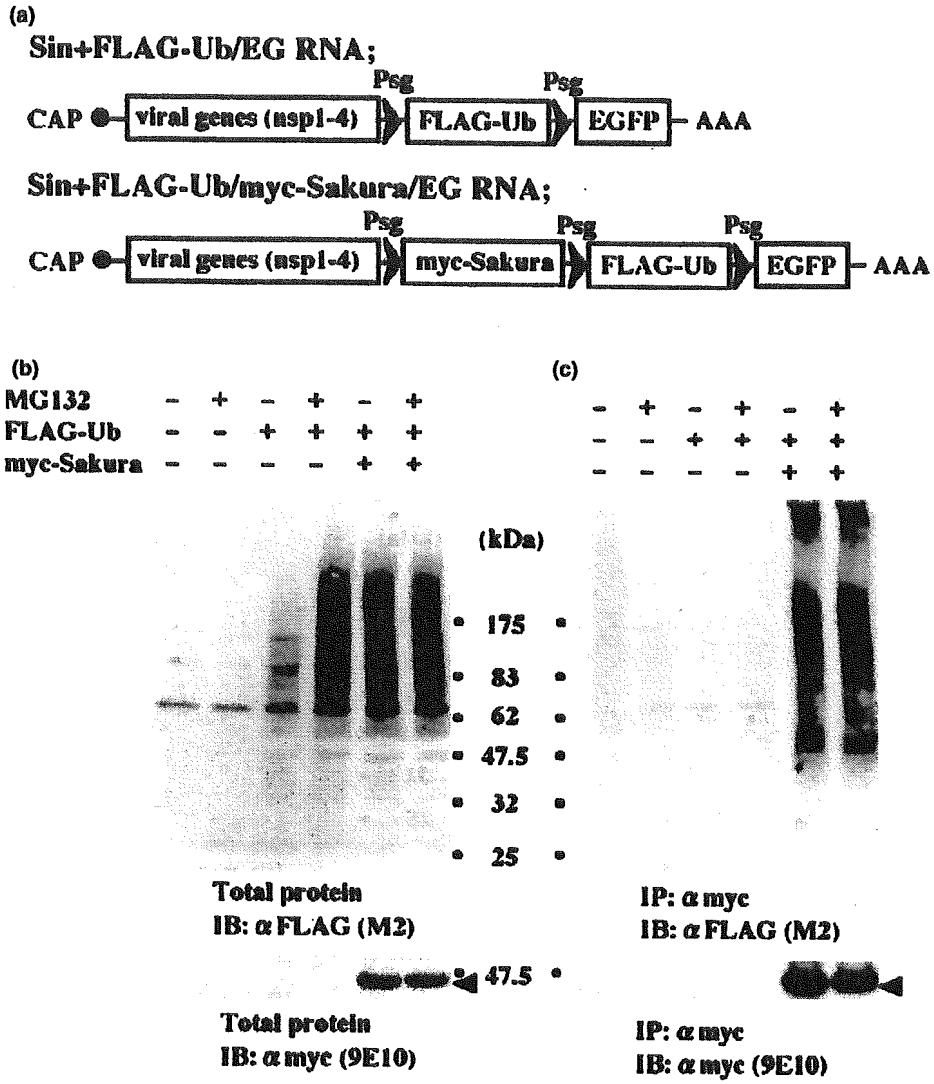

Fig. 4 Ubiquitination assay of Sakura protein in cortical culture. (a) Structures of recombinant Sindbis virus RNA for FLAG-Ub expression and for coexpression of myc-Sakura and FLAG-Ub are shown. The viral transcription and translation essential elements (Psg, nsp1–4, CAP, AAA signals) and a marker protein (EGFP) are shown in the RNA constructs as referred in the legend for Fig. 4. (b) Overexpression of myc-Sakura and/or FLAG-Ub proteins in cortical culture. Total cell lysates were subjected to western blotting using the anti-FLAG (M2) antibody (top) and anti-myc (9E10) antibody (bottom). (c) Ubiquitinated protein(s) in the immunocomplexes with myc-Sakura. The protein complex immunoprecipitated with anti-myc polyclonal antibody were subjected to immunoblotting with anti-FLAG antibody for ubiquitination (top) and anti-myc antibody (9E10) for Sakura (bottom). Closed triangles indicate Sakura immunoreactivity.

were subjected to the fractionation, these mutant proteins were recovered predominantly in the cytoplasmic fraction. The C-terminal deletion mutants of Momo and Sakura exhibited the same subcellular distributions as their wild types (data not shown). These data indicate that the N-terminal domain of Momo and Sakura was responsible for their membrane association. The cytoplasmic recovery of endogenous Momo and Sakura was quite low in the tissue preparation, however (see Fig. 2). The higher cytoplasmic recovery in HEK293 cells might result from their overexpression in the heterologous non-neural cells.

Palmitoylation of Sakura

Both Momo and Sakura carry the putative signal sites for palmitoylation. To confirm their palmitoylation, we transfected the eukaryotic expression vectors carrying myc-tagged Momo and Sakura cDNAs into HEK293 cells and then allowed cells to incorporate [^{3}H]palmitic acid. HEK293 cells

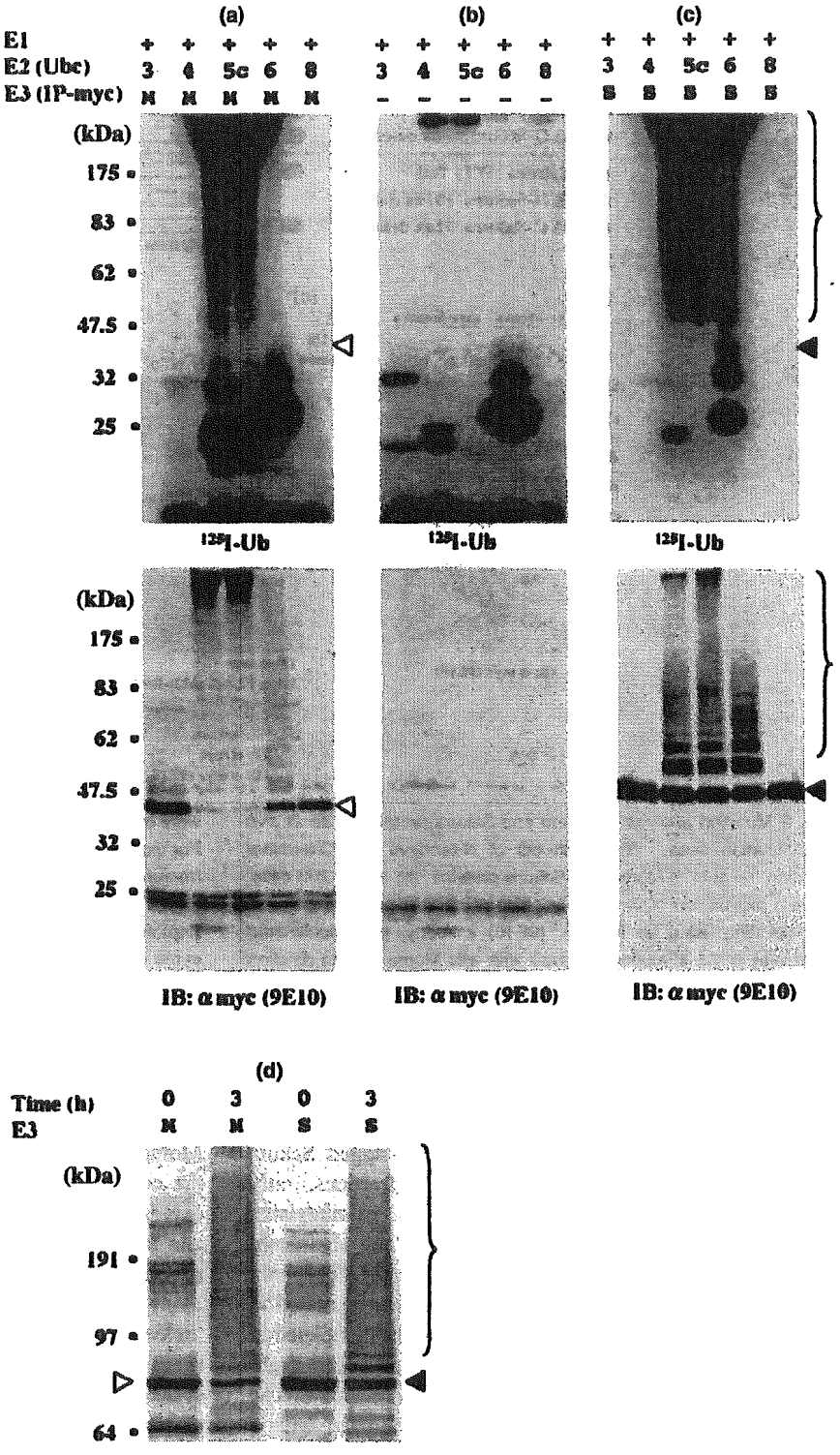

Fig. 5 Detection of an E3 ligase activity of Momo and Sakura by *in vitro* ubquitination assay. Cultured cortical cells were infected with Sin + myc-Momo/EG (a), SinEGdsp as control (b) or Sin + myc-Sakura/EG (c) (see Materials and methods). Cells were treated with MG132 for 6 h before harvesting. The protein samples immunoprecipitated with a myc-antibody (IP-myc) are indicated by myc-Momo (M), an endogenous protein control (–), and myc-Sakura (S). In the upper panels, polyubiquitinated signals were detected at high molecular regions by autoradiography. In parallel, one-third of the sample of all experiments was applied for immunoblotting with an anti-myc (9E10) antibody to detect myc-Momo or myc-Sakura in the immunoprecipitates (lower panels). The original positions (kDa) of Momo and Sakura are indicated by open and closed triangles, respectively. E1; recombinant Ub activating enzyme, E2; recombinant Ub conjugating enzymes of UbcH3 (3), UbcH4 (4), UbcH5 (5c), UbcH6 (6) and UbcH8 (8). (d) Ubiquitination reaction was reconstituted *in vitro* with the bacterial recombinant Momo (M) and Sakura (S). They were produced in *E. coli* as a fusion protein of maltose binding protein and purified. *In vitro* ubiquitination assay was carried out for 3 h in the presence of the recombinant mouse E1 and UbcH4, and followed by immunoblotting using the anti-maltose binding protein antibody. The zero time point is used as a control.

