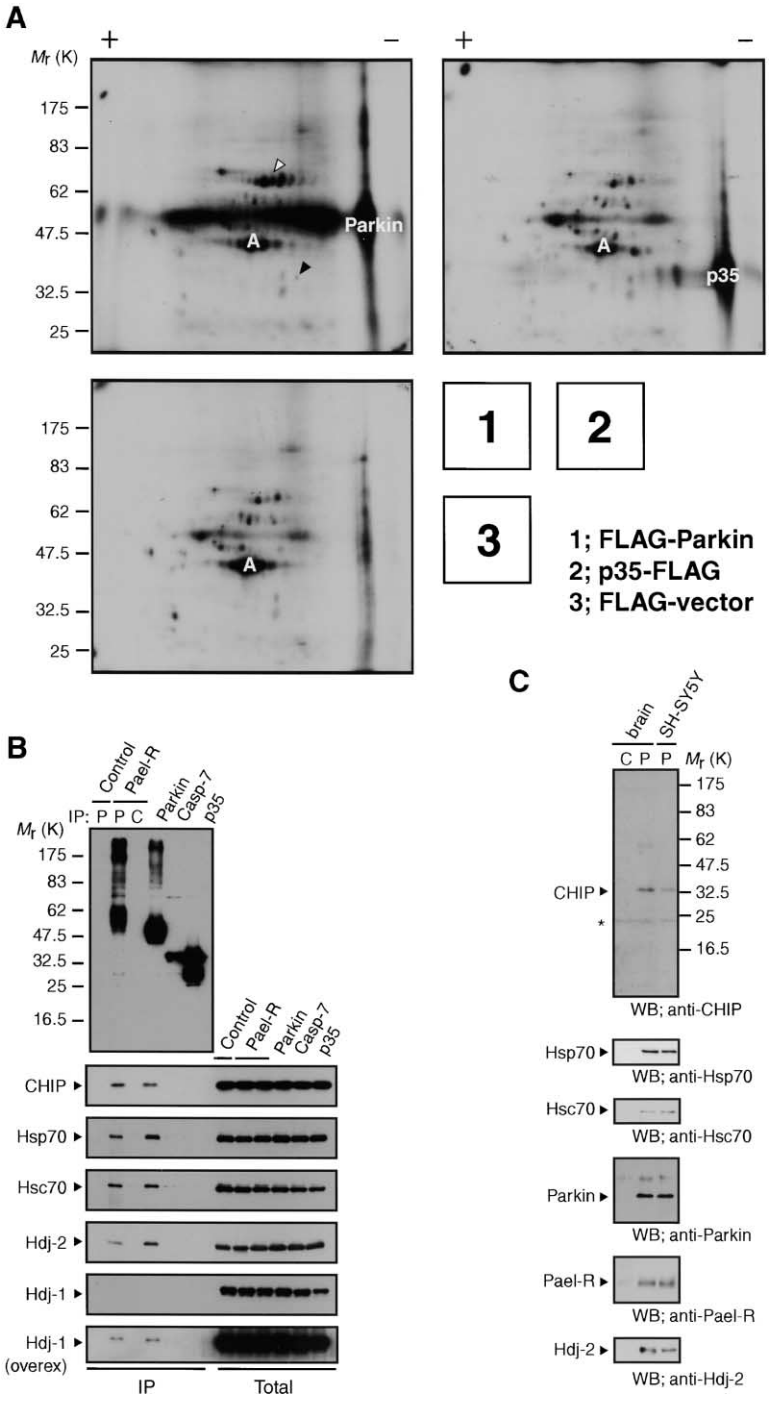

Figure 1. Identification of Parkin Interacting Proteins

(A) N-terminal FLAG-tagged Parkin (FLAG-Parkin) and C-terminal FLAG-tagged p35 (p35-FLAG) were immunoprecipitated from ^{35}S-labeled lysate from stably transfected SH-SY5Y cells using anti-FLAG antibody (Ab) coupled to agarose beads. Immunoprecipitated protein was separated by 2D electrophoresis. The arrowheads indicate immunoprecipitated proteins from FLAG-Parkin-expressing cells, but not p35-FLAG-expressing cells or SH-SY5Y cells bearing an empty vector (FLAG-vector). A, Actin.

(B) Vector plasmid (Control) or FLAG-tagged Pael-R, Parkin, Caspase-7 (Casp-7), and p35 cDNA (1.0 μg each) were transfected into 293T cells (1.7×10^6). Immunoprecipitate (IP) of Pael-R with anti-Pael-R (P), or isotype-matched control IgG (mouse IgG_{2b}, C), and immunoprecipitate of the other proteins with anti-FLAG Ab, as well as total soluble lysate (Total; 5% input of immunoprecipitation for CHIP, 10% input for other proteins), were analyzed by Western blotting using Abs specific to each of the indicated proteins. overex, overexposure for detection of Hdj-1 signal. A representative result from two experiments is shown.

(C) Endogenous interaction of Parkin with CHIP, Hsp70, and Pael-R in brain tissue and cultured cells. Lysate from human brain (10 mg soluble protein) or cultured SH-SY5Y cells (6×10^7) was immunoprecipitated with rabbit anti-Parkin serum (P) or preimmune serum (C). The immunoprecipitate was analyzed by Western blotting (WB) using anti-CHIP, anti-Hsp70 anti-Hsc70, anti-Hdj-2, or anti-Pael-R Ab. The asterisk indicates IgG_L.

SY5Y cells demonstrated two different Parkin binding proteins (Figure 1A, arrowheads). Mass spectrometry and Western blot analysis revealed that these proteins (represented by the open and closed arrowheads) corresponded to Hsp70 and CHIP, respectively (data not shown). Analyses of immunoprecipitates of FLAG-tagged proteins (Pael-R, Parkin, caspase-7, and a baculovirus caspase inhibitor p35) expressed in 293T cells revealed that Parkin and the Parkin substrate Pael-R formed complexes with CHIP, Hsp/Hsc70, and the cofactor Hdj-2 (and weakly to Hdj-1) (Figure 1B). Caspase-7 and p35 were not associated with the complexes. Moreover, coimmunoprecipitation assays indicated that endogenous Parkin, Pael-R, CHIP, Hsp/Hsc70, and Hdj-2 also form a multiprotein complex (Figure 1C).

A previous study observed that CHIP was localized at the ER and that when proteasome was inhibited, CHIP was localized at protein inclusions termed aggresomes (Meacham et al., 2001). A cytochemical study of SH-SY5Y cells stably expressing FLAG-Parkin showed that endogenous as well as exogenous Parkin localized at

