CHIP Is a U-box-dependent E3 Ubiquitin Ligase

IDENTIFICATION OF Hsc70 AS A TARGET FOR UBIQUITYLATION*

Jihong Jiang‡‡, Carol A. Ballinger§, Yaxu Wu, Qian Dai‡, Douglas M. Cyr**, Jörg Höhfeld‡‡, and Cam Patterson‡‡‡§§

From the ‡Program in Molecular Cardiology and Lineberger Comprehensive Cancer Center, the †Department of Pharmacology, and the **Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, North Carolina 27599-7075, the ‡Department of Pharmacology and Toxicology and $Sealy Center for Molecular Cardiology, University of Texas Medical Branch, Galveston, Texas 27599, and the ‡‡Institute for Cell Biology, University of Bonn, Bonn, Germany D-53121

Proper folding of proteins (either newly synthesized or damaged in response to a stressful event) occurs in a highly regulated fashion. Cytosolic chaperones such as Hsc/Hsp70 are assisted by cofactors that modulate the folding machinery in a positive or negative manner. CHIP (carboxyl terminus of Hsc70-interacting protein) is such a cofactor that interacts with Hsc70 and, in general, attenuates its most well characterized functions. In addition, CHIP accelerates ubiquitin-dependent degradation of chaperone substrates. Using an in vitro ubiquitylation assay with recombinant proteins, we demonstrate that CHIP possesses intrinsic E3 ubiquitin ligase activity and promotes ubiquitylation. This activity is dependent on the carboxyl-terminal U-box. CHIP interacts functionally and physically with the stress-responsive ubiquitin-conjugating enzyme family UBC5. Surprisingly, a major target of the ubiquitin ligase activity of CHIP is Hsc70 itself. CHIP ubiquitylates Hsc70, primarily with short, noncanonical multibiquitin chains but has no appreciable effect on steady-state levels or half-life of this protein. This effect may have heretofore unanticipated consequences with regard to the chaperoning activities of Hsc70 or its ability to deliver substrates to the proteasome. These studies demonstrate that CHIP is a bona fide ubiquitin ligase and indicate that U-box-containing proteins may comprise a new family of E3s.

Multifunctionality is a common feature of highly conserved eukaryotic proteins and often becomes evident when these proteins are analyzed in dissimilar species and in different cellular systems. The cytosolic heat shock proteins Hsp90 and Hsc/Hsp70 are one such set of proteins. Heat shock proteins have evolved as chaperones to catalyze the proper folding of nascent proteins but have accrued additional functions that include renaturation of proteins damaged under stressful conditions, targeting proteins for degradation, participation in cellular signaling events, and regulation of developmental processes (1). Similarly, the discovery that ubiquitylation of nascent proteins can occur cotranslationally suggests that the ubiquitin-proteasome system initially evolved as an error-checking system to mark improperly or unnecessarily synthesized proteins for destruction (2). This same system is now recognized to assist in the timely destruction of mature proteins once their functions are no longer needed.

A coordinated interaction between the ubiquitin-proteasome and chaperone systems is indicated by the observations that a significant proportion of newly synthesized proteins are either chaperone-associated (3) or rapidly degraded via the ubiquitin-proteasome pathway (4). Manipulations of the chaperone system can alter the balance between folding and degradation of chaperone substrates (5), suggesting that substrate interactions with chaperones (and consequently their commitment either toward the folding pathway or to their degradation via the ubiquitin-proteasome machinery) serve as an essential post-translational protein quality control mechanism within eukaryotic cells. The partitioning of proteins to either one of these mutually exclusive pathways is referred to as “protein triage” (6).

It is well accepted that chaperones play a central role in the triage decision (7–9); however, less well understood are the events that lead to the cessation of efforts to fold a substrate and the removal of the substrate to the terminal degradative pathway. It is possible that chaperones and components of the ubiquitin-proteasome pathway exist in a state of competition for these substrates and that repeated cycling of a substrate on and off a chaperone maintains the substrate in a soluble state and increases, in a stochastic fashion, its likelihood of interactions with the ubiquitin machinery (6). However, some data argue for a more direct role for the chaperones in the degradation process (10). Plausible hypotheses to explain these observations include direct associations between the chaperone and ubiquitin-proteasome machinery to facilitate transfer of a substrate from one pathway to the other or conversion of the chaperone itself to a ubiquitylation complex (11). It is also entirely possible that several quality control pathways may exist and that the endogenous triage decision may involve components of each of these hypotheses.

