Chapter 11
CHIP: A Co-chaperone for Degradation by the Proteasome

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Abstract Protein homeostasis relies on a balance between protein folding and protein degradation. Molecular chaperones like Hsp70 and Hsp90 fulfil well-defined roles in protein folding and conformational stability via ATP dependent reaction cycles. These folding cycles are controlled by associations with a cohort of non-client protein co-chaperones, such as Hop, p23 and Aha1. Pro-folding co-chaperones facilitate the transit of the client protein through the chaperone mediated folding process. However, chaperones are also involved in ubiquitin-mediated proteasomal degradation of client proteins. Similar to folding complexes, the ability of chaperones to mediate protein degradation is regulated by co-chaperones, such as the C terminal Hsp70 binding protein (CHIP). CHIP binds to Hsp70 and Hsp90 chaperones through its tetratricopeptide repeat (TPR) domain and functions as an E3 ubiquitin ligase using a modified RING finger domain (U-box). This unique combination of domains effectively allows CHIP to network chaperone complexes to the ubiquitin-proteasome system. This chapter reviews the current understanding of CHIP as a co-chaperone that switches Hsp70/Hsp90 chaperone complexes from protein folding to protein degradation.

Keywords CHIP · STUB1 · Ubiquitin · Proteasome

Introduction to Ubiquitin Modification and Proteasomal Degradation of Proteins

The ubiquitin proteasome system is a highly conserved mechanism through which eukaryotic cells facilitate the controlled enzymatic degradation of unwanted proteins (Amm et al. 2014; Ciechanover 1998). The ubiquitin and proteasome systems work in concert to regulate protein levels in eukaryotic cells (Roos-Mattjus and Sistonen 2004; Wolf et al. 2004; Lecker et al. 2006). The proteasome not

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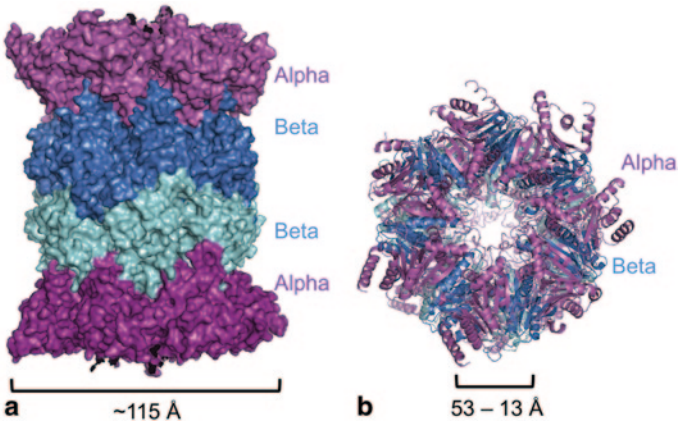

Fig. 11.1 Structure of the 20S proteasome. The proteasome forms a barrel shaped structure in which the proteolytic residues are deep within the central channel through which substrates to be degraded must pass. The 20S subunit is formed of two heptameric ring structures composed of beta subunits, which is bounded by single rings of a similar heptameric ring structure composed of alpha subunits. **a** Surface rendering of a side view and **b** cartoon representation of the top view of the 20S core catalytic particle of the proteasome. The alpha and beta subunits are shown in shades of purple and blue respectively. The image was generated using Pymol (DeLano Scientific). PDB code: 1FNT

only regulates the degradation of incorrectly folded proteins, but is also important for the degradation of proteins whose expression needs to be temporally regulated (such as cell cycle kinases) (Wagner et al. 2011). The proteasome is a large (>2 MDa), multiprotein complex that comprises the major non-lysosomal degradation machinery for cytosolic and nuclear proteins (Fig. 11.1). The structure resembles a barrel-like assembly with a central proteolytic cavity through which substrate proteins are degraded (Bedford et al. 2010; Tanaka 2009). The proteasome can be divided into two components, namely the 20S core proteasome and the 19S regulatory components. A single 20S core proteasome associates with two 19S regulatory particles to form the active 26S proteasome (Murata et al. 2009; Walz et al. 1998). The location of the proteolytic sites within the central cavity of the 20S proteasome allows protein degradation to be compartmentalised (da Fonseca and Morris 2008; Heinemeyer et al. 2004). The core proteasome particle contains stacked ring structures built from 7 copies of either an alpha or beta subunit (Fig. 11.1). The inner two heptameric beta rings form the central catalytic component of the proteasome. This central unit is bounded on either side by an equivalent outer ring structure made up of 7 copies of the structural alpha subunit (Tanaka 2009; Unverdorben et al. 2014; da Fonseca and Morris 2008). Whereas the beta subunit complex contains the catalytic protease sites, the outer ring of alpha subunits serves as a 'gate' to restrict unregulated entry of proteins into the catalytic cavity (da Fonseca and Morris 2008). The alpha subunit rings also act as the docking sites for interaction with the regulatory 19S particle of the

proteasome. The 19S regulatory subunit is a protein complex that functions to activate the 20S particle for protein degradation (Unverdorben et al. 2014). The size of the central pore of the proteasome ranges between 50–13 Å and entry is restricted by the alpha subunit gate. The 19S proteasome is required to open the gate in the 20S proteasome, thereby permitting entry of substrate proteins into the catalytic sites within the proteasome core (da Fonseca and Morris 2008; Sledz et al. 2013; Unverdorben et al. 2014). Polyubiquitinated proteins targeted for degradation need to be deubiquitinated and delivered to the proteolytic active site of the proteasome that is buried within the 20S core particle. The substrate is thought to be partially unfolded during translocation into the cavity. Within the proteasome core, substrate peptide bonds are hydrolysed by nucleophilic attack dependent on catalytic threonine residues that extend into the cavity from the beta subunits. The resultant peptides released from the proteasome range between 4 and 25 residues (Babbitt et al. 2005; Ortega et al. 2005; Goldberg et al. 1997).

