Animal Cells and Systems
Vol. 14, No. 1, March 2010, 1–10

Taylor & Francis
Taylor & Francis Group

CHIP promotes the degradation of mutant SOD1 by reducing its interaction with VCP and S6/S6′ subunits of 26S proteasome

Jin-Sun Choi[a] and Do Hee Lee[b]*

[a]*College of Pharmacy, Chungnam National University, Daejeon, Korea;* [b]*Department of Biotechnology, College of Natural Science, Seoul Women's University, Seoul, Korea*

(*Received 26 October 2009; final version received 1 February 2010*)

Previously we showed that CHIP, a co-chaperone of Hsp70 and E3 ubiquitin ligase, can promote the degradation of mutant SOD1 linked to familial amyotrophic lateral sclerosis (fALS) via a mechanism not involving SOD1 ubiquitylation. Here we present evidence that CHIP functions in the interaction of mutant SOD1 with 26S proteasomes. Bag-1, a coupling factor between molecular chaperones and the proteasomes, formed a complex with SOD1 in an hsp70-dependent manner but had no direct effect on the degradation of mutant SOD1. Instead, Bag-1 stimulated interaction between CHIP and the proteasome-associated protein VCP (p97), which do not associate normally. Over-expressed CHIP interfered with the association between mutant SOD1 and VCP. Conversely, the binding of CHIP to mutant SOD1 was inhibited by VCP, implying that the chaperone complex and proteolytic machinery are competing for the common substrates. Finally we observed that mutant SOD1 strongly associated with the 19S complex of proteasomes and CHIP over-expression specifically reduced the interaction between S6/S6′ ATPase subunits and mutant SOD1. These results suggest that CHIP, together with ubiquitin-binding proteins such as Bag-1 and VCP, promotes the degradation of mutant SOD1 by facilitating its translocation from ATPase subunits of 19S complex to the 20S core particle.

Keywords: CHIP; VCP; Bag-1; 26S proteasome; ATPase subunits; SOD1

Introduction

Misfolding of mutant proteins is a likely cause of a number of neurodegenerative diseases e.g., Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). A hallmark of such disparate neurologic disorders is the deposit of protein aggregates in the brains of affected patients (Stefani and Dobson 2003). However, how the protein aggregation functions in disease pathogenesis is controversial. Although it was generally assumed that large protein aggregates are responsible for neuronal cell death, recent studies have challenged this notion and proposed that the formation of inclusion body or the deposit of aggregates is the cellular mechanism to sequester aberrant and often toxic proteins. Oligomeric structures of misfolded proteins, not the more compacted large aggregates, are now suggested as the real culprits in neurodegeneration (Soto and Estrada 2008). Cells prevent the accumulation of misfolded proteins inside the cells by employing a vast array of chaperones as well as the ubiquitin-proteasome system (UPS), which promote either refolding or rapid elimination of these potentially harmful proteins (Goldberg 2003). Less is known about how these processes are regulated and which factors recognizing misfolded proteins are involved.

CHIP (*c*arboxyl terminus of *H*sc70 *i*nteracting *p*rotein) is a highly conserved protein with dual functions. CHIP functions as a co-chaperone of Hsp70, interacting with the chaperones through its TPR (tetratricopeptide repeat) domain, and also possesses an E3 ligase activity which is mediated by the U-box domain (McDonough and Patterson 2003). Thus CHIP can provide a link between the chaperones and UPS and probably regulates the balance between protein refolding and degradation in the cells. Reportedly CHIP mediates the degradation of numbers of disease proteins linked to neurodegeneration – e.g., α-synuclein (Shin et al. 2005) and polyglutamine (polyQ) proteins (Jana et al. 2005; Al-Ramahi et al. 2006; Choi et al. 2007). Notably CHIP promotes the degradation of mutant SOD1 without increasing its ubiquitylation (Choi et al. 2004; Urushitani et al. 2004).

Findings that CHIP interacts with the S5a subunit of proteasomes and co-localizes with the proteasomes in cells have led to a suggestion that CHIP also participates in substrate delivery to the proteasomes (McDonough and Patterson 2003). Bag-1, another co-chaperone of Hsp70, has also been implicated in the sorting of substrates to proteasomes (Demand et al. 2001; Alberti et al. 2002). CHIP and Bag-1 can directly interact and possibly function as partners in substrate

*Corresponding author. Email: do_lee@swu.ac.kr

ISSN 1976-8354 print/ISSN 2151-2485 online
© 2010 Korean Society for Integrative Biology
DOI: 10.1080/19768351003765145
http://www.informaworld.com

delivery to proteasomes (Demand et al. 2001). VCP (valosin-containing protein; p97) is a member of the AAA class of proteins and is identified as a proteasome-associated protein (Doss-Pepe et al. 2003). VCP is co-localized with the protein aggregates in neuronal cells expressing polyQ proteins or mutant SOD1 (Kakizuka 2008). Interestingly, VCP is suggested to play a dual role in aggregate formation and clearance, perhaps depending on the nature of interacting proteins (Kobayashi et al. 2007).

To determine whether CHIP functions together with such ubiquitin-binding proteins (e.g., Bag-1 and VCP) for the degradation of mutant SOD1, we investigated their physical interaction as well as their effects on SOD1 degradation *in vivo*. Interestingly CHIP interfered with the interaction between mutant SOD1 and VCP and, conversely, VCP decreased the association between CHIP and SOD1, suggesting that they competitively interact with the common substrates. Over-expressed Bag-1, which formed a complex with mutant SOD1, stimulated the interaction between VCP and CHIP. Finally, CHIP specifically reduced the association between mutant SOD1 and S6/S6′ ATPase subunits of 26S proteasomes, which may account for this co-chaperone's ability to promote degradation of the aggregation-prone proteins such as mutant SOD1.