were subjected to the above subcellular fractionation and the crude membrane fraction was solubilized with a mild detergent of Triton X-100 (1%). Myc-tagged Sakura in the cytoplasmic and membrane fractions was immunoprecipitated with the anti-myc antibody, and the immunoprecipitates were subjected to SDS–PAGE followed by autoradiography or immunoblotting for the anti-myc antibody. [^{3}H]palmitic acid incorporation into the protein fraction was confirmed in the presence of an inhibitor for endogenous fatty acid synthesis (Fig. 8a). Film autoradiography revealed a protein band in

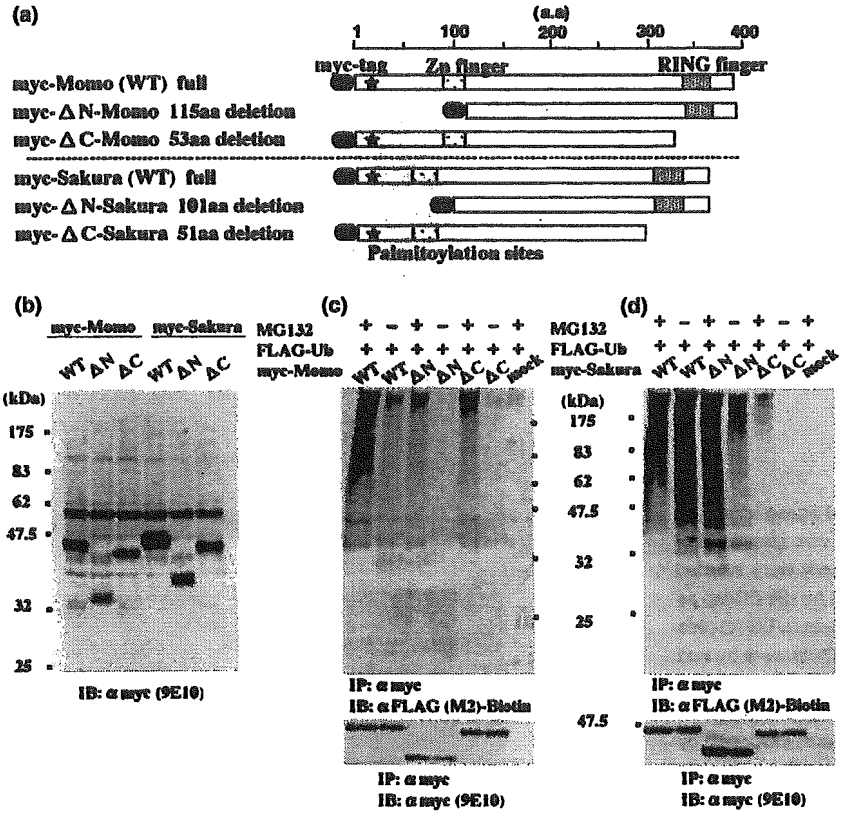

Fig. 6 Mutation analysis of Momo and Sakura proteins in an *In vivo* ubiquitination assay. (a) Constructs of N-terminal and C-terminal deletion mutants of Momo and Sakura proteins. (b) In HEK293 cells, protein expression of these deletion mutants was confirmed by immunoblotting using an anti-myc (9E10) antibody. (c) Ubiquitinated protein(s) in the immunocomplexes with wild Momo and its deletion mutants. The cells coexpressed FLAG-Ub with wild myc-Momo (WT), N-terminal deletion mutant (ΔN) or C-terminal deletion mutant (ΔC) were treated with or without 10 μM of MG132 for 6 h before harvesting. For experimental control, the pCi + myc vector was mock transfected. Immunoprecipitates with an anti-myc polyclonal antibody were subjected to western blotting with a biotinylated anti-FLAG (M2) antibody (top) and with an anti-myc (9E10) antibody (bottom). (d) Ubiquitination in the immunocomplexes of myc-Sakura and its deletion mutants was monitored similarly.

the membrane fraction that was linked to [^{3}H]palmitic acid and matched the size of Sakura (Fig. 8b). Although immunoblotting confirmed the presence of myc-tagged Sakura both in the membrane and cytoplasmic fractions, only membrane-bound Sakura appeared to be palmitoylated (Fig. 8c). In contrast, we failed to obtain any significant palmitoylation signal for myc-tagged Momo (data not shown). As the expression of Momo was quite restricted in neurons, palmitoylation of Momo might not be completed in the non-neuronal cells. Alternatively, the myc tagging might cause steric hindrance against palmitoylation of Momo. The palmitoylation of Momo remains to be examined with a different approach.

Discussion

We characterized novel membrane-associating E3 Ub ligases with a RING finger domain, Momo and Sakura. Both E3 Ub ligases are structurally homologous to each other but had distinct distributions in the brain as well as in HEK293 cells. Momo mRNA and protein expression was pronounced in the cerebellar gray matter and Sakura mRNA and protein expression was more limited to the cerebellar white matter and lower brain stem. The mRNA and protein distribution patterns suggest that neurons and oligodendrocytes are likely to express Momo and Sakura, respectively. Although molecular targets of these Ub ligases remain to be identified, these enzymes appear to have a primal role in the nervous system. Structural analysis revealed several unique functional domains; putative palmitoylation sites, double zinc finger structure and RING finger domain. Consistent with possession of the RING finger domain, Momo and Sakura exhibited Ub ligase activity *in vitro*. In particular, self-ubiquitination of Momo and Sakura was detected *in vitro* assay as reported in other E3 ligases (Fang *et al.* 2000; Yang *et al.* 2000).

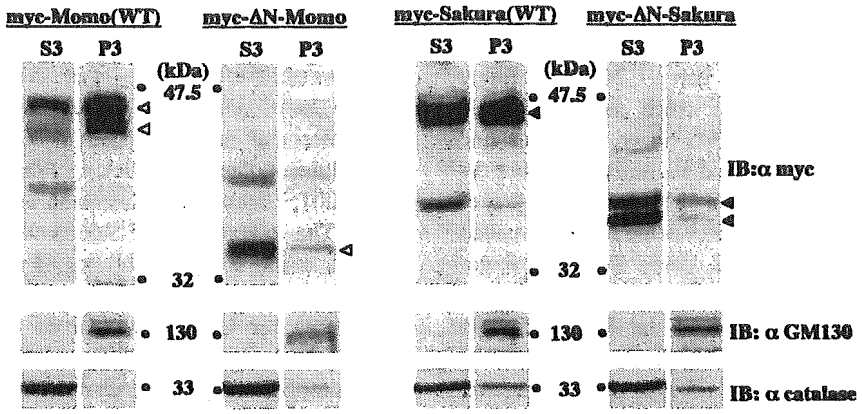

Fig. 7 Subcellular localization of Momo and Sakura proteins in HEK293 cells. The cells were transfected with wild myc-Momo (WT), myc-ΔN-Momo (the N-terminal deletion mutant), wild myc-Sakura (WT) and myc-ΔN-Sakura (the N-terminal deletion mutant). After 42-h post-transfection, cellular proteins were extracted and followed by subcellular fractionation into microsomal fraction (P3) and cytoplasmic fraction (S3). Open triangles mark myc-Momo immunoreactivity and closed triangles indicate myc-Sakura immunoreactivity. Subcellular fractionation was confirmed by their immunoreaction with the anti-GM130 (for membrane fraction) and anti-catalase (for cytoplasmic fraction) antibodies (middle and bottom panels).