Degradation of proteins by the proteasome is preceded by the conjugation of ubiquitin to the substrate via a series of sequential enzyme catalysed reactions (Fig. 11.2) (Hershko and Ciechanover 1998). Ubiquitin is a small, abundant protein (~8 kDa) found in all eukaryotic cells which, when added to proteins in a polyubiquitin chain, functions as the degradation signal (Smith 1988; Johnson et al. 1992; Johnson et al. 1995). Ubiquitin is initially activated by conjugation to an ubiquitin activating enzyme (E1) in an ATP dependent manner via a thioester linkage (Lee and Schindelin 2008). Ubiquitin is subsequently transferred via an ubiquitin conjugating enzyme (E2) intermediate to the substrate protein targeted for degradation (Olsen and Lima 2013). This reaction is catalysed by an ubiquitin ligase enzyme (E3) and results in the formation of a peptide bond between a glycine residue in the C-terminus of ubiquitin and lysine residues within the substrate protein (Scheffner et al. 1995; Wilkinson 2000). There are a range of different E2 and E3 isoforms that may combine for different substrate proteins, suggesting a diverse and discriminatory recognition system for ubiquitin conjugation (Spratt et al. 2012). This process may be repeated a number of times, often involving the conjugation of subsequent ubiquitin molecules to lysines within ubiquitin itself, leading to the formation of covalently linked polyubiquitin chains. A fourth enzyme may also be involved in this cascade. Known as E4, this protein acts as an ubiquitin chain elongation enzyme to catalyse the assembly of polyubiquitin chains on protein substrates (Koegl et al. 1999). While monoubiquitination may induce changes in activity or subcellular localisation of proteins, the conjugation of a polyubiquitin chain to a substrate protein is required for degradation by the proteasome (Johnson et al. 1992). Ubiquitin contains 7 lysines residues (K6, K11, K27, K29, K33, K48, and K63), in addition to its N-terminus, which act as potential sites of conjugation. The lysine residue involved in the bond can also impact on the outcome of ubiquitination (Hershko and Ciechanover 1998). K48 linked ubiquitin chains, where the covalent linkage of the ubiquitin chain is via the K48 residue of ubiquitin, is the canonical signal for proteasomal degradation (Jacobson et al. 2009). K48 linkage is regulated

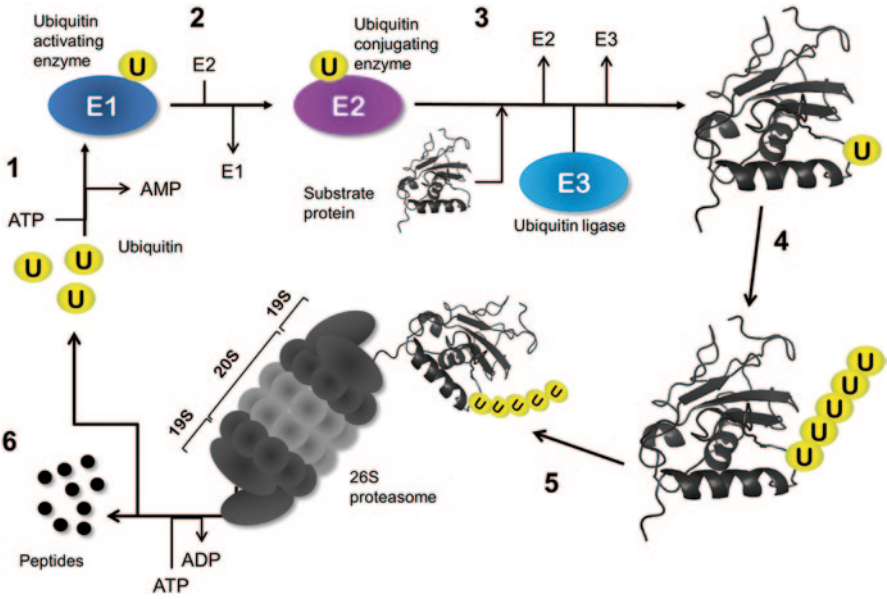

Fig. 11.2 Degradation of proteins via the ubiquitin-proteasome system. Conjugation of polyubiquitin chains to substrate proteins is catalysed as part of an ATP dependent enzyme catalysed cascade that precedes protein recognition and degradation by the proteasome. (*1*) The process is initiated by the activation of ubiquitin via conjugation to an ubiquitin activating enzyme (E1) via a thioester bond. (*2*) The ubiquitin moiety is subsequently transferred to ubiquitin conjugating enzyme (E2), which (*3*) subsequently forms a complex with a specific ubiquitin ligase (E3) and a substrate protein. (*4*) The E3 ligase transfers the ubiquitin to lysine residues within the substrate protein. This cycle is repeated multiple times to generate substrate proteins linked to polyubiquitin chains. (*5*) Polyubiquitination, particularly via K48 linkages, is the signal for transfer of substrate proteins to the proteasome. (*6*) At the proteasome, ubiquitinated substrates interact with the 19S regulatory particle which deubiquitinates them and passes them into the central cavity of the core 20S proteasome. Here in the active site, proteins are degraded and peptides released. Ubiquitin molecules can subsequently be recycled. 19S: regulatory particle, 20S: core particle

by members of the Ubc4, Ubc5 and Ubc7 E2 ubiquitin conjugating enzymes. In contrast, K63 ubiquitination may have a regulatory function (Jacobson et al. 2009) and is catalysed by the Ubc13 E2 in complex with other Ubc proteins (e.g. Uev1a) (Hofmann and Pickart 1999; Sun and Chen 2004). Most often, the selectivity of protein degradation is controlled by the E3 ligase. These E3 ligases integrate with the cellular molecular chaperone system, which is used as the recognition system of the misfolded substrate during this process. In this way, the numerous different E3 isoforms, each of which may be specific for certain protein substrates, are able to utilise the innate ability of chaperones to capture a range of misfolded substrates, to target specific proteins for degradation (Kriegenburg et al. 2012; Esser et al. 2004).