Materials and methods

Plasmid construction

Generation of FLAG-tagged SOD1 – WT, A4V and G93A – and Xpress-tagged CHIP mammalian expression constructs – WT (amino acids 1–303), ΔTPR (amino acids 128–303) and ΔU-box mutants (amino acids 1–215) – were described in a previous report (Choi et al. 2004). To generate HA-tagged Bag-1 and HHR23A constructs, cDNA clones for Bag-1 and HHR23A obtained from 21st Century Frontier Human Gene Banks (http://genbank.kribb.re.kr/) were inserted into pcDNA3.1 vector containing the N-terminal HA tag. The coding sequence for VCP was cloned from human liver cDNA library by the PCR method and then introduced into the mammalian expression vector containing the N-terminal GST tag. All expression constructs were verified by sequencing from both directions.

Antibodies

The following antibodies were used in this study. Mouse anti-FLAG antibody and anti-FLAG M2 affinity gel were purchased from Sigma. Rabbit anti-HA antibody (Santa Cruz Biotechnology), mouse anti-Xpress antibody (Invitrogen), mouse anti-GST (Sigma) and sheep anti-human Cu/Zn-superoxide dismutase (SOD1) (Calbiochem) were also used. Antibodies against 19S proteasomal subunits were from Boston Biochem and Calbiochem.

Cell culture

BOSC 23 cells, a derivative of HEK293T cells, were cultured in DMEM (Gibco) supplemented with 2 mM L-glutamine, 0.24% HEPES (Sigma), 0.375% sodium bicarbonate, 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 10% fetal bovine serum (Hyclone) and maintained in 5% CO_2 at 37°C.

Transfection of the cultured human cells

For transient transfection, BOSC cells (1.4×10^6 per 60 mm plate) were plated, grown overnight, and transfected with the indicated expression constructs using Lipofectamine reagent (Invitrogen). The transfection mixture was prepared in the following steps. Unless otherwise specified, 1 μg of plasmid DNA was added into 200 μl of DMEM (without antibiotics or FBS) and mixed well. Lipofectamine reagent (8 μl) was added to the DNA/DMEM mixture and then incubated at room temperature for 15 min. The mixed solution was added to the cells and then incubated at 37°C. After 4 hours of transfection, the cells were washed twice with serum-free DMEM, added with fresh 10% FBS-DMEM and then further incubated for up to 48 h at 37°C.

Immunoprecipitation, GST pull-down assay and immunoblotting

After 48 h of transfection, cells were washed twice with PBS and lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1% glycerol and 1% Nonidet P-40) supplemented with protease inhibitor mixture (Complete-MINI™; Roche) at 4°C. The resulting lysate was centrifuged at $13{,}000 \times g$ for 20 min and then the supernatant was subjected to immunoprecipitation or GST pull-down assay. For immunoprecipitation of FLAG-tagged proteins, the lysate was mixed with 30 μl of anti-FLAG M2 affinity gel for 5 h at 4°C. For GST pull-down assay, the lysate was incubated with 25 μl of glutathione-Sepharose 4B bead (Amersham) for 4–6 h at 4°C. After protein binding and washing, the beads were boiled in an equal volume of 1 × SDS sampling buffer and the eluted proteins were applied to 8–10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membrane, probed with primary and secondary antibodies, and then visualized by using colorimetric reagents (Promega) or the ECL detection system (PIERCE).

Two-dimensional gel electrophoresis (2-DE)

SOD1 (G93A) proteins expressed in BOSC cells were immunoprecipitated as described above. Proteins co-immunoprecipitated with mutant SOD1 were eluted from the anti-FLAG beads by incubating with 30 μl of equilibration buffer (8 M urea, 2% CHAPS, 180 mM DTT, 2% IPG buffer) and then applied onto 2-D gels (the first dimension: 18 cm, IPG pH 3–10NL; the second dimension: 10–12.5% SDS-PAGE). After electrophoresis, the resolved proteins were transferred to PVDF membrane and subsequently subjected to immunoblot analysis as described above.

Results

CHIP mediates poly-ubiquitylation of Hsp70-associated with mutant SOD1

We have previously demonstrated that CHIP promoted the degradation of SOD1 without apparently increasing SOD1 ubiquitylation (Choi et al. 2004). Similarly, Urushitani et al. (2004) revealed that CHIP did not promote the SOD1 ubiquitylation but instead mediated the poly-ubiquitylation of SOD1-interacting proteins, including Hsc70/Hsp70 and CHIP itself. However, which SOD1-interacting proteins were the predominating species was not systematically investigated, and their role(s) in the degradation of SOD1 has not been fully investigated. To better resolve and identify the mutant SOD1-associated proteins that were ubiquitylated by CHIP, we employed 2-DE and Western-blot analysis. As shown in Figure 1 (upper panel), 2-DE/Western-blot analysis with anti-HA antibody (to detect poly-ubiquitin conjugates) demonstrated that CHIP over-expression increased the ubiquitin conjugation (indicated by boxes) to a certain protein with apparent molecular weight of 75 kDa, which was most likely Hsp70 – the major mutant SOD1-interacting protein (Choi et al. 2004). The result was confirmed by 2-DE/Western-blot analysis with anti-Hsp70 antibody (Figure 1, lower panel). Apart from Hsp70, however, we did not reproducibly observe other poly-ubiquitylated proteins in the immunoprecipitates. Our results confirmed again the previous findings that Hsp70 is the major SOD1-interacting protein ubiquitylated by CHIP.