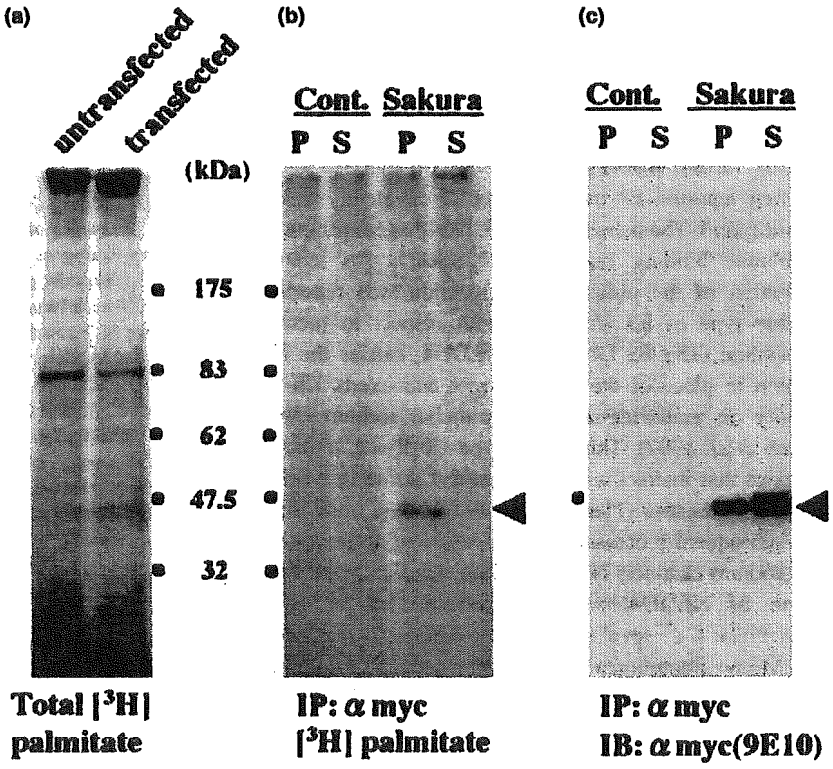

Fig. 8 Palmitoylation of Sakura protein in HEK293 cells. (a) Incorporation of [^{3}H]palmitate was detected by autoradiography of total protein extracts. Untransfected cells were used as a negative control for metabolic labeling. (b) Proteins in the crude membrane (P) fraction and cytoplasmic component (S) were immunoprecipitated with an anti-myc polyclonal antibody. The radioactivity of [^{3}H]palmitate in the immunoprecipitates appeared by a film autoradiography and is marked with a closed triangle. (c) An authentic position of immunoreactive myc-Sakura on an immunoblot is indicated by a closed triangle.

The RING finger sequence of Momo exhibited a 42% amino acid identity and 64% similarity to that of IAP (inhibitor of apoptosis) (Strausberg 2002), and that of Sakura 51% identity and 70% similarity to that of IAP. Both Momo and Sakura, however, lack the BIR domain of IAP, which interacts with caspases (Deveraux *et al.* 1997; Roy *et al.* 1997). A human orthologue of Momo was recently isolated and termed as hRFI (human RING Finger homologue to IAP type) and its anti-apoptotic activity was examined based on its structural similarity to IAP (Sasaki *et al.* 2002). In

contrast, we failed to detect an influence of Momo or Sakura overexpression on the cell survival of neurons (data not shown). Momo and Sakura also carry C2C2 zinc finger motifs at their N-terminal portion, which contain common cysteine repeats among B-box, RING finger, and other zinc fingers. According to the amino acid alignment therefore the zinc finger region of Momo shares a 32% identity and that of Sakura shares a 30% identity to the cysteine finger, so-called FYVE, of human EEA1 (Mu *et al.* 1995). Both zinc finger regions of Momo and Sakura, however, lack essential RRHH amino acid residues in the core of the FYVE domain (Stenmark and Aasland 1999). Accordingly, we classified this domain as a conventional C2C2 zinc finger structure. Mutation analysis revealed that the N-terminal domain of Momo and Sakura influenced the ubiquitination reaction. In addition, the lack of a C-terminal RING finger in Momo did not fully inhibit the reaction. In this context, we cannot rule out the possibility that the N-terminal zinc finger structure functions as an E3 Ub ligase. Alternatively, this zinc finger domain might be involved in protein–protein interactions to associate with other components (Kuroda *et al.* 1996; Rodgers *et al.* 1996; Lyngso *et al.* 2000).

Although the indirect interaction of E3 Ub ligases with cell membranes via substrates or other associating proteins is often reported (Kavsak *et al.* 2000; Shenoy *et al.* 2001; Soubeyran *et al.* 2002; Plant *et al.* 2000), information on the direct membrane anchor of E3 Ub ligases is quite limited. Among various RING finger-type E3 Ub ligases, Sakura is the first reported E3 molecule that is covalently linked to palmitic acid. The palmitoylation is likely to contribute to the membrane binding capability of Sakura. An interesting regulation of the ubiquitination reaction was reported on another type of E3 Ub ligase with respect to membrane association. The E3 Ub ligase, NEDD4, carries the HECT domain in place of the RING finger and exerts Ub ligase activity on amiloride-sensitive epithelial sodium channels (Staub *et al.* 1997). This HECT-type Ub ligase contains C2 domains that interact with phosphatidyl serine in a calcium-dependent manner (Plant *et al.* 1997). Thus, ubiquitination and subsequent proteasomal degradation of amiloride-sensitive sodium channels is regulated by the membrane translocation of NEDD4, which is induced by an elevated intracellular Ca^{2+} level (Staub *et al.* 2000). Although Sakura and Momo immunoreactivities were enriched in the membrane fraction of the brain preparations (Fig. 2), they were recovered in both cytoplasmic and membrane fractions when it was overexpressed in HEK293 cells. This observation suggests that the palmitoylation of Sakura, and potentially that of Momo, might be regulated to control their subcellular distributions and substrate recognition (Plant *et al.* 2000). The regulatory mechanism of the palmitoylation remains to be characterized, however.

In the nervous system, an impaired Ub-proteasome pathway is often implicated in neurodegenerative diseases in which there is accumulation of abnormal protein deposits in the nervous system (Paulson 1999; Saigoh *et al.* 1999; Bence *et al.* 2001; Chung *et al.* 2001a). Recent studies indicate that protein ubiquitination serves not only as a signal for proteasomal degradation but also as a routing code for intracellular molecular trafficking (Shih *et al.* 2000; Dupre *et al.* 2001; Hicke 2001; Katzmann *et al.* 2001; Polo *et al.* 2002). The degradation of Sakura ubiquitination products was not sensitive to an inhibitor of the conventional 26S proteasome, potentially suggesting the use of unconventional lysine sites for Ub ligation. Given the distributions and activities of these Ub ligases, future studies should elucidate their contribution to molecular trafficking as well as to protein degradation.

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Review

CHIP: a quality-control E3 ligase collaborating with molecular chaperones

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Abstract

It is notable that both the chaperone and ubiquitin–proteasome systems are required for removal of aberrant cellular proteins to ensure protein homeostasis in cells. However, the entity that links the two systems had remained elusive. Carboxyl-terminus of Hsc70 interacting protein (CHIP), originally identified as a co-chaperone of Hsc70, has both a tetratricopeptide repeat (TPR) motif and a U-box domain. The TPR motif associates with Hsc70 and Hsp90, while the U-box domain executes a ubiquitin ligase activity. Thus, CHIP is an ideal molecule acting as a protein quality-control ubiquitin ligase that selectively leads abnormal proteins recognized by molecular chaperones to degradation by the proteasome. Accumulating evidence from in vitro studies indicates that this is apparently the case. Here, we present and discuss several unresolved but critical issues related to the molecular mechanism and in vivo roles of CHIP.

Keywords: Protein quality control; Ubiquitin ligase; Molecular chaperone; U-box; CHIP

1. Introduction

Over 30% of newly-synthesized cellular proteins are discarded without being properly folded (Schubert et al., 2000). Even when proteins are normally formed into tertiary structures, the high density of protein

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molecules in the cytosol increases the spontaneous denaturation and consequently the likelihood of partially folded or unfolded proteins to undergo off-pathway reactions, such as aggregation. In addition, environmental stresses such as heat, oxidation (i.e. formation of free radicals), and ultraviolet, could result in the production of impaired proteins. However, those proteins with non-native or aberrant structures are rapidly removed inside the cells. In this regard, the cellular apparatus monitoring the "normality" of proteins in the cell is usually referred to as "the protein quality control system" (Wickner, Maurizi, & Gottesman, 1999). This monitoring machinery is considered an integral cellular component involved in maintaining cell survival and homeostasis, because it prevents the accumulation of abnormal proteins, formation of toxic inclusion bodies, followed by cell death, as seen in various neurodegenerative diseases. Indeed, there are growing lines of evidence of the potential link between failure of the protein quality control and neurodegeneration (Sherman & Goldberg, 2001). Hence, it is important to characterize the quality control mechanism of the cell for our understanding of the molecular basis underlying neurodegenerative diseases.