Our laboratory has recently reported the cloning and characterization of CHIP (carboxyl terminus of Hsc70-interacting protein) (12), a tetratricopeptide repeat (TPR)1-containing pro-

---

* These studies were supported by National Institutes of Health Grants HL03658, GM61728, and HL6519 (to C. P.) and GM56981 (to D. M. C.); grants from the Cystic Fibrosis Foundation (to D. M. C.); and Deutsche Forschungsgemeinschaft Grant He1518/5–1 (to J. H.).

§§ To whom correspondence should be addressed: University of North Carolina at Chapel Hill, Division of Cardiology, 324 Burnett-Womack Bldg., Chapel Hill, NC 27599-7075. Tel.: 919-843-8477; Fax: 919-966-1743; E-mail: cpatters@med.unc.edu.

---

1 The abbreviations used are: TPR, tetratricopeptide repeat; HA, hemagglutinin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; Ub, ubiquitin.
CHIP Is a U-box-dependent E3 Ubiquitin Ligase

MATERIALS AND METHODS

Antibodies—The following antibodies were used for immunoblotting: mouse anti-HA (Roche Molecular Biochemicals), mouse anti-ubiquitin (Babco), mouse anti-His (Novagen), mouse anti-Hc/Hs70 (StressGen), and rabbit anti-CHIP (12). Mouse anti-Hc/Hs70/His70 and mouse anti-Myc antibody-agarose conjugates (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for immunoprecipitations.

Transient Transfections—COS-7 cells were transiently transfected using FuGene according to the protocol of the manufacturer (Roche Molecular Biochemicals), pcDNA3-CHIP, pcDNA3-CHIP His260A, pcDNA3-CHIP 3TPR (amino acid residues 32–145 deleted), and pcDNA3-CHIP ΔU-box (residues 196–303 deleted) were transfected with vectors expressing HA- and Myc-tagged ubiquitin (generous gifts of Ron Kopito, Stanford University). In some experiments, transfected cells were treated with MG-132 (25 μM) or vehicle for 2.5 h or were subjected to heat shock at 42°C before harvesting.

Western Blotting and Immunoprecipitations—Transfected cells were harvested 48 h after transfection and lysed with radioimmunoprecipitation buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM Na3VO4) supplemented with protease inhibitors and 50 mM N-ethylmaleimide. Cell lysates were clarified by centrifugation at 16,000 × g for 10 min, and protein concentration was determined. For immunoprecipitations, equal amounts of lysate proteins were incubated with 10–15 μg mouse monoclonal antibody-agarose conjugates at 4°C for 2 h. The beads were washed five times with lysis buffer. Immunoprecipitated proteins or cell lysates were mixed with 3× SDS sample buffer and were separated by SDS-PAGE. Western blotting was performed as described (12) with the appropriate antibodies.

In Vitro Ubiquitylation Reactions—Bacterially expressed CHIP, CHIP-H260A, and CHIP ΔU-box and/or Hsc70 was incubated in the presence of 4 μM CHIP or CHIP mutants, 0.1 μM purified rabbit E1 (Calbiochem), 2.5 μg/ml ubiquitin (Sigma) or His-tagged ubiquitin (Calbiochem), and equivalent activities of E2 enzyme in 20 mM MOPS, pH 7.2, 100 mM KCl, 5 mM MgCl2, 5 mM ATP, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride for 4 h at 30°C. Samples were analyzed by SDS-PAGE and immunoblotting performed with appropriate antibodies.

S-protein Pull-down Assays—Bacterially expressed S-tagged CHIP and His-tagged E2 were incubated for 1 h at 4°C in 1 ml of binding buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% Triton X-100, 25 μg/ml bovine serum albumin), and then incubated with 10 μl of S-protein-agarose (Novagen) for 30 min at room temperature. Precipitates were washed three times with binding buffer and subjected to 15% SDS-PAGE and immunoblotting with anti-His antibody.

Pulse-Chase Experiments—Transiently transfected COS-7 cells were preincubated in a methionine-free, cysteine-free medium and then metabolically labeled with 50 μCi/ml [35S]methionine for 20 min (PerkinElmer Life Sciences) and chased at different time points. Myc-Hc/Hs70 was immunoprecipitated with mouse anti-Myc antibody and identified by SDS-PAGE, followed by autoradiography.