Mutant SOD1 interacts with Bag-1

Although we and other groups have demonstrated that Hsp70 was predominantly ubiquitylated by CHIP, it was still unclear how the poly-ubiquitylated Hsp70 acts to promote the proteasomal degradation of SOD1. Bag-1 is the nucleotide-exchange factor of Hsp70 and is believed to function in the unloading of client proteins from the chaperone. In addition to interaction with chaperone molecules, Bag-1 also associates with the proteasome via its ubiquitin-like (UBL) domain in an ATP-dependent manner and possibly functions as a coupling factor between the chaperones and the proteasomes (Alberti et al. 2003). To understand whether Bag-1 plays a role in the CHIP-mediated degradation of mutant SOD1, we examined the interaction between mutant SOD1 and Bag-1 and tested the effect of Bag-1 over-expression on the levels of mutant SOD1 proteins. As expected, Bag-1 strongly associated with mutant SOD1 (G93A) in an Hsp70-dependent manner (Figure 2A). However, Bag-1 alone did not change the cellular level of G93A SOD1, whereas CHIP effectively down-regulated mutant G93A SOD1 (Figure 2B). Contrary to the previous report (Demand et al. 2001), even co-expression of Bag-1 with CHIP failed to enhance the CHIP-mediated SOD1 degradation (Figure 2B). These results suggest that this ubiquitin-domain protein primarily participates in the recognition of mutant SOD1 but not in the degradation.

Bag-1 stimulates the interaction between CHIP and VCP

VCP (valosin-containing protein; also known as p97 and cdc48p) is a member of the AAA ATPase superfamily and is involved in a variety of cellular activities including degradation of misfolded proteins by UPS. VCP recruits the substrates not only by directly recognizing misfolded domains but also by indirectly interacting with poly-ubiquitylated forms (Halawani and Latterich 2006). Interestingly, VCP interacts with proteasomes, perhaps with the aid of other proteins containing ubiquitin-like domains (e.g., HHR23 proteins, human orthologs of yeast Rad23 proteins), and participates in the degradation of poly-ubiquitylated substrates (Doss-Pepe et al. 2003). These attributed functions led to speculation that VCP possibly plays a role in CHIP-mediated SOD1 degradation. To prove this possibility, we examined the interaction between VCP and mutant SOD1 protein. Like Bag-1, VCP also strongly interacted with mutant SOD1 (A4V and G93A) in the cells (Figure 3A). Interestingly, an ubiquitin-like domain protein HHR23A (a human homolog of yeast Rad23), which enables the interaction between VCP and 26S proteasomes (Besche et al. 2009), also presented in the SOD1 immunoprecipitates (Figure 3A). In contrast to CHIP, which effectively down-regulated G93A SOD1 (Figure 2B), VCP did not cause any change in the level of mutant SOD1 (Figure 3B). The above results implied that VCP, like Bag-1, is not directly involved in the degradation of mutant SOD1 but instead in the recognition of misfolded proteins. We then hypothesized that Bag-1 may provide a physical link between the chaperone complex recognizing

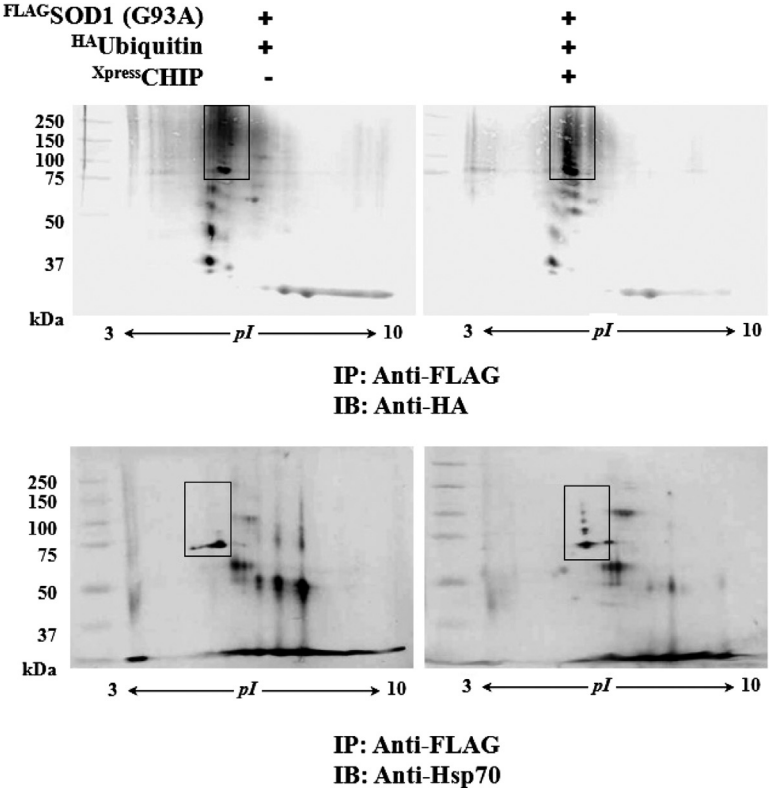

Figure 1. Identification of SOD1-interacting proteins that are ubiquitylated by CHIP. After co-transfection of BOSC23 cells with FLAG-tagged SOD1 and HA-tagged ubiquitin, SOD1 proteins were isolated by anti-FLAG affinity gels and the co-immunoprecipitated proteins were resolved by 2-DE. The proteins were then transferred to PVDF membrane and probed with anti-HA antibody (upper panel; to detect poly-ubiquitylated proteins) or anti-Hsp70 antibody (lower panel).

misfolded proteins (including CHIP/Hsp70) and the proteasome-associated complex (including VCP) transferring the poly-ubiquitylated substrates into the proteasomes. While CHIP could interact with Bag-1 (data not shown; Demand et al. 2001) and Bag-1 associated with VCP (Figure 3C), CHIP did not directly bind to VCP under normal conditions (Figure 3D; Hatakeyama et al. 2004). Over-expression of Bag-1, by contrast, stimulated the interaction between CHIP and VCP and enhanced the formation of an *in vivo* ternary complex of CHIP/Bag-1/VCP (Figure 3D).