2. Chaperones and ubiquitin–proteasome system in protein quality control

Most cellular proteins in eukaryotic cells are targeted for degradation by the 26S proteasome, a eukaryotic ATP-dependent protease, usually after they have been covalently attached ubiquitin in the form of a polyubiquitin chain with linkages involving lysine 48 (K48-linked polyubiquitin chain) functioning as a degradation signal (Glickman & Ciechanover, 2002). This ubiquitylation reaction is catalyzed by a cascade system, consisting of activating (E1), conjugating (E2), and ligating (E3) enzymes. Of these ubiquitylating enzymes, E3s are believed to exist as molecules with a large diversity, presumably in hundreds of species, and play a critical role in the selection of substrate for degradation, because each distinct E3 usually binds a protein substrate with a degree of selectivity for ubiquitylation. At present, the ubiquitin–proteasome system is considered to play a key role in protein homeostasis by catalyzing the immediate destruction of misfolded or impaired (i.e. abnormal) cellular proteins (Bercovich et al., 1997; Jungmann, Reins, Schobert, & Jentsch, 1993; Lee, Sherman, & Goldberg, 1996). A related issue is when to call a protein "abnormal". "Abnormal proteins" tend to have exposed hydrophobic regions, which are then recognized by molecular chaperones such as Hsp70 and Hsp90. Molecular chaperones try to prevent these abnormal proteins from irreversible aggregation, and assist in their conversion to a properly folded and functional state. However, when these chaperones fail to refold the abnormal proteins, the ubiquitin–proteasome system disposes of unfolded, non-functional proteins. Then, how does the ubiquitin–proteasome system recognize these non-functional proteins, akin to our understanding of "garbage"? In general, the ubiquitin-protein ligase (E3) is responsible for determining what is to be degraded, that is, substrates are specifically recognized by E3 proteins (Glickman & Ciechanover, 2002). Therefore, it is rational that there exists an E3 protein that is associated or cooperates with molecular chaperones.

3. TPR motif and U-box domain of CHIP

Recently, a 35-kDa protein called carboxyl-terminus of Hsc70 interacting protein (CHIP) was identified as a candidate for a ubiquitin ligase that plays a role in the protein quality control. First, CHIP was identified during screening a library with a cDNA fragment coding tetratricopeptide repeat (TPR) motifs (Ballinger et al., 1999) (Fig. 1). Subsequent studies revealed that CHIP possessed two characteristic domains; one is the TPR domain at its amino terminus, which serves as the protein–protein interaction domain, and particularly this domain has been found to interact with members of heat shock proteins. The TPR domains of phosphatase 5, cyclophilin 40, and FKBP52 bind to Hsp90, assisting in folding hormone receptors and kinases (Young, Moarefi, & Hartl, 2001). Hip (Hsc70-interacting protein) binds to the ATPase domain of Hsc/Hsp70 by its TPR domain and increases the affinity for substrates by stabilizing the ADP-bound state of Hsc/Hsp70. Hop (Hsc70–Hsp90 organizing protein) connects Hsc70 with Hsp90, facilitating the cooperation between these two chaperones (Frydman, 2001). As for CHIP, it is indeed associated with the carboxyl-terminus of Hsp70, Hsc70, and

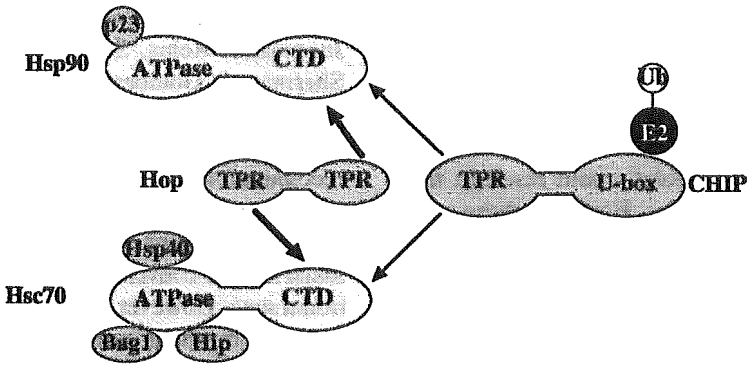

Fig. 1. Association of CHIP with molecular chaperones. CHIP associates with the carboxyl-terminus of Hsp90 and Hsc70 through its TPR motif at the N-terminus. Many other co-chaperones are also associated with Hsp90 and Hsc70. Binding of CHIP to Hsp90 detaches p23. Binding of CHIP to Hsc70 attenuates the effect of Hsp40 on Hsc70 ATPase activation.

Hsp90 through its TPR and adjacent charged domain (Ballinger et al., 1999; Connell et al., 2001; Meacham, Patterson, Zhang, Younger, & Cyr, 2001). CHIP attenuated the Hsp40-stimulated ATPase activity of Hsc70. Consequently, addition of CHIP diminished the refolding activity of the Hsc70–Hsp40 complex for denatured substrates (Ballinger et al., 1999).

Another important and unique domain that is not found in other TPR containing proteins is the U-box domain at the carboxyl-terminal region (Fig. 1). The U-box domain was first recognized in the yeast Ufd2 protein, which performs "E4" activities, i.e. recognizes oligoubiquitin chains and elongates them further, producing polyubiquitin chains (Koegl et al., 1999). Moreover, the tertiary structure of the U-box resembles that of the RING-finger domain, which is responsible for E3 activities of many ubiquitin ligases such as c-Cbl, mdm2, Parkin (Aravind & Koonin, 2000). Therefore, there is a great interest in whether the novel CHIP protein mediates interactions between the chaperone system and the ubiquitin–proteasome system.

4. CHIP is a chaperone-dependent ubiquitin ligase

Subsequent studies revealed that this is true. In cells transfected with CHIP and glucocorticoid receptor (GR), a well-known substrate of Hsp90 for its maturation, CHIP promoted dissociation of p23, a co-chaperone of Hsp90, from Hsp90, and at the same time ubiquitylated GR (Connell et al., 2001). While dissociation of p23 was U-box-independent, the ubiquitylating activity was U-box-dependent, indicating the two characteristic domains of CHIP attenuate GR mediated signaling separately but cooperatively. Another study indicated that CHIP is also involved in the quality control of endoplasmic reticulum (ER) resident proteins. In cells, CHIP ubiquitylated CFTR (cystic-fibrosis transmembrane-conductance regulator), a well-known Hsc70 substrate, in cooperation with Hsc70 in both U-box- and TPR motif-dependent manners (Meacham et al., 2001). Finally, the ubiquitylation assay using firefly luciferase as a model substrate directly demonstrated that CHIP is a chaperone-dependent E3 that selectively polyubiquitylates unfolded proteins in a completely reconstituted in vitro system (Murata, Minami, Minami, Chiba, & Tanaka, 2001). Luciferase was ubiquitylated by CHIP, together with the E2 enzyme Ubc4 or 5, only when it was heat-denatured in the presence of Hsp90 or Hsc70/Hsp40 complex, and CHIP did not ubiquitylate native luciferase or luciferase that was denatured in the absence of these molecular chaperones. Thus, CHIP functions as a "quality-control E3" involved in the selective ubiquitylation of target proteins by recognizing the non-native state in a molecular chaperone-assisted manner.

5. Interaction between molecular chaperones and CHIP

How does CHIP discriminate between what is to be degraded and what will be properly folded? For

CHIP to ubiquitylate its target proteins, it is required that targets are first captured by molecular chaperones. However, the substrates of Hsp90 and those of Hsc/Hsp70 are presented to CHIP in a quite different way. It is shown that CHIP and p23 compete for binding to Hsp90, although the binding sites of these in Hsp90 are different, that is, the former binds to the C-terminus while the latter binds to the N-terminus of Hsp90 (Connell et al., 2001; Young et al., 2001). Since p23 stimulates the ATP-hydrolysis dependent dissociation of Hsp90-substrate complex, CHIP seems to stabilize polypeptides captured by Hsp90, facilitating ubiquitylation of the substrate.

Another feature of Hsp90 is that it has two independent substrate-binding sites at its N-terminal and C-terminal regions. The chaperone activity of the N-terminal substrate binding site is dependent on its ATPase activity and is inhibited by the specific ansamycin drug, geldanamycin (GA), whereas the C-terminal substrate binding site is GA-insensitive (Minami, Nakamura, Emori, & Minami, 2001), although ATPase activity was also recently reported in the C-terminal region (Marcu, Chadli, Bouhouche, Catelli, & Neckers, 2000). CHIP can effectively ubiquitylates denatured substrates captured by the C-terminal chaperone site of Hsp90 without any assistance of the N-terminal chaperone site and the ATPase activity (Murata et al., 2001). These facts suggest that substrates chaperoned by the N-terminal region are delicately balanced between folding and degradation with the help of co-chaperones, whereas those chaperoned by the C-terminal region are more susceptible to ubiquitylation by CHIP.As for Hsc70, CHIP diminishes the substrate affinity of Hsc70 by inhibiting the Hsp40-enhanced ATPase activity (Meacham et al., 2001). Nevertheless, both Hsc70 and Hsp40 are required for denatured luciferase to be effectively ubiquitylated by CHIP (Murata et al., 2001). BAG-1, another co-chaperone of Hsc70, also plays a role in facilitating the degradation of CHIP substrates. BAG-1 connects Hsc70/CHIP complex with the proteasome by binding simultaneously to Hsc70 and to the 26S proteasome(Demand, Alberti, Patterson, & Hohfeld, 2001; Luders, Demand, & Hohfeld, 2000), probably Rpn1 subunit of 19S complex considering the cases of RAD23, Dsk2, and Ubp6 (Elsasser et al., 2002; Leggett et al., 2002), through its BAG domain and ubiquitin-like domain, respectively. Attachment of K 11-linked polyubiquitin chain, which does not serve as a degradation signal, to BAG-1 by CHIP enhances the association of BAG-1 with the proteasome (Alberti et al., 2002). How CHIP chooses between the two different modes of ubiquitylation is a quite intriguing matter but has yet to be determined. At the same time, BAG-1 promotes the release of ubiquitylated substrates from Hsc70. As a result, BAG-1 stimulates the degradation of chaperone substrates in conjunction with CHIP.