Mutagenesis—A point mutation of histidine to alanine at position 260 of CHIP was generated with the QuikChang™ XL site-directed mutagenesis kit (Stratagene) with mutagenic primers 5′-CGCAAGGA-CATCGAGGAGGCCCTGCAGCGTGTGGG-3′ and 5′-CCCACACGCT-TGACAGCGCT-GACGGGCTCCTCAGTGCTTGCGG-3′ to create the plasmid CHIP-H260A. Point mutations were also created in HA-tagged ubiquitin to convert lysines 29, 48, and 63 to arginine (singly and in combination) by similar methods.

RESULTS

CHIP Functions as a U-box-dependent E3 Ubiquitin Ligase—Having previously demonstrated that CHIP elicits the ubiquitylation and proteasome-mediated degradation of ectopically expressed glucocorticoid receptor, a model chaperone substrate (13), we wanted to determine whether expression of CHIP affected the ubiquitylation of endogenous proteins. We overexpressed CHIP in COS cells with a hemagglutinin-tagged ubiquitin (HA-Ub), in the absence or presence of MG-132, a selective inhibitor of proteasome activity. A slowly migrating smear of high molecular weight proteins detectable by Western blotting for the HA epitope was enhanced in cells overexpressing CHIP, and the accumulation of these species was accentuated in the presence of the proteasome inhibitor (Fig. 1). We can
conclude that these high molecular weight species represent proteins ubiquitylated with HA-tagged ubiquitin in the presence of CHIP, because these high molecular weight species were not detected in cells treated similarly but transfected with a Myc-tagged ubiquitin instead of HA-Ub. These experiments indicate that CHIP can serve as a ubiquitylation factor for endogenous proteins; moreover, CHIP appears to be a rate-limiting factor for these ubiquitylation events.

We reconstituted ubiquitylation reactions with recombinant proteins to determine how CHIP participated in ubiquitylation. We used an approach similar to that used to characterize the ubiquitin ligase activity of the RING finger protein c-Cbl, in which ubiquitylation in the absence of a specific substrate is tested as an indication of multiubiquitin chain assembly that occurs either freely, on co-purifying proteins, or on the ubiquitin ligase itself (19). Bacterially expressed CHIP and UBCH5a, an E2 ubiquitin-conjugating enzyme, were incubated in a reaction containing ubiquitin and E1, and these reaction mixtures were probed by Western blotting with an anti-ubiquitin antibody. Ubiquitylation activity, as assessed by the detection of high molecular weight multiubiquitin chains, was only detected in reactions containing E1, UBCH5a, and CHIP in the presence of ubiquitin (Fig. 2A). Thus, CHIP is sufficient to serve as a ubiquitin ligase in these reconstitution experiments. Because the U-box of CHIP is predicted to have a modified RING finger-like structure, we determined whether the U-box (contained in the carboxyl-terminal residues 196–303) was necessary for this ubiquitin ligase activity by performing similar reactions with a protein lacking this motif. In comparison with full-length CHIP, the truncated protein lacking the U-box had no ubiquitin ligase activity (Fig. 2B). We also found that BAG-1, which is also a chaperone cofactor that contains a ubiquitin-like domain at its amino terminus and participates in interactions between Hsc70 and the proteasome (20), could not replace CHIP in these reactions. In addition, identical results demonstrating the ubiquitin ligase activity of CHIP were obtained in similar in vitro reconstitution assays using a ubiquitin-glutathione S-transferase fusion as an artificial substrate for ubiquitylation (data not shown). Thus, CHIP has all of the characteristics of a ubiquitin ligase, and this activity is U-box-dependent.

**FIG. 1.** CHIP increases ubiquitylation of proteins in vivo. COS-7 cells were transiently transfected with HA-Ub or Myc-Ub with or without CHIP and treated with 25 μM MG-132 for 2.5 h as indicated. HA-Ub conjugates were separated by SDS-PAGE, and Western blotting (WB) was performed with an anti-HA antibody. Cell lysates were blotted simultaneously with anti-CHIP and anti-β-actin antibodies (lower panels). MW, molecular weight; NS, nonspecific.