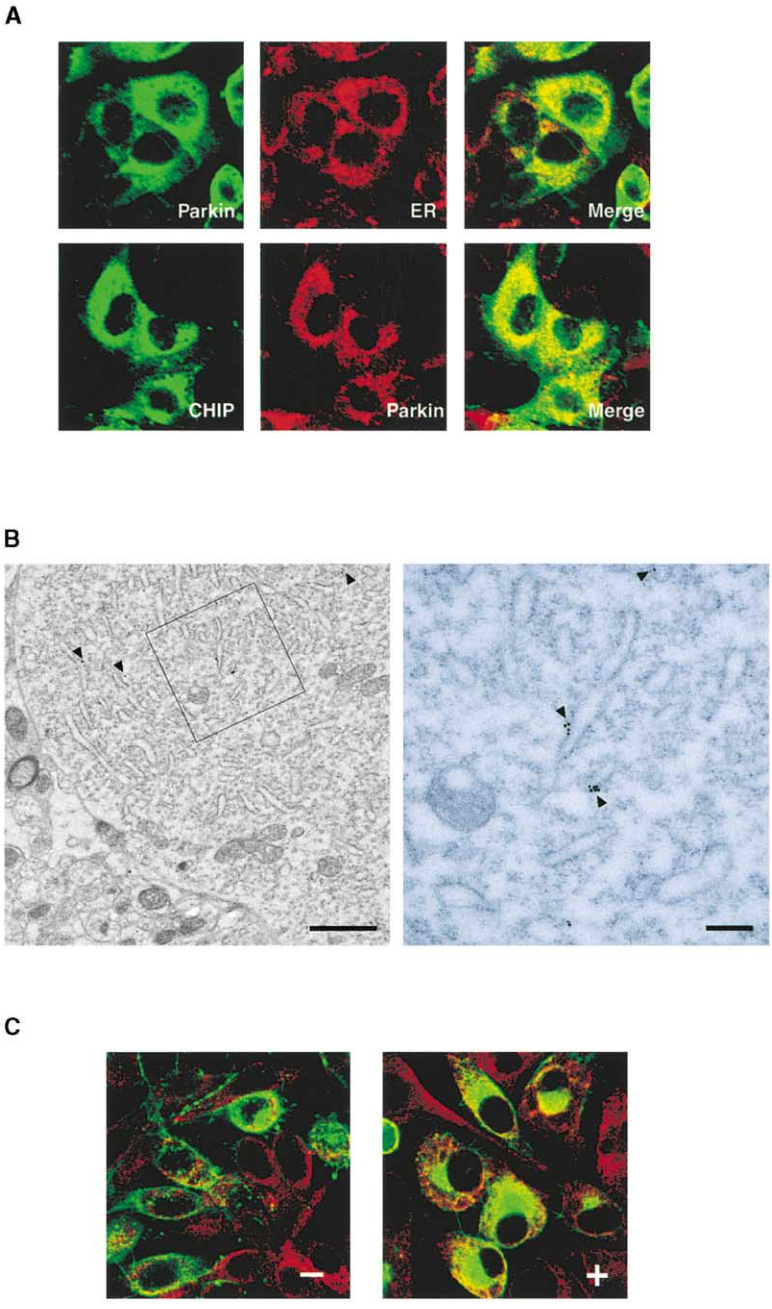

Figure 2. Subcellular Localization of CHIP and Parkin

(A) Immunolocalization of Parkin and CHIP in SH-SY5Y cells stably expressing FLAG-Parkin. Localization of Parkin and CHIP was visualized with anti-Parkin (upper, green; lower, red) or anti-CHIP Ab (lower, green) and by counterstaining the ER with anti-BiP Ab (upper, red). Areas of colocalization of Parkin and CHIP in the ER are represented in yellow.

(B) Ultrastructural analysis of Parkin immunoreactivity in rat dopaminergic neurons in the substantia nigra. Magnification of the box in the left panel is shown (right). The arrowheads indicate the immunogold particles showing the localization of Parkin on the ER surface. Scale bar, 1 and 0.2 μm.

(C) After 20 hr of transfection with plasmid carrying Pael-R, SH-SY5Y cells were incubated with (+) or without (–) lactacystin (1 μM) for 12 hr. Cells expressing Pael-R were visualized using anti-Pael-R Ab (green). Localization of endogenous CHIP in the cells was visualized by staining with anti-CHIP Ab (red).

the ER (Figure 2A, upper). Consistent with an earlier report, immunoelectron microscopic analysis also revealed that Parkin immunoreactivity was localized on the membrane of the ER, as well as in the cytoplasm, on the Golgi membrane, and at the synapse in dopaminergic neurons of the substantia nigra (Figure 2B, data not shown) (Zarate-Lagunes et al., 2001). Moreover, colocalization of endogenous CHIP and Parkin was observed at the ER (Figure 2A, lower). ER localization of Parkin was also supported by an in vivo association of Parkin and Hdj-2, which is localized on the cytoplasmic face of the ER membrane (Figures 1B and 1C) (Meacham et al., 1999; Terada and Mori, 2000). Expression of Pael-R was noted on the cytoplasmic membrane (Figure 2C, left). Treatment with a proteasome inhibitor, lactacystin, prevented cell-surface localization of Pael-R, resulting in Pael-R accumulation within the ER as well as the appearance of γ-tubulin immunopositive perinuclear inclusions, i.e., aggresomes (Figure 2C, right, data not shown) (Kopito, 2000). During Pael-R accumulation, colocalization of Pael-R with endogenous CHIP was observed in both the ER and in the aggresomes, suggesting that these proteins, together with Parkin, might form a complex involved in ER-associated degradation (ERAD).

Using coimmunoprecipitation and in vitro binding assays, we examined which regions of CHIP and Hsp70 bind to Parkin (Figures 3A–3C). The CHIP U box mutant (211–304 aa) and ΔTPR mutant both contain the U box, and both of these mutants as well as the full-length CHIP bound strongly to Parkin. However, the U box

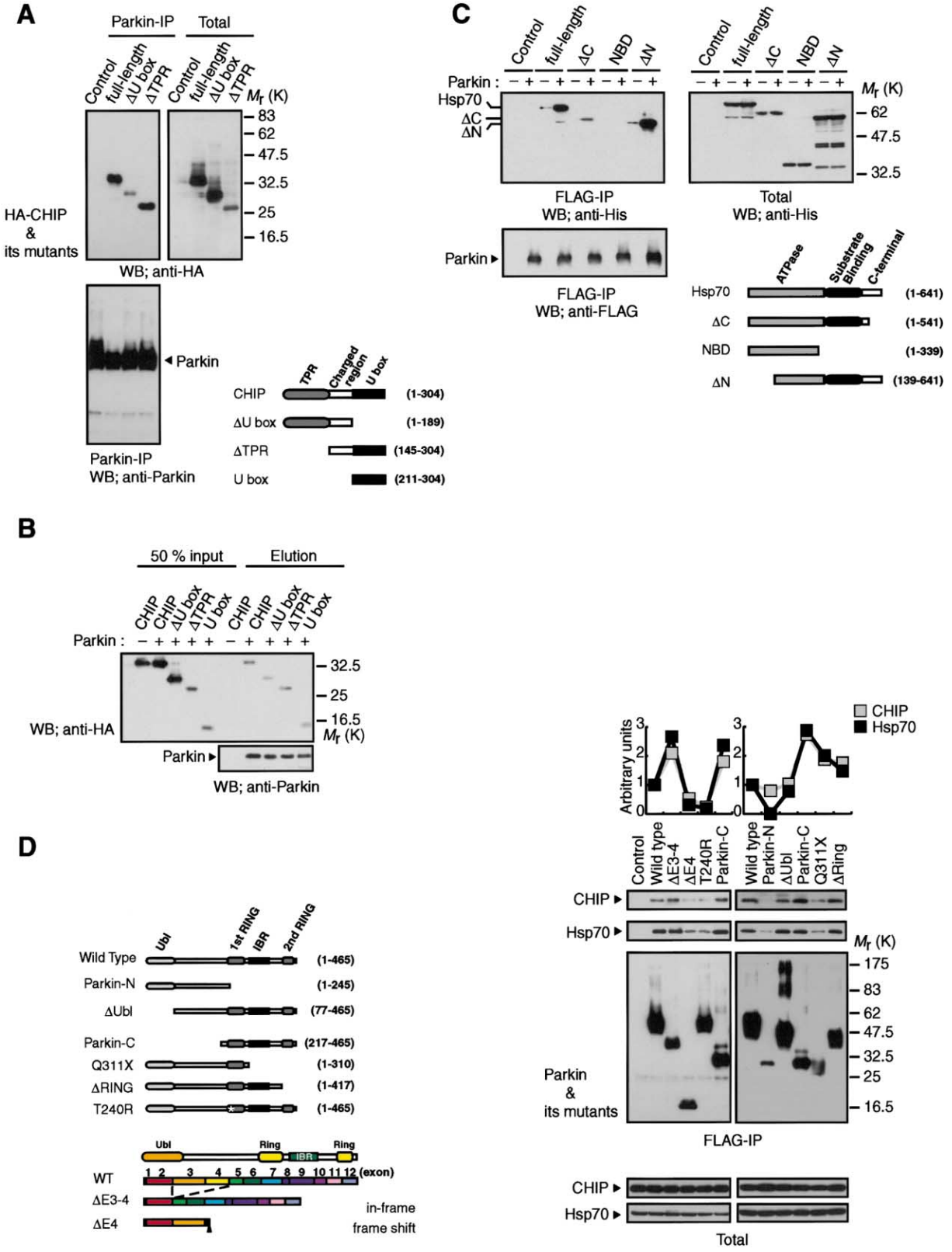

Figure 3. Interaction Domain Analysis of Parkin, CHIP, and Hsp70

(A) Domain analysis of CHIP. Lysate from 293T cells (1.7×10^6) transfected with Parkin (1 μg) and Hemagglutinin (HA)-tagged CHIP (1 μg), deletion constructs of CHIP (1 μg), or an empty vector (Control, 1 μg) was immunoprecipitated with anti-Parkin Ab (Parkin-IP), then Western blotted (WB). The lower panel on the right diagrammatically represents CHIP and the mutants used to determine the Parkin binding domain.

deletion mutant (ΔU box), containing the TPR domain and the charged linker region, also retained significant Parkin binding activity. Although these results did not reveal a specific binding domain of CHIP with Parkin, it is conceivable that mutants containing the U box domain are capable of direct binding to Parkin, while mutants containing the TPR domain, without the U box domain, can bind to Parkin directly or indirectly through Hsp/Hsc70 (see Figure 4). On the other hand, full-length Hsp70, ΔC, and ΔN, but not the NBD mutant, associated with Parkin, indicating that Hsp70 binds to Parkin via the region containing its substrate binding domain.