Molecular Chaperones and the Ubiquitin-Proteasome System

A molecular chaperone is a protein that participates in the conformational regulation and folding of a range of substrate proteins, known as client proteins. Chaperones function under physiological conditions to maintain protein homeostasis, and are also vital during or after stressful conditions to prevent or reverse the potentially disastrous consequences of protein aggregation for the cell (Agashe and Hartl 2000; Landry and Gierasch 1994; Hartl 1996; Welch and Brown 1996; Fedorov and Baldwin 1997; Ellis 1997). Many molecular chaperones are members of the heat shock protein family (HSP). In particular, the Hsp70 and Hsp90 chaperones, as part of multiprotein complexes, regulate both *de novo* and stress-related protein folding and stability. Hsp70 and Hsp90 are ATP-dependent chaperones who participate in protein folding cycles that involve multiple phases of client binding and release (Wegele et al. 2004). The activities of both chaperone complexes is dependent on interactions with a number of protein cofactors, known as co-chaperones. A co-chaperone is defined as a non-client accessory protein that lacks intrinsic chaperone activity, but functions to modulate the activity of a known chaperone (Caplan 2003). Co-chaperones act at every stage of the chaperone folding cycle and control progression of the client protein through these cycles by a range of mechanisms, including regulation of ATPase activity, direct protein-protein interactions and posttranslational modifications. Co-chaperones may be selective for one particular family of chaperones, or may interact with multiple chaperone families (Li et al. 2012).

The Hsp70-Hsp40 chaperone complex is one of the main foldase complexes in the cell, participating in both *de novo* and stress-related protein folding (Landry and Gierasch 1994; Cheetham et al. 1994; Strickland et al. 1997; Hiromura et al. 1998). Hsp40 co-chaperones deliver client proteins to Hsp70 and regulate the affinity of Hsp70 for these client proteins by stimulating the ATPase activity of Hsp70 (Cheetham et al. 1994). The stimulation of Hsp70 ATPase converts Hsp70 into the high affinity substrate binding form and leads to binding of the client protein by Hsp70 and prevention of misfolding or aggregation. This form of Hsp70 is ADP bound and is stabilised by another co-chaperone, known as Hsc70/Hsp70 interacting protein (Hip). Client proteins are subsequently released from Hsp70 via nucleotide exchange, which returns the Hsp70 to the ATP bound form, which has low affinity for the substrate. This stage is catalysed by the GrpE (in prokaryotes) or BAG1/HspBP1 (in eukaryotes) co-chaperones (Hohfeld 1998; Chang et al. 2010; Mao et al. 2006; Kabani et al. 2002).

A subset of client proteins will be passed from the Hsp70 chaperone complex to the Hsp90 chaperone complex. Hsp90 is also an ATP-dependent chaperone, but its function primarily relates to the maintenance of protein stability of labile client proteins prior to their activation (Prodromou et al. 1997; Panaretou et al. 1998) The Hsp90 complex is also able to stabilise a number of mutated client proteins, thereby preventing their degradation (Whitesell et al. 1998). Hsp90 is constitutively

dimerised at the C-terminus and exists in an open conformation (resembling a V shape) when inactive (Ali et al. 2006). Once bound by Hsp90, the client protein then transitions through the Hsp90 cycle, a process which involves the sequential interaction with a range of co-chaperones (McLaughlin et al. 2002; Li et al. 2012). The early stages of Hsp90 mediated folding involve the transfer of client proteins between Hsp70 and Hsp90, catalysed by the co-chaperone Hop (Frydman and Hohfeld 1997; Brinker et al. 2002; Siligardi et al. 2004). Hop is a member of the TPR (tetratricopeptide repeat) domain containing co-chaperones that is able to simultaneously bind to both Hsp70 and Hsp90 (Prodromou et al. 1999). The TPR domain is a protein-protein interaction module that is found in a wide range of proteins (Allan and Ratajczak 2011). TPR domains are comprised of multiple copies of a TPR motif, which gives rise to a particular alpha helical structure. The TPR motif is loosely defined by a 34 residue degenerate consensus sequence (Tpr-Leu-Gly-Tyr-Ala-Phe-Ala-Pro). Therefore, while the primary sequence varies substantially between different TPR domains, the overall structure is conserved. Most TPR domains are comprised of three TPR motifs, each of which contributes 6 alpha helices, which pack together to form an alpha helical amphipathic groove. This groove is the site of interaction with the target peptide (Brinker et al. 2002). TPR containing chaperones bind to the C terminal EEVD motifs contained in both Hsp70 and Hsp90 (Odunuga et al. 2003; Blatch and Lassle 1999; Van Der Spuy et al. 2000). Hop binds to the complex as a monomer via one EEVD motif in the Hsp90 dimer, while the other EEVD motif may be bound by a peptidyl prolyl isomerase (PPIase) leading to the formation of the asymmetric intermediate complex. The binding of ATP and the late co-chaperones p23 stimulate conformational change in Hsp90 to the closed conformation, with a concomitant dissociation of Hop from the complex (Prodromou et al. 2000). ATP hydrolysis by Hsp90 is subsequently stimulated by Aha1, which returns Hsp90 to the open conformation and results in release of the client protein (Lotz et al. 2003). Co-chaperones like Hop, p23 and Aha1 are considered general co-chaperones, as they constitute the core co-chaperones required for the transition of general client proteins through the cycle. In addition, there exist a range of other co-chaperones that may associate with Hsp90 complex for specific functions, such as regulation of specific classes of client proteins (like Cdc37) or post-translational modification (e.g. PP5) (Li et al. 2012).

The term chaperone is associated with protein folding. However, chaperones also participate in the degradation of proteins via the proteasome (Kriegenburg et al. 2012; Esser et al. 2004; Kettern et al. 2010; Kastle and Grune 2012). This role for chaperones is opposing, yet complementary, to their role in promoting protein folding and is consistent with a role as regulators of global protein homeostasis (Hohfeld et al. 2001; Imai et al. 2003). Molecular chaperones are thought to be able to identify and capture misfolded protein substrates in order for them to be directed to the proteasome (Bercovich et al. 1997; Kriegenburg et al. 2012; Meimaridou et al. 2009). The involvement of chaperones, particularly Hsp70 and Hsp90, in proteasomal mediated protein degradation is also regulated by co-chaperones, including the Hsp70/Hsp90 co-chaperone, carboxyl terminus of Hsp70-interacting protein (CHIP) (Murata et al. 2001; McDonough and Patterson 2003).