**FIG. 2.** CHIP is an E3 ubiquitin ligase, and the U-box of CHIP is required for its E3 ligase activity. A, in vitro ubiquitylation reactions were performed with purified Ub, E1, the E2 UBCH5a, and CHIP as indicated. Following SDS-PAGE, blots were probed with anti-ubiquitin and anti-CHIP antibodies, as indicated. B, in vitro ubiquitylation reactions were performed with purified ubiquitin, E1, and E2 UBCH5a. CHIP, a fragment of CHIP lacking the U-box motif (∆U-box), or BAG-1 was used in the reactions, as indicated. Ubiquitin conjugates were resolved by SDS-PAGE and detected by anti-ubiquitin or anti-CHIP antibodies. Brackets denote slowly migrating modified forms of CHIP.

The UBCH5 Ubiquitin-conjugating Enzyme Family Interacts Functionally with CHIP—Ubiquitin ligases interact functionally with specific E2 ubiquitin-conjugating enzymes to facilitate substrate recognition and ubiquitin transfer (21). We tested the ability of CHIP to assemble mult ubiquitin chains in the presence of a panel of ubiquitin-conjugating enzymes. We found that the three mammalian members of the UBCH5 family (UBCH5a, -b, and -c) were able to interact functionally with CHIP in the ubiquitylation process (Fig. 3A). E2-20k, UBCH7, UBCH3, and E2-25k were ineffective in facilitating CHIP-mediated multiubiquitin chain assembly. The UBCH5 E2 enzymes are structurally very similar to one another (22) and are closely related structurally to the yeast E2 enzymes UBC4 and UBC5, which are stress-activated ubiquitin-conjugating enzymes that preferentially mediate degradation of short lived and damaged proteins (23). Based on the functional cooperativity observed between CHIP and UBCH5 proteins, we tested whether these proteins directly interacted, since physical E2-E3 interactions are thought to determine substrate specificity for ubiquitylation (24). Indeed, we found in co-precipitation experiments that recombinant CHIP and UBCH5a are physically associated with one another under conditions that efficiently promote ubiquitylation in vitro (Fig. 3B).

**CHIP Is Autoubiquitylated in Vitro**—In the course of the ubiquitylation reactions, we noted the presence of discrete slowly migrating complexes when immunoblotting for CHIP (brackets in Figs. 2 and 3). These bands were present only when reactions were performed in the presence of E1, E2, CHIP, and ubiquitin (Fig. 2A) and did not occur in reactions containing the
CHIP Is a U-box-dependent E3 Ubiquitin Ligase

42941

A. in vitro ubiquitylation reactions were performed with ubiquitin, E1, CHIP, and equivalent activities of different E2 ubiquitin-conjugating enzymes, as indicated. Ubiquitin conjugates and CHIP were detected with respective antibodies. B, recombinant CHIP (containing an S-tag) and His-tagged UBCH5a were co-incubated, and the association between S-tagged CHIP and His-tagged UBCH5a was measured using an S-protein pull-down assay. UBCH5a was detected with an anti-His antibody.

U-box-deleted form of CHIP (Fig. 2B). Together, these findings suggested to us that CHIP undergoes autoubiquitylation. To ascertain that this is indeed the case, we compared the molecular weights of these complexes when ubiquitylation reactions were performed with ubiquitin or with a polyhistidine-tagged Ub. The modified forms of CHIP migrated more slowly in reactions containing His-Ub, indicating that they reflect multoubiquitin-modified CHIP (data not shown). Autoubiquitylation has been described for other E3 ligases (25, 26) and may provide a means of autoregulation of ubiquitin ligase activity.

Hsc70 Is an Endogenous Target for CHIP-mediated Ubiquitylation—Based on several lines of reason, we considered whether Hsc70 itself was a target for ubiquitylation mediated by CHIP. First, Hsc70 is a functional interaction partner with CHIP (12, 14). Second, we noted that the most prominent ubiquitin-conjugated proteins detected when CHIP is overexpressed appear at 80 kDa and above (Figs. 1 and 4A), which would be consistent with these bands representing ubiquitin-modified forms of Hsc70. However, until now ubiquitin-mediated modification of the cytosolic chaperones has not been described. Therefore, to test this possibility, we examined ubiquitylation of endogenous Hsc70 in COS cells transfected with CHIP or empty vector. First, we asked whether ubiquitylated proteins were present in Hsc70 immunoprecipitates. Ubiquitylated proteins (detected by HA blotting) were only present in immunoprecipitates of COS cells co-transfected with CHIP (Fig. 4A). Surprisingly, blotting of Hsc70 immunoprecipitates with Hsc70 demonstrated that at least some of these bands were ubiquitin-Hsc70 conjugates (Fig. 4B). The bands disappeared when immunoprecipitates were treated with ubiquitin-carboxyl hydrolase, indicating that these higher bands did indeed contain ubiquitin (not shown). These retarded bands did not represent substrates bound to Hsc70, since they did not release when immunoprecipitates were incubated with ATP (Fig. 4B). We also could demonstrate that this effect was independent of overexpression of HA-Ub, since Hsc70 could be modified as efficiently in cells only expressing endogenous ubiquitin (Fig. 4C).