Such different mechanism of ubiquitylation and subsequent degradation may reflect the difference in the properties of the substrates between Hsp90 and Hsc70. In other words, the significance of ubiquitylating Hsp90 substrates and that of ubiquitylating Hsc70 substrates may be quite distinct in vivo. In any case, functional analysis of CHIP has emphasized for the first time a realistic situation where an unfolded protein is handled by the ubiquitin–proteasome system as well as molecular chaperones.

6. Substrates of CHIP

What are the natural substrates of CHIP in vivo? The present understating is based on the results of studies employing either cells that overexpress CHIP cDNA or in vitro reconstitution system. However, the levels of Hsc70 are about 6 times greater than those of Hdj-2, which are about 10 times greater than those of CHIP in HEK293 cells (Meacham et al., 2001). Therefore, one cannot conclude that GR and CFTR are the natural substrates of CHIP since overexpression of CHIP could ubiquitylate these proteins unphysiologically if only they were associated with chaperones. The physiological conditions under which CHIP is greatly upregulated and whether CHIP works constitutively or only under such conditions in cells is still unclear.

Another issue is whether CHIP has any specificity for targets. It is known that both ErbB2 and HIF-1α are the substrates of Hsp90 and that GA treatment promotes ubiquitin–proteasome-dependent degradation of both proteins, probably because GA treatment abolishes the chaperone function of Hsp90 and causes abnormal conformation of the substrates (Buchner, 1999). Nevertheless, while CHIP is involved, at least to some extent, in the GA-induced degradation of ErbB2 (Xu et al., 2002), HIF-1α seems to be degraded

independent of CHIP on GA treatment (Isaacs et al., 2002). Other factors that regulate the activity of CHIP may exist or CHIP itself may have some ability to recognize substrates directly. In fact, CHIP was able to ubiquitylate Raf-1 in the absence of chaperones in in vitro reconstituted assay (Demand et al., 2001), suggesting direct recognition of substrates by CHIP could occur. However, the mechanism is yet to be understood.

At this stage, all of the Hsp90 substrates and Hsc/Hsp70 substrates could be the candidates for the physiological substrates of CHIP in vivo, but whether ubiquitylating activity of CHIP toward every substrate is really important for the cellular function still remains elusive.

7. Possible role of CHIP in neurodegenerative diseases

Although CHIP is expected to play a general role in the protein quality control, its expression level varies in various mouse tissues. The mRNA levels of CHIP are high in the skeletal muscles, heart, pancreas, and the brain (Ballinger et al., 1999). However, the protein of CHIP is highly expressed in the brain (Murata et al., unpublished data). Since neurons do not regenerate themselves, it is conceivable that the protein quality control system is more important in the brain than in other tissues. In fact, a number of neurodegenerative diseases appear to result from failure of the protein quality control system (Sherman & Goldberg, 2001) (Fig. 2). Expansions of CAG trinucleotide repeats coding polyglutamine (polyQ) tracts are responsible for Huntington's disease, and spinobulbar ataxia (SCA) types 1, 2, 3, 6, 7, and 17 (Zoghbi & Orr, 2000). In rare cases, familial amyotrophic lateral sclerosis is caused by mutations of the superoxide dismutase 1 (SOD1) gene (Julien, 2001). Mutations of α-synuclein gene cause autosomal dominant Parkinson's disease (Polymeropoulos et al., 1997). The products of these mutated genes are prone to misfolding, and the formation of insoluble protein aggregates in neurons is a common hallmark of these diseases (Sherman & Goldberg, 2001).

Interestingly, molecular chaperones as well as ubiquitin and the proteasome are recruited to the inclusion bodies, indicating the attempt of the quality control system to prevent or remove protein deposits (Sherman & Goldberg, 2001). Recently, Parkin was found to

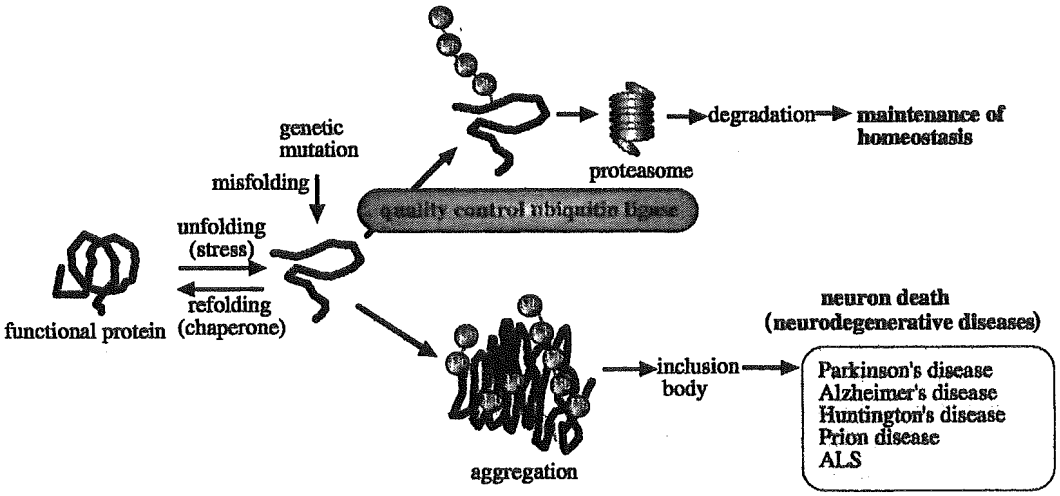

Fig. 2. Concept of protein quality control ubiquitin ligase in the pathogenesis of neurodegenerative disease. Misfolded proteins are found when the cell is exposed to various environmental stresses such as heat and oxidation. In hereditary diseases, genetically mutated gene products are prone to misfold. Molecular chaperones try to refold such misfolded or unfolded proteins in cooperation with co-chaperones. However, when the capacity for refolding is inadequate, these proteins are subjected to proteasomal degradation. Ubiquitin ligase plays a critical role in protein quality control. Proteins that evade refolding and degradation form aggregates, resulting in the formation of toxic inclusion bodies. CHIP is an attractive candidate E3 that could prevent the accumulation of such abnormal proteins.

be the causative gene of autosomal recessive juvenile Parkinsonism (Kitada et al., 1998). Subsequently, Parkin was characterized as a RING-finger type ubiquitin ligase (Shimura et al., 2000) and is currently considered to participate in the ER quality control since Parkin is upregulated by ER stress and mutant Parkin product failed to ubiquitylate Pael Receptor, one of its known targets, followed by unfolded protein response and cell death (Imai et al., 2001). The relationship between CHIP and Parkin is intriguing. Parkin on its own cannot fully ubiquitylate Pael receptors, and CHIP is required for efficient ubiquitylation of Pael receptors in cooperation with Parkin (Imai et al., 2002). Hsc70 and Hsp40 are associated with Pael receptors since the receptor seems to be prone to misfolding. However, in ubiquitylating Pael receptors, CHIP does not cooperate with Hsc70, but rather detaches Hsc70 from Parkin and the receptor. CHIP and Hsc70 seem to bind directly to the RING-finger domain of Parkin competitively. A principal question in this regard is what determines the pathways through which CHIP ubiquitylates target proteins. One explanation is that CHIP substrates, which are usually unfolded or misfolded and prone to form aggregations, should be degraded rapidly and efficiently without fail. In this context, it may be reasonable that CHIP associates with Parkin, which has ubiquitin-like domain in its amino-terminal region and may also target its substrates to the proteasome, like BAG-1 does. Thus, CHIP may not need to make complex with Hsc70/BAG-1 in this case.

Considering its role as a quality-control ligase, preferential expression in the brain, and association with Parkin, CHIP may also be involved in the pathogenesis of neurodegenerative diseases, but the concrete evidence is yet to be presented.

8. Concluding remarks

The recent studies have revealed that CHIP is a ubiquitin ligase that ubiquitylates and promote degradation of unfolded and misfolded proteins in a chaperone-assisted manner. However, there are still major unanswered questions about CHIP. One question is "how does CHIP change folding machinery to degradation machinery?" Does it require another trigger factor? Is upregulation of CHIP sufficient for the switching, as shown by overexpression experiments? Alternatively, only hopelessly misfolded proteins that spend excess time for refolding by chaperones may have a chance to encounter CHIP and be ubiquitylated. Another question is "how important is CHIP physiologically?" Studies thus far have indicated potential importance of CHIP in cellular function, but this prediction mostly relies on the results of CHIP overexpression or in vitro analysis. Thus, the biological relevance of CHIP is still unclear. Further efforts should be made to define the in vivo role of CHIP in more physiological situations.