Binding assays using CHIP or Hsp70 and various Parkin mutants revealed that both CHIP and Hsp70 bound preferentially to wild-type Parkin and to all Parkin mutants bearing the complete first RING finger motif (Figure 3D). This result indicates that mutations of the first RING finger motif (e.g., the T240R mutant) affect the recruitment of CHIP and Hsp70.

CHIP Promotes Dissociation of Chaperone Complex from the Pael-R/Parkin Complex

Since CHIP has been characterized as an Hsp/Hsc70- and Hsp90 binding protein, we expected that the interaction between Hsp/Hsc70 and Parkin would be enhanced by CHIP (Ballinger et al., 1999; Connell et al., 2001; Meacham et al., 2001). An in vivo binding assay, however, revealed a significantly reduced amount of Hsp/Hsc70 coimmunoprecipitated with Parkin in the presence of CHIP (Figure 4A). Overexpression of CHIP inhibited the interaction of endogenous Hsp/Hsc70 and Hdj-2 with Parkin and Pael-R in a dose-dependent manner (Figure 4B). For an unknown reason, the protein level of Parkin was moderately decreased when it was coexpressed with CHIP (Figures 4A and 4B). To avoid a confounding effect of protein levels on binding activity, we performed in vitro binding assays using recombinant protein. First, we investigated whether CHIP affects the ability of Parkin and Hsp70 to bind to Pael-R (Figure 4C). Competitive inhibition of binding between Pael-R and Parkin was not observed; however, binding between Hsp70 and Pael-R was attenuated in the presence of excessive CHIP. Conversely, we examined whether Hsp70 affects the binding between Parkin and CHIP. In this binding assay, no competition was observed between formation of the Parkin/CHIP complex and binding of Hsp/Hsc70 to Parkin (Figure 4D). Finally, we compared the binding affinity of CHIP for Parkin with that of Hsp70 (Figure 4E). We found a lower affinity of binding between Hsp/Hsc70 and Parkin when recombinant protein rather than endogenous protein was used, regardless of whether or not ATP was present (data not shown). This suggests that Hsp/Hsc70 might require a cofactor(s) to bind with full activity. To resolve this problem, we used immunopurified FLAG-Parkin, which we expected would bind to the complete chaperone complex, from SH-SY5Y cells stably expressing FLAG-Parkin. As expected, a substantial amount of Hsp70 was coprecipitated with FLAG-Parkin in the absence of ATP, and a slightly lesser amount was coprecipitated in the presence of 2 mM ATP. The addition of excessive recombinant CHIP to this complex, however, resulted in the complete dissociation of Hsp70 from Parkin. These in vivo and in vitro results suggest that CHIP regulates the binding affinity of Hsp/Hsc70 for Pael-R and Parkin.

CHIP Promotes the Release of Hsp70 from the Parkin-Pael-R Complex during Unfolded Protein Stress

Previously, we reported that the accumulation of unfolded protein leads to upregulation of Parkin and that the ubiquitin-ligase activity of Parkin protects neurons from damage induced by unfolded Pael-R (Imai et al., 2000, 2001). These results prompted us to seek evidence of a functional association between CHIP and Parkin, as well as Hsp70 and Parkin, during stress induced by unfolded protein. Treatment with a specific *N*-glycosylation inhibitor, tunicamycin, effectively induced unfolded protein stress and led to upregulation of protein levels of Parkin, CHIP, Hsp70, and BiP (GRP78), but not Hdj-2, in a time-dependent manner (Figure 5, left). To examine

---

The numbers in parentheses indicate the corresponding amino acid residues of CHIP. TPR, tetratricopeptide repeat. A representative result from three experiments is shown.

(B) Domain analysis of CHIP using recombinant proteins. Recombinant HA-CHIP, -ΔU box, -ΔTPR, or -U box (12 pmol each) was added to recombinant GST (12 pmol; –) or GST-Parkin (12 pmol; +) on GSH Sepharose beads (Amersham Biosciences). After 1 hr of incubation at room temperature to allow binding, the GSH beads were washed four times with PreScisson cleavage buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) containing 10 μg/ml bovine serum albumin (BSA). Immobilized Parkin was eluted by cleavage with PreScisson protease (Amersham Biosciences). Coeluted CHIP and its variants were analyzed by Western blotting with anti-HA Ab. A representative result from three experiments is shown.

(C) Domain analysis of Hsp70. Lysates from 293T cells (1.7×10^6) transfected with (+) or without (−) FLAG-Parkin (1 μg) in combination with 6 × histidine-tagged (His-) Hsp70 (full-length, 0.8 μg), its deletion constructs (0.8 μg), or an empty vector (Control, 0.8 μg) were immunoprecipitated with anti-FLAG Ab (FLAG-IP), then Western blotted (WB). The lower panel on the right diagrammatically represents Hsp70 and the mutants used to determine the Parkin binding domain. The numbers in parentheses indicate the corresponding amino acid residues of Hsp70. A representative result from three experiments is shown.

(D) Interaction of CHIP with Parkin and Parkin mutants. Lysate from 293T cells (1.7×10^6) transfected with an empty vector (Control, 1 μg), FLAG-tagged Parkin (Wild-type, 1 μg), or Parkin mutants (1–3 μg) was immunoprecipitated with anti-FLAG Ab (FLAG-IP) and then Western blotted (WB) using Abs against the indicated proteins. A representative result from two experiments is shown. The graphs indicate the intensity of the bands for the coprecipitated CHIP and Hsp70, which is normalized against that of the band for each Parkin derivative. The values are expressed as fold increase of the amount of the coprecipitated proteins with wild-type Parkin. The panel on the left diagrammatically represents Parkin and the Parkin mutants used in this experiment. The numbers in parentheses indicate the corresponding amino acid residues of Parkin. The asterisk indicates the site of a point mutation. Ubl, ubiquitin-like domain; RING, RING-finger motif; IBR, in between RING fingers; ΔE3-4, deletion of exons 3 and 4; ΔE4, deletion of exon 4. The arrowhead indicates a stop codon introduced by the frameshift due to the exon 4 deletion. Poor expression of the Q311X mutant was always observed, probably due to the unstable conformation of the protein, even though we transfected cells with 3-fold the amount of cDNA used in other samples.