CHIP contains TPR domains at its amino terminus and the U-box domain at its carboxyl terminus. To determine the requirements of each of these domains in these ubiquitylation effects, we created CHIP expression vectors lacking the TPR (ΔTPR) or U-box (ΔU-box) domains. These truncated proteins were expressed in levels equivalent to wild-type CHIP when transfected in COS cells (not shown). In contrast with the wild-type protein, neither of the truncated proteins induced ubiquitylation of Hsc70 (Fig. 4D). Thus, the effect of CHIP on Hsc70 is specific and requires both the Hsc70-binding domain and the U-box domain. The total amount of ubiquitylated Hsc70 was estimated in COS cells expressing endogenous levels of CHIP and in cells overexpressing CHIP. Approximately 1% of Hsc70 was ubiquitylated under endogenous conditions (primarily in the monoubiquitylated form, although additional modifications could be observed on overexposure of blots), and 11% of Hsc70 was ubiquitylated when CHIP was overexpressed (data not shown). Although only a minor fraction of Hsc70 is ubiquitylated under standard conditions, this still represents a large amount of protein, since Hsc70 accounts for ~1% of all proteins within COS cells.

If CHIP indeed ubiquitylates Hsc70 via its E3 ubiquitin ligase activity, then we should be able to recapitulate this ubiquitylation reaction in a reconstituted reaction with purified proteins. To test this, reactions with or without recombinant E1, E2 (UBCH5a), and CHIP were used to assess for ubiquitin modification of Hsc70. Ubiquitylation of Hsc70 was observed in these in vitro reactions and only occurred in the presence of ubiquitin, E1, E2 (UBCH5a), and CHIP (Fig. 5A). Consistent with our previous observations of multoubiquitin chain assembly, the U-box domain of CHIP was required for this activity. Replacement of ubiquitin by N-methylated ubiquitin blocked formation of all but the singly modified forms of Hsc70, indicating that Hsc70 is ubiquitylated at a single site by CHIP. In addition, we found that CHIP-mediated ubiquitylation of Hsc70 required members of the UBCH5 family of ubiquitin-conjugating enzymes (Fig. 5B). Taken together with the data in Fig. 4, these experiments demonstrate that Hsc70 is a target for ubiquitylation and that CHIP functions as a ubiquitin ligase to catalyze this reaction in vitro and in vivo.

Ubiquitylation of Hsc70 has, to our knowledge, not been described previously and immediately raises several questions as to the cellular consequences of such an event (see discussion below). Previously, we have shown that chaperone substrates targeted for ubiquitylation by CHIP are rapidly degraded in vivo (13, 14). However, we did not observe decreases in steady-state levels of Hsc70 when CHIP is overexpressed (for example, see Fig. 4). To test the stability of Hsc70 in the presence of increased levels of CHIP, we co-transfected CHIP with an amino-terminal Myc-tagged Hsc70 expression plasmid (so we could measure the effects on Hsc70 only in transfected cells), and pulse-labeled the cells with [35S]methionine, followed by immunoprecipitation. (In preliminary experiments, we found that Myc-tagging had no effect on the ability of Hsc70 to interact with CHIP.) In COS cells overexpressing CHIP, we detected labeled bands that corresponded to the ubiquitylated forms of Hsc70 (arrow, Fig. 6). However, the stability of Hsc70 was not significantly affected by CHIP-induced ubiquitylation. Although we cannot exclude the possibility that a small (and, in
CHIP Is a U-box-dependent E3 Ubiquitin Ligase

Fig. 4. CHIP stimulates ubiquitylation of Hsc70. A, COS-7 cells were transiently transfected with HA-Ub with or without CHIP and treated with 25 μM MG-132 for 2.5 h before harvesting. Lysates were immunoprecipitated with anti-Hsc70 antibody. Lysates and immunoprecipitated proteins were immunoblotted for HA. B, COS-7 cells were transfected and immunoprecipitated as in A, except that some cells were harvested and incubated for 30 min in radioimmune precipitation buffer containing 2.5 mM ATP, as indicated. Immunoprecipitated proteins were immunoblotted for Hsc70. C, COS-7 cells were transiently transfected with or without CHIP and HA-Ub as indicated and immunoprecipitated and immunoblotted as in B. D, COS-7 cells were transiently transfected with CHIP or its mutants as indicated. Cell lysates were immunoblotted for Hsc70.