The Carboxyl Terminus of Hsp70-Interacting Protein (CHIP)

The carboxyl terminus of Hsp70-interacting protein (CHIP; also known as STIP1 homology and U-box containing protein 1 or STUB1) has dual functions, one as a co-chaperone of Hsp70 and Hsp90, and the other as an E3 ubiquitin ligase to regulate proteasomal degradation of chaperone client proteins (McDonough and Patterson 2003; Ballinger et al. 1999). In this way, CHIP is a major link between chaperone mediated folding and protein degradation. CHIP is distinguished from the other Hsp90 co-chaperones in that it is primarily involved in tuning the chaperone complexes towards protein degradation, rather than supporting protein folding (Demand et al. 2001). The CHIP gene is conserved in a range of eukaryotes, being demonstrated or predicted to exist in the genomes of the human, monkey, mouse, zebrafish, fruit fly, frog, and even the genome and transcriptome of the recently sequenced Coelacanth (*Latimeria sp.*) (Tastan Bishop et al. 2014). CHIP knockout mice were viable and displayed normal development, suggesting that CHIP is not an essential gene (Morishima et al. 2008; Dai et al. 2003). However, there was increased peripartum mortality of CHIP null mice compared to wild type mice. This was attributed to wasting of the thymus, which is an indicator of reduced ability to cope with stress. The link between peripartum death of CHIP null mice and stress was subsequently supported by the fact that CHIP-/- mice were temperature sensitive and that induction of stress in these animals induced apoptosis in multiple organs after challenge (Dai et al. 2003). CHIP overexpression activated the stress response by specifically inducing trimerization and nuclear translocation of HSF-1 and activation of HSE containing stress responsive promoters, like Hsp70 (Dai et al. 2003). This suggests that the role of CHIP is not exclusively linked to protein degradation, but also involves regulation of the stress response.

Notwithstanding its role in activation of the stress response, CHIP appears to be a master regulator of protein degradation via chaperones, although it is by no means the only co-chaperone associated with protein degradation. The Hsp40 isoform, Hsj1 (DNAJB2) (Chapple et al. 2004; Westhoff et al. 2005; Gao et al. 2011) and the nucleotide exchange factor, BAG-1, both have defined roles in proteasome-mediated protein degradation (Luders et al. 2000; Alberti et al. 2002; Alberti et al. 2003; Elliott et al. 2007). In addition, there are other E3 ubiquitin ligase proteins (e.g. Ubr1, Cul5, Parkin, Mdm2) that may associate with Hsp90 and/or Hsp70 chaperone complexes to target client proteins for ubiquitination and degradation (Nillegoda et al. 2010; Eisele and Wolf 2008; Ehrlich et al. 2009). These proteins are able to induce ubiquitination in the absence of CHIP, although there is also evidence that inhibition of these E3 ligases can affect protein folding and degradation even in the presence of CHIP. It is clear that functional redundancy exists between the E3 ligases and is possible that multiple members will collaborate as a complex to control degradation of specific proteins (Morishima et al. 2008).

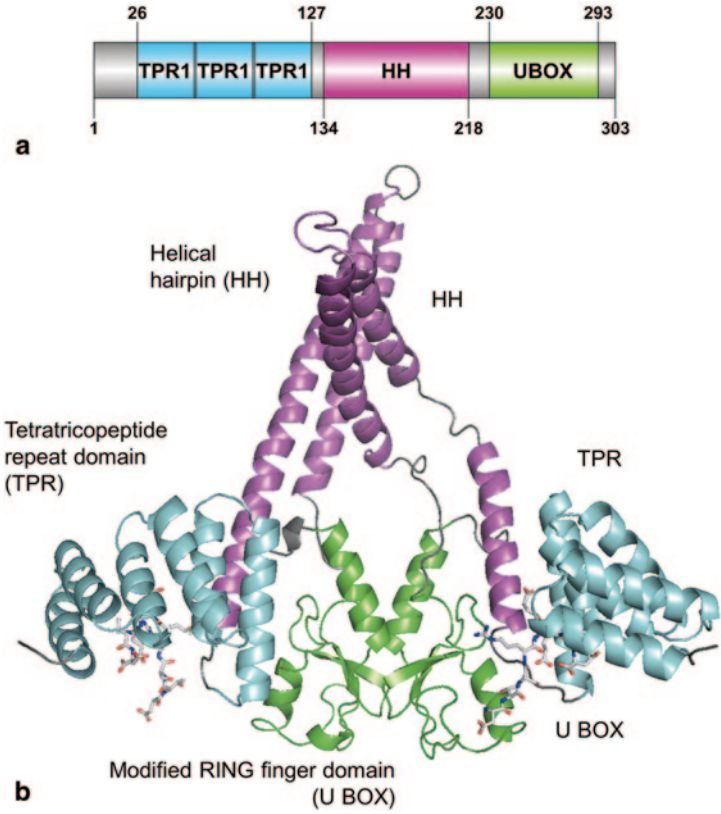

Fig. 11.3 Domain architecture and structure of CHIP. **a** Domain structure of CHIP showing the N-terminal TPR domain (composed of 3 TPR motifs) and the C terminal U-box separated by the helical hairpin region. **b** The asymmetric dimer structure observed in the crystal structure of murine CHIP. The U-box domain (green) is the point of dimerization. The structure of the TPR (cyan; helices 1–6) and U-box domains are largely conserved between the two protomers. The helical hairpin (magenta, helices 7–8) region differs substantially. The C-terminal MEEVD peptide from Hsp90 in shown in stick format and coloured grey. The image was generated using Pymol (DeLano Scientific). PDB code: 2C2L