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Dissecting Various ATP-Dependent Steps Involved in Proteasomal Degradation

Proteolysis by archaeal 20S proteasomes and the PAN (proteasome-activating nucleotidase) regulatory complex, a homolog of the eukaryotic 19S AAA ATPases, requires ATP hydrolysis through multiple steps. ATP hydrolysis, activated by binding of substrates to PAN, is utilized for substrate unfolding, gate opening of 20S proteasomes, and substrate translocation.

Selective proteolysis requiring metabolic energy is a critical part of cellular regulation. In eukaryotic cells, the 26S proteasome is an ATP-dependent protease complex and consists of the central 20S proteasome and two outer 19S regulatory particles. The 20S proteasome is a barrel-like particle formed by the axial stacking of four rings made up of two outer α rings and two inner β rings, being associated in the order of $\alpha\beta\beta\alpha$. The active sites reside in a chamber formed by the centers of the abutting β rings. The 19S particle can be divided into the lid and base complexes, and the latter consists of six proteasomal ATPases, Rpt1-6, and a few additional Rpn (non-ATPase) subunits (Glickman et al., 1998). Recent studies have provided immense information on the roles of the ATPases in the base, but details of the molecular mechanisms underlying energy consumption are still largely unknown.

An archaeal ATPase complex named PAN has been shown to stimulate the degradation of proteins by archaeal 20S proteasomes (Zwickl et al., 1999). It provides advantages for mechanistic studies in the energy-requiring proteolysis, based on its homo-oligomeric structure and the ability of recognizing and unfolding of globular proteins in vitro. In this issue of *Molecular Cell*, Benaroudj et al. probed the roles of PAN in several distinct steps during proteolysis using model substrates such as SsrA-tagged GFP and casein. They first found that PAN's ATPase activity is stimulated in a similar fashion by globular and unfolded substrates and even by the short recognition peptide (SsrA). Then, they proposed that energy emitted by PAN is utilized in three processes including substrate unfolding, gate opening in the 20S particle, and polypeptide translocation. In general, the concept proposed for PAN would be applicable to the eukaryotic proteasomal ATPases and also to the ATPases of prokaryotic proteases such as ClpA, ClpX, HslU, Lon, and FtsH, since all these belong to the AAA^+ family of ATPases (Ogura and Wilkinson, 2001).

Substrate recognition/binding

Lam et al. (2002) recently provided evidence that Rpt5 interacts with the polyubiquitin chains, functioning as a degradation signal by eukaryotic 26S proteasomes, and that this interaction is modulated by ATP hydrolysis. It has also been shown that a non-ATPase subunit, Rpn10, binds polyubiquitin chains. Thus, it remains unknown whether the two substrate-trapping mechanisms function redundantly or independently. On the other hand, PAN and ATPase subunits or domains of prokaryotic ATP-dependent proteases directly recognize and bind substrates without ubiquitin and ATP, although the molecular basis for such recognition remains undefined.

Unfolding of substrates

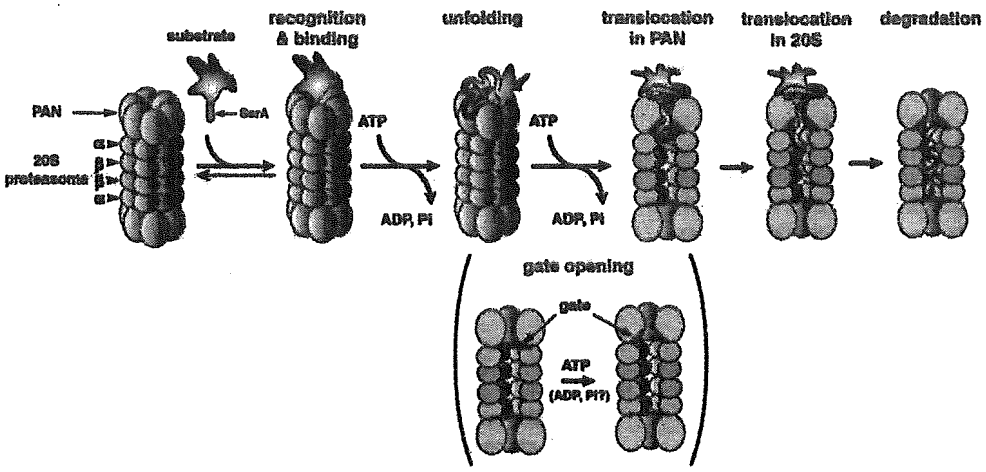
Protein substrates must be unfolded before they are translocated to the proteolytic chambers, since the opening of the α ring is usually quite narrow. It has been demonstrated that the unfolding is sequential and directional, and that it requires ATP hydrolysis. Using engineered substrates, it has been shown that PAN-mediated unfolding occurs even when translocation is inhibited (Navon and Goldberg, 2001), suggesting that proteins are unfolded on the surface of PAN before translocation (Figure).

Gate opening

It has been indicated that Rpt2 in yeast appears to be important in opening of the axial channel in the α ring (Kohler et al., 2001). However, unlike the eukaryotic 20S particles, archaeal proteasomes appear open to small peptides; the gating of these particles, which is crucial for entry of polypeptides, is also regulated by ATP (Benaroudj et al., 2003). Artificial opening of the gate achieved by N-terminal deletion of the 20S α subunits, e.g., $\Delta\alpha$(2-12)20S proteasome, facilitates degradation of unfolded proteins without PAN and ATP, while degradation of these substrates by the wild-type 20S requires both ATP and PAN. Thus, PAN appears to open the gate in the α ring in an ATP-dependent manner. Surprisingly, the $\Delta\alpha$(2-12)20S proteasome degrades unfolded substrates several-fold faster than the wild-type 20S proteasome in the presence of PAN and ATP.

Translocation of unfolded substrates

Degradation of GFP-SsrA, which has already been unfolded by PAN, by the $\Delta\alpha$(2-12)20S proteasome still requires ATP hydrolysis by PAN. This energy-requiring step is most likely translocation of unfolded substrates. The rate of ATP hydrolysis by PAN is independent of whether PAN has to catalyze substrate unfolding, since



Schematic Diagram of PAN- and Proteasome-Mediated Proteolysis with Special Emphasis on ATP-Dependent Steps

Proteolytic steps of the SsrA (cyan)-tagged substrate by PAN and 20S proteasome are shown schematically. It is uncertain when opening of the gate (green) of the α ring occurs and whether it requires ATP hydrolysis or just binding. Although translocation through the PAN ring and that within the α ring should be coupled, these steps are separately depicted to indicate more clearly the differences in energy dependence between these steps.

the amount of ATP hydrolysis per protein degraded is similar with the globular and unfolded substrates.

These results raise several fundamental questions on the roles of regulatory ATPases of proteases. The fact that the 20S proteasome with α subunits in an open state can degrade unfolded substrates efficiently suggests that the gate opening is sufficient for the rapid degradation of unfolded substrates. It is also possible, however, that deletion of the N-terminal in α subunits might cause extremely wide opening of the gate. How wide is the gate opened during degradation in wild-type proteasomes? A recent report indicates that the pore of the proteasome allows the concurrent passage of at least three stretches of a polypeptide chain (Lee et al., 2002). Whether the gate opening indeed requires ATP hydrolysis has not yet been verified experimentally, although it is ATP dependent. It is also unclear when the gate is opened and whether the size of the opened gate differs according to substrate bound to PAN.

Even after unfolding of globular GFP-SsrA by PAN, its degradation by the Δα(2-12)20S proteasome requires ATP hydrolysis by PAN. This strongly suggests that ATP hydrolysis is required for translocation but not gate opening in this case, since the gate is already open by the N-terminal deletion. However, it is worth noting that PAN is not required for the degradation of unfolded protein by the Δα(2-12)20S proteasome, indicating that its translocation occurs without energy. Consequently, it is likely that the role of ATP in substrate translocation differs, depending on the degree of unfolding by PAN. What is the reason for different ATP dependency in degradation of acid-denatured substrates and that of PAN-unfolded substrates by the Δα(2-12)20S proteasome? It is most likely that the latter requires cycles of ATP hydrolysis in order to pass polypeptides through the central pore of the PAN ring (active and probably directional translocation in PAN), whereas polypeptide movement from the gate of the α ring to the catalytic β subunits is achieved by simple diffusion (passive translocation in 20S). This may suggest that the active translocation through the channel of PAN is rather inefficient. Alternatively, the state of PAN-unfolded substrates may be completely different from that of acid-denatured ones, causing different requirement of ATP hydrolysis.