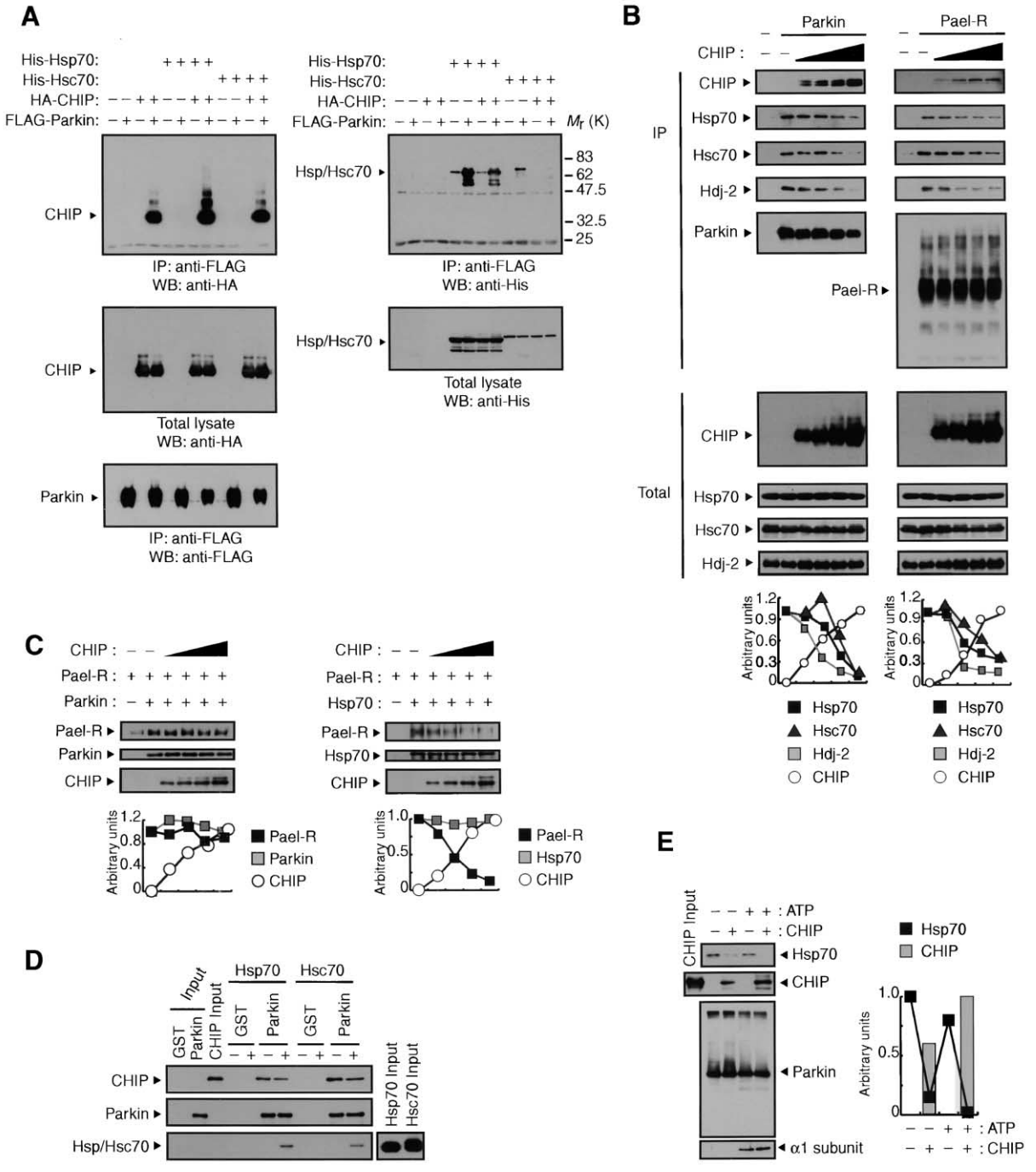

Figure 4. Parkin Forms a Complex with CHIP and Various Chaperones

(A) Vector plasmid (–), FLAG-Parkin (1 μg), HA-CHIP, 6 × histidine-tagged (His-) Hsp70 (0.8 μg), or His-Hsc70 cDNA (1 μg) was transfected into 293T cells (1.7×10^6) as indicated. Immunoprecipitate of anti-FLAG Ab (FLAG-IP) and total soluble lysate (10% input of immunoprecipitation) was analyzed by Western blotting using Abs specific for each tag. The total amount of plasmid DNA used for transfection was adjusted with the vector plasmid. A representative result from three experiments is shown.

(B) Vector plasmid (–) or increasing amounts of CHIP cDNA (0.5, 1, 1.5, and 2 μg) together with FLAG-tagged Pael-R (1 μg) or Parkin cDNA (1 μg) was transfected into 293T cells. The total amount of plasmid DNA used for transfection was adjusted with the vector plasmid. Immunoprecipitate of anti-FLAG Ab and total soluble lysate (Total, 10% input of immunoprecipitation) was analyzed by Western blotting using Abs specific to each of the indicated proteins. A representative result from three experiments is shown. The graphs indicate the intensity of the bands for the coprecipitated Hsp70, Hsc70, Hdj-2, or CHIP, normalized against that of each band for Parkin or Pael-R. The values for Hsp70, Hsc70, and Hdj-2 are expressed as fold increase in the amount of the coprecipitated protein with Parkin or Pael-R in the absence of CHIP. The amount of CHIP is expressed as fold increase of that in the transfection with 2 μg of CHIP cDNA (lane 6 in each panel).

(C) Recombinant GST-Parkin (Parkin, 1.5 μg; left) or His-Hsp70 (Hsp70, 1.5 μg; right) was incubated with or without increasing amounts of His-CHIP (CHIP, 0.5, 1, 2, and 3 μg) in the presence of in vitro-translated and purified Pael-R. Parkin, Hsp70, Pael-R, or CHIP, precipitated

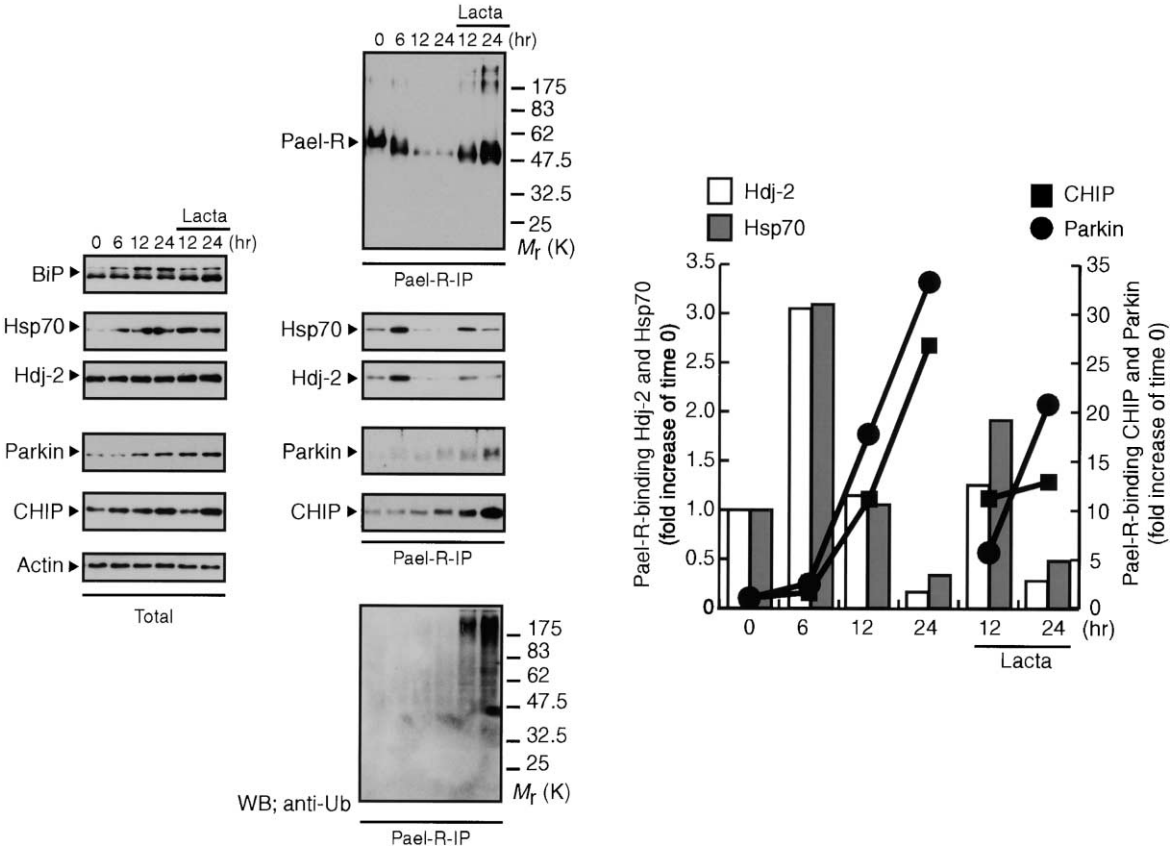

Figure 5. Association of Parkin, CHIP, and Chaperones with Unfolded Pael-R during ER Stresses

SH-SY5Y cells stably expressing Pael-R (10^7 cells in each lane) were treated with tunicamycin (10 μg/ml) in the presence or absence of lactacystin (Lacta, 10 μM) for the indicated periods. Following this, cells were treated with 0.25% trypsin for 5 min at 37°C, then washed with phosphate-buffered saline containing aprotinin (50 μg/ml) three times. One percent Triton X-100-soluble lysate from each sample was subjected to immunoprecipitation with an anti-Pael-R mAb, which recognizes the extracellular domain of Pael-R. The immunoprecipitate (Pael-R-IP) and the soluble lysate (Total) were subjected to Western blot analysis for the indicated proteins. The graph indicates the intensity of the bands for the coprecipitated Hsp70, Hdj-2, Parkin, or CHIP, normalized against that of each band for the precipitated Pael-R. The experiment was repeated three times, and typical results are shown. The total amount of protein loaded in each case was estimated from the results of experiments using anti-actin Ab. Ub, ubiquitin.

whether the interactions between molecular chaperones and the Parkin-Pael-R complex are affected by ER stress, we analyzed the immunoprecipitate of Pael-R at several time points during the ER stress induction (Figure 5, center). To eliminate cell-surface Pael-R and immunoprecipitate only the unfolded species, the cells were pretreated with trypsin for 5 min. This treatment was sufficient for complete elimination of plasma membrane-associated Pael-R (data not shown). The amount of CHIP associated with unfolded Pael-R was increased under ER stress. On the other hand, the amount of Pael-R-associated Hsp 70 and Hdj-2 was transiently increased 6 hr after the addition of tunicamycin, but then was markedly decreased 24 hr later, despite a dramatic upregulation in Hsp70 levels. The decrease in the amount of Hsp70 associated with Pael-R during ER

with GSH Sepharose or anti-Hsp70 Ab-Protein G Sepharose complex, were detected using Abs specific to each of the indicated proteins. A representative result from four experiments is shown. The graphs indicate the relative intensity of each band for GST-Parkin, His-Hsp70, coprecipitated Pael-R, and coprecipitated His-CHIP. The values are expressed as in (B).