Structure of CHIP

CHIP is a 35 kDa protein, expressed as a single isoform containing an N terminal TPR domain together with a U-box domain linked via a long helical region (Ballinger et al. 1999). The crystal structure of murine CHIP was determined in 2005 by Pearl and colleagues (Zhang et al. 2005b). This structure demonstrates that CHIP is dimerised at the C-terminus via the U-box domains (Fig. 11.3). The murine CHIP homodimer from this study was shown to be asymmetrical, with the two monomers of the dimer adopting different structures. The structural difference

in the two monomers is predominantly in the structure of a long helical region that links the TPR and U-box domains. This region, termed the helical hairpin, is formed from two continuous antiparallel alpha helices in an extended conformation in one protomer. In contrast, in the other monomer, the helical hairpin adopts a bent conformation and the seventh helix is split into two helices (as opposed to being a single continuous helix) (Fig. 11.3). C-terminal to the helical hairpin domain is the U-box domain, which is composed of beta hairpins separated by alpha helices. Interestingly, the structure of the helical hairpin and U-box region of *D. rerio* CHIP present a symmetrical dimer that differs from the asymmetric dimer observed in the crystal structure of the full length mouse CHIP (Xu et al. 2006). This discrepancy may be a consequence of the absence of the TPR domains in this structure, or may reflect the fact that crystal structures are static and may capture only one form of a dynamic structure. Consistent with this, recent studies demonstrate that the full length human CHIP homodimer appears highly flexible in solution (Graf et al. 2010). Specific changes in the CHIP conformation were noted upon interaction with either chaperones or E2 enzymes. Binding of Hsp70/Hsp90 chaperones or chaperone-derived peptides to CHIP promoted stabilisation of the TPR domains, while distinct changes were observed upon interaction with E2 conjugating enzymes (UbcH5a and Ubc13) (Graf et al. 2010).

Interaction of CHIP with Chaperones and E2 Ligases

The crystal structure of the CHIP dimer in complex with peptides from both Hsp90 and enzymes of the ubiquitin pathway demonstrates how the structure of CHIP has evolved to allow simultaneous interaction with chaperones and proteasomal substrates (Zhang et al. 2005a). The dimeric E3 ubiquitin ligase CHIP bound with its TPR domain the C-terminus of molecular chaperones Hsp70 and Hsp90 and with its U-box region E2 ubiquitin-conjugating enzymes (Zhang et al. 2005b; Ballinger et al. 1999; Xu et al. 2008). This unique combination of domains allows CHIP to bind to both chaperones Hsp70 and Hsp90, via its TPR domain, and to interact with the proteasome by acting as an E3 ligase using its U-box domain, effectively crosslinking the chaperones to the proteasome via ubiquitination of substrates for degradation.

CHIP was originally identified as co-chaperone for Hsp70 and Hsp90 in a screen for novel TPR-containing proteins (Ballinger et al. 1999). CHIP interacts with the C terminal EEVD motifs in both Hsp90 and Hsp70 via its TPR domains (Ballinger et al. 1999), a feature in common with other TPR containing co-chaperones, like Hop, PP5 and Hip (Allan and Ratajczak 2011; Brinker et al. 2002; Cortajarena and Regan 2006; Odunuga et al. 2003). The CHIP monomer contains only a single TPR domain which can bind indiscriminately to both Hsp90 and Hsp70. CHIP can bind two molecules of Hsp70 in a dynamic and flexible complex in which both CHIP and Hsp70 move independently of each other and is predicted to provide space

to accommodate additional client proteins (Smith et al. 2013). This independent movement is demonstrated by the fact that only the extreme C terminal residues (IEEVD) of Hsp70 appear to be involved in the interaction with CHIP. The IEEVD motif of Hsp70 was also required for ubiquitination of Hsp70, although a reduction in the length of the C terminal tail preceding the IEEVD led to reduced capacity to ubiquitinate Hsp70 (Smith et al. 2013). Although the TPR motif is the chaperone binding site of CHIP, there is evidence to suggest that allosteric interactions with the U-box domain are required for CHIP association with Hsp70 (Matsumura et al. 2013). Despite this, there was little difference in the affinity of binding to CHIP between full length Hsc70 and the C terminal IEEVD peptide (Smith et al. 2013). There are conflicting reports regarding the affinity of the interaction between CHIP and different chaperones, with reports that both Hsp70 binding (Kundrat and Regan 2010a) and Hsp90 binding to CHIP (Stankiewicz et al. 2010) is the greater affinity of the two.

As an E3 ubiquitin ligase, CHIP also interacts with members of the E2 family of ubiquitin conjugating enzymes during ubiquitination of substrates. These interactions between E2 and E3 proteins are highly specific and will ultimately determine the nature of ubiquitination that occurs, as well as the identity of the substrate protein. CHIP has been shown to interact with specific E2 from the UBCH5 family (which are involved in K48 mediated ubiquitination which promotes proteasomal degradation) (Cyr et al. 2002; Wiederkehr et al. 2002; Xu et al. 2008) and Ubc13 (which regulates K63 ubiquitination and has a regulatory role) (Alberti et al. 2002; Jiang et al. 2001; Murata et al. 2001; Zhang et al. 2005b). CHIP also displays E4 ligase activity, in that it can catalyse the extension of polyubiquitin chains on substrate proteins (Murata et al. 2001; Murata et al. 2003; Jiang et al. 2001; Koegl et al. 1999). CHIP interacts with E2 enzymes via its U-box domain. The U-box is composed of 70 amino acids and is structurally similar to the RING finger domains found in other ubiquitin ligases (Ohi et al. 2003). U-box containing ligases are distinct from HECT and RING finger E3 ligases and appear to associate almost exclusively with chaperones during ubiquitination of client proteins (Hatakeyama et al. 2004b; Hatakeyama et al. 2001; Kriegenburg et al. 2012).

CHIP binding can inhibit both Hsp90 and Hsp70- thereby preventing both protein folding by Hsp70 and conformational regulation of client proteins by Hsp90. CHIP blocked the Hsp40 mediated stimulation of Hsp70 ATPase activity and attenuated the function of Hip (Ballinger et al. 1999). The consequence of this is that CHIP promotes accumulation of the ATP-bound form of Hsp70. This prevented substrate binding and refolding of denatured luciferase *in vitro* (Ballinger et al. 1999). CHIP therefore regulates ubiquitination of Hsp70 client proteins through regulation of client protein affinity. CHIP did not affect ATP or ADP association, but blocked the Hsp40 mediated stimulation of Hsp70 ATPase activity (Stankiewicz et al. 2010). The consequence of this is that, indirectly, CHIP promotes an ATP bound form of Hsp70, one which has low affinity for client proteins. CHIP also works in concert with certain members of the Hsp70 nucleotide exchange family of proteins, including the Bcl2-associated athanogene (BAG). BAG-1

could bind simultaneously to both Hsp70 and the 26S proteasome (Luders et al. 2000). In doing so, BAG-1 induces the release of ubiquitinated client proteins from Hsp70 to the proteasome.