In case of ClpXP, a bacterial ATP-dependent protease, it was shown that unfolding was the slowest step during the degradation of GFP-SsrA and therefore determined the rate of degradation (Kim et al., 2000). However, Benaroudj et al. (2003) claimed that the unfolding does not appear to be the rate-limiting step in degradation by archaeal proteasomes, because globular and acid-denatured GFP-SsrA are degraded at apparently similar rates. They also suggested that the rate-limiting and major ATP-hydrolyzing step occurs after protein unfolding, and thus probably is the translocation into the 20S particle. If, in the presence of PAN and ATP, the rate of degradation of globular proteins by Δα(2-12)20S proteasome, however, is much faster than that by wild-type proteasomes, then the gate opening could be the rate-limiting process, but this point has not been discussed.

How much ATP is consumed during the unfolding step? Benaroudj et al. (2003) suggest that it is small. Precise measurement of ATP consumption may be possible using modified substrates, described by Novan and Goldberg (2001), that can be unfolded by PAN but cannot be translocated into the 20S proteasome. Further probing and dissection of the molecular mechanism of PAN should provide not only a better understanding of ATP-dependent proteolysis but also insights into the common mechanism of the AAA^+ family of ATPases in protein remodeling.

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Spotlight on Heat Shock Proteins

Proteasomes and Molecular Chaperones

Cellular Machinery Responsible for Folding and Destruction of Unfolded Proteins

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ABSTRACT

Molecular chaperones recognize proteins of nonnative structure, prevent them from irreversible intracellular aggregation, and then act with regulatory cochaperones in the conversion of proteins to be properly folded and in a functional state. However, not every nonnative protein is folded successfully. Those proteins that are not accurately folded/refolded are then directed to the ubiquitin-proteasome system (UPS) for destruction. Both chaperones and proteasomes act jointly together for selective removal of proteins with aberrant structure so as to keep protein homeostasis in cells. Though the precise nature of the cooperative linkage between chaperone and UPS pathways remains largely elusive so far, accumulating evidence from in vivo and in vitro studies shed some light on the molecular mechanisms that link proteasomes and molecular chaperones. This review focuses on how unfolded proteins are handled by these two machineries.

INTRODUCTION

In the intracellular environment, the concentration of proteins could rise leading to their aggregation as either partially folded or unfolded proteins. Over one third of the newly synthesized cellular proteins cannot fold correctly, even though they are normally synthesized without mutations of their genes or errors in the translation process. This phenomenon is provisionally called DRiPs (defective ribosomal products).[1] In addition, even when proteins are synthesized and folded correctly as functional proteins with a normal tertiary structure, they are often damaged by unfavorable environmental stresses such as heat shock, oxidization, and chemical modification.[2] The cells is equipped with two surveillance systems to manage these off-pathway reactions; the molecular chaperones and the ubiquitin-proteasome system (UPS), which serve to prevent accumulation of abnormal proteins formed through the protein biosynthetic pathway or postsynthesis damage.

Molecular chaperones play a role in maintaining protein homeostasis by regulating protein folding. They recognize nascent polypeptides and unstructured regions of proteins; e.g., exposed hydrophobic stretches of amino acids. Then cooperating with many cochaperones that complement their functions, chaperones refold nonnative proteins and prevent their irreversible aggregation with other proteins.[3-5] On the other hand, the UPS also participates in the regulation of protein homeostasis by selective destruction of misfolded/unassembled and impaired proteins generated in eukaryotic cells.[2] Namely, most of these unwanted proteins are covalently attached to ubiquitin, a highly conserved small protein consisting of 76 amino acids, in the form of a poly-ubiquitin chain functioning as a marker for proteolytic degradation by the 26S proteasome.[6,7]

It is also possible for molecular chaperones and proteasomes to act together in preventing aggregation and accumulation of abnormal proteins, thus maintaining protein homeostasis in cells; both of them are capable of recognizing common substrates under non-native states and are required for removal of aberrant cellular proteins to ensure protein homeostasis in cells. In this regard, the cellular apparatus monitoring the "normality" of proteins in the cell is usually referred to as "the protein quality control system".[8] This control system is highly flexible under constitutively changing environments, and thus the fate of unfolded proteins, i.e., either refolding or degradation, is largely dependent on environmental conditions. These facts indicate that regulation of intracellular balance of protein refolding and degradation is a critical issue for cells exposed to stressful environment, thus there is a special interest regarding the interplay between the chaperone system and the UPS pathway.

THE PROTEASOME

The proteasome is a principal machinery that participates not only in the "regulated proteolysis" responsible for selective and rapid destruction of a diverse array of biologically important cellular proteins, but also in massive degradation of abnormal proteins generated in the cells.[9,10] It is a large multisubunit complex, consisting of a central catalytic 20S proteasome (alias CP, core particle), and two terminal regulatory subcomplexes, which are attached to both ends of the central portion in opposite orientations. The 20S proteasome is composed of two copies of 14 different subunits, seven distinct α type subunits and seven distinct β type subunits, arranged in a particle as four hetero-heptameric rings, $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$, with C2 symmetry. Three β-type subunits of each inner ring have catalytically active threonine residues at their N-terminus (in which of β1, β2, and β5 corresponding to caspase-like, trypsin-like, and chymotrypsin-like activities, respectively), and these active sites face the interior of the cylinder and reside in a chamber formed by the centers of the abutting β rings.[11]

Interestingly, the center of the α-ring of the 20S proteasome is almost closed, preventing free penetration of proteins into the inner surface of the β-ring on which the proteolytically active sites are located. Substrates gain access to the active sites only after passing through a narrow opening corresponding to the center of the α rings and the amino-termini of the α subunits form an additional physical barrier for substrates to reach the active sites. To activate this latent 20S proteasome, three types of regulatory complexes; PA28, PA200, and PA700, are described to date. Although PA28 and PA700 play a prominent role in the quality control system, the role of recently identified PA200 appears to be rather restricted in DNA repair.[12]

PA700. PA700 (alias RP, regulatory particle or 19S complex), associates with either or both ends of the 20S proteasome in an ATP-dependent manner, producing the enzymatically active 26S proteasome with a molecular mass of ~2.5 Mda. The 26S proteasome is responsible for ATP-dependent degradation of a wide variety of cellular proteins tagged with a polyubiquitin chain that serves as a degradation signal and certain proteins in a manner independent on ubiquitylation.[9,10] PA700 is a 700-kDa protein complex composed of ~20 subunits with sizes of 25-110 kDa, which are organized into two distinguishable subcomplexes; the base and the lid.[10] The base is made up of six AAA-type ATPases ($Rpt_{1\text{-}6}$) and two large regulatory components; Rpn1 and Rpn2, while the lid contains multiple non-ATPase ($Rpn_{1\text{-}n}$) subunits. Most functions of multiple lid proteins are largely unknown, except for Rpn10, which functions as an acceptor for polyubiquitylated proteins, and Rpn11, a deubiquitylating enzyme involved in reutilization of ubiquitin. Furthermore, Rpn1, 2, and/or 10 also are responsible for interactions with various proteins with an ubiquitin-like domain.

Since the channel of the dilated 20S proteasome is cramped, only almost completely denatured proteins are permitted to access the catalytic sites within the chamber of the β-ring. Denaturation of the substrate primarily requires utilization of the energy liberated by ATP consumption. The base-complex, thought to bind ATP-dependently to the outer α-ring of the central 20S proteasome, seems to be involved in opening the gate of the α-ring for entry of the protein substrate. Finally, ATP energy is used for intra-molecular traffic of substrates so that they can penetrate the channel of the α- and β-rings of the 20S proteasome.[15]

It is worth emphasizing that the base complex has a putative unfoldase activity ensuring that the folded substrates are denatured before accessing the catalytic site. The base complex contains a site(s) that recognizes and interacts with unfolded proteins, represses their aggregation, and reactivates some of them, indicating that PA700 functions as a molecular chaperone, independent of the 20S proteasome. Indeed, the PA700 is demonstrated to retain the folding activity of partially denatured model proteins. However, it is unclear whether ATP hydrolysis is required for this process, because antagonizing results have been reported; one requiring ATP energy[16] and the other not.[17] In addition to facilitating proteasome-mediated proteolysis, PA700 itself shows several nonproteolytic roles, such as nucleotide excision repair,[18] stimulation of transcription elongation by RNA polymerase II,[19] and dissociation of the Cdc2-cycline B complex.[20] Though the precise natures of the nonproteolytic processes are not yet clear, one reasonable hypothesis is that chaperone activity of this complex is responsible for these phenomena.

PA28. PA28 or the 11S regulator (REG) is another activator of the latent 20S proteasome, which enhanced only the peptidase activity in an ATP-independent manner.[21,22] However, it fails to enhance the hydrolysis of large protein substrates with native or denatured structures, even when they are polyubiquitylated, indicating that PA28 does not play a central role in the initial cleavage of protein substrates. Unlike PA700, association of PA28 with the 20S proteasome does not require energy.