(D) Recombinant GST (0.3 μg) or GST-Parkin (1 μg) was immobilized on GSH Sepharose beads, then recombinant 6 × histidine-tagged (His-) CHIP (0.3 μg) was added together with (+) or without (–) His-Hsp70 (2 μg) or His-Hsc70 (2 μg). After 3 hr of incubation at room temperature, the GSH beads were washed four times with PreScisson cleavage buffer containing 10 μg/ml BSA. Immobilized Parkin was eluted by cleavage with PreScisson protease. A representative result from five experiments is shown.

(E) FLAG-Parkin, immunopurified from stably expressing FLAG-Parkin SH-SY5Y cells (10^7 cells in each lane), was immobilized on anti-FLAG affinity gel in the presence or absence of 2 mM ATP. The complex was incubated with recombinant CHIP (1 μg) in the presence or absence of 2 mM ATP overnight at 4°C and then eluted with FLAG peptide. The eluted fraction was analyzed by Western blotting with anti-Hsp70, anti-Parkin, anti-CHIP, and anti-proteasome, α-type 1 subunit. A representative result from three experiments is shown. The graph indicates the relative intensity of each band for coprecipitated Hsp70 (fold increase in the absence of ATP and CHIP) and CHIP (fold increase in the presence of ATP and CHIP), normalized against each precipitated Parkin.

stress might be due to the marked degradation of Pael-R via ERAD. In fact, proteasome inhibition by lactacystin suppressed the reduction of Pael-R and dramatically increased high molecular weight ubiquitin immunoreactivity in the Pael-R immunocomplex. However, even when Pael-R degradation was suppressed by lactacystin, the association between Hsp70 and Pael-R was attenuated with a similar time course, despite the significant increase in the protein levels of both Pael-R and Hsp70 (Figure 5, center). Compared with the rapid induction of Hsp70, Parkin and CHIP were slowly induced and levels were still increasing 24 hr after the addition of tunicamycin (Figure 5, left). These results strongly suggest that CHIP promotes the release of Hsp70 and Hdj-2 from the Parkin-Pael-R complex under ER stress, thereby enhancing the Parkin E3 activity.

CHIP Promotes and Hsp70 Inhibits Pael-R Ubiquitination by Parkin In Vitro

Since the association between Parkin-Pael-R complex and Hsp70 or CHIP is affected by ER stress, we examined whether CHIP and Hsp70 might be involved in Parkin-mediated ubiquitination and degradation of Pael-R using purified recombinant proteins (Figure 6A). Although an in vitro ubiquitination assay revealed that Pael-R is polyubiquitinated by GST-Parkin, this reaction was incomplete when a limited amount of E2 was used. Under these conditions, we performed in vitro ubiquitination assays of Pael-R in the presence of recombinant CHIP, Hsp70, Hsp40 (Hdj-2), and/or Ubc4, which has demonstrated E2 activity toward CHIP in vitro (Figure 6) (Hatakeyama et al., 2001; Murata et al., 2001). Notably, in the presence of both Parkin and Ubc4, CHIP dramatically enhanced the ubiquitination of Pael-R in a dose-dependent manner (Figure 6B, lanes 16–18). The CHIP-mediated in vitro ubiquitination of Pael-R requires Parkin, Ubc6ΔC (C-terminal truncated Ubc6), Ubc7, and Ubc4: in the absence of any one of these molecules, the reaction did not occur (Figure 6B). Moreover, mutations in the U box domain (ΔU, H261A, P270, or HP/AA) abrogated CHIP-mediated enhancement of Pael-R polyubiquitination (Figure 6C, compare lanes 8–11 with lane 7) (Hatakeyama et al., 2001). In light of these findings, it appears that optimal ubiquitination of Pael-R requires Parkin, Ubc6/7, wild-type CHIP, and Ubc4, as demonstrated by the disappearance of nonubiquitinated Pael-R when all of these components were present.

Next, we analyzed the effect of Hsp70 and Hsp40 (Hdj-2) on the in vitro ubiquitination of Pael-R. Although CHIP is reported to be a chaperone-dependent E3, our experiments revealed that Hsp70 (and Hsp40) were not essential for CHIP-mediated Pael-R ubiquitination (Murata et al., 2001). On the contrary, ubiquitination of Pael-R by Parkin and CHIP was inhibited by Hsp70 and Hsp40 in a dose-dependent manner, as shown by significant retention of the unmodified form of Pael-R (Figure 6D).

CHIP and Hsp70 Suppress Cell Death Due to the Presence of Unfolded Pael-R

Next, we used transient transfection assays to investigate whether CHIP and Hsp70 influence cell death due to accumulation of unfolded Pael-R (Figure 7). Overexpression of Pael-R reduced cell viability to approximately 70% of that observed in mock-transfected cultures. Coexpression of Parkin improved viability to 83%, and combined expression of Parkin and CHIP had a pronounced pro-survival effect, increasing cell viability to 97% of that in mock-transfected cultures. Treatment with MG-132 (10 μM) for 12 hr abolished the combined Parkin and CHIP effect on cell viability. Whether or not CHIP variants were present, coexpression of Hsp70 and Parkin suppressed cell death significantly to as great an extent as Parkin and CHIP coexpression. The effect of the combinations of CHIP, Hsp70, and/or Parkin on cell death is additive (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/10/1/55/DC1). There was a strong inverse correlation between the cell death-suppressing effects of Parkin, CHIP, and Hsp70 and the amount of unfolded Pael-R observed in 1% Triton X-100-insoluble fractions, as shown in the upper and middle panels of Figure 7. Interestingly, overexpression of wild-type CHIP together with Parkin strongly decreased the amount of soluble as well as insoluble Pael-R. Treatment with MG-132 inhibited the Pael-R degradation-promoting effect of CHIP in both fractions (Figure 7, compare lane 5 with lane 4). These findings suggest that CHIP inhibits endogenous chaperone activity for Pael-R folding and promotes the degradation of Pael-R, resulting in cell death suppression. While Hsp70 coexpression with Parkin decreased insoluble Pael-R in the presence of either wild-type or mutant and presumably inactive CHIP (ΔU box mutant, ΔU; ΔTPR mutant, ΔT; see Figure 3A), it canceled the CHIP effect on soluble Pael-R (Figure 7, compare lanes 6–8 with lane 4, soluble and insoluble fractions). The effect of CHIP and Hsp70 on the soluble and insoluble Pael-R without Parkin overexpression was essentially the same: CHIP decreased the amounts of both soluble and insoluble Pael-R whereas Hsp70 decreased that of only insoluble Pael-R (see Supplemental Figure S2 at http://www.molecule.org/cgi/content/full/10/1/55/DC1). These results suggest that Hsp70 associates with unfolded Pael-R, thereby promoting the synthesis of properly folded, soluble Pael-R and protecting Pael-R from degradation by CHIP.

Taken together, these results indicate that the protein degradation pathway involving Parkin and CHIP works in coordination with the molecular chaperone system involving Hsp70 to suppress unfolded protein stress-induced cell death.