Addition of CHIP is also sufficient to modify the co-chaperone complement of the Hsp90 complex and induce degradation of canonical Hsp90 client proteins (Connell et al. 2001). CHIP did not affect the ATPase activity of Hsp90 (Stankiewicz et al. 2010), but CHIP binding did reduce Hop binding and prevented binding of p23 completely (Ballinger et al. 1999). Displacement of p23 relies on the presence of the CHIP TPR domains, while the ubiquitination of glucocorticoid receptor is U-box dependent; demonstrating defined functions for the two different domains, but that cooperation between them is required for protein degradation. This is interesting, as p23 and CHIP bind to different termini of the chaperone. CHIP may antagonise the action of p23, thereby stabilising the substrate protein within the complex for ubiquitination. CHIP activity does not require the N terminal domain of Hsp90 and an interaction with the C terminal region of Hsp90 is sufficient to allow CHIP-mediated ubiquitination of the client protein. This may suggest that client proteins that associate with different Hsp90 regions may be differentially susceptible to ubiquitination by CHIP.

The ability of CHIP to induce degradation requires the presence of the substrate protein in a denatured form, in addition to the chaperone, as demonstrated by the fact that denatured, but not native, luciferase could be ubiquitinated *in vitro* in the presence of Ubc4/5 (E2 enzyme) and the Hsp90 or Hsp70 complex (Murata et al. 2001). In this way, Hsp70/Hsp90 are involved in the recognition and delivery of substrates for ubiquitination. Indeed, binding of CHIP to the Hsp70 EEVD motif was favoured when Hsp70 was in the ADP form, which has higher affinity for client proteins (Matsumura et al. 2013). In addition, Hsp70-bound peptides are preferentially targeted for degradation by CHIP compared to Hsp90 bound substrates (Qian et al. 2006; Stankiewicz et al. 2010). Therefore, the chaperones would act as sensors for denatured protein substrates which could subsequently be targeted for degradation by CHIP. Interestingly, CHIP also mediates the degradation of Hsp70 itself, once the misfolded client proteins have been degraded.

CHIP Substrates and Human Disease

The role of CHIP as a major regulator of proteasome-mediated degradation has been cemented by the recent identification of numerous protein substrates that are dependent on CHIP for proteasomal degradation (Table 11.1). One of the best described client proteins for CHIP is CFTR, upon which many of the early fundamental studies on CHIP function were performed. An updated list of CHIP substrate proteins includes a number of transcription factors, signalling intermediates and cytoskeletal or structural proteins (Table 11.1). The substrates, many of which are known client proteins of either Hsp90 or Hsp70, fulfil important

Table 11.1 Selection of the proteins targeted for proteasomal degradation by CHIP

Substrate/Client	Classification	Disease association	Reference
Androgen receptor (AR)	Receptor	Cancer	Sarkar et al. (2014)
Cystic fibrosis transmembrane conductance regulator (CFTR)	Receptor	Cystic fibrosis	Younger et al. (2004); Meacham et al. (2001)
Katanin-p60	Cytoskeleton		Yang et al. (2013)
Profilin	Cytoskeleton		Choi et al. (2014)
Tau	Cytoskeleton	Neurodegeneration	Elliott et al. (2007); Dickey et al. (2007); Hatakeyama et al. (2004a); Petrucelli et al. (2004); Shimura et al. (2004)
Alpha-synuclein	Cytoskeleton	Neurodegeneration	Kalia et al. (2011); Shin et al. (2005)
Keratin	Cytoskeleton		Loffek et al. (2010)
Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)	Enzyme		Wang et al. (2012)
Histone deacetylase 6 (HDAC6)	Enzyme		Cook et al. (2012)
Nitric-oxide synthase (NOS)	Enzyme	Neurodegeneration	Chen et al. (2009); Peng et al. (2004)
NAD(P)H:quinone oxidoreductase 1 (NQO1)	Enzyme		Tsvetkov et al. (2011)
V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (Her2)	Receptor	Cancer	Zhou et al. (2003); Xu et al. (2002)
Phosphatidylinositol 3-kinase P85 (P13K)	Signalling intermediate	Cancer	Ko et al. (2014)
Interferon regulatory factor 1 (IRF-1)	Signalling intermediate		Gao et al. (2013)
Liver kinase B1 (LKB1)	Signalling intermediate	Cancer	Gaude et al. (2012)
Phosphatase and tensin homolog (PTEN)	Signalling intermediate	Cancer	Ahmed et al. (2012)
Protein kinase B (PKB/Akt)	Signalling intermediate	Cancer	Su et al. (2011)
TNF receptor-associated factor 2 (TRAF2)	Signalling intermediate		Jang et al. (2011b)
Met receptor	Signalling intermediate	Cancer	Jang et al. (2011a)
Immature BCR-ABL	Signalling intermediate	Cancer	Tsukahara and Maru (2010)

Table 11.1 (continued)

Substrate/Client	Classification	Disease association	Reference
MAPK/ERK kinase kinase 2 (MEKK2)	Signalling intermediate		Maruyama et al. (2010)
Apoptosis signal-regulating kinase 1 (ASK1)	Signalling intermediate		Gao et al. (2010); Hwang et al. (2005)
Leucine-rich repeat kinase 2 (LRRK2)	Signalling intermediate	Neurodegeneration	Ding and Goldberg (2009); Ko et al. (2009)
Eukaryotic translation initiation factor 5A (eIF5A)	Transcription factor		Shang et al. (2014)
Estrogen Receptor (ER)	Transcription factor	Cancer	Fan et al. (2005)
Glucocorticoid receptor (GR)	Transcription factor		Wang and DeFranco (2005); Galigniana et al. (2004); Connell et al. (2001)
Tumour protein 53 (p53)	Transcription factor	Cancer	Wang et al. (2011); Sisoula et al. (2011); Muller et al. (2008); Esser et al. (2005)
V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (c-Myc)	Transcription factor	Cancer	Paul et al. (2013)
Forkhead transcription factor p (FOXp)	Transcription factor		Chen et al. (2013)
Hypoxia-inducible factor 1 alpha (HIF-1alpha)	Transcription factor	Cancer	Luo et al. (2010)
Forkhead transcription factor 1 (FoxO1)	Transcription factor		Li et al. (2009)

roles in fundamental cellular processes. What is also striking is that many of these proteins are linked with diseases including cancer and neurodegenerative diseases like Alzheimer's disease. CHIP may therefore be of therapeutic value given the potential ability to regulate the degradation of disease relevant proteins like tau or p53 (Hatakeyama et al. 2004a; Petrucelli et al. 2004; Shimura et al. 2004; Esser et al. 2005).