PA28 is composed of two subunits, named PA28α and PA28β, that share ~50% amino acid identity. These subunits assemble into a heteroheptameric ring with nearly equal stoichiometric amounts of PA28α and PA28β, which are mainly located in the cytoplasm of the cell.[23] PA28α and PA28β are up-regulated by γ-interferon and other available evidence indicates that the heteropolymer of PA28 (α and β) is involved in the processing of intracellular antigens, generating MHC class I ligands.[24] Indeed, simultaneous disruption of both genes encoding PA28α/β suggests that PA28α/β is not a prerequisite for antigen presentation in general, but plays an essential role in the processing of certain antigens.[25] Interestingly, other studies reported the existence of the 'hybrid proteasome' that contains both PA28 and PA700,[26] in which PA28 and PA700 associate either end of the 20S proteasome.[27,28] γ-interferon induces the production of the hybrid proteasome in cells, indicating that it might play an important role in the efficient production of MHC class I ligands.[26]

Subsequent studies identified the third member of this family, named PA28γ, which forms a homopolymer predominantly present in the nucleus.[29] Analyses of mice deficient in the PA28γ gene revealed that PA28γ functions as a regulator of cell proliferation and body growth in mice.[30]

Of particular interest is that PA28 is considered as an effective component responsible for refolding of the thermally denatured firefly luciferase mediated by the Hsp90 and Hap70/40 chaperone team in reticulocyte lysate.[31] In fact, purified Hsc70, Hsp40 and PA28 were necessary and sufficient to fully reconstitute Hsp90-initiated refolding. PA28 may be the physical link between Hsp90-dependent capture of unfolded proteins and Hsc70- and ATP-dependent refolding process. Moreover, PA28 binding unfolded luciferase could combine with the 20S proteasome, suggesting that PA28 may serve as a coupling factor between protein folding and degradation. In other words, PA28 serves as a molecular carrier that transfers the heat-denatured protein from the Hsp90 captured state to the Hsp70/Hsp40-dependent refolding process, suggesting that PA28 is a multi-functional complex in the protein quality control system. Although no homolog of PA28 has been detected in lower eukaryotes such as fungi, other molecules, such as Cdc48 and Hsp104, might substitute PA28 in these organisms, though the counterpart of Hsp104 has not yet been identified in higher eukaryotes.

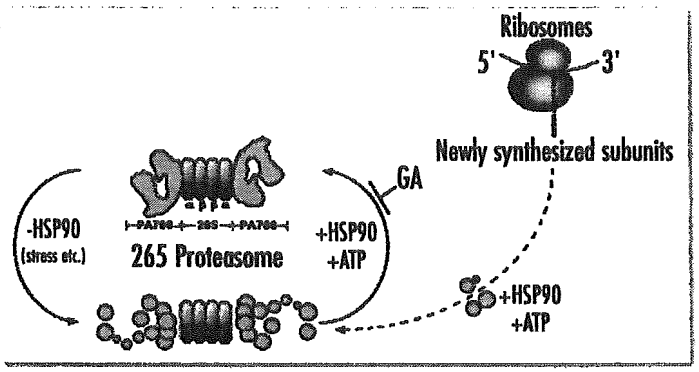

Figure 1. Model for the role of Hsp90 in the assembly and maintenance of the 26S proteasome. Dysfunction of Hsp90 causes rapid dissociation of regulatory PA700 complex of the 26S proteasome, without affecting the catalytic integrity of 20S proteasome. Dysfunction of Hsp90; e.g., loss of Hsp90, in the cell may occur under environmental stress, because Hsp90 is immensely required for refolding of unfolded cellular proteins. Hsp90 accelerates the 26S proteasome assembly in a manner requiring ATP energy, which can be prevented by geldanamycin (GA), an Hsp90 inhibitor. Hsp90 may be involved in the biogenesis of the 26S proteasome, although no evidence has been presented to date. See text for details.

MOLECULAR CHAPERONES

The molecular chaperones are a set of conserved protein families, divided into several functionally distinct classes of proteins that collaborate with a set of cochaperones. Among them, Hsp70 and Hsp90 play general roles in protein quality control in eukaryotes. It is also worth noting that the molecular chaperones that interact with UPS, are rather limited to Hsp70 and Hsp90. This section will discuss the roles of Hsp70 and Hsp90 with a special reference to the proteasome.

Hsp70

20S Assembly. A member of the Hsp70 family protein, Hsc73 is essential for the final maturation steps of the 20S proteasome from the 16S precursor complex.[32] Removal of Hsc73 results in an immediate formation of aggregates of this precursor indicating that Hsc73 keeps the assembly of intermediate complex in a soluble and probably partially unfolded state to allow subunit processing and correct folding. Considering these notions, Hsc73 might regulate the amount of 20S complex under stressful conditions.

BAG-1 (BCL-2 Binding Athanogene-1). BAG-1, one of the cochaperones of Hsp70, belongs to a family of proteins with an integral ubiquitin-like (UBL) domain capable of binding to Rpn10 or -Rpn1/2 of the 26S proteasome and acts as a coupling factor between Hsp70 and the 26S proteasome.[33] In addition, BAG-1 can stimulate the release of proteins captured by Hsp70 based on its nucleotide exchange activity through BAG domain.[34] It is possible that these chaperone-recruiting and chaperone-regulating activities can stimulate the transfer of Hsp70-captured substrates to the 26S proteasome and accelerate their degradation.

CHIP (Carboxyl-Terminus of Hsc70 Interacting Protein). CHIP, originally identified as a cochaperone of Hsp70,[35] can associate with Hsp70 and Hsp90 through the amino-terminal TPR domain and adjacent charged domain.[36,37] Since CHIP attenuates stimulation of ATPase activity of Hsc70 by Hsp40, addition of CHIP diminishes the refolding activity of the Hsc70-Hsp40 complex for denatured substrates.[35] CHIP also uniquely carries a U-box domain on its carboxyl-terminal region among many TPR-containing proteins. The U-box domain has a tertiary structure that resembles the RING-finger domain of ubiquitin-protein ligase (E3), which covalently attaches ubiquitin to target proteins, designating them for destruction by the proteasome.[38] As anticipated from its tertiary structure, CHIP executes E3 ubiquitin ligase activity upon specific substrates; CHIP ubiquitylates substrates of Hsp70 and Hsp90 and stimulates their degradation by the proteasome.[35,36,39] Consequently, CHIP appears to be chaperone-dependent E3 that ubiquitylates Hsp90-captured unfolded proteins.[40] Thus, CHIP is an ideal molecule acting as a protein quality-control E3 that selectively leads abnormal proteins recognized by molecular chaperones to degradation by the proteasome. It should be emphasized that CHIP is the first molecule that realizes the expected situation where an unfolded protein is handled by the UPS pathway as well as molecular chaperones.

Since both BAG-1 and CHIP are molecules that integrate molecular chaperones and UPS, cooperation of these two cofactors appears to reflect the fate of chaperone-captured proteins. CHIP converts Hsp70/Hsp90 chaperones into substrate recognition factors of a functional ubiquitin ligase complex, whereas BAG-1 supports binding of the Hsp70 complex to the proteasome and triggers the release of ubiquitylated substrates from Hsc/Hsp70 for their transfer to the proteasome. Since BAG-1 and CHIP bind to different domains of Hsp70, these two cochaperones are able to associate simultaneously with Hsp70. CHIP also regulates the association of BAG-1 with proteasome by K 11-linked polyubiquitylation of BAG-1.[41] The formation of the ternary chaperone-cofactor complex might accelerate the degradation of chaperone-captured unfolded proteins by the UPS pathway.[42-45]

Hsp90

The molecular chaperone Hsp90 is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of total cellular proteins even in conditions of nonstress. Hsp90 is an evolutionarily conserved protein and contributes to a wide variety of fundamentally common and species-specific processes in cells.[46] For example, it is essential for maintenance of functional integrity of various fragile proteins, such as steroid hormone receptors and many of protein kinases.[47] It is also notable that Hsp90 functions as a protein-folding machinery collaborating with other chaperone molecules, such as Hsp70 and Hsp40, and cochaperones containing p23 and Hop.[48,49] Indeed, Hsp90 can bind nonnative proteins through N- and C-terminal domains for refolding.[50]

In addition, Hsp90 appears to be closely linked to the protein degradation in the cell. Hsp90 also shows direct interaction with the proteasome and might possess regulatory roles, other than determination of the fate of unfolded proteins that cooperate with cochaperones, PA28 and CHIP.[31,40] Initially, Hsp90 was considered to inhibit the 20S proteasome[51,52] and also to protect it from oxidative stress.[53] Proteomics analysis of proteasome-interacting proteins revealed physical interactions between Hsp70 and Hsp90 with the 26S proteasome.[54] Evidence for a functional interplay between Hsp90 and PA28 also indicates that Hsp90 appears to compensate the loss of PA28 function in MHC class I antigen processing, suggesting that Hsp90 and PA28 operate either redundantly or specifically for generation of MHC class I ligands.[55] However, the biological relevance of these interactions is not clear at this stage.

We have recently shown that Hsp90 participates in the ATP-dependent assembly of the 26S proteasome, as depicted in the model shown in Figure 1.[56] These findings may provide new mechanistic insight into the cooperative interactions between the molecular chaperone and proteolysis systems. In the same study, we