CHIP and VCP bind to mutant SOD1 competitively

The above results prompted us to propose a model that CHIP/Hsp70 is the critical factor that recognizes and determines the rate of degradation of misfolded SOD1 proteins by influencing their subsequent transfer to the proteasomes with the aid of other proteins, such as Bag-1 and VCP. Based on this model, we speculated that the chaperone complex (including CHIP) and the proteasome-associated components (including VCP) may compete for interaction with the common substrates, i.e., mutant SOD1 proteins. To explore this possibility, we first tested the effect of CHIP over-expression on the interaction between VCP and mutant SOD1. As shown in Figure 4A, CHIP over-expression strongly interfered with the interaction between VCP and mutant SOD1 (G93A). Both the TPR (tetratricopeptide repeats) domain for chaperone binding and the U-box domain for E3 ligase acitivity were needed for CHIP's inhibitory effects on VCP-SOD1 association (Figure 4B). Conversely, VCP over-expression also exerted a similar effect on the complex formation of CHIP and mutant SOD1 although a greater amount of VCP was needed to inhibit the CHIP–SOD1 interaction (Figure 4C). These results supported our notion that the chaperone complex and the proteasome-associated factors compete for a common substrate and that over-expressed CHIP shifts the partitioning of mutant SOD1 into the chaperone complex and reduces the interaction between mutant SOD1 and VCP.

CHIP reduces the interaction of mutant SOD1 with S6/S6′ ATPase subunits of 26S proteasomes

Misfolded proteins linked to neurodegenerative disorders often form aggregates – whether the large protein

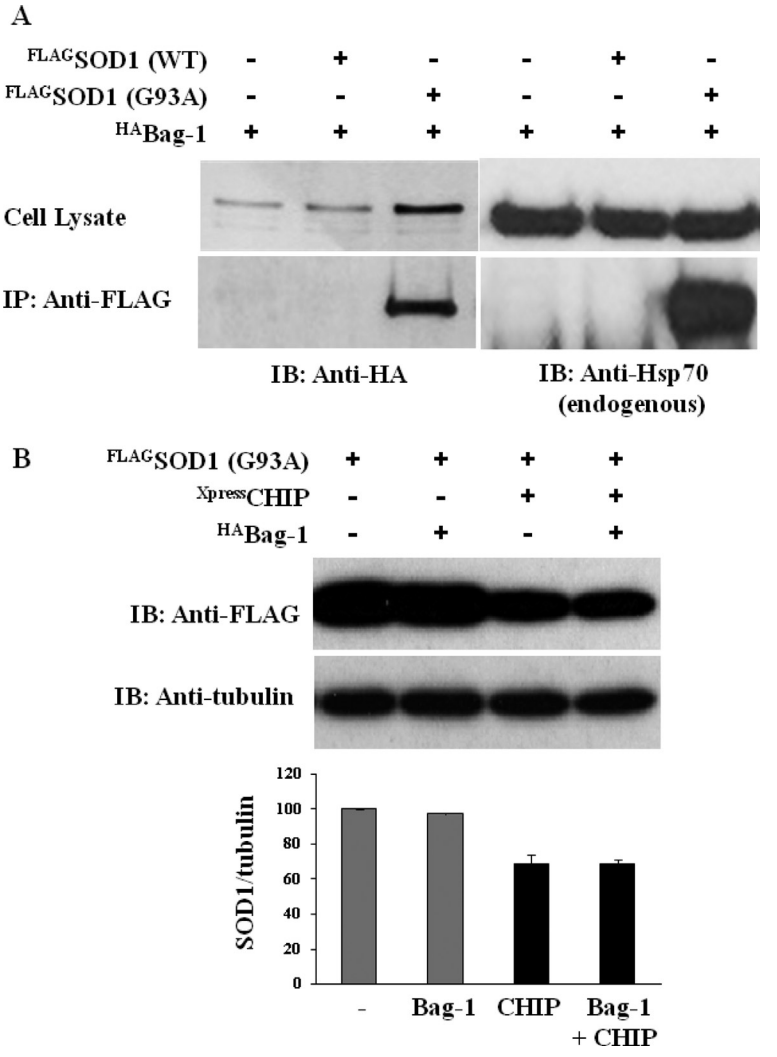

Figure 2. Mutant SOD1 associates with Bag-1 in an Hsp70-dependent manner. (A) After isolation of FLAG-tagged SOD1, the presence of Bag-1 and Hsp70 in the immunoprecipitates was determined by immunoblotting with anti-HA antibody and anti-Hsp70 antibody, respectively. (B) Bag-1 over-expression had no effect on the cellular level of SOD1 (G93A). The effects of over-expressed Bag-1 and CHIP (separately or together) on the level of SOD1 (G93A) were examined by immunoblot analysis with anti-FLAG antibody. Tubulin was used as a loading control. Similar results were obtained from three independent experiments.

aggregates are the actual species causing toxicity is still a matter of debate – inside the neuronal cells and cause proteasomal dysfunction, presumably by sequestering the proteasomal components and other proteins including molecular chaperones. For example, α-synuclein and synphilin linked to Parkinson's disease (PD) have been shown to associate tightly with S6′ and S6 19S proteasomal ATPase subunits, respectively (Snyder et al. 2003; Marx et al. 2007). Such strong interaction of proteasomal subunits with misfolded proteins may cause the inhibition of proteolytic activities of proteasomes. To study whether mutant SOD1 also binds to 26S proteasomes tightly, we examined the association between mutant SOD1 and individual subunits of 19S complex. We were particularly interested in the interaction between the misfolded proteins and ATPase subunits located in the base subcomplex of the 19S regulatory particle, which play important roles in recognition, unfolding and transferring of substrates to the catalytic 20S complex (Elsasser and Finley 2005) (Figure 5D). As shown in Figure 5A, mutant SOD1 (G93A) strongly associated with all eight 19S subunits we tested (similar results were obtained with A4V mutant: data not shown). Interestingly, CHIP over-expression selectively inhibited the interaction of G93A SOD1 with S6 and S6′ subunits, while it did not affect the binding of SOD1 to other 19S subunits (Figure 5A). S6 and S6′ subunits, like VCP, are members of the AAA ATPase superfamily and are essential for the binding of poly-ubiquitylated

substrates to proteasomes (Lam et al. 2002). Our results showing that CHIP over-expression specifically inhibits the interaction between mutant SOD1 and ATPase proteins involved in the recognition of poly-ubiquitylated proteins (i.e., VCP, S6 and S6′ subunits) suggest that CHIP prevents the aggregation of misfolded proteins on ATPase subunits. Such effects of CHIP on proteasome–substrate interaction required both TPR and U-box domains, which was similar to the result shown in Figure 4B.