In particular, the role of CHIP in neurodegenerative diseases, where protein aggregates are a hallmark, has been a main focus of research. CHIP can bind directly to and ubiquitinate the protein tau, actions that mediate the dissolution of tau aggregates (Hatakeyama et al. 2004a; Petrucelli et al. 2004; Shimura et al. 2004). These tau aggregates are associated with the pathology of Alzheimer's disease (Kosik and Shimura 2005; Medeiros et al. 2011; Martin et al. 2011; Salminen et al. 2011). CHIP may also regulate the aggregation of tau via ubiquitination of other client

proteins, like HDAC6 and Akt (Cook et al. 2012; Dickey et al. 2008). Therefore overexpression of CHIP may represent a therapeutic strategy to prevent neuronal cell death and ameliorate the symptoms and onset of the disease (Dickey et al. 2007; Sahara et al. 2005). The role of CHIP in cancer has been relatively less well studied than neurodegeneration. However, CHIP also controls the proteasomal degradation of a number of important oncogenic transcription factors or signalling intermediates, including p53, PTEN, Akt and c-Myc (Paul et al. 2013; Kajiro et al. 2009; Ahmed et al. 2012). These classes of proteins often act as nodes for the activation of a host of downstream proteins in the cellular reactions that lead to oncogenesis. Therefore, CHIP may in fact indirectly regulate a larger cohort of cellular proteins via degradation of central transcription factors or signalling intermediates. Indeed, analysis of the function of CHIP in breast cancer has demonstrated that the protein can regulate cellular responses, many of which are considered cancer hallmarks. Overexpression of CHIP blocked oncogenic signalling pathways, inhibited cancer associated processes like cell migration and anchorage independent growth, and induced cell death. Conversely, depletion of CHIP protein levels increased tumour formation and metastasis in mouse models (Kajiro et al. 2009; Choi et al. 2014; Sarkar et al. 2014).

In addition to classical substrate proteins, CHIP also ubiquitinates the chaperones Hsp70 and Hsp90 on multiple solvent exposed, but clustered lysine residues (6 in Hsp70 and 13 in Hsp90) (Kundrat and Regan 2010b). The polyubiquitination of these chaperones by CHIP occurs via K6, K11, K48, and K63 linkages. The canonical signal for protein degradation is ubiquitination via K48 linkages, and it is known that CHIP can mediate degradation of Hsp70 via this mechanism (Jiang et al. 2001). This reduction in Hsp70 plays a central regulatory role to return Hsp70 levels to basal after the induction of the stress response. However, non-canonical ubiquitin linkages (like K6, K11 and K63) have not been demonstrated to induce protein degradation, but may mediate other functions. In some experiments, ubiquitination via K63 resulted in recruitment of Hsp70, Hsp90 and BAG-1 to the proteasome but did not lead to their degradation (Alberti et al. 2002; Jiang et al. 2001). This suggested that K63 linkage may be a proteasome targeting sequence and represent a mechanism by which CHIP uses the chaperone to deliver its clients to the proteasome (Saeki et al. 2009; Chen and Sun 2009).

Ubiquitination of substrates by CHIP does not always lead to proteasomal degradation via the canonical K48 ubiquitiation. There are some examples in the literature than demonstrate a role for CHIP in non-canonical ubiquitination of substrates. One example is the protein, sirtuin, which underwent non-canonical CHIP-mediated ubiquitination that culminated in its stabilisation and promotion of DNA repair (Ronnebaum et al. 2013). CHIP also mediated T cell activation by ubiquitination of CARMA1 (Caspase recruitment domain (CARD) containing membrane-associated guanylate kinase protein 1), a receptor important in antigen receptor linked NF-kappaB signalling. The CHIP mediated ubiquitination of CARMA1 via K27 was determined to be important for activation of this pathway (Wang et al. 2013).

Hsp70 and Hsp90: To Degrade or to Refold?

The chaperone folding and ubiquitin-proteasome degradation pathways work competitively during protein homeostasis (Marques et al. 2006). The major question that remains unanswered is how is it determined whether proteins enter refolding or degradation pathways? Pro-folding chaperone complexes are largely associated with the co-chaperone Hop, which facilitates entry of client proteins from the Hsp70 complex into the Hsp90 complex (Siligardi et al. 2004). In contrast, chaperone complexes containing CHIP are considered to be pro-degradation complexes. Hop and CHIP cannot bind simultaneously to the Hsp90 complex, which indicates that the complexes controlling either protein folding or protein degradation are mutually exclusive and possibly competitive (Kundrat and Regan 2010a). Therefore, the simplest mechanism to control the choice of folding or degradation may be via regulation of the levels of the specific co-chaperone (Marques et al. 2006). Indeed, simply increasing the CHIP concentration by overexpression increased proteasomal degradation of client proteins, including hormone receptors (Connell et al. 2001; Adachi et al. 2007).