Fig. 5. A, CHIP acts as an E3 ligase for Hsc70, and the U-box of CHIP is required for its E3 ligase activity. In vitro ubiquitylation reactions were performed as in Fig. 2 except that 2 μM purified Hsc70 was included in the reactions. Me-Ub, methyl-ubiquitin. B, UBCH5 family members couple with CHIP in its ligase activity for Hsc70. In vitro ubiquitylation reactions were performed as in Fig. 3A except that 2 μM purified Hsc70 was included in the reactions. Ubiquitin conjugates of Hsc70 were resolved by SDS-PAGE and detected with an anti-Hsc70 antibody.

In these analyses, unmeasurable proportion of Hsc70 is susceptible to degradation after ubiquitylation by CHIP, the stability of the ubiquitin-modified forms of Hsc70 in this assay argues that the ubiquitylation of Hsc70 induced by CHIP (in contrast with the activity of CHIP on glucocorticoid receptor and cystic fibrosis transmembrane conductance regulator) is not sufficient to target Hsc70 for degradation and may instead have other effects on Hsc70 function.

Because Hsc70 plays a role in protection against thermal and other types of stress, we asked whether the ubiquitylation of Hsc70 by CHIP was modified under conditions of thermal stress. Although the levels of Hsc70 were increased after heat shock, this had no effect on the amount of ubiquitylated Hsc70 that accumulated (Fig. 7A). We also tested the ubiquitin linkages that are used to assembly mult ubiquitin chains on Hsc70, reasoning that the short length of the chains and their failure to signal degradation of Hsc70 may indicate that the ubiquitin-ubiquitin bonds are forming via residues other than lysine 48; in particular, we tested the importance of lysines 29 and 63, given existing evidence that linkages via these residues serve functions other than signaling degradation (27). We found that mutation of lysine 48 to arginine did not decrease the ubiquitylation of Hsc70 and in fact enhanced this effect (Fig. 7B).

Likewise, single mutations of lysine 29 or 63 did not prevent Hsc70 ubiquitylation. However, when both lysines 29 and 63 were mutated together, with or without mutation of lysine 48, only a single ubiquitin residue was added to Hsc70, and monoubiquitylation of Hsc70 under these circumstances was either relatively unstable, possibly because it is a better target for deubiquitylating enzymes, or conjugated inefficiently. Taken together, these studies argue that Hsc70 is normally ubiquitylated by CHIP via noncanonical ubiquitin chains that utilize either lysine 29 or 63 of ubiquitin and that do not target Hsc70 for proteasome-mediated degradation.

Evidence for Mechanistic Differences between U-box and RING Finger E3 Activities—RING fingers contain conserved cysteine residues that coordinate Zn²⁺ ions to stabilize the structure of the motif as well as a conserved aromatic residue (such as Trp408 in Cbl) that participates in E2 activation and ubiquitylation (17, 19). This residue of the RING finger directly interacts with the cognate E2 enzyme and probably determines E3-E2 selectivity (28), and its mutation invariably abolishes ubiquitin ligase activity of proteins containing RING fingers (19). Proteins predicted to contain U-boxes also contain a conserved aromatic residue (usually tryptophan or histidine) at this position (17); in the case of CHIP, the corresponding residue is a histidine at position 260. We mutated this residue to alanine in order to determine whether the conserved aromatic residue of U-box-containing proteins served a role similar to the analogous amino acid in RING fingers. However, in contrast to expectations derived from mutagenesis of RING finger domains, we found that ubiquitylated species in general, and ubiquitylated forms of Hsc70 in particular, accumulated in COS cells expressing the H260A mutant with efficiency almost equal to that of cells expressing the wild-type protein (Fig. 8A).