Discussion

CHIP was originally cloned as a Hsp/Hsc70 binding protein; however, its U box has since been characterized as a ubiquitin chain formation-catalyzing domain (Ballinger et al., 1999; Connell et al., 2001; Meacham et al., 2001). The function of mammalian U box proteins has recently been shown to depend on specific E2 activity, which is similar in character to E3 activity (Hatakeyama et al., 2001; Jiang et al., 2001; Murata et al., 2001). E3 enzymes are thought to determine substrate specificity for ubiquitination. CHIP has been implicated in the ubiquitination of unfolded CFTR and glucocorticoid receptor (Connell et al., 2001; Meacham et al., 2001). However,

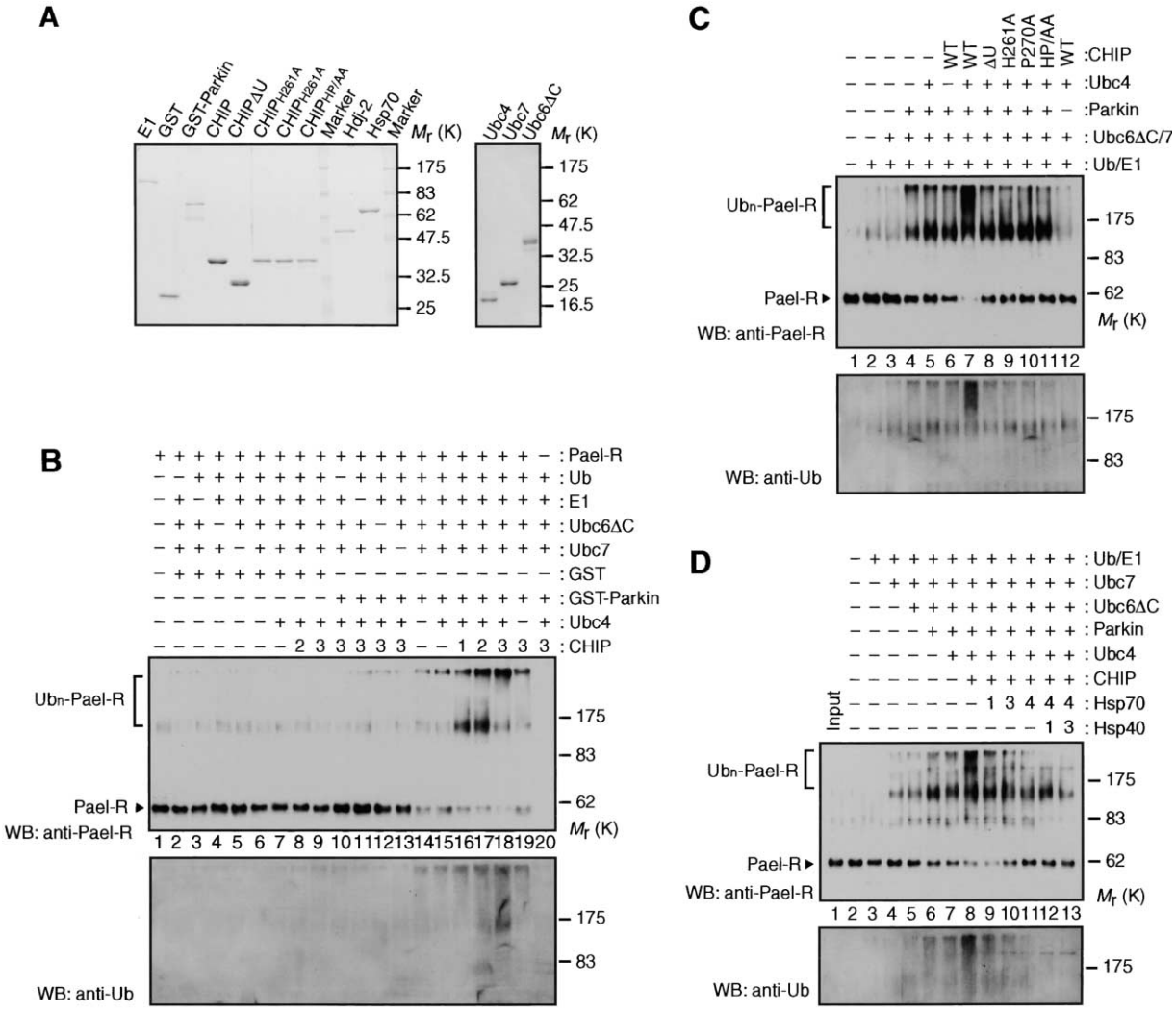

Figure 6. CHIP Promotes, While Hsp70 Inhibits, Parkin-Mediated Ubiquitination of Pael-R In Vitro

(A) Recombinant proteins used in the in vitro ubiquitination assay were produced in bacteria or baculovirus-infected insect cells. Coomassie-stained gels of the proteins purified by affinity and ion exchange chromatography are shown. A smaller band (~60 kDa) in the lane of GST-Parkin is a C-terminal truncated form of GST-Parkin that was copurified in the purification process.

(B) In vitro ubiquitination assay of Pael-R using recombinant CHIP, Parkin, and E2s (Ubc4, Ubc6ΔC, and Ubc7). Pael-R-FLAG generated in rabbit reticulocyte lysate was immobilized on the anti-FLAG affinity gel. Pael-R on the gel was incubated at 30°C for 90 min (lane 1), or Pael-R (lanes 2–19) or mock (lane 20) was treated 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 or anti-Ub Ab.

(C) In vitro ubiquitination assay of Pael-R using recombinant wild-type CHIP (WT), CHIP lacking the U box domain (ΔU), or a U box variant in which histidine 261 (H261A), proline 270 (P270A), or both residues (HP/AA) are replaced by an alanine residue. The ubiquitination reaction was performed as in (B).

(D) In vitro ubiquitination assay of Pael-R using increasing amounts of recombinant Hsp70 (1, 3, and 4 μg) and Hsp40 (Hdj-2; 1 and 3 μg). The ubiquitination reaction was performed as in (B). The purified Pael-R used in the assay was also shown as "Input." These ubiquitination assays in were performed at least twice, and representative data are shown.

it is unclear whether E1, E2, and CHIP combined can fully ubiquitinate these substrates. Recently, in vitro data by Murata et al. showed that the combination of E1, E2, CHIP, and a chaperone can ubiquitinate heat-denatured luciferase (Murata et al., 2001). These findings support the idea that CHIP functions as an E3 with the help of molecular chaperones, which identify and capture substrates for degradation.

In this study, we found that CHIP potentiates Parkin-mediated Pael-R ubiquitination and subsequent degradation through two different mechanisms: promotion of the release of Hsc/Hsp70 from Parkin and Pael-R, and E4-like, ubiquitination-enhancing activity. CHIP promotes the release of Hsp/Hsc70 and Hdj-2 from both Parkin and a substrate of Parkin, Pael-R (Figures 4B and 4C). Both CHIP and Hsp/Hsc70 preferentially interact with the first RING finger motif of Parkin in vivo. Although an in vitro binding assay revealed that CHIP can bind to Parkin directly, binding could also be mediated by Hsp/Hsc70 since both CHIP and Parkin can directly bind

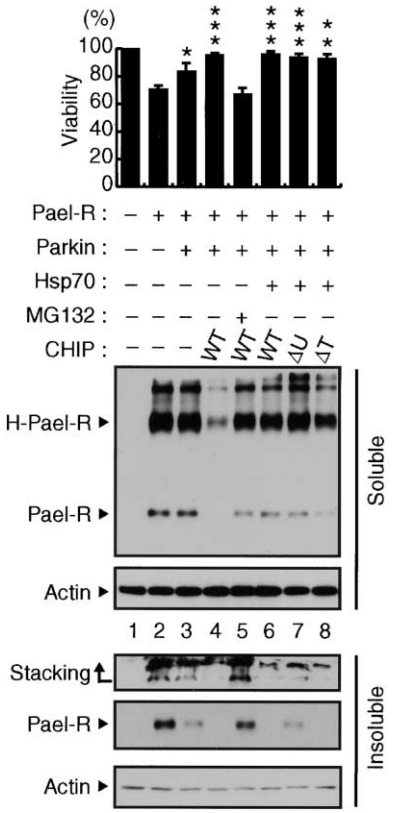

Figure 7. CHIP and Hsp70 Help Parkin Suppress Accumulation of Unfolded Pael-R In Vitro

Upper, SH-SY5Y cells on 96-well plate after 36 hr of transfection with either an empty vector, CHIP (WT), CHIP mutants (ΔU box mutant, ΔU; ΔTPR mutant, ΔT; see Figure 3B), Parkin, Hsp70, and/or Pael-R were incubated with or without MG-132 (10 μM) for 12 hr. Following this, cell viability was assessed by mitochondrial dehydrogenase activity using WST-1 reagent (Roche Diagnostics) and expressed as a percentage of the viability of the vector control. Experiments were performed in triplicate. Representative results (mean ± SD) from one out of four independent experiments are presented.