Hop and CHIP compete with each other for binding to Hsp70 and Hsp90, which could determine whether pro-folding or pro-degradation complexes form. There is evidence that these associations are regulated by post-translational modification of the C terminal region of these chaperones. Phosphorylation of sites in the C-terminus of either Hsp70 or Hsp90 blocked CHIP binding and promoted association of Hop with the chaperones. This observation is particularly important in the context of cancer, where increased levels of phosphorylated Hsp70 and Hsp90 chaperones have been linked with high cell proliferation rates (Muller et al. 2013). Indeed, many of the kinases that phosphorylated Hsp90, including CK1, CK2 and GSK3β, are linked to the cell cycle or mitogenic signalling pathways. Hop has also been shown to be upregulated in numerous cancers, suggesting that increased levels of this co-chaperone may out-compete CHIP for chaperone binding in these cells (Willmer et al. 2013; Ruckova et al. 2012). The net consequence of this would be to create a cellular environment that promoted protein folding over degradation. The fact that this is observed in cancer cells may explain the dependency of proteins on the Hsp70-Hsp90 folding complex and support the high cellular growth rates observed in most malignancies.

However, protein folding also predominates under physiological conditions. This is as a result of a greater concentration of chaperone complexes containing Hop or Hip, than CHIP or BAG-1 (Kundrat and Regan 2010a). Despite this, ubiquitination of substrates by CHIP proceeds at a basal level under physiological conditions. Recent studies by the Regan group suggest that the switch to degradation of protein clients was largely determined by partitioning of the client protein between Hsp70 and Hsp90 chaperone complexes (Kundrat and Regan 2010a). Hsp70 and Hsp90 can both bind CHIP, but the purported greater affinity of the CHIP-Hsp70 interaction predicts that this is the dominant complex that mediates the degradation pathway (Kundrat and Regan 2010a). Therefore, the interaction

between Hsp90 and CHIP is predicted to play a minor role in direct protein triage decisions. This is supported by the fact that CHIP has a preference for ubiquitination of Hsp70-bound client proteins compared to Hsp90-bound substrates (Stankiewicz et al. 2010). Rather, degradation of Hsp90 clients is proposed to be induced indirectly by inhibition of the Hsp90 complex, meaning that client proteins associate with the Hsp70 complex for an extended duration, leading to increased potential for degradation via CHIP (Kundrat and Regan 2010a). This is consistent with the observation that the time spent by the client protein in the Hsp70 complex determines its stability (Matsumura et al. 2013). Interestingly, the TPR containing co-chaperone, DNAJC7, which has been proposed to catalyse retrograde transfer of client proteins from Hsp90 back to Hsp70 (Brychzy et al. 2003), can interact directly with CHIP (Hatakeyama et al. 2004b). Hsp90 inhibition using compounds that induce the stress response (such as 17-AAG) lead to increased Hsp70 levels, which could subsequently associate with clients released from the non-functional Hsp90 complex and trigger their degradation via CHIP. The fact that CHIP is also known to induce both the expression and turnover of Hsp70 as part of the stress response, supports the major role for Hsp70 over Hsp90 in this process (Qian et al. 2006).

The increase in substrate degradation in response to higher levels of CHIP could also be explained by the fact that increased levels of CHIP result in increased levels of Hsp70-CHIP degradation complexes to a concentration that exceeds Hsp70-Hop-Hsp90 folding complexes (Kundrat and Regan 2010a). The addition of CHIP to the Hsp90 complex induces a similar response to treatment with GA, promoting dissociation of stabilising co-chaperones, like p23, and promoting proteasomal degradation of the client protein (Connell et al. 2001; Whitesell and Cook 1996). This suggests that, in addition to an increase in Hsp70-CHIP complexes, increased CHIP levels may also block Hsp90 complexes and push client proteins towards association with Hsp70.

The balance between folding and degradation can also be controlled by naturally occurring regulators of CHIP. The activity of CHIP is regulated by BAG-1 and HspBP1, which are both nucleotide exchange factors for Hsp70 (Kabani et al. 2002; Alberti et al. 2003). CHIP can bind directly to the proteasome (Connell et al. 2001; Meacham et al. 2001), or it may interact with the proteasome via BAG-1. BAG-1 binds simultaneously to CHIP and the 26S proteasome, thereby recruiting a complex that delivers CHIP bound complexes to the proteasome (Luders et al. 2000). Interestingly, the association of BAG-1 with the proteasome is mediated in part by CHIP-mediated ubiquitination of BAG-1 (Alberti et al. 2002). HspBP1 is a negative regulator of CHIP activity (Alberti et al. 2004). HsBP1 interacts with the Hsp70 ATPase domain (Raynes and Guerriero 1998) and induces conformational changes in the chaperone (McLellan et al. 2003), leading to binding of CHIP to the C terminal site of Hsp70. This complex abolished the CHIP mediated ubiquitination and degradation of the substrate protein, CFTR (Alberti et al. 2004). Hsp40 and client protein (steroid receptors) preferentially associate with BAG-1 over HspBP1. HspBP1, not BAG-1, also reduced binding of Hsp70 to client proteins and inhibited the activity of steroid hormone receptors at both high and low concentrations. In

contrast, the effect of BAG-1 on steroid receptor function was concentration dependant, being stimulatory at low levels of BAG-1 and inhibitory at higher concentrations of BAG-1 (Knapp et al. 2014).

Conclusions

While the molecular mechanisms that are involved in defining the balance between protein folding and protein degradation are not fully understood, the existence of CHIP suggests that chaperones actively participate in protein degradation via the proteasome. This suggests that an as yet undefined mechanism exists to determine which pathway, folding or degradation, should be followed under certain conditions. Recent studies have demonstrated that CHIP mediated the degradation of a wide range of cellular proteins, which signifies a central role for this co-chaperone in protein degradation. Many of these client proteins are important factors in a range of human diseases; an association that suggests CHIP may be a putative drug target. The potential applications of CHIP to human disease are likely to be largely restricted to those that involve either the overexpression or activation of CHIP. While some experiments demonstrate that CHIP depletion results in degradation of CHIP substrates, other reports demonstrate that CHIP clients remain unaffected in a CHIP depleted background. This hints at functional redundancy whereby other E3 ligase factors may compensate for the loss of CHIP. These data may also suggest that some substrates are more reliant on CHIP for their degradation, whereas others may be promiscuous with respect to the E3 ligase required for their degradation. The application of CHIP as a drug target will be limited until we are able to define the mechanisms which regulate whether chaperones function in protein folding or protein degradation.

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