Similarly, when recombinant proteins were tested in an in vitro reconstitution assay, we found that the H260A mutant, although not as active as the wild-type protein, still possessed considerable ubiquitin ligase activity when compared with CHIP lacking the U-box (Fig. 8B). These results indicate that the U-box motif, while also serving a ubiquitin ligase function, may mediate interactions with ubiquitin-conjugating enzymes and facilitate ubiquitylation events through mechanisms different from those that occur with RING finger-containing ubiquitin ligases.
in vitro and were pulse-labeled with [35S]methionine and chased at different time points, as indicated. Myc-Hsc70 was immunoprecipitated and detected by autoradiography. Results shown are representative of three independent experiments.

**Fig. 6.** CHIP does not decrease the half-life of Hsc70. COS-7 cells were transiently transfected with Myc-Hsc70 with or without CHIP. Cells were pulse-labeled with [35S]methionine and chased at different time points, as indicated. Myc-Hsc70 was immunoprecipitated and detected by autoradiography. Results shown are representative of three independent experiments.

**Fig. 7.** Factors affecting modification of Hsc70 by CHIP. A, COS-7 cells overexpressing CHIP, as indicated, were subjected to heat shock at 42 °C for the indicated times. Cell lysates were immunoprecipitated and blotted with an antibody to Hsc70 to determine the effects of heat shock on CHIP-mediated Hsc70 ubiquitylation. B, COS-7 cells were transiently transfected with CHIP, as indicated, and with hemagglutinin-tagged wild-type (WT) Ub or with HA-ubiquitin containing mutations of lysine to the indicated arginine (R) residues. Immunoprecipitation was performed with an anti-Hsc70 antibody, followed by immunoblotting with an anti-hemagglutinin antibody to detect ubiquitylated species of Hsc70.

**DISCUSSION**

Previous studies have linked U-box-containing proteins with ubiquitylation (13, 14, 16, 29). In this report, we demonstrate through several lines of evidence that the U-box protein CHIP is a bona fide ubiquitin ligase that regulates ubiquitylation activity in vitro and in vivo via interactions with the UBCH5 family of ubiquitin-conjugating enzymes. To date, two additional U-box-containing proteins have been characterized. NOSA is a developmentally regulated protein found in Dictyostelium that affects the ubiquitylation of a subset of proteins in this species (29). The means by which NOSA exerts its activities are not certain, but it seems likely that this protein is also a ubiquitin ligase. A multiubiquitin chain extension activity (or E4 activity) was attributed to a second U-box-containing protein, UFD2 (16). Based on our observations, we would predict that the E4 activity is similar or identical in some respects to ubiquitin ligase activity; studies to test whether UFD2 directly interacts with E2 enzymes and elicits ubiquitin chain extension in an E2-dependent manner will be helpful in addressing this issue. Nonetheless, it is reasonable to hypothesize that many, if not most, proteins containing U-boxes will be ubiquitin ligases, and therefore that the U-box family will form a new family of ubiquitin ligases similar to the RING finger and HECT domain families (30).

We found that CHIP interacts functionally with the UBCH5 family of ubiquitin-conjugating enzymes. The association of CHIP with this family of E2 enzymes may be instructive with regard to the cellular role of CHIP, insofar as the related S. cerevisiae proteins, UBC4 and UBC5, are up-regulated in response to stress and are protective against stress-mediated injury (23, 31). In addition, UBC4 and UBC5 are required for the selective degradation of short lived and abnormal proteins (23). It has previously been postulated that UBC4/UBC5/UBCH5-dependent proteolysis is mechanistically linked with the function of molecular chaperones such as Hsc70 (29). The experiments presented here support this hypothesis and indicate that interactions with CHIP are at least one means by which this linkage occurs. Based on these studies and our previous reports indicating that CHIP targets chaperone substrates for ubiquitin-dependent degradation (13, 14), we propose that CHIP, via its ubiquitin ligase activity as well as its ability to interact with and modify the function of the stress-induced molecular chaperones (12, 13), participates in the stress response by partitioning chaperone substrates (which may, under endogenous conditions, be proteins that have been damaged under harsh conditions) toward the stress-associated degradative pathway.

We have previously shown that model chaperone substrates glucocorticoid receptor and cystic fibrosis transmembrane conductance regulator are ubiquitylated and degraded by a CHIP-dependent pathway (13, 14). It is therefore quite surprising that a major endogenous target of CHIP-mediated ubiquitylation is Hsc70 itself. One possible reason for this activity is that CHIP targets Hsc70 for ubiquitin-dependent degradation. This does not seem to be the case, however, since steady-state levels of Hsc70, as well as its half-life, are not affected by CHIP overexpression (Figs. 5 and 7) (findings that stand in contrast with our previous observations that overexpression of CHIP...
decreases steady-state levels of chaperone substrates (13, 14). We cannot exclude entirely the possibility that Hsc70 is being targeted for degradation by CHIP, since it is conceivable that only a small population of Hsc70 molecules are available for ubiquitin-dependent degradation; however, the stability of ubiquitylated forms of Hsc70 detected in our pulse-chase experiments would again argue against this interpretation. A second possible explanation for these observations is that Hsc70 is being ubiquitylated artifactually; although such an interpretation would not discount the attribution of ubiquitin ligase activity to CHIP, we think it unlikely insofar as this effect can be observed in two independent assays (in vitro and in vivo) and does not happen to other abundantly expressed proteins such as β-actin (Fig. 1).

Several aspects of CHIP-mediated Hsc70 ubiquitylation events are atypical and may therefore provide clues to the functional role of this activity. First, these ubiquitylated forms are readily observed even in the absence of proteasome inhibition (in contrast to ubiquitin conjugates of chaperone substrates that are elicited by CHIP (13)), suggesting that they are not recognized as a degradation signal by the proteasome. Second, the major ubiquitylated forms of Hsc70 detected in our assays have relatively short ubiquitin chains (less than four). In general, it is thought that once ubiquitylation begins, multiple ubiquitin residues are added rapidly, so our observations would be contrary to this dogma (32). It is conceivable that the predominance of short chains is due to steric changes in Hsc70 that interfere with its recognition by antibodies; this would not, however, explain the stability of these ubiquitin conjugates. Another, more intriguing possibility is that shorter multiubiquitin chains, perhaps involving atypical (other than lysine 48-linked) chains, are being formed on Hsc70 and that these chains do not serve as recognition signals for the proteasome but somehow alter the function of Hsc70, serve as a targeting sequence, or otherwise alter cellular signaling events. Recent studies indicate that noncanonical ubiquitin linkages (involving lysine 29 and lysine 63) do occur. Rather than serving as degradation markers, these linkages mediate signaling events (particularly under conditions of cellular stress) and participate in replicative DNA repair, endocytosis of plasma membrane proteins, and ribosomal function (27, 31, 33–37). Interestingly, the yeast homologues of the UBCH5 family, UBC4 and UBC5, are the E2s implicated in catalyzing noncanonical ubiquitin linkage formation in yeast (31). Because lysines 29 or 63 of ubiquitin are required for Hsc70 ubiquitylation by CHIP, we can infer that Hsc70 is indeed ubiquitylated in a noncanonical fashion, which may provide important clues to the cellular role of ubiquitylated Hsc70.

If ubiquitylation of Hsc70 is not targeting this chaperone for degradation but is instead serving another purpose, what might that be? Several possibilities can be considered. Ubiquitylation may target a portion of Hsc70 molecules to a particular subcellular location, as is the case for uracil permease (36). Another possibility is that ubiquitin modification may alter the ATPase activity of Hsc70 or its affinity for substrates, either of which would affect its activity as a chaperone in ways that may be relevant in the context of protein triage decisions that are being affected by CHIP. Last, it is possible that ubiquitin modification serves to target Hsc70 to the proteasome, yet due to the nature of the ubiquitin linkages formed (or for other reasons), Hsc70 is resistant to proteasome-mediated degradation. Such a scenario has interesting implications, since Hsc70 has recently been linked physically to the proteasome via intermediary interactions with BAG-1 (20), and we have previously shown that CHIP links Hsc70 functionally with the proteasome (13, 14). Several proteins, including BAG-1 itself and the hPLIC ubiquitylation factors, contain ubiquitin-like domains that are thought to facilitate interactions between these proteins and the proteasome, although these ubiquitin-like domain-containing proteins themselves are not thought to be substrates for proteasome-mediated degradation (20, 38). It is conceivable that short ubiquitin chains serve a similar purpose when attached to Hsc70 and provide an additional means by which CHIP links the chaperone and proteasome machinery.

Acknowledgments—We thank Ron Kopito and Kazuhiro Iwai for generous provision of reagents.

REFERENCES