Lower, cells on 12-well plate were treated as described above, lysed in fractionation buffer containing 1% Triton X-100, and separated into soluble and insoluble fractions (Imai et al., 2001). Each fraction was subsequently Western blotted using anti-Pael-R (upper panel) or anti-actin (lower panel) Ab. The ~120 kDa form of Pael-R (H-Pael-R), which is probably an SDS-resistant dimer of Pael-R, is shown in the panel of the soluble fraction. Heavily ubiquitinated Pael-R, which could not migrate into the separating gel of SDS-PAGE and stayed in the stacking gel, is shown in the upper panel of the insoluble fractions. The unmodified form of insoluble Pael-R, detected by prolonged exposure to the X-ray film, is indicated in the middle panel of the insoluble fractions. A representative result from three experiments is shown.

Asterisk, significantly different from Pael-R alone, $p < 0.05$ (Student's t test); two asterisks, $p < 0.01$; three asterisks, $p < 0.005$.

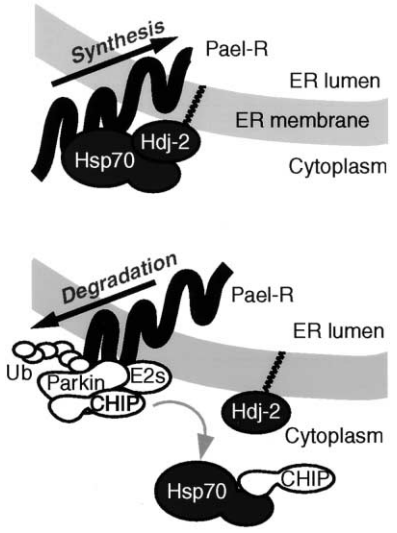

Figure 8. Hypothetical Model Showing How CHIP Might Participate in Pael-R Degradation

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 is then upregulated and binds to Hsp70, promoting the release of Hsp70 and Hdj-2 from Pael-R. CHIP also associates and cooperates with Parkin and E2s, such as Ubc4, Ubc6, and Ubc7, on the ER surface to promote ubiquitination of Pael-R.

to Hsp/Hsc70 (Figures 4C and 4D). Our in vitro data suggest that CHIP competes with Hsp70 in binding to Parkin, probably via suppression of the ATPase activity of Hsc/Hsp70 (Figure 4E). Although the affinity of a chaperone for a specific protein in vivo can be determined by various factors such as the presence of co-chaperones and the ratio of available ATP to ADP, our in vivo experiment of molecular kinetics of the unfolded Pael-R complex shown in Figure 5 is consistent with the in vitro binding data presented in Figure 4.

Although Murata et al. (2001) have reported CHIP to be a chaperone-dependent E3, CHIP was observed to promote the ubiquitination of Pael-R in a chaperone-independent manner in vitro in our study (Figure 6). Moreover, the addition of excess Hsp70 (and Hsp40) negatively affected the ubiquitination reaction induced by Parkin (data not shown), as well as that induced by Parkin and CHIP (Figure 6D). The purified Pael-R used in the in vitro experiment was generated in rabbit reticulocyte lysate, which possibly contained chaperone proteins. However, Hsp/Hsc70 and Hsp90 were not detectable in Western blot analysis of a purified fraction of Pael-R (data not shown), indicating that CHIP functioned independently of Hsp/Hsc70 and Hsp90 in this experiment.

We could not detect intrinsic differences between Hsp70 and Hsc70 in our in vivo and in vitro experiments. Hsp70, however, seemed to bind to Parkin with higher affinity than Hsc70 in the overexpression cell assays (Figure 4A). Furthermore, Hsp70 seemed to promote the binding of CHIP to Parkin, in spite of a simultaneous dissociation of Hsp70 from Parkin. Our in vitro experiments failed to reproduce this phenomenon, suggesting that other cofactor(s) or specific conditions might be required for this to occur. Indeed, marked induction of Hsp70 occurs in the face of unfolded protein stress (Figure 5). Given that overexpression of Hsp70 in cells mimics induction of Hsp70, the CHIP-recruiting activity

of Hsp70 for Parkin might reflect an elegant unfolded protein response in vivo.

In this study, we found that CHIP promotes Parkin-mediated Pael-R ubiquitination and subsequent degradation. In vitro ubiquitination assays suggested that only a combination of both Parkin and its cofactor CHIP function as a ubiquitin ligase, which is able to sufficiently ubiquitinate Pael-R in vivo (Figure 6). This idea is supported by our previous observation that bacterially produced recombinant Parkin protein shows only moderate E3 activity in in vitro ubiquitination assays (Imai et al., 2001). This function of CHIP is reminiscent of a novel ubiquitination factor, E4 (Koegl et al., 1999). A yeast U box protein, Ufd2, has been identified as a multiubiquitin chain assembly factor (E4). E4 is known to interact with an oligo-ubiquitinated artificial substrate and promote efficient ubiquitin chain polymerization of the substrate by cooperating with E1, E2, and E3. The properties of Ufd2, however, are different from those of CHIP in at least two respects. First, direct interaction between Ufd2 and E3 is not observed, although CHIP directly interacts with Parkin. Second, Ufd2 does not seem to require its specific partner E2, while Ubc4 is necessary for CHIP to exert its E4-like function. Further characterization of CHIP and other mammalian U box proteins is necessary to clarify the existence of mammalian E4.

Under normal conditions, most nascent polypeptides associate with Hsp/Hsc70 and other co-chaperones. This is essential to ensure the proper folding and intracellular localization of newly synthesized polypeptides (Frydman and Hartl, 1996). Under unfolded protein stress, Hsp70 is one of the most rapidly inducible proteins (Bush et al., 1997; Zimmermann et al., 2000). By retarding protein degradation and promoting proper refolding, Hsp70 functions as a major cellular defense molecule against the accumulation and aggregation of damaged protein caused by a diverse array of stress conditions (Bukau and Horwich, 1998; Glover and Lindquist, 1998; Johnson and Craig, 1997). In fact, it acts as an inhibitory factor that suppresses the ubiquitination of Pael-R mediated by Parkin in vitro, and Hsp70 enhances the efficiency of folding of overexpressed Pael-R in vivo. On the other hand, Hsp70 can transfer unrecoverable polypeptides to the degradation complex. Taking our results into consideration, steady-state cell conditions might allow Hsp70 and co-chaperone Hdj-2 to recruit newly synthesized Pael-R, thereby suppressing unwanted aggregation and promoting translocation into the ER (Figure 8, upper). Even when Parkin is already in association with the Pael-R-chaperone complex, Hsp70 might inhibit its E3 activity. Upon unfolded protein stress, cells might rapidly induce Hsp70, thereby suppressing the aggregation and accumulation of unfolded Pael-R, with subsequent induction of CHIP and Parkin. CHIP appears to associate with Hsp70, promoting dissociation of Hsp70 from Pael-R, thus activating Parkin function and E2 activity (Figure 8, lower). The Parkin complex appears to recruit the proteasome-complex in an ATP-dependent manner, facilitating the degradation of Pael-R (Figure 4E). According to the hypothesis shown in Figure 8, an elevation of CHIP is a decisive event in promoting the transition from the substrate (nascent peptides)-chaperone complex to the substrate (unfolded peptides)-disassembly complex.

There are growing lines of evidence to indicate that overexpression of Hsp70 has a beneficial effect on several disease models of misfolded protein-associated neurodegeneration, including Parkinson's disease, CAG repeat disease, and ALS. This strongly suggests a functional link between the protein refolding and degradation pathways (Auluck et al., 2002; Sherman and Goldberg, 2001). According to the protein triage model for protein quality control, the fate of damaged proteins, i.e., refolding or degradation, depends on the kinetics of the proteins' interaction (binding and release) with molecular chaperones and proteases (Wickner, et al. 1999). CHIP, which appears to play a key role in the fate of unfolded Pael-R, might be an excellent therapeutic target for the treatment of AR-JP and related neurodegenerative diseases.

Experimental Procedures

Plasmids, Antibodies, and Proteins

Expression plasmids for human Parkin, Parkin mutants, Pael-R, mouse CHIP, CHIP mutants, and E2s (Ubc4, Ubc6ΔC, and Ubc7) are described elsewhere (Imai et al., 2001; Hatakeyama et al., 2001). cDNA for the deletion form of the ubiquitin-like domain of Parkin (ΔUbl; 77–465 aa) was obtained by PCR. Full-length cDNA of human Hsp70 and Hsc70 was kindly provided by N. Nukina. cDNA of Hdj-2 was a kind gift from S. Kato. Recombinant GST-Parkin, GST-HA-CHIP, GST-HA-CHIP mutants, 6 × His-tagged (His-) CHIP, His-E2s, and His-Hsp70, as well as His-Hsc70 and His-Hdj-2, were produced in the *E. coli* strain, BL21(DE3)pLysS (Novagen). Human E1 cDNA was cloned by RT-PCR, then His-E1 protein was generated and purified in the baculovirus expression system (Invitrogen). Anti-Parkin monoclonal and polyclonal Abs and anti-Pael-R monoclonal Ab are described elsewhere (Imai et al., 2000, 2001). Rabbit anti-CHIP polyclonal Abs were raised against recombinant GST-CHIP. Anti-BiP (N-20), anti-Hsp70 (K-20), and anti-Hsc70 (K-19) Abs were purchased from Santa Cruz Biotech. Anti-KDEL (10C3) and anti-Hdj-1 (SPA-400) were purchased from StressGen. Anti-Hdj-2 (KA2A5.6), anti-FLAG (M2), anti-HA (3F10), anti-Proteasome 20S α-type 1 subunit (539145), and anti-actin (C4) Abs were obtained from NeoMarkers, Sigma, Roche Diagnostics, CALBIOCHEM, and Chemicon, respectively.