Next, we tested whether VCP over-expression has a similar effect on the interaction between 26S proteasomes and misfolded proteins. VCP over-expression did not inhibit the protein interaction, and the association of G93A SOD1 with S6/S6′ subunits of 19S regulatory particle was not affected (Figure 5C). These results support our notion that CHIP/Hsp70 are the rate-determining factors in the proteasomal degradation of mutant SOD1 and suggest that CHIP, together with AAA ATPases, e.g., VCP and S6/S6′ subunits, is involved in the translocation of substrates into the 20S core particle.

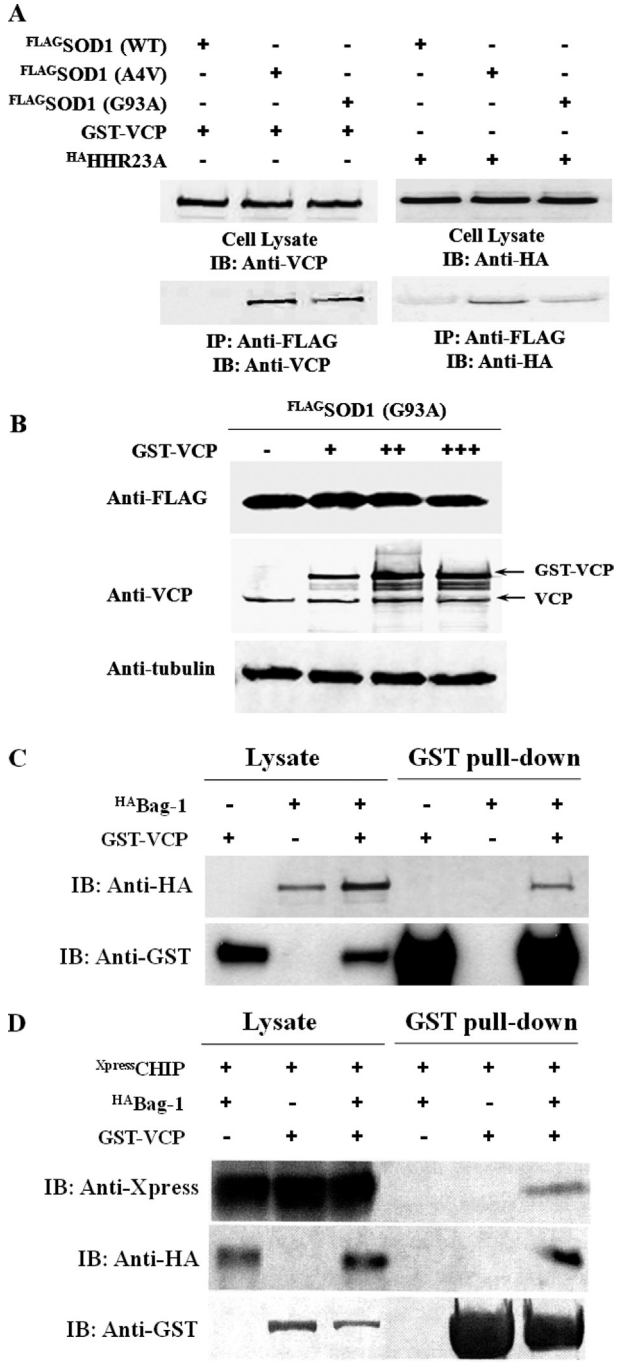

Figure 3. (*Continued*)

Discussion

Through its ability to interact with both the chaperone machinery and UPS, CHIP plays a pivotal role in protein triage decisions especially for the misfolded proteins implicated in neurodegenerative diseases. The exact roles of CHIP in the degradation of individual proteins, however, seem to vary depending on the nature of the substrates. Upon interaction with E2 proteins, such as UbcH5 family members, CHIP catalyzes ubiquitin conjugation to the misfolded proteins presented by the hsp70/hsp90 chaperone machinery and thereby promotes their proteasomal degradation. However, this co-chaperone is also involved in the unloading of the substrates from chaperone complex (Imai et al. 2002). CHIP over-expression, while promoting poly-ubiquitylation, also leads to the accumulation of polyglutamine protein ataxin-1 in the insoluble fraction in cells (Choi et al. 2007). The role(s)

Figure 3. Interaction between mutant SOD1, VCP, HHR23A and CHIP. (A) Mutant SOD1 (G93A) bound to VCP and HHR23A. After affinity isolation of SOD1, the presence of VCP and HHR23A in the immunoprecipitates was determined by immunoblotting with anti-VCP antibody and anti-HA antibody, respectively. (B) VCP over-expression did not affect the cellular level of SOD1 (G93A). The effects of over-expressed VCP (0, 1, 2, and 3 μg of DNA) on the level of SOD1 (G93A) were assessed by immunoblot analysis with anti-FLAG antibody. Similar results were obtained from three independent experiments. (C) Bag-1 formed a complex with VCP in the cells. GST-tagged VCP was isolated from cell lysate by GST pull-down and the presence of Bag-1 in the complex was determined by immunoblotting. (D) Over-expressed Bag-1 induced the interaction between CHIP and VCP, which did not associate in the absence of Bag-1. After co-transfection of BOSC cells with Xpress-tagged CHIP, GST-VCP or HA-tagged Bag-1 (as indicated), GST pull-down experiments were carried out and the presence of Bag-1 and CHIP was examined by immunoblot analysis.

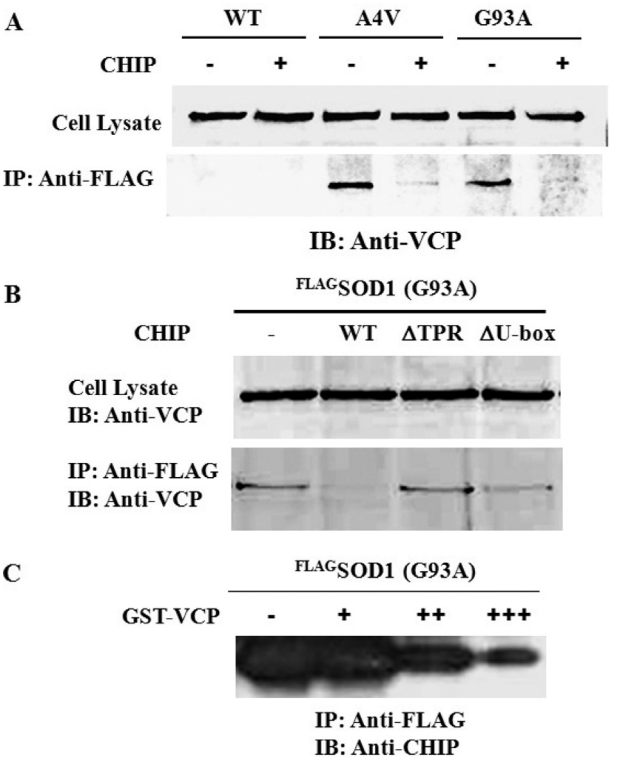

Figure 4. CHIP and VCP competitively bind to mutant SOD1. (A) Over-expressed CHIP interfered with the interaction between mutant SOD1 and VCP without affecting the cellular level of VCP. (B) Both the TPR domain (chaperone binding) and the U-box domain (E3 ligase activity) were necessary for the inhibitory effect of CHIP on the association of mutant SOD1 and VCP. (C) VCP over-expression (0, 1, 2, and 3 μg of DNA) also reduced the interaction between SOD1 and CHIP.

of CHIP in the proteasome-mediated degradation of mutant SOD1 is not yet clearly understood because it promotes the degradation of mutant SOD1 without increasing the ubiquitin conjugation; instead CHIP enhances ubiquitylation of the SOD1-associated proteins such as Hsp70 (Figure 1; Urushitani et al. 2004).

Previously CHIP has been proposed to function in substrate delivery to 26S proteasomes (McDonough and Patterson 2003). However, it is unlikely that CHIP is the sole factor in mediating the transfer of ubiquitylated substrates from the chaperone complex to the proteolytic machinery, instead, additional proteins must be present for efficient delivery. Interestingly, CHIP catalyzes ubiquitin conjugation to several other protein functions in the chaperone machinery – i.e., Hsp70/Hsc70 and Bag-1 – without influencing their half-lives (Jiang et al. 2001; Alberti et al. 2002). Co-chaperone Bag-1 interacts with proteasomes through its ubiquitin-like (UBL) domain in an ATP-dependent manner, which enables this protein to act as a 'shuttling

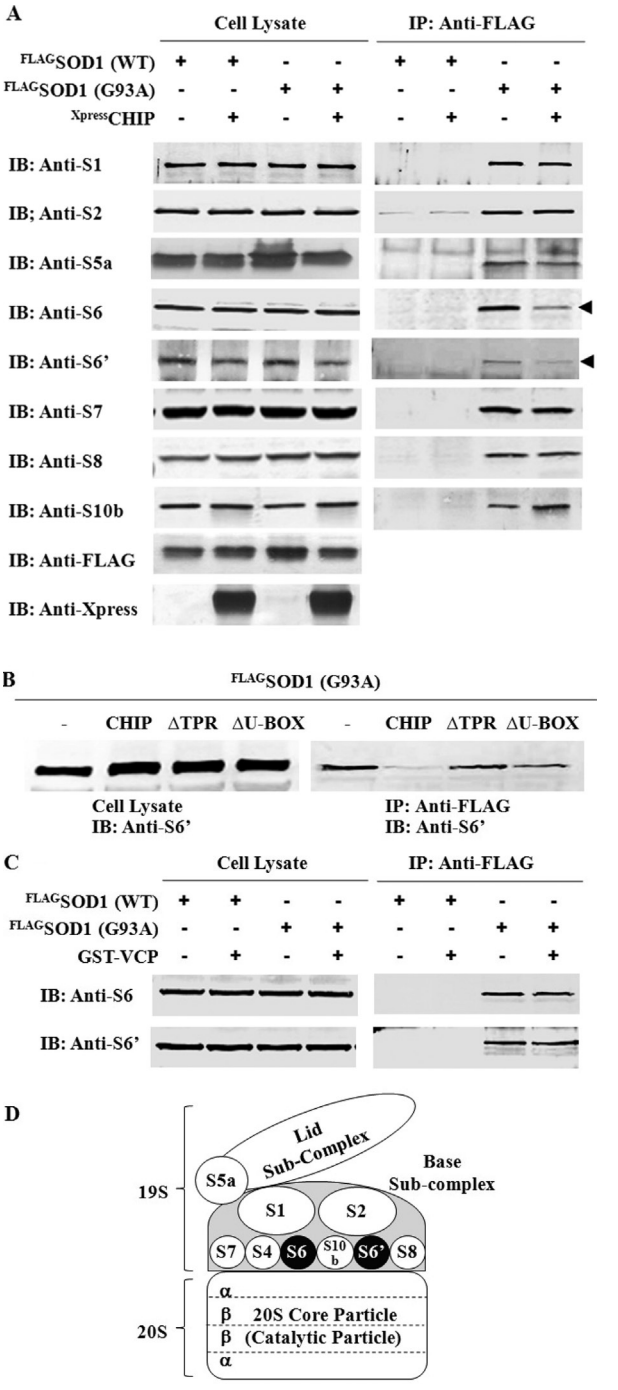

Figure 5. CHIP reduces the association between mutant SOD1 and ATPase subunits of 19S regulatory particle of proteasomes. (A) Mutant SOD1 (G93A) associated with 19S regulatory complex of proteasomes and CHIP selectively reduced the interaction between S6/S6′ subunits and SOD1 (G93A). (B) Both the TPR domain and the U-box domain were needed for the inhibitory effect of CHIP on the association between mutant SOD1 and the S6′ subunit of the 19S particle. (C) VCP over-expression did not affect the association of SOD1 and S6/S6′ subunits. (D) Organization of 19S subunits in 26S proteasomes. S6 and S6′ ATPase subunits (filled circles) are located in the base sub-complex along with S4, S7, S8 and S10b subunits.

factor' between the chaperone and the proteasome complex. Importantly, CHIP-mediated ubiquitylation of Bag-1 further stimulates its association with the proteasome (Alberti et al. 2002). By analogy, our finding that CHIP promotes ubiquitylation of mutant SOD1-associated Hsp70 could imply that such a modification facilitates the binding of CHIP/Hsp70/Bag-1 ternary complex (together with substrates) to the proteasome. It is noteworthy that Bag-1 stimulates the interaction between CHIP/Hsp70 chaperone complex and VCP (p97), which do not normally associate. Over-expressed Bag-1 may cause a stoichiometric transfer of the ubiquitylated substrates from the chaperone complex (CHIP/Hsp70) to the proteolytic complex (VCP/26S proteasome). Notably, the effects of Bag-1 on the half-lives of the client proteins differ greatly. For example, Bag-1 alone is sufficient to promote the degradation of polyglutamine protein ataxin-1 (Choi et al. 2007), while it inhibits the degradation of Hsp70-associated Tau protein (Elliott et al. 2007). When co-expressed with CHIP, Bag-1 further increases the CHIP-mediated degradation of GR (Demand et al. 2001). These results suggest that additional factors may function in CHIP/Bag-1-mediated protein triage decisions and can tilt the protein folding–refolding machinery toward the degradation machinery.

Although its interaction with mutant SOD1 and/or other co-chaperones has not been systematically studied, involvement of VCP in the proteasomal degradation of mutant SOD1 has already been suggested. For example, VCP directly binds to Dorfin, an E3 ligase catalyzing ubiquitylation of mutant SOD1, and thereby affects its activity in cultured cells. Moreover, VCP and Dorfin co-localize in the neuronal inclusions of ALS (Ishigaki et al. 2004). VCP may function as an 'uncoupling factor' that transfers ubiquitylated substrates from the shuttling factors to the proteasomes, which does not occur passively without the concerted actions of other regulatory factors involved in the recognition and delivery of substrates (Doss-Pepe et al. 2003). Our findings that CHIP and VCP bind competitively to mutant SOD1 and that both TPR and U-box domains are necessary for the CHIP's inhibitory effect on SOD1-VCP association could be explained by the assumption that binding of misfolded proteins to 'uncoupling factor' (i.e., VCP) may be an intermediate step in the process of substrate delivery. Interestingly a recent study demonstrated that VCP, which does not have UBL domains, can be isolated together with the 26S proteasomes in the presence of a functional UBL domain derived from HHR23B (human homolog of Rad23) proteins (Besche et al. 2009). Our finding that mutant SOD1 also bound to HHR23A protein together with VCP supports our model that VCP – together with UBL proteins – mediates the transfer of poly-ubiquitylated mutant SOD1 to the proteolytic machinery.

The finding that aggregated α-synuclein – a major component of Lewy body formed in Parkinson's disease (PD) – can inhibit 26S proteasomal activities by selectively interacting with the S6′ subunit of 19S complex (Snyder et al. 2003) suggests that the formation of a stable complex between misfolded proteins and the 19S regulatory particle can be a major cause of proteasomal dysfunction. Similarly synphilin-1, an α-synuclein interacting protein, associates and co-localizes with 19S ATPase subunits in cultured cells and in brains of PD patients (Marx et al. 2007). Polyglutamine proteins (e.g., ataxin-7) also associate with proteasomal components (Matilla et al. 2001). Our findings that mutant SOD1 can interact with several 19S subunits are consistent with such observations. Interestingly, α-synuclein interacts selectively with the S6′ subunit but not with S10b or S14 (Rpn12) subunits (Snyder et al. 2003). On the other hand, mutant SOD1 (Figure 5A) and polyglutamine protein ataxin-7 (Matilla et al. 2001) interact with multiple proteasomal subunits, indicating that these misfolded proteins probably recruit the entire 26S complex into the protein aggregates. Such observations can argue that a main function of CHIP in the degradation of misfolded proteins is to prevent accumulation of 26S proteasomes in the inclusions. Our results showing that CHIP specifically reduced the association of mutant SOD1 and S6/S6′ subunits but not the other 19S subunits (Figure 5A), however, raises the possibility that the actual role of CHIP is to facilitate the translocation of substrate from 19S regulatory particles to 20S catalytic core particles and to rapidly eliminate the aggregation-prone polypeptides. S6 (Rpt3/PSMC4) and S6′ (Rpt5/PSMC3) subunits are members of the AAA ATPase superfamily and exhibit chaperone-like activities including recognition of poly-ubiquitin signal and substrate unfolding (Lam et al. 2002; Marx et al. 2007). Interestingly, a recent study has revealed that COOH terminal residues of 19S ATPase subunits, particularly that of the S6′ (Rpt5) subunit, induce gate-opening of 20S core particle and stimulate peptide hydrolysis (Smith et al. 2007). Perhaps a prolonged association between these ATPase subunits and mutant SOD1 proteins impairs the ATPase-induced activation of the 20S catalytic core and causes proteasomal dysfunction.

Although it is still plausible that CHIP plays a direct role in ubiquitin conjugation to mutant SOD1, our study suggests that CHIP promotes the degradation of mutant SOD1 by facilitating the translocation of substrates from 19S regulatory particles (for recognition and unfolding) to 20S core particles (for degradation). Further investigation including *in vitro*

studies using purified proteins, the co-sedimentation analysis of mutant SOD1 and proteasomal subunits and knock-down experiments of ubiquitin-binding proteins involved in this process should provide more insights into the functions of CHIP, Bag-1 and VCP in the degradation of mutant SOD1.

Acknowledgements

We are grateful to Ms. Hye-Won Park and Ms. Hyo-Sun Kim for their assistance. This work was supported by a special research grant from Seoul Women's University (2008) to D.H.L.

References

Alberti S, Demand J, Esser C, Emmerich N, Schild H, Hohfeld J. 2002. Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. J Biol Chem. 277:45920–45927.

Alberti S, Esser C, Höhfeld J. 2003. BAG-1-a nucleotide exchange factor of Hsc70 with multiple cellular functions. Cell Stress Chap. 8:225–231.

Al-Ramahi I, Lam YC, Chen HK, de Gouyon B, Zhang M, Pérez AM, Branco J, de Haro M, Patterson C, Zoghbi HY, Botas J. 2006. CHIP protects from the neurotoxicity of expanded and wild-type ataxin-1 and promotes their ubiquitination and degradation. J Biol Chem. 281: 26714–26724.

Besche H, Haas W, Gygi S, Goldberg AL. 2009. Isolation of mammalian 26S proteasomes and p97/VCP complexes using the ubiquitin-like domain from HHR23B reveals novel proteasome-associated proteins. Biochemistry. 48:2538–2549.

Choi JS, Cho S, Park SG, Park BC, Lee DH. 2004. Co-chaperone CHIP associates with mutant Cu/Zn-superoxide dismutase proteins linked to familial amyotrophic lateral sclerosis and promotes their degradation by proteasomes. Biochem Biophys Res Commun. 321: 574–583.

Choi JY, Ryu JH, Kim HS, Park SG, Bae KH, Kang S, Myung PK, Cho S, Park BC, Lee DH. 2007. Co-chaperone CHIP promotes aggregation of ataxin-1. Mol Cell Neurosci. 34:69–79.

Demand J, Alberti S, Patterson C, Höhfeld J. 2001. Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. Curr Biol. 11:1569–1577.

Doss-Pepe EW, Stenroos ES, Johnson WG, Madura K. 2003. Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. Mol Cell Biol. 23:6469–6483.

Elliott E, Tsvetkov P, Ginzburg I. 2007. BAG-1 associates with Hsc70-Tau complex and regulates the proteasomal degradation of Tau protein. J Biol Chem. 282: 37276–37284.

Elsasser S, Finley D. 2005. Delivery of ubiquitinated substrates to protein-unfolding machines. Nat Cell Biol. 7:742–749.

Goldberg AL. 2003. Protein degradation and protection against misfolded or damaged proteins. Nature. 426:895–899.

Halawani D, Latterich M. 2006. p97: the cell's molecular purgatory? Mol Cell. 22:713–717.

Hatakeyama S, Matsumoto M., Yada M., Nakayama KI. 2004. Interaction of U-box-type ubiquitin-protein ligases (E3s) with moleudar chaperones. Genes Cells. 9:533–548.

Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI, Takahashi R. 2002. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell. 10:55–67.

Ishigaki S, Hishikawa N, Niwa J, Iemura S, Natsume T, Hori S, Kakizuka A, Tanaka K, Sobue G. 2004. Physical and functional interaction between Dorfin and Valosin-containing protein that are colocalized in ubiquitylated inclusions in neurodegenerative disorders. J Biol Chem. 279:51376–51385.

Jana NR, Dikshit P, Goswami A, Kotliarova S, Murata S, Tanaka K, Nukina N. 2005. Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. J Biol Chem. 280:11635–11640.

Jiang J, Ballinger CA, Wu Y, Dai Q, Cyr DM, Höhfeld J, 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.

Kakizuka A. 2008. Roles of VCP in human neurodegenerative disorders. Biochem Soc Trans. 36:105–108.

Kobayashi T, Manno A, Kakizuka A. 2007. Involvement of valosin-containing protein (VCP)/p97 in the formation and clearance of abnormal protein aggregates. Genes Cells. 12:889–901.

Lam YA, Lawson TG, Velayutham M, Zweier JL, Pickart CM. 2002. A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. Nature. 416:763–767.

Marx FP, Soehn AS, Berg D, Melle C, Schiesling C, Lang M, Kautzmann S, Strauss KM, Franck T, Engelender S, et al. 2007. The proteasomal subunit S6 ATPase is a novel synphilin-1 interacting protein--implications for Parkinson's disease. FASEB J. 21:1759–1767.

Matilla A, Gorbea C, Einum DD, Townsend J, Michalik A, van Broeckhoven C, Jensen CC, Murphy KJ, Ptácek LJ, Fu YH. 2001. Association of ataxin-7 with the proteasome subunit S4 of the 19S regulatory complex. Hum Mol Genet. 10:2821–2831.

McDonough H, Patterson C. 2003. CHIP: a link between the chaperone and proteasome systems. Cell Stress Chap. 8:303–308.

Shin Y, Klucken J, Patterson C, Hyman BT, McLean PJ. 2005. The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways. J Biol Chem. 280:23727–23734.

Smith DM, Chang SC, Park S, Finley D, Cheng Y, Goldberg AL. 2007. Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry. Mol Cell. 27:731–744.

Snyder H, Mensah K, Theisler C, Lee J, Matouschek A, Wolozin B. 2003. Aggregated and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. J Biol Chem. 278:11753–11759.

Soto C, Estrada LD. 2008. Protein misfolding and neurodegeneration. Arch Neurol. 65:184–189.

Stefani M, Dobson CM. 2003. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J Mol Med. 81:678–699.

Urushitani M, Kurisu J, Tateno M, Hatakeyama S, Nakayama K, Kato S, Takahashi R. 2004. CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70. J Neurochem. 90:231–244.