Cultured Cells, Transfection, Immunoprecipitation, and Western Blot Analysis

Neuroblastoma SH-SY5Y cells were transfected with linearized pcDNA3-FLAG-Parkin, pcDNA3-p35-FLAG, pcDNA3-Pael-R, or pcDNA3-FLAG vector. Neomycin-resistant cells were isolated and cloned. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 200 μg/ml G418. Human embryonic kidney 293T and SH-SY5Y cells were transfected and used for immunoprecipitation, Western blot, immunocytochemistry, and cell death assay as described elsewhere (Imai et al., 2000, 2001). The total amount of plasmid DNA used for transfection was constant by adding an appropriate amount of the vector plasmid. Cell lysis, fractionation, immunoprecipitation, and Western blot were carried out as described elsewhere (Imai et al., 2000, 2001). The densitometric analysis was performed using Quantity One software (BioRAD).

Immunoelectron Microscopic Analysis

Sections of the substantia nigra from Wistar male rats (8 weeks) were incubated with rabbit affinity-purified anti-Parkin Ab. The sections were then incubated with 10 nm colloidal gold-conjugated secondary Ab, counterstained with uranyl acetate and lead citrate, and observed using an electron microscope (LEO 912AB, LEO, Germany).

Protein Purification and Identification

SH-SY5Y cell lines were stably transfected with FLAG-Parkin (SH-SY5Y/FLAG-Parkin), p35-FLAG, or a FLAG-vector construct. Cells

(1×10^7) were starved for 1 hr in methionine/cysteine-free DMEM (M/C-free DMEM) containing 5% FCS and 10 μM lactacystin. They were then labeled for 3 hr at 37°C with 200 μCi/ml ^{35}S-methionine/cysteine in M/C-free DMEM containing 5% FCS and 10 μM lactacystin. Cells were washed four times with phosphate buffered saline and lysed. The lysate was affinity-purified with anti-FLAG M2 affinity gel (Sigma). FLAG-protein complexes eluted with FLAG peptide were resolved using a 2D immobilized-pH-gradient (range, pH 4–7)/SDS-PAGE (9% acrylamide gel) and visualized after exposure to X-ray film. For identification of specific proteins, SH-SY5Y/FLAG-Parkin cells (6×10^8) were pretreated with 10 μM MG-132 for 3 hr and then lysed. Affinity purification with anti-FLAG gel and 2D-electrophoresis was performed as described above. The purified protein was used for mass spectrometry or Western blot analysis.

In Vitro Ubiquitination Assay

Pael-R-FLAG was produced using TNT quick coupled transcription/translation systems (Promega) and immunopurified using anti-FLAG affinity gel. The in vitro ubiquitination assay was performed as described elsewhere with minor modifications (Imai et al., 2000, 2001). In brief, Pael-R-FLAG immobilized on the anti-FLAG affinity gel was incubated at 30°C for 90–120 min in 40 μl of reaction solution (50 mM Tris-HCl [pH 7.2], 120 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, and 0.5 mM dithiothreitol) containing 5 μg of bovine ubiquitin (Sigma), 0.15 μg of His-E1, 0.2 μg of His-Ubc6ΔC, 0.1 μg of His-Ubc7, 0.3 μg of His-Ubc4, and 1.5 μg of GST-Parkin and/or 1 μg of CHIP with the GST moiety removed with PreScisson protease, unless otherwise described. The total amount of proteins in each reaction mixture was adjusted with BSA (fraction V, Nacalai Tesque). After the incubation, Pael-R-FLAG on the gel was washed three times with the reaction solution and then subjected to Western blot analysis.

Acknowledgments

We would like to thank K. Takio, H. Nakayama, and R. Araya for their assistance in the mass spectrometry analysis. This work was funded by research grants from RIKEN BSI, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, a Grant-in-Aid for Encouragement of Young Scientists from the Japan Society for the Promotion of Science, as well as grants from the Ministry of Health, Labor, and Welfare, Japan.

Received: February 1, 2002
Revised: June 25, 2002

References

Auluck, P.K., Chan, H.Y.E., Trojanowski, J.Q., Lee, V.M.-Y., and Bonini, N.M. (2002). Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. Science *295*, 865–868.

Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y., and Patterson, C. (1999). Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol. Cell. Biol. *19*, 4535–4545.

Bukau, B., and Horwich, A.L. (1998). The Hsp70 and Hsp60 chaperone machines. Cell *92*, 351–366.

Bush, K.T., Goldberg, A.L., and Nigam, S.K. (1997). Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J. Biol. Chem. *272*, 9086–9092.

Connell, P., Ballinger, C.A., Jiang, J., Wu, Y., Thompson, L.J., Hohfeld, J., and Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat. Cell Biol. *3*, 93–96.

Frydman, J., and Hartl, F.U. (1996). Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. Science *272*, 1497–1502.

Glover, J.R., and Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell *94*, 73–82.

Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K.I. (2001). U box proteins as a new family of ubiquitin-protein ligases. J. Biol. Chem. *276*, 33111–33120.

Hershko, A., Ciechanover, A., and Varshavsky, A. (2000). The ubiquitin system. Nat. Med. *6*, 1073–1081.

Imai, Y., Soda, M., and Takahashi, R. (2000). Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J. Biol. Chem. *275*, 35661–35664.

Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001). An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell *105*, 891–902.

Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol. *10*, 429–439.

Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J., and Patterson, C. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J. Biol. Chem. *276*, 42938–42944.

Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549–552.

Johnson, J.L., and Craig, E.A. (1997). Protein folding in vivo: unraveling complex pathways. Cell *90*, 201–204.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature *392*, 605–608.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635–644.

Kopito, R.R. (2000). Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. *10*, 524–530.

Krüger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Genet. *18*, 106–108.

Meacham, G.C., Lu, Z., King, S., Sorscher, E., Tousson, A., and Cyr, D.M. (1999). The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. EMBO J. *18*, 1492–1505.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., and Cyr, D.M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat. Cell Biol. *3*, 100–105.

Mizuno, Y., Hattori, N., and Matsumine, H. (1998). Neurochemical and neurogenetic correlates of Parkinson's disease. J. Neurochem. *71*, 893–902.

Morett, E., and Bork, P. (1999). A novel transactivation domain in parkin. Trends Biochem. Sci. *24*, 229–231.

Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. *2*, 1133–1138.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., et al. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045–2047.

Sherman, M.Y., and Goldberg, A.L. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron *29*, 15–32.

Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat. Genet. *25*, 302–305.

Terada, K., and Mori, M. (2000). Human DnaJ homologs dj2 and dj3, and bag-1 are positive cochaperones of hsc70. J. Biol. Chem. *275*, 24728–24734.

Wickner, S., Maurizi, M.R., and Gottesman, S. (1999). Posttransla-

tional quality control: folding, refolding, and degrading proteins. Science *286*, 1888–1893.

Zarate-Lagunes, M., Gu, W.J., Blanchard, V., Francois, C., Muriel, M.P., Mouatt-Prigent, A., Bonici, B., Parent, A., Hartmann, A., Yelnik, J., et al. (2001). Parkin immunoreactivity in the brain of human and non-human primates: an immunohistochemical analysis in normal conditions and in Parkinsonian syndromes. J. Comp. Neurol. *432*, 184–196.

Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L., and Dawson, T.M. (2000). Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc. Natl. Acad. Sci. USA *97*, 13354–13359.

Zimmermann, J., Erdmann, D., Lalande, I., Grossenbacher, R., Noorani, M., and Furst, P. (2000). Proteasome inhibitor induced gene expression profiles reveal overexpression of transcriptional regulators ATF3, GADD153 and MAD1. Oncogene *19*, 2913